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CELLR4

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Repair, Replacement, Regeneration & Reprogramming

The Official Journal of The Cure Alliance

VERDUCI INTERNATIONAL
Miami, Florida, USA
http://www.cellr4.org - e-mail: info@cellr4.org

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Miami, Florida, USA

Editorial Board

Aims and Scope

The journal CellR^4 is a multidisciplinary, cross-platform "open source" e-publication in the areas of cell repair, replacement, regeneration, reprogramming and differentiation. CellR^4 also serves as the official journal of The Cure Alliance (www.thecurealliance.org) and of the Fondazione Cure Alliance Onlus, two linked non-profit international organizations that include physicians, scientists, patients, patient advocates, business and philanthropy leaders, with the mission to promote collaborative efforts worldwide, while addressing and working to resolve impediments and challenges on the path to develop cures for diseases now afflicting humankind. In this direction, the publication will also serve as a shared communication platform to discuss challenges and opportunities on the path to develop new treatments. Many of these topics are shared by most translational efforts in the fields of cell therapy and regenerative medicine, independently from the specific disease area they are targeting. Therefore CellR^4 will also have sections on regulatory, legal, and ethical issues, as well as global collaborative platforms and funding opportunities of interest. CellR^4 is grateful to the Editorial Board for their enthusiastic support to this initiative and is looking forward to welcoming your contributions to the journal.

NOTE: Any editorial compensation has been donated to The Cure Alliance and Fondazione Cure Alliance Onlus, in support of their strategic mission.

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MESSAGE FROM THE EDITOR-IN-CHIEF

Welcome to the first issue of CellR^4, a multidisciplinary, cross-platform "open source" e-publication in the exponentially growing fields of cell repair, replacement, regeneration, reprogramming and differentiation. The journal will be published primarily on-line with a dedicated website and will also serve as the official journal of The Cure Alliance (www.thecurealliance.org) and Fondazione Cure Alliance Onlus, two linked non-profit international organizations that include physicians, scientists, patients, patient advocates, business and philanthropy leaders, with the mission of promoting collaborative efforts worldwide, while addressing and working to resolve impediments and challenges on the path to develop cures for diseases now afflicting humankind. In this direction, the publication will also serve as a shared communication platform to discuss challenges and opportunities on the path to the development of new treatments. Many of these topics are shared by most translational efforts in the fields of cell therapy and regenerative medicine, independently from the specific disease area they are targeting. CellR^4 will, thus, also have sections on regulatory, legal, and ethical issues, as well as global collaborative platforms and funding opportunities of interest. I am grateful to the founding editorial board for their enthusiastic support of this initiative, and I am looking forward to your contributions to the journal and to the fields of cell repair, replacement, regeneration and reprogramming/differentiation. Any editorial compensation will be entirely donated to The Cure Alliance and the Fondazione Cure Alliance Onlus, in support of their strategic mission to promote collaborative efforts and resolve challenges and impediments to the development of clinically relevant treatment strategies.

Camillo Ricordi
Editor-in-Chief

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MOVING TOWARDS A DETENTE IN THE STEM CELL DEBATE

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The Nature Editorial titled "Smoke and Mirrors"[1] on the Second International Vatican Adult Stem Cell meeting was ironically itself a collection of smoke and mirrors beginning with the lack of a named author. The editorial focused on an Italian parliamentary decision to allow stem cell treatment for a child with a devastating and lethal disease that had been stopped by regulators against the parents and physicians wishes[2]. In actuality, the Vatican is a State separate and independent from Italy, and balanced, compassionate, and realistic regulatory oversight was not questioned at the Vatican meeting.

The editorial opted for prejudicial phrases such as: "sick children were paraded". Instead the meeting was inclusive of concerns from patients. The author states that the Vatican is "naïve". A less sensational description of the Vatican's position is altruism. The editorial states that the meeting was "shamelessly choreographed" "with stem cell companies and scientists desperate to hawk a message". This denigrates all speakers at the Vatican conference including a Nobel Laureate as well as other academic researchers not affiliated with companies marketing adult stem cells. In contrast, the World Stem Cell Summit was praised for including scientists, doctors, patients, and stem cell companies[3]. Perhaps it could be said that the Nature editorial was itself shamelessly choreographed to hawk a message.

Demagoguery is the antithesis of Science. No person or institution should have their freedom of speech or ethical concerns ridiculed or made to fear collaboration especially from a scientific journal. On the other hand, there are historical concerns and lessons why religion, business, science, and State require transparency, independence, and separation. Unfortunately, the Nature editorial missed the opportunity to discuss these transcendent concepts.

The etymology of the word education has been partially obscured by the passage of time, but in Portuguese, perhaps the most Latin of the Romance languages, the word "educado" means polite. "Educational" editorials that avoid emotional trigger words and promote détente of the stem cell debate are in the best interest of patients, science, and humanity.

Conflicts of Interest: All three authors work with both adult and embryonic stem cells and were speakers at the Second Vatican Adult Stem Cell Conference.

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TOWARDS A CONSTRUCTIVE DEBATE AND COLLABORATIVE EFFORTS TO RESOLVE CURRENT CHALLENGES IN THE DELIVERY OF NOVEL CELL BASED THERAPEUTIC STRATEGIES

Camillo Ricordi

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Soon after the Cell Transplant Society (CTS) was formed in 1991, with E. Donnall Thomas, Thomas E. Starzl and Paul E. Lacy serving as Honorary Presidents, we were worned at that time of CTS first congress (1992) about the potential risk of overregulation by FDA of the emerging field of cellular therapies.

I was told how many of the breakthroughs of the past century including bone marrow transplantation and liver transplantation could not have been possible under the current FDA rule and that the fortune of bone marrow transplantation and liver or organ transplantation in general, as well as of the patients who have benefited from these prcedures, has been indeed to be able to remain out of such rule. A recent article by Scott Gottlieb, former deputy director of FDA, "How The FDA Can Cost You Your Life" (Wall Street Journal, October 3rd 2011) highlights some of these concerns in the area of devices, but many of the same considerations could apply to cellular therapies. The same day this article was published, the keynote address by Andrew Grove at the World Stem Cell Summit in Pasadena, CA, outlined how the problem started when Congress allowed FDA to extend their regulatory oversight to "efficacy" beyond "safety" (1962). If the only consideration or performance indicator is safety and risk avoidance without considering other factors, such as the morbidity and mortality of a disease condition, the resulting course of action could be doing nothing, or imposing years of additional "efficacy" demonstrations for the proposed strategy, in experimental model systems which could often reveal themselves irrelevant to the clinical setting in which an actual human cell product will be eventually used.

I am honored to have been able to serve as chairperson of the steering committee of the NIH Clinical Islet Transplant Consortium, which just completed the first Phase III trial towards registration of the first metabolically active cell product in the US. I am also proud to have served on the FDA Biologic Modifiers Committee and I am proud to have directed one of the first cGMP human cell processing facilities in the US that was approved by FDA to ship cell products across state barriers and that is now one of 4 cGMPs in the US certified for processing more than minimally-manipulated human cell products. However, I am also deeply concerned by the cost and time requirements for any clinical trial to move forward, for many reasons including immediately life threatening conditions for which an alternative effective treatment does not exist.

In this editorial I am expressing my personal opinion and not that of NIH or the CIT consortium, but I believe that dialogue and understanding each other positions (tolerance if you wish) could be a starting point towards a much needed "resetting" of the regulatory environment for cellular therapies, to allow and promote innovative trials while maintaining the centrality of patient safety. Mandatory reporting and monitoring/audits by DSMBs will then continue to assess efficacy and safety. The introduction of "efficacy" in the definition of compassionate use is arguable as well, because if it would be efficacious (in addition to safe) shouldn't it be allowed beyond compassionate use? Just a thought ... I am happy that the debate will continue during the upcoming congress of The Cell Transplant Society, now 22 years old (http://www.cts2013.org/).

Clinical trials in cellular therapies have witnessed a significant growth in the number of applications targeted and patients treated, while a series of impediments and challenges on the path of translation of cell based therapies have emerged and are going well beyond patient safety concerns or risk/benefit considerations. Some regulatory environments have been largely unprepared to address such challenges, often limiting innovation and the development of new treatments.

An increasing number of scientists, clinician, patients and patient advocates are expressing concerns on whether an excess of caution from regulators may result in unacceptable delays or even completely pre-

venting the development of selected cell, stem cells and reprogrammed cells therapies from reaching patients.

Comments made by John Gurdon after winning this year's Nobel Prize in Physiology and Medicine are clearly pointing to these growing concerns: "I think patients would be happy to take the risk of using their own cells given the choice," Gurdon told a press conference in London, criticising the US Food and Drug Administration for placing "immense conditions on approval" *(Aldhous, P. and A. Coghlan, Medicine Nobel: good choice, but will cures come soon?, in New Scientists. 2012, Reed Business Information Ltd.)*.

The costs related to health care delivery are becoming unsustainable, while the prevalence of chronic degenerative disease conditions is dramatically increasing. Paradoxically, also the costs and time imposed to reach regulatory approval for a new therapeutic strategy, a new molecular entity or cell based product have dramaticlly increased in recent decades and now even minimally manipulated, autologous cell products face the possibility to become regulated as drugs in several countries.

As an opening editorial, I would like to raise some concerns for the polarizing and highly emotional debate which is now dividing the scientific community regarding regulatory environment, translational strategies, compassionate release, safety vs. efficacy preclinical data that should be required before allowing pilot clinical testing and reimbursement issues, to name a few.

We therefore decided to dedicate a significant space in the next issues of CellR[4] to invited as well as submitted, unsolicited papers/letters/commentaries expressing different points of view both from the scientific community, ethical, legal experts and from patients/patient advocacy groups, to discuss in a constructive and productive way the elements that could be of assistance to better understanding existing concerns and help us develop a team effort to better resolve current impediments and obstacles on the path to cures, while preserving patient safety and risk-benefit ratios, which should remain central to all efforts considered.

Below I outlined selected topics, issues and controversies that have ben raised in recent months, by different editorials, articles, blogs and meetings. Since commentaries have been accepted in very selective way by some journals, for reasons that are not apparent, we believe it is important to offer an opportunity to voice opposing views and different opinions, with the objective to provide a constructive forum for confrontation, discussion and resolution of current strategic differences on the path to develop novel treatment strategies and on what are the options to make them available to patients worldwide in the fastest, most efficient and safest way possible.

We would like to promote a constructive debate, where different opinions could be voiced, with a special emphasis on trying to reach a balanced and tolerant understanding of the concerns and possible solutions proposed by opposing positions, trying to avoid the arrogant and dogmatic statements that occasionally have characterized recent editorials and opinion papers.

Points and questions to be adressed by submitted papers/commentaries for the next issues of CellR[4] include the following:

- **To what extent can the FDA regulate a physician's ability to treat a patient with that patient's own stem cells?** A growing number of physicians routinely offer treatments involving Autologous Stem Cells (ASCs) to their patients which can be performed in their offices. Autologous adult stem cells, used to treat a variety of conditions, are harvested from the patient, processed, and returned to the same patient. It is no surprise that moving ASCs from laboratories to physician offices raises complex questions of law. At first glance, the idea of the FDA regulating our own cells looks like an outrageous invasion of individual privacy and denial of personal autonomy. If patients weigh risks and benefits of medical treatments every day, why prevent them from doing so with their own cells? This question is especially compelling where a patient has few or no effective therapies, and limited or no access to experimental treatments. That a treatment may be more risky in the hands of untrained or unskilled doctors is not unique to autologous adult stem cell therapies; this problem pervades medical practice. The FDA should re-examine its HCT/P (Human Cell Therapy/Products) regulations especially as applied to physicians treating patients with their own cells. Extracting a patient's cells for subsequent reinjection undoubtedly carries risk – but so does banking one's own blood or freezing eggs for later use. Conditioning the extent of regulation on the degree of manipulation may make sense on paper but is vague and confusing in practice,

especially in the dynamic field of cellular therapies. In an age of relentless cost inflation and limited therapies for debilitating illness, it makes no sense to deprive patients of autologous therapies because their physician lacks the resources – and patients lack the time – to satisfy the premarketing requirements that oppress even Merck and Johnson & Johnson. The FDA is obligated to protect the public health as well as individual patients. Critical to this mission is striking the proper balance of risks and benefits, where the benefits include facilitating medical innovation. In the context of adult stem cell regulation, especially autologous cells, it is time for that risk-benefit balance to be recalibrated. *(Adapted from Mary Ann Chirba, J.D., D.Sc., M.P.H. and Alice A. Noble, J.D., M.P.H.; http://lawprofessors.typepad.com/healthlawprof_blog/2013/06/mary-ann-chirbajd-dsc-mph-and-alice-a-noble-jd-mph-our-bodies-ourcells-fda-regulation-of-autologous-ad.html#more*).

- **Should cellular therapies be regulated like drugs or medicinals and/or when should this shared regulatory pathway occur? Stem cell therapies, even autologous ones, should be regulated, but should those regulations be redesigned to fit the parties and products being regulated?** It makes no sense for the FDA to insist that a practicing physician who is treating an individual patient must conform to the same premarketing and manufacturing requirements that bind large-scale, commercial pharmaceutical manufacturers that produce drugs in bulk for mass distribution. Moreover, the agency should not monopolize risk-benefit calculations to the exclusion of patients who, with the counsel of their physicians, want to make their own calls about using their own cells to treat their own conditions. Preventing them from doing so is already leading many patients to assume other and perhaps greater forms of risk, such as seeking treatments in foreign clinics that may or may not be up to the task. Suing an agency is usually an uphill and often losing battle and it is doubtful that it could do much to lower regulatory hurdles. Some form of regulation is needed, but the FDA must recognize that its current HCT/P framework is ill-suited to many kinds of cellular therapies. It could revamp its HCT/P framework entirely, but that will take time. In the near term, the agency should reach beyond existing expert advisory committees and public comment sessions. It should engage in a true collaboration with a wider group of physicians and surgeons who are already using or stand ready to use various types of autologous adult stem cell therapies, and the patients who have had or want treatment. It can also look to the guidelines of relevant organizations, such as the American Association of Blood Banks or various physician organizations. Only then can the FDA get a firm handle on what kinds of techniques and treatments present tolerable levels of risk when balanced with the need for innovation and the basic right of patients to use their own cells. After all, patients are the ones who must bear the burdens of illness, not the regulators, judges or attorneys. (Adapted from *Mary Ann Chirba, J.D., D.Sc., M.P.H. and Alice A. Noble, J.D., M.P.H.; http://lawprofessors.typepad.com/healthlawprof_blog/2013/06/mary-ann-chirbajd-dsc-mph-and-alice-a-noble-jd-mph-our-bodies-ourcells-fda-regulation-of-autologous-ad.html#more)*.

- **Would the weakening of regulatory standards for propagated adult stem cell interventions greatly increase patient risk?** While most agree that changes are definitely needed at the FDA in some respects related to stem cells such as expanded compassionate use of stem cells for patients with fatal diseases and a push for more openness, some argue that the weakening of regulatory standards for propagated adult stem cell interventions would greatly increase patient risk. Should the extent of regulation be conditioned on the degree of manipulation since it is operationally (not just on paper) extremely important from a patient safety perspective and it makes sense that stem cells manipulated in different ways and to different degrees should be subject to different regulations? *(Adapted from commentary to the above article by Paul Knoepfler, UC Davis)*.

- **Should smaller companies providing cell/stem cell products be subject to the same regulations as large multinational companies, or should the degree of regulation be proportional to the developmental stage of the cell product, the risk/benefit of proposed therapy and the relative patient size of the clinical trials considered?** Some argue that the law variable should not depend on the size of the entity that should be following that law and that just as small and large drug manufacturers of pill (chemical) drugs have to follow the same rules to provide data on safety and efficacy, smaller companies selling stem cell drug interventions should have to follow the same rules and laws as big compa-

nies. The argument is that doing otherwise put the growing number of patients treated by stem cell clinics (now in the thousands and growing) at great risk? On the other hand should it be considered that if the standards are defined by regulations that only large multinational companies can afford, this could severily limit innovation and development of new treatment strategies by smaller entities and academic centers?

- **Should all reguatory agencies have time limits to respond to IND applications or requests for inspection of cGMP Facilities?** There are increasing concerns for special emphasis commissions, expert groups and/or regulatory agencies that have no time limits to respond to requests for approval of an IND or a cell processing facility: a system in which there are requirements and guidelines, but no time frame for evaluation and eventual approval of applicants. Inspection and accreditation of cGMP facilities can possibly sit for years, with the facilities ready to operate but unable to do so, for an open ended delay in regulatory inspections, while a few individuals in a region may control what and who can perfom cell therapies in that geographical area, opening the possibility that special interest groups could define criteria, access and development of novel treatments.

- **If a subject has a terminal disease or one for which there were no approved efficacious treatments, would it be ethical to receive and be charged for unproven stem cell treatments?** … with the following caveats:
  1. It is safe to the best of our knowledge *(http://www.plosone.org/article/info:doi/10.1371/journal.pone.0047559)*; 2.The patient and/or their caretakers understand that this is experimental; 3. The price of treatment includes profit for the hospital/clinic the doctors and other researchers involved. Would there be a problem with a full disclosure like that? Why are there researchers who personally profit and otherwise benefit from ongoing research funding point their fingers at others when ignoring their own inherent conflicts?

"Smoke and mirror" is the title of a now famous (or infamous depending on the point of view) editorial on Nature *(Nature 2013 April 18; 496: 269-270 doi: 10.1038/496269b)* which was reported to represent the voice of the international scientific community position against a decree from the Italian Parliament, approved after media and patients' families pressure in favor of the Stamina Foundation Onlus. The decree is now allowing for a limited time controlled clinical trials of stem cell therapies within public hospitals, with a stringent regulatory oversight. There will be approximately 100 subjects treated, affected by genetic or orphan diseases, who will be allowed to start or continue a compassionate therapy, at no charge to the patients. The procedures will be performed with the cell line produced by Stamina at the Brescia Hospital's GLP laboratory, where hospital's biologists also routinely prepare bone marrow for transplant into leukemic children. For those who are not familiar with the saga surrounding the Stamina Foundation trials in Italy, selected arguments raised against Stamina and the (unedited) responses from Stamina Foundation have been included at the end of this article (Appendix).

While some scientists and physicians are concerned with the possible abuse of a potentially more permissive regulatory system, for example by for-profit entities or private clinics that may push for open-ended clinical treatments in the absence of a clear validation of the results and/or analysis or the efficacy of the proposed strategies, other scientists and physicians are concerned with the misleading and arrogant way that some arguments have been presented to the public. There are concerns that small group of scientists could selectively filter information available to the public, by controlling editorial boards or publishing anonimous editorials, while impeding commentaries of opposing views.

The surprising decision by the Italian Parliament in a way led the way to a wake up call, when actually what appeared at a first glance to be the overwhelming and almost unanimous position of some very vocal esponents of the scientific community, was blown away by the public response, including scientists in the Italian Parliament. In fact, the Italian Parliament passed virtually unanimousy a decree that was approved at the House with 504 yes votes and only one no (4 abstensions) and that was also approved by the Senate with 259 yes votes, 2 no (4 abstensions). The approval of this decree that offers a limited window of opportunity for cellular therapies to be tested in some patients clearly made the public opinion voice heard, but still divides the scientific community.

While some voices proposed that science was united against this decree that was overwhelmingly approved by the Italian Parliament, opposing commentaries were blocked from publication, for no apparent reasons, but generating the misleading appearance that scientists on one side and politicians and the general public on the other side, were deeply divided on issues of cellular therapies and how to better explore potentially novel therapeutic strategies.

In the mean time Japan may also be moving towards a dramatic retooling of the country's drug authorization framework that could produce the world's fastest approval process specifically designed for regenerative medicine. This special track for cell based therapies will create a novel, separate approval channel for regenerative medicine where companies will have to demonstrate efficacy in pilot studies of as few as ten patients in one study, if the change is dramatic enough. If efficacy can be "surmised," the treatment will be approved for marketing. At that stage, the treatment could be approved for commercial use and for national insurance coverage. Following approval, there will be a post-market surveillance period of five to seven years, after which the treatment will be evaluated again for safety and efficacy. Every patient must be entered in a registry during that period. If the therapies prove inefficacious or unsafe, approval can be withdrawn. *(http://www.nature.com/nm/journal/v19/n5/full/nm0513-510.html*).

On the other hand, concerns have been also expressed on ways in which the system could be abused by some commercial, for-profit interests, which should not be allowed to have an open ended opportunity to offer compassionate or unproven treatments without mandatory reporting and documentation of the results, without a Data Safety Monitoring Board or equivalent auditing/monitoring entity. We could indeed work collectively on a solution that could protect patient safety while allowing innovation and translational efforts to occur, avoiding any abuse of the system.

We therefore welcome your contributions to future issues of CellR[4] on these topics as well as any other relevant scientific contribution, from original papers, reviews, case reports and letters, to opinion papers on current challenges and topics of shared strategic interest, on how to be as efficient as possible on the path to identify and deliver clinically relevant strategies to cure disease conditions now afflicting humankind.

APPENDIX

Selected Arguments Against Stamina Stromal Mesenchymal Stem Cell Trials And Responses (S) From The Stamina Foundation

- **MSC suitable for use in compassionate therapy must be produced in a GMP lab or facility.** (S) We disagree. The aim of a compassionate therapy should be to promptly treat a patient affected by a very serious disease, threatening his life or compromising his social life, for which no official or already approved cure is available. If the cells have to be produced in a GMP facility, the time necessary to develop the GMP methodology would be too long to treat such compassionate cases who, in the meantime, would be destined to die or to compromise their health to a point of no return.
- **MSC produced in a GLP lab don't guarantee to be safe.** (S) In our opinion the production in GLP is absolutely suitable, and the final cell product should be released for clinical use once is properly analyzed to guarantee: (1) Microbiological sterility and purity of the preparation, without the presence of undesired cells (hematopoietic, macrophages, etc.); (2) Characterization of the stem cells through their CDs; (3) Telomerase activity to exclude the presence of tumor cells; (4) The cells' viability; (5) DNA genetic analysis; (6) Cell number
- **Fetal Bovine Serum (FBS) does not comply with GMP and should be banned.** (S) We don't agree. FBS free media should be proven to allow to obtain the same cell products before replacing validated FBS based media.
- **The production methodology has to be fully disclosed to Authority.** (S) We don't agree. We would rather propose to supply the list of all the cultural media, the components used and the characteristics of the final cell product to be administered.
- **MSC can induce cancer.** (S) At this regard, MSC are recognized as safe cells also by EMA, while Embryonic and IPS cells are not (January 14, 2011; EMA/CAT/571134/2009; Committee for Advanced Therapies – CAT). We underline that telomerase activity is assessed on every cell line.
- **Cells could induce a rejection reaction.** (S) STAMINA stem cells do not express the HLA DR, therefore they don't need any immunosuppression of the patient. They can be used both in autologous and allogenic transplant, regardless the race and the sex of the donor and the patient. In case patient suffers from a genetic disease, the transplant should always be allogenic. In case of allogenic use, the donor should always be subject to proper analysis, similar to the ones usually performed in case of organ transplant, except for the tissue compatibility.

- **MSC cannot be useful for diseases which are so different from each other; they are not "magic".** (S) We don't agree. MSC are not chemical drugs which act by interacting with specific receptors. MSC are like a "Drug-store"; in fact they can deliver a lot of chemical substances, including proteins, enzymes, growth factors, as required. Moreover, they can substitute dead cells and help repair other cells, so performing a global regenerating effect. They can modulate the immune system, by increasing Treg and regulate various cytokines. They have also been shown to partially inactivate bacteria and viruses. Due to all such activities, it's comprehensible why they can be active and give benefits in hundreds of disease conditions.
- **MSC trials should undergo three clinical phases for each single disease indication.** (S) Due to the fact that MSCs like Stamina's have proven to be safe and that theoretically the number of rare and orphan diseases are some thousands, it is recommended that each cell preparation should only undergo clinical Phase I trials (i.e., 10-40 subjects depending on the prevalence of the disease condition) and then go directly to a Phase IV post marketing. This would save a lot of patients.

BIOGRAPHICAL SKETCH AND FINANCIAL DISCLOSURE
CAMILLO RICORDI, M.D.

Editor-in-Chief

Camillo Ricordi, M.D. is the Stacy Joy Goodman Professor of Surgery, Distinguished Professor of Medicine, Professor of Biomedical Engineering, and Microbiology and Immunology at the University of Miami (UM), Florida, where he serves as Director of the Diabetes Research Institute (DRI) and the Cell Transplant Center. Dr. Ricordi also serves as Responsible Head of the Human Cell Processing Facility, an NIH funded cGMP (current Good Manufacturing Practices) facility that has been providing Human Cell Products for research and clinical applications at UM, in Florida and worldwide since 1993.

Dr. Ricordi was president of the Cell Transplant Society (1992-94), co-founder and chairman of the National Diabetes Research Coalition (Chairman 1997), co-founder and president (1999-2001) of the International Association for Pancreas and Islet Transplantation (IPITA), and a member of the council of The Transplantation Society (2002-2008). He also served on the council of the American Society of Transplant Surgeons (2000-2002), on the National Institutes of Health (NIH-NIAID) Expert Panel on clinical approaches for tolerance induction, on the FDA Biologic Response Modifiers Advisory Committee, on the NIH/NCRR Islet Cell Resources (ICRs) Executive Committee, on the NIH-NIDDK Strategic Planning Committee and on the NIH-NIAID Expert Panel on Transplantation Research. He is currently serving as Chairperson of the Clinical Islet Transplant Consortium (NIDDK-NIAID). He has also been serving on several NIH study sections in addition to serving as a reviewer for several international funding agencies.

Dr. Ricordi has received numerous honors and awards, including the 2001 Nessim Habif World Prize in Surgery (University of Geneva) for developing a technology that significantly contributed to the advancement of a surgical field. He was awarded the 2002 Outstanding Scientific Achievement Award and delivered the Lilly Lecture at the 2002 Congress of the American Diabetes Association. He also delivered the opening plenary (Galileo Lecture) at the European Association for the Study of Diabetes (EASD) Congress in Rome (2008). In 2009 Dr. Ricordi was Knighted by the President of the Republic of Italy in the highest Order of the Republic (the Order of Merit) with the Knighthood decoration of Cavaliere Ufficiale and in 2010 he was only surgeon and one of the few ever inducted into the Association of American Physicians (AAP). In 2011 Dr. Ricordi received the D-Life's Top Award for making the biggest difference in diabetes in 2010 (international web-based public vote competition).

Dr. Ricordi is currently serving on the editorial boards of *CellR[4] (Editor-in Chief), Cell Transplantation* (Co-Editor-in-Chief) and also served on the boards of *American Journal of Transplantation* (Associate Editor), *Transplantation*, *Transplantation Proceedings*, *Tissue Engineering*, and *Graft* (Editor-in-Chief, 1998-2002).

Dr. Ricordi serves as President of the Cure Alliance (www.thecurealliance.org) and Chairman of the Diabetes Research Institute Federation (www.diabetesresearch.org), coordinating and promoting cure focused research at over 24 leading institutions worldwide, while further developing the Telescience platform technology to eliminate geographic barriers to scientific collaboration. These initiatives now allow scientists and project teams from around the world to synergize efforts and work together like if they are in the same physical space. Dr. Ricordi has authored over 700 scientific publications.

FINANCIAL DISCLOSURE. As an inventor, Dr. Ricordi has been awarded 18 patents. He was founding scientist, holds stocks/stock options and serves on the Scientific Advisory Board of several Biotechnology Companies, including Converge Biotech, Inc., Ophysio, Inc., Betagenon, Inc., Lipogems, Inc., Axelera, Inc., Betalogics Inc. and Neva Scientific, LLC. Dr. Ricordi has waived any compensation, royalty or equity for any islet cell processing technology he has developed to facilitate "open source" access to the inventions and related technologies. He has also waived editorial compensation for serving as Editor-in-Chief of CellR[4], to redirect them as donations to The Cure Alliance.

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25 YEARS OF THE RICORDI AUTOMATED METHOD FOR ISLET ISOLATION

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Keywords: Pancreatic islets; Diabetes; Islet transplantation; Automated method; History.

Abstract

The year 2013 marks the 25th anniversary of the Automated Method for islet isolation. The dissociation chamber at the core of the Automated Method was developed by Dr. Camillo Ricordi in 1988 to enhance the disassembling of the pancreatic tissue via a combined enzymatic and mechanical digestion while preserving endocrine cell cluster integrity. This method has ever since become the gold standard for human and large animal pancreas processing, contributing to the success and increasing number of clinical trials of islet transplantation worldwide. Herein we offer an attempt to a comprehensive, yet unavoidably incomplete, historical review of the progress in the field of islet cell transplantation to restore beta-cell function in patients with diabetes.

"*....This cell has a small and polygonal structure. Its cytoplasm is perfectly brilliant and free from granules, with a distinct nucleus that is round and of discrete dimension. This type of cell clusters, generally in large number, is diffusely scattered in the glandular parenchyma. These clusters have generally a diameter of 0.1-0.24 mm and may be easily distinguished in fragmented pancreatic preparations…*"[1]. With these words, the German anatomist and anthropologist ***Paul Langerhans*** described for the first time the pancreatic islets in his dissertation published in 1869 summarizing the results of his research as medical student in Berlin in the laboratory of ***Rudolf von Virchow***'s[1]. In 1893, the French pathologist and histologist ***Gustave-Édouard Laguesse*** associated the Langerhans' name to that of islets ("*îlots de Langerhans*") having observed similar structures in the human pancreas[2]. In those years, insulin had not yet been discovered (until 1922, by ***Fredrik Bating*** and ***Charles Best*** from London, Ontario) and part of the scientific discussion was polarized on whether the pancreas produced or not "a substance able to destroy glucose". Thus, the research was primarily focused on attempts to demonstrate that pancreatic fragments transplanted into diabetic animals could cure the disease. The first success was reported in the medical literature in 1882 by doctors ***Oscar Minkowski*** and ***Joseph von Mering*** at the University of Strasburg demonstrating transient improvement of glycosuria following subcutaneous transplantation of autologous pancreatic fragments in a pancreatectomy-induced diabetic dog[3]. The following year (1883), doctor ***P. Watson Williams*** and surgeon ***William H. Harsant*** at Bristol Royal Infirmary in the UK attempted the first transplant of three fresh sheep pancreatic fragments in the subcutaneous space of a 15-year old boy with ketoacidosis, who eventually died and histopathological assessment demonstrated "fibrous stroma" in the grafts[4].

In the subsequent years, most of the research aimed at demonstrating the hypothesis of the presence in the pancreas of beneficial secretion through transplantation of fragments in sited alternative to the subcutaneous space. In 1896, Italian surgeon ***Roberto Alessandri*** at the Royal University in Rome reported for the first time the transplant of autologous pancreatic fragments in the dog spleen after pancreatectomy, though without achieving measurable graft function[5]; similar results were reported by others[6-8]. ***Alessandri*** also tried for the first time the intrahepatic site with poor success[5, 8]. Despite these failures, the attempts increased and it is noteworthy the series of small pancreatic fragment allografts performed into liver, spleen, peritoneal cavity and subcutaneous space of experimental animals by doctor ***Donato Ottolenghi*** in 1901 in

Turin, Italy[7]; despite the extremely small size of the fragments utilized, all grafts underwent necrosis and resorption within a couple of days, even if in a few cases preserved insular tissue was observed at histopathological evaluation. In 1903, doctor ***James Allan*** at Glasgow Royal Infirmary attempted another xenotransplant using feline pancreatic fragments in a patient with diabetes who died two weeks later with ketoacidosis[9]. The British surgeon ***Frederick Charles Pybus***, in light of initial success obtained with adrenal grafts in the treatment of Addison disease, attempted in 1916 the transplant of allogeneic pancreatic tissue into patients with diabetes[10]; considering that previous attempts with xenogeneic tissue had failed, he recovered a human pancreas immediately after the death of a trauma victim and transplanted slices into the abdominal subcutaneous space of two males with diabetes (32 and 37 years old, respectively). Despite transient reduction of glycosuria in one patient, none of the grafts yielded reversal of diabetes[10]. The disappointing outcome and the subsequent discovery and implementation of insulin therapy for diabetes tempered the interest and limited the development of further research on the transplantation of pancreatic tissue in the following years.

An important intuition that impacted significantly the progress of experimental research was the hypothesis that function and viability of the endocrine pancreatic graft was impaired by the presence of exocrine acinar tissue. In order to overcome the potential detrimental effect of acinar tissue in pancreatic grafts, two hypothetical solutions were developed. One of the approaches considered transplanting a tissue "enriched" of islets such as fetal or neonatal pancreas, since the development of the exocrine and endocrine pancreas is not synchronous with endocrine cells appearing early during organogenesis[11] and being able to synthesize and secrete insulin and glucagon in the period preceding exocrine differentiation. Studies in experimental animals demonstrated that the survival of the graft was influenced by donor age[11-13] and that survival over 56 days could be achieved with fetal and neonatal pancreatic grafts implanted in the cheek of hamsters[13]. The other methodological approach pursued was to separate the endocrine tissue from the exocrine component before the transplant.

The idea to physically separate the endocrine component from the exocrine pancreas was originally proposed by Russian doctor ***Leonid W. Ssobolew*** from Saint Petersburg in 1902[14], but was not pursued at least for almost 60 years before the isolation of the islets of Langerhans was reported in the medical literature[15]. In fact, the development of pancreatic islet isolation techniques was characterized by a first era relying on microdissection under the microscope as described in 1964 by doctor ***Claes Hellerstöm*** at Uppsala University in Sweden[15], though with poor results both in terms of yields and quality (namely, substantial functional impairment). Considering the paucity of endocrine pancreatic tissue, the research focused on transplantation in experimental animal models did not progress much in this period. A renewed impulse to the field followed the discovery of the action of collagenases on pancreatic fragments and the introduction of the enzymatic processing. Polish doctor ***Stanisław Moskalewski*** described in 1965 a novel method isolate the islets from the minced Guinea pig pancreas using collagenase action that resulted in loss of acinar tissue and freeing of pancreatic islet clusters[16]. His method was further improved by U.S. doctors ***Paul E. Lacy*** and ***Mery Kastianovsky*** at Washington University in Saint Louis, MO, with the introduction of intra-ductal injection of cold saline buffer to obtain the distension of the pancreas prior to mincing and enzymatic digestion followed by had-picking under the dissecting microscope[17]. Clearly, this approach did not allow obtaining adequate islet yields for transplant experiments into animals, with the purification step being the limiting factor of the process. A step forward toward overcoming this hurdle was the introduction of density gradient purification that was initially based on sugar or albumin. Subsequently, the use of discontinuous gradient purification with Ficoll by ***Arnold Lindall*** *and Coll*. at University of Minnesota contributed to achieving higher yields after islet isolation even though initially the cell product obtained with this technique was not functional[18]. Only when Ficoll was dialyzed and lyophilized in doctor ***Lacy***'s laboratory vital islets could be obtained for experimental transplant studies. Indeed, Lacy established the two phases of islet cell processing: (i) islet cluster dissociation and dispersion followed by (ii) islet purification from the pancreas. The technique became the standard for rodent islet isolation for the following decade that led to remarkable volume of studies addressing pancreatic islet metabolism, physiology and immunobiology. In 1972, U.S. doctors ***Walter F. Ballinger II*** and ***Lacy*** reported the first successful reversal of experimental diabetes in rats following intraperitoneal implantation of 400-600 islets and also that retrieval of transplanted tissue resulted in the reoccurrence of diabetes, as well as the presence of both alpha and beta-cells in explanted tissue at histopathological analysis[19]. A further step forward in experimental islet transplant was the 1973 study by doctor ***Charles B. Kemp*** *and*

Coll. at Washington University describing that the technique of islet embolization into the liver of recipient rats through the portal vein improved the efficiency of transplanted islets compared to the intraperitoneal site with recovery of glucose homeostasis within 2-3 days from implant[20]. This study set the basis for the choice of the intraportal islet infusion technique in the clinical setting that still today remains the transplant site of choice.

The islet isolation technique developed in the rat by doctor ***Lacy*** resulted in a significant increase of experimental studies in rodents. However, for several years the attempts to extend the Lacy isolation protocol to large animal pancreas (*i.e*., dog, nonhuman primate and human) yielded poor results with no reports of purified islet cell preparations until 1977[21]. In the mid 1970's the approach of avoiding the purification process for large animal pancreas because of the big islet loss gained favor. Several reports in the literature describe attempts to use pancreatic fragments containing unpurified islets to cure experimental diabetes in large animals[22,23].

An important innovative approach to enhance the isolation protocol for large animal pancreas was described by doctors ***Atsushi Horaguchi*** and ***Ronald C. Merrell*** at Stanford University using a dog model[24]. Their protocol consisted of three phases: (i) the cannulation of pancreatic duct with intra-ductal injection of collagenase solution to better digest the fibrotic structures; (ii) mechanical dissociation with digestion at 37°C; and (iii) filtration of the pancreatic digest through a 400 μm filter mesh[24]. With this approach, islet recovery was estimated of 57% with a purification of approximately 10%: thus, it became possible obtaining adequate islets for transplantation from a single donor. In the same period, doctors ***Garth L. Warnock***, ***Ray V. Rajotte*** and ***A.W. Procyshyn*** at the University of Alberta demonstrated that improvements in the technique of pancreatic micro-fragment transplantation into the splenic sinusoids could result to the achievement of sustained normoglycemia in diabetic dogs[25,26]. This allowed for the development in experimental models of immunosuppression as well as of cryopreservation protocols by doctor ***Rajotte*** *and Coll*. for the storage pancreatic fragments at –196°C[27].

At the end of 1970's, the technique by Horaguchi and Merrell applied to the human pancreas led to initial attempts of unpurified pancreatic micro-fragment transplantation in the clinical arena by doctors ***John S. Najarian***, ***David E.R. Sutherland***, ***Arthur J. Matas, Fred C. Goetz*** *and Coll*. at the University of Minnesota[28,29], though resulting in poor metabolic control[30] and did not solve the numerous clinical issues associated with inadequate immunosuppression, suboptimal endocrine mass transplanted *vis-à-vis* the complications associated with the lack of purification of the grafted tissue (namely, portal hypertension and disseminated intravascular coagulation).

The first report of successful transplantation of allogeneic pancreatic fragments into patients with Type 1 diabetes was reported in 1979 by doctors ***Felix Largiadèr***, ***E. Kolb*** and ***Ulrich Binswanger*** at Zurich University[30,31]; one of the patients, a 22 years old with T1D and severe retinopathy and nephropathy underwent simultaneous allogeneic kidney along intrasplenic pancreatic micro-fragments (obtained from two donors) transplantation under anti-lymphocyte serum, azathioprine, cyclophosphamide and prednisone treatment and showed sustained normoglycemia 1 year post-transplant. Over the months post-transplant, besides improvement of renal graft function, a progressive reduction of exogenous insulin requirements was observed, with achievement of insulin independence by 8 months that lasted for ten months, when rejection of the kidney was associated with hyperglycemia recurrence. The patients died a month later and intrasplenic pancreatic islets could be detected in the necroptic specimens[30,31]. More substantial, both in terms of numbers and measurable success, was the clinical experience with autologous intrahepatic islet transplantation as a palliative treatment of pain in patients with chronic pancreatitis undergoing total pancreatectomy performed by doctors ***Najarian***, ***Sutherland***, ***Matas*** and ***Goetz*** at the University of Minnesota[28,29,32,33].

In the 1980's, new progress were reported with the islet isolation techniques from dog and human pancreata by doctor ***Daniel H. Mintz***'s group at the University of Miami and doctor ***Derek W. Gray*** *and Coll*. at Oxford University[34-37]. Briefly, the protocol consisted of intraductal injection collagenase injection, dispersion of the pancreatic tissue by mechanical agitation and passing through a series of graded needles followed by purification using filtration and centrifugation on density gradient solution – a method that would allow yields of approximately purity of 20-40% from human glands[35,36]. The technique showed some promise in canine islet transplantation models, particularly for the autografts while it required more than one donor in the allografts[37-39]. Using modifications of this method, doctors ***Rodolfo Alejandro***, ***Daniel H. Mintz*** *and Coll*. at the University of Miami initiated in 1985 a pilot clinical trial of 5 allogeneic islet transplantation in four C-peptide negative patients with Type 1 diabetes with evidence of retinopathy, nephropathy and neuropathy as islet after kidney (IAK) and simultaneous islet-kidney (SIK) transplantation (negative

serum crossmatch and ABO compatible donor:recipient combination)[36,40]. Novelty introduced in the field through this clinical trial include: preservation of the pancreas by hypothermic pulsatile perfusion with cryoprecipitated silica-treated human plasma, the use of transhepatic portal vein cannulation for three procedures with monitoring of portal vein pressure before and after islet infusion, as well as modulation of islet immunogenicity by treatment with anti-Ia monoclonal antibody *in vitro*. The longest islet graft function was measured for 26 and 18 weeks in two transplants, and graft failure invariable occurred in all cases possibly consequent to inadequate levels of immunosuppression[36].

During the same years modifications in the canine islet isolation procedures allowed to obtain adequate numbers in volumes of pancreatic tissue to be safely infused in the portal system without inducing portal hypertension while treating diabetes were introduced by doctors ***Mark S. Cattral***, ***Warnock***, ***Norman M. Kneteman***, and ***Rajotte*** at the University of Alberta by combining intraductal perfusion with collagenase of the pancreas, gentle dissociation and purification of density gradients[41,42].

A turning point for clinical islet transplantation was the introduction of the "Automated Method" of pancreas dissociation by doctor ***Camillo Ricordi***, who, after obtaining his medical degree from the University of Milan, received an NIH Research Trainee Award in 1986 to join doctor Lacy's team at Washington University. The method consisted of a mechanically enhanced enzymatic digestion based on a dissociation/filtration chamber allowing pancreatic fragments and islets freed from gland to be removed promptly from the system to avoid overdigestion while preserving cluster integrity (Figures 1 and 2). The method was first published in 1988[43] and has represented ever since the gold standard for virtually all research centers working on human[44] and large animal islets[45], besides its application also for the isolation of other tissues[46]. Shortly after the introduction of the automated method, the initial success with islet transplantation in humans were reported by doctors ***David W. Scharp***, ***Lacy***, ***Ricordi*** *and Coll*. at Washington University with a first series of patients with T1D and established or incipient nephropathy to ascertain if insulin independence could be attained and if immunosuppression could be discontinued one year after transplantation without rejection. Three subjects received approximately

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Automated Method for Isolation of Human Pancreatic Islets

CAMILLO RICORDI, PAUL E. LACY, EDWARD H. FINKE, BARBARA J. OLACK, AND DAVID W. SCHARP

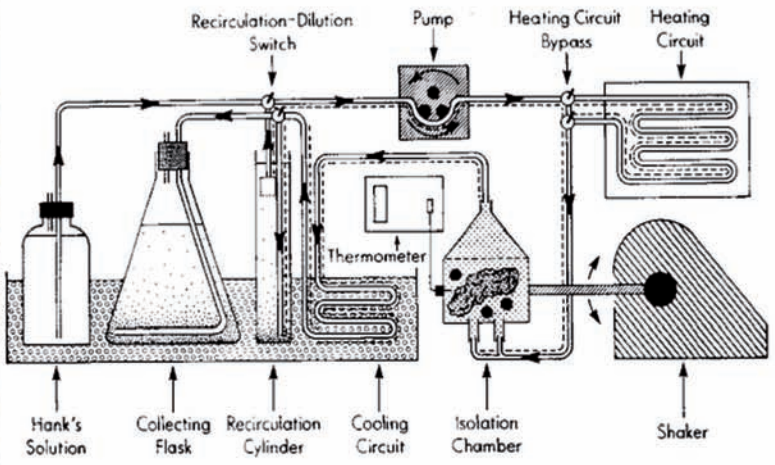

Figure 1. The headings of the manuscript describing the Automated Method and a picture of Dr. Ricordi with one of the first human pancreas shipments from NDRI that was processed in Saint Luis.

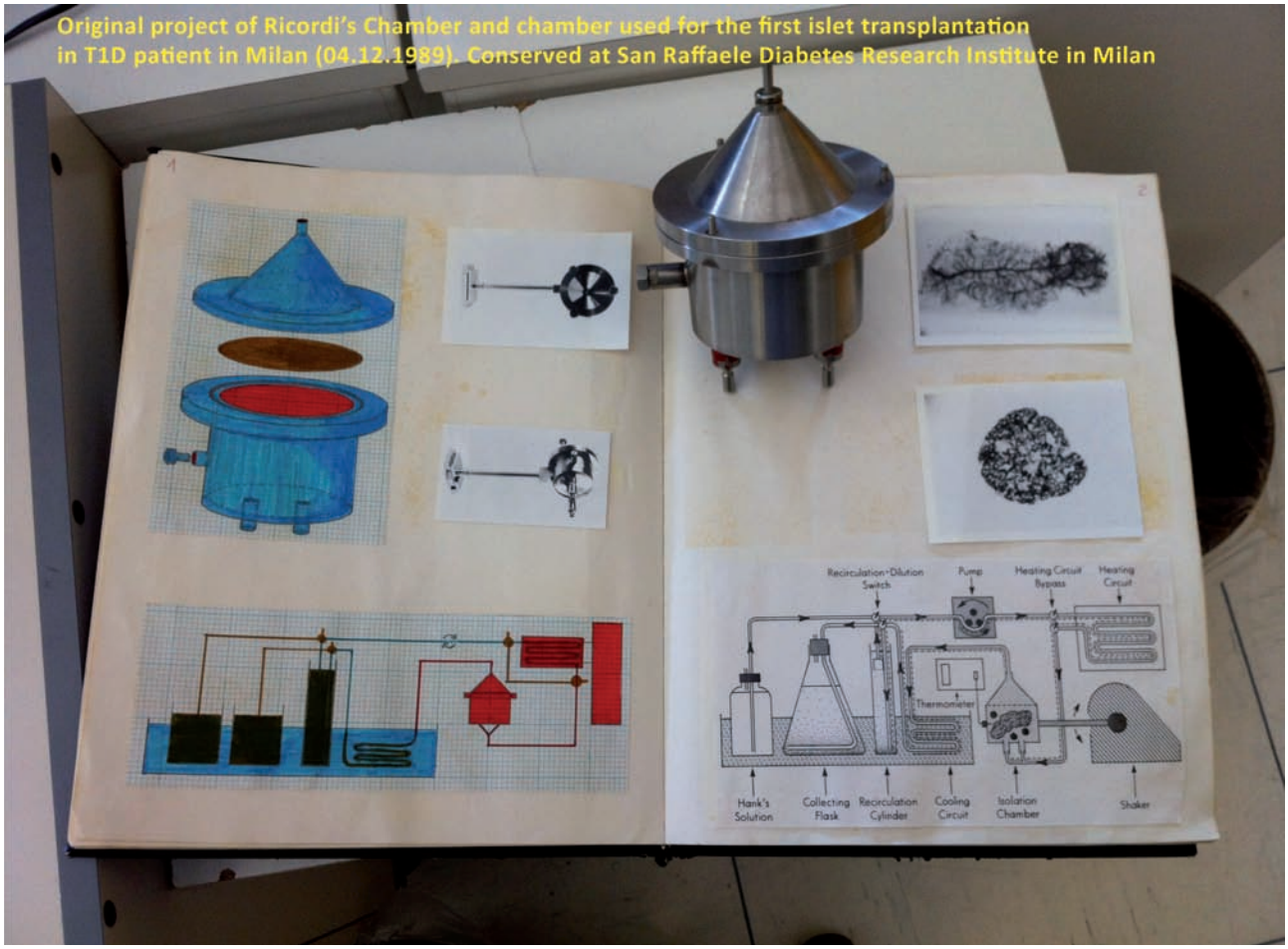

Figure 2. One of the initial prototypes of the stainless steel Dissociation Chamber and Dr. Ricordi's Laboratory Notebook describing the concept and drafts of the Automated Method for islet isolation. Photo by L. Piemonti (Milan, 3rd March 2013).

6,000 islet equivalents per kg of body weight but all lost graft function despite ongoing immunosuppressive regimen (azathioprine, prednisone and cyclosporine)[47]; additional islet transplants were performed with some degree of success thereafter[47-49]. Using a more elaborated islet isolation technique[50], doctors ***Warnock***, ***Rajotte*** *and Coll*. at the University of Alberta reported a good production of C-peptide in two recipients with T1D following SIK (from the same donor). They received conventional triple immunosuppression that had to be reduced in both cases due to CMV infection 6 week post-transplant with subsequent loss of islet graft function.

Further improvements were introduced in the late 1980's. Amongst these were techniques aimed at optimizing the efficiency of the purification of large animal and human islets using a semiautomated method of density gradient separation using computerized cell separator by doctors ***Stephen P. Lake*** *and Coll*. from the Leicester Royal Infirmary in UK[51] and doctors ***Alejandro*** and ***Mintz*** from the University of Miami[52]; improved gradients that incorporated cold preservation solutions by student ***Barbara J. Olack*** and Coll. at Washington University[53]; techniques to stain islet cell clusters with zinc dye by doctor ***R. Alejandro*** *and Coll*.[54] and by doctor ***W. A. Hansen*** and Coll. from Hagedorn Research Laboratory in Gentofte, Denmark; as well as the publication of consensus papers aimed at standardizing human islet assessment[55] contributed to progress in the field.

In the 1990, the introduction of novel techniques to improve the efficiency of isolation techniques resulting in high yields of pancreatic islets contributed to the development of numerous clinical protocols around the World. In the early 1990's, doctor ***Ricordi*** moved to the University of Pittsburgh to direct the Cellular Transplantation Division of the Transplantation Institute headed by doctor ***Thomas Starzl***. Also doctors ***Alejandro*** and ***Mintz*** from the

University of Miami joined forces with doctors Ricordi and Starzl's Team to help optimizing protocols and accelerate the progress in the field of islet transplantation. The first series of sustained insulin independence was obtained in nine patients undergoing excision of liver and pancreas (that would result in surgery-induced diabetes) and receiving allogeneic liver and islet transplantation from the same cadaveric donor; the first clinical case of sustained insulin independence following allogeneic islet transplantation was a 15 years old girl whose visceral organs were excided for cancer who received multi-visceral organ (liver, small bowel and islet) transplantation[56,57]. These trials allowed improving the transplant technique with the introduction of the use of slow infusion (by gravity) of the islet graft kept in suspension into an infusion bag to reduce the risk of portal hypertension[56,58].

In 1990, doctors ***Scharp***, ***Lacy*** *and Coll*. at Washington University reported the first case of transient exogenous insulin independence obtained following transplantation of 800,000 cultured allogeneic islets with 95% purity (pool of two allogeneic islet preparations) isolated using the automated method into a patient with T1D receiving Minnesota anti-lymphocyte serum, azathioprine and cyclosporine[48]. Ten days after transplantation, the patient achieved normoglycemia (albeit with glucose intolerance) and could discontinue exogenous insulin therapy for 2 weeks[48]. Insulin independence following islet transplantation from a single donor obtained using the automated method was reported by doctor ***Carlo Socci*** *and Coll*. at the San Raffaele Institute in Milan, Italy in a patient with T1D transplanted on April 25 1990[59]. Subsequently, insulin independence and/or consistent graft function after islet transplantation was reported by Centers across the World using also cryopreserved[60] along to fresh allogeneic islets, paving the way for a possible clinical application of cellular therapies to restore beta-cell function in patients with T1D.

In 1994, German doctors ***Bernard J. Hering***, ***Reinhard G. Bretzel*** *and Coll*. at Justus-Liebig University in Giessen reported 30% insulin independence after allogeneic islet transplantation in patients treated with cyclosporine and steroids and receiving an anti-oxidant therapy and intense intravenous insulin management in the peritransplant period[61]. In 1997, doctors ***Antonio Secchi***, ***Socci***, ***Guido Pozza*** *and Coll*. at the San Raffaele Institute in Milan reported a 45% insulin independence rate by administering elevated islet numbers (~11,000 islet equivalents per kg of body weight) under cyclosporine and steroid immunosuppression[62]. Also, ***Alejandro***, ***Ricordi, Joshua Miller, Mintz,*** *and Coll*. at the University of Miami reported long-term (6 years) function in patients with T1D recipients of allogeneic islets and kidneys[40], two of which maintained detectable C-peptide for 13 years[63]. The development of better purified and characterized enzyme blends characterized by reduced endotoxin contamination[64-67] resulted in higher reproducibility, when compared to other collagenase blends available at the time (i.e., Collagenase P), contributing significantly to achieving improved islet yields from human pancreata[68-70]. At the same time, initial attempts of fetal porcine islet cell cluster transplantation were performed in uremic patients with diabetes receiving a renal allograft by doctors ***Carl G. Groth, Olle Korsgren, Anita Tibell*** *and Coll*. at the Karolinska Institute in Stockholm, Sweden[71]. The grafts were implanted intra-hepatically or under the renal capsule under conventional immunosuppression with anti-thymocyte globulin or 15-deoxyspergualin, and showed detectable c-peptide in the urines for up to 200-400 days[71] without evidence of porcine endogenous retrovirus infections[72]. In 1992 doctor ***Ricordi*** funded the Cell Transplantation Society (CTS) during the first meeting that was held in Pittsburgh, PA. The CTS that is now a section of The International Transplantation Society (TTS), has steadily grown gaining an important role for advancements of the field of islet transplantation and for cellular transplantation by and large.

The Islet Transplantation Registry (http://www.med.uni-giessen.de/itr/) collected the experience on a total of 267 transplants performed in several Centers voluntarily reporting the outcome of their trials from 1990 until 2001[73]. Collectively, the outcome was overall rather modest with only 12.4% of the 267 transplants achieving insulin independence for periods greater than a week and only 8.2% for over one year[73]. A new era in the field of islet transplantation begun with the introduction of the 'Edmonton Protocol' in 1999 by doctors ***A.M. James Shapiro***, ***Jonathan R.T. Lakey***, ***Edmond A. Ryan***, ***Gregory S. Korbutt***, ***Ellen Toth***, ***Garth L. Warnock***, ***Norman M. Kneteman***, and ***Ray V. Rajotte*** at the University of Alberta in Canada[74]. The trial showed 100% insulin independence at 12 months in 7 consecutive individuals with brittle T1D and was characterized by: (i) implantation of large numbers of freshly isolated (no culture) cadaveric allogeneic islets (mean of 11,574 islet equivalents/kg per recipient obtained from multiple donors), (ii) the use of human albumin instead of fetal bovine serum in the media utilized for cell processing, and (iii) the use of a steroid-free immunosuppressive regimen based on sirolimus, tacrolimus and anti-IL-2 antibody[74]. This protocol was subse-

quently reproduced in a multicenter international trial[75] that yielded 58% insulin independence at one year and revealed the important impact of the center experience in islet cell processing and patient management on clinical outcomes[76].

In the meanwhile, the 'Edmonton Protocol' was adopted with or without various modifications at several institutions contributing to increasing the numbers of islet transplants performed worldwide. Overall, the results from these single center trials showed consistent achievement of metabolic improvements with normalization of HbA1c and glycemic excursions using low exogenous insulin doses and complete insulin independence after transplantation of adequate islet numbers, paralleled by a significant reduction/elimination of severe hypoglycemic episodes and improved quality of life in patients with unstable diabetes receiving islet transplant alone (ITA), islet-after kidney (IAK) or simultaneous islet-kidney (SIK) transplantation[50,75,77-93].

At the evaluation of the extended follow up of patients receiving the 'Edmonton Protocol' it was revealed that progressive loss of insulin independence occurred over time, with need to reintroduce exogenous insulin resulting in excellent metabolic control long term and HbA1c. While approximately 80% of the patients showed sustained graft function (measured as persistence of detectable C-peptide), only 10% of study subjects maintained independence from exogenous insulin five years after transplantation[94]. The possible causes of the progressive loss of graft function are multifold[95]. Since 2005 to date, the clinical research has focused on the major objectives of obtaining and maintaining the longest possible time high rates of insulin independence with lowest islet numbers. At the present time, in at least 5 centers (namely, Edmonton, Minneapolis, Geneva, Lille and Milan) have been reported proportions of insulin independence between 40-50% at five years after transplantation[96-99], which are not too far from those of pancreas transplant alone. Furthermore, the data available through the CITR confirm an overall trend toward an improvement of insulin independence at 3 years (approximately 44% of patients) following islet transplantation.

The islet transplant community is rapidly growing. The Collaborative Islet Transplant Registry (CITR; www.citregistry.org) has been established in 2001 and has been collecting data from over thirty Centers in North America, Europe and Oceania through self-reporting their activity. In the most recent CITR Report[100], a total of 571 recipients received 1,072 islet infusions from 1,187 donor pancreata, the majority of transplant being ITA. The availability of pooled data from different centers is invaluable as it allows analyses of clinical outcomes and provide insights on potential variables associated with higher success rates of islet transplantation[93, 101-105]. Notably, the community is likely much larger than what currently captured by the CITR, since several Centers in Europe, Asia and South America that have and/or are performing islet transplantation trials in recent years do not necessarily report to the Registry; these include, amongst others: the trial by doctors ***Frantisek Saudek*** *and Coll*. at Prague University in Czech Republic[106]; by doctor ***Shinichi Matzumoto*** *and Coll*. at Kyoto University[107,108]; the trial by doctor ***Pablo F. Argibay*** *and Coll*. in Argentina[109]; the trial by doctor ***Mari Cleide Sogayar*** *and Coll*. in Brazil[110]; and the trial introducing Campath-1H in SIK transplant recipients by doctor ***Janming Tan*** *and Coll*. at Xiamen University in China[111].

Amongst the several progresses of the recent years, it is worth mentioning at least a few that contributed moving the field forward. The introduction of CD25 targeting in islet transplant recipients by doctor ***Philip Morel*** *and Coll*. at the University of Geneva[112]. The development of islet transplant consortia to maximize efficiency of the transplant programs, such as the Portland/Minneapolis, the Huddinge/Giessen, the Swiss-French GRAGIL[89,113-115], the Miami/Huston and Miami/Dallas[116-120] the Miami/Washington DC[121, 122]; the Geneva/Athens[123] networks. The introduction of iodixanol-University of Wisconsin solution density gradients to enhance large animal[124-126] and later human[127] islet separation by doctor ***Michel P.M. van der Burg*** *and Coll*. at Leiden University in the Netherland. The utilization of cultured islets by the University of Minnesota and University of Miami groups[128-130]. The implementation of anti-CD3 antibody at induction by doctor ***Bernard J. Hering*** *and Coll*. at the University of Minnesota[128]. The attempts at inducing hematopoietic chimerism in islet transplant recipients by combined bone marrow-derived stem cell and islet transplantation at the University of Miami[63,131,132]. The invaluable report describing the histopathological features of bioptic specimens obtained from a patient who passed away >13 years of insulin independence following islet transplantation by doctor ***Thierry Berney*** *and Coll*. at the University of Geneva in Switzerland[133]. The understanding of the critical role of stress-activated signal transduction pathways occurring in the pancreas and islets due to ischemia following donor cerebral death, organ preservation and islet isolation resulting in amplification of acute, nonspecific inflammation on islet yields, quality and immunogenicity reported by several research groups, in-

cluding doctors ***Stephen Paraskevas, Laurence Rosenberg*** *and Coll*. in Montreal, Canada[134], ***Saida Abdelli, Christophe Bonny*** *and Coll*. at the University of Lausanne in Switzerland[135,136], ***Rita Bottino, Massimo Trucco and Jon D. Piganelli*** *and Coll*. at the University of Pittsburgh[137], amongst others[138,139]. The discovery of islet production of tissue factor, described by doctors ***Lisa Moberg, Bo Nillson*** *and Coll*. at the Karolinska Institute (140), MCP-1/CCL2 described by doctors ***Lorenzo Piemonti, Federico Bertuzzi*** *and Coll*. at the San Raffaele Institute in Milan, Italy[141-144], CD40 described by doctor ***Ricardo L. Pastori*** and Coll. at the University of Miami[145], amongst others. The description that targeting the TNF pathway after transplantation may be beneficial to improve islet engraftment in rodents by doctors ***Alan C. Farney*** *and Coll*. in 1993 at the University of Minnesota[146], which was substantiated by the demonstration of the significant release of pro-inflammatory cytokines such as TNF-alpha and the induction of endothelial cell activation upon intrahepatic embolization of allogeneic rat islets or inert beads which could be partially reduced via macrophage depletion as shown by doctors ***Rita Bottino, Luis Fernandez, Camillo Ricordi, Luca Inverardi*** *and Coll*. at the University of Miami in 1998[147], thus justifying the subsequent introduction of TNF-alpha signaling blockers in clinical islet transplant recipients by the University of Miami[130,131,148] and University of Minnesota[97,129], which retrospectively was confirmed as one of the key factors associated with the success of islet transplantation[101]. The demonstration of synergy when combining blockade of TNF-alpha and IL-1-beta signaling by doctors ***Morihito Takita, Shinichi Matsumoto, Marlon F. Levy*** *and Coll*. at Baylor Research Institute in Dallas, TX[149]. The description of the triggering of an *instant blood mediated inflammatory reaction* involving coagulation factors, platelets and leukocytes immediately after intra-portal islet implantation by doctors ***William Bennet, Carl G. Groth, Olle Korsgren*** *and Coll*. at the Karolinska Institute[150], which led to implementation of targeted anti-inflammatory strategies to improve islet engraftment and survival including the introduction of novel anti-inflammatory treatment targeting CXCR1/2 by doctor ***Piemonti*** *and Coll*. at the San Raffaele Institute (151). The optimization of pancreas preservation solution containing perfluorocarbons to improve oxygenation during cold storage ('Two Layer Method') by doctors ***Yoshikazu Kuroda, T Kawamura, Yoichi Saitoh*** *and Coll*. at Kobe University in Japan (152-157). The introduction of trypsin and protease inhibitors during pancreas preservation by doctor ***Kuroda*** *and Coll*. in 1988 and other groups[158-162]. The introduction of a controlled pancreatic distension to increase the reproducibility of islet isolation techniques by doctors ***Jonathan R. Lakey, Ray V. Rajotte*** *and Coll*. in Edmonton[163]. The recent evaluation of extra-hepatic transplantation sites for islet cells, including intramuscularly[164] and subcutaneous space[165] by doctors ***Keith Reemtsma, Collin J. Wever, Mark A. Hardy*** *and Coll*. at Columbia University in the late 1970's, unfortunately without function, followed more recently by better results reported with the intramuscular site by doctor ***Ehab Rafael*** *and Coll*. at the Karolinska Institute[166] and by doctor ***Sabrina Dardenne*** *and Coll*. at the University of North France in Lille[167]; as well as new pilot experiments with an intra-bone marrow site by doctor ***Piemonti*** *and Coll*. in Milan[98,168], and the use of intra-peritoneal site mainly for initial clinical attempts to transplant encapsulated porcine or human islets to confer immunoprotection by doctors ***Robert B. Elliot, Christina Buchanan*** *and Coll*. at the University of Auckland in New Zealand[169], and by doctors ***Giuseppe Basta, Giovanni Luca, Riccardo Calafiore*** *and Coll*. at the University of Perugia in Italy[170,171], amongst others. The use of noninvasive imaging techniques to detect islet grafts using paramagnetic beads for magnetic resonance imaging in experimental animals by doctor ***Frantisek Saudek*** *and Coll*. in Prague[172,173] and in the clinical settings by doctors ***Christian Toso, Thierry Berney*** *and Coll*. in Geneva[174], and the use of positron emission tomography by doctors ***Olof Eriksson, Torbjörn Lundgren*** *and Coll*. in Sweden[175]. The use of supplemental islet infusion and or the use of exenatide in patients developing graft dysfunction by the Miami group[148,176,177]. The introduction of novel approaches to seal the tract of trans-hepatic catheterization of the portal vein to reduce the risk of hemorrhage and improve safety of the islet transplant procedure by doctors ***Tatiana Froud, Ricordi, Alejandro*** *and Coll*. at the University of Miami[178]. The introduction of exenatide treatment for islet transplant recipients to favor engraftment by the University of Illinois at Chicago and the Miami groups[179,180]. The use of living-related segmental pancreas donor by doctor ***Shinichi Matsumoto*** *and Coll*. at Kyoto University[107,108]. The introduction of Campath-1H as lympho-depleting agent by doctor ***Tan*** *and Coll*. at Xiamen University[111], the University of Miami[132] and the University of Alberta[99,181].

The field of islet transplantation has significantly evolved since the initial attempts by doctors ***Minkowski*** and ***von Mering*** in 1882, with remarkable acceleration over the last three decades thanks to the incredible effort of the research community

across the Worlds yielding to the achievement of steady improvements in the cell processing and transplantation techniques, patient management and immunotherapy protocols utilized (Figure 3). Preservation of beta-cell function is reproducibly currently attained in recipients of islet autografts, a therapeutic option that should be considered for individuals undergoing total pancreatectomy for non-malignant conditions and as recently reported also for malignant condition[182]. Restoration of beta-cell function can be obtained by transplantation of allogeneic islets in both nonuremic (ITA) and uremic (SIK and IAK) subjects with diabetes, allowing for long-term sustained function and associating with improved metabolic control even when required exogenous insulin (*i.e.*, suboptimal islet mass transplanted or development of graft dysfunction). The introduction of ***Ricordi***'s Automated Method twenty-five years ago has definitely given a remarkable impulse to the field, contributing to the expansion of the number of transplants performed worldwide since the early 1990's. Islet transplantation has been approved as a reimbursable procedure in several Countries, including Australia, Canada (selected provinces), France, Italy, Switzerland, the National Institute for Health and Clinical Excellence in the United Kingdom, Sweden and the Nordic Network. In the U.S.A., autologous islet transplantation is currently reimbursed. The completion of registration trials by the Clinical Islet Transplant consortium (CIT-06 and CIT-07) will likely lead to biological licensure by the U.S. Food and Drug Administration shortly. This is an important step, as islet transplant activity in the United States has been severely restricted by limited access to research funds, with the exception of a joint Medicaid/Medicare initiative that is currently supporting the islet-after-kidney trial (CIT-06).

We are currently experiencing an exciting stage of innovation and renewed promise for cellular-based therapies to restore beta-cell function. Novel extra-hepatic transplant sites and tissue engineering approaches are being explored which may allow for improved engraftment and sustained function with cadaveric human, xenogeneic or stem-cell derived islet cells in the near future. It has been and still is an exceptional journey!

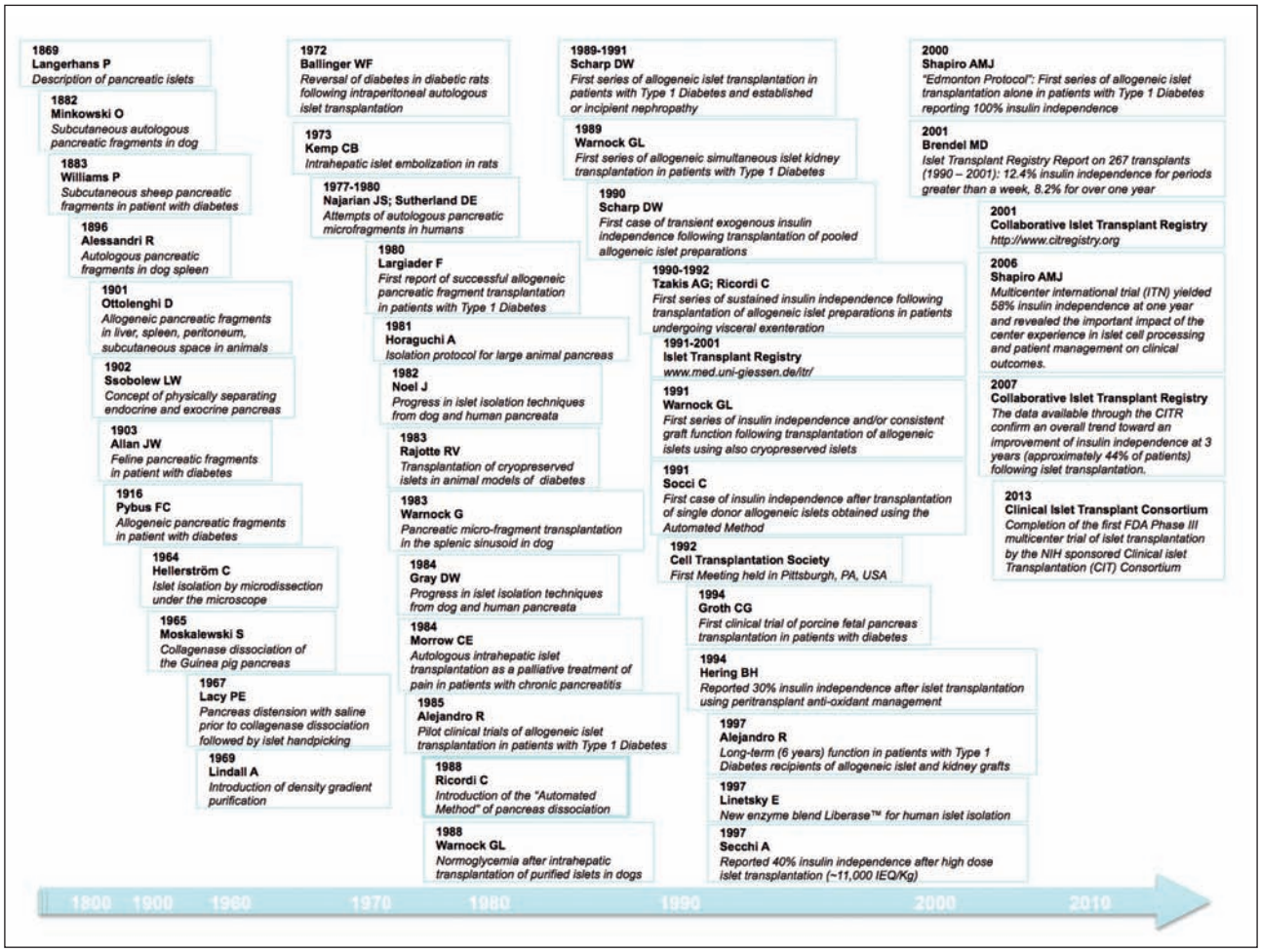

Figure 3. Timeline of pancreatic islet transplantation.

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CELL TRANSPLANT SOCIETY

ABSTRACTS FROM THE 12TH CONGRESS

ORALS

201 - CORRECTION OF DUCHENNE MUSCULAR DYSTROPHY BY GENOME EDITING WITH ENGINEERED NUCLEASES

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Duchenne Muscular Dystrophy (DMD) is a relatively common degenerative disease that results from mutation of the gene encoding the dystrophin protein. The genetic nature of DMD has led to substantial interest in gene therapy-based approaches to this disease, including several clinical trials. However, these therapies typically require the random integration of exogenous DNA into the genome or the lifelong re-administration of transient gene therapy vectors, both of which have significant safety and practical concerns. Furthermore, these strategies have been limited by an inability to deliver the large and complex dystrophin gene sequence. An exciting alternative to these approaches is the targeted editing of the human genome to repair the endogenous mutant dystrophin gene. This concept represents a potential cure to DMD without the need for random integration of or repeated exposure to foreign biological material.

The focus of our work is to develop and implement strategies for directed modification of the genome for the treatment of genetic disorders. Engineered nucleases, including zinc finger nucleases (ZFNs), TALE nucleases (TALENs), and CRISPR/Cas9 constitute powerful tools for coordinating the site-specific manipulation of genomic DNA sequences. The ZFN and TALEN technologies have been developed by biomolecular engineering of novel enzymes comprised of synthetic DNA-binding domains fused to the catalytic domain of a restriction endonuclease. Engineering of the DNA-binding domain to target specific sites in the human genome can be used to direct nuclease activity and endogenous DNA repair machinery to any locus of interest. More recently, the RNA-guided nuclease Cas9, which has natural role in bacterial adaptive immunity, has been used in human cells as a method to direct nuclease activity to new targets without protein engineering. Using any of these systems, site-specific nuclease-mediated DNA cleavage can be used to frameshift or excise gene sequences via DNA re-ligation. Alternatively, DNA sequences can be added or exchanged at targeted loci via the nuclease-mediated enhancement of homologous recombination. Our goal is to use these genome editing technologies to repair mutated DNA sequences responsible for genetic diseases such as DMD.

We have engineered and optimized ZFNs, TALENs, and CRISPR/Cas9 systems that can mediate efficient manipulation of the dystrophin gene sequence in human cells. This includes the direct correction mutations or the introduction of dystrophin cDNA into the endogenous dystrophin locus under control of the natural promoter. We have used these approaches to restore dystrophin expression in human muscle progenitor cells derived from DMD patients as well as dermal fibroblasts that can be reprogrammed to the myogenic lineage. Substantial levels of dystrophin are expressed in the DMD cells following genetic correction. We further show that these nucleases are non-toxic with minimal off-target effects. Corrected cells have been transplanted into immunodeficient mice. This project represents an exciting new avenue for DMD therapy that can permanently correct the underlying genetic mutations.

202 - ENGINEERING IMMUNOLOGICAL TOLERANCE

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Prophylactic and therapeutic induction of antigen-specific immune tolerance has potential value in protein replacement therapies, cell transplant therapies, and prevention and treatment of autoimmunity. For example, in protein replacement therapy, a patient usually expresses a mutant form of the protein of interest (for example, factor VIII in hemophilia A), or expresses no protein at all, and as such the patient has not developed immunological tolerance to the replacement form and frequently develops immunity versus the replaced protein (approx. 30% incidence in factor VIII). As another example, in type 1 diabetes, the patient developed humoral and more importantly cellular autoimmunity versus pancreatic islet beta cell antigens. Thus, there exists a pressing need for antigen-specific tolerogenic vaccine technology to deliver such antigens (e.g., factor VIII prophylactically to induce pre-treatment tolerance; islet antigens therapeutically to re-introduce tolerance after early presentation of the disease).

Antigens derived from apoptotic cells are known to drive tolerance via deletion or anergy of reactive T cells. Reasoning that a large number of erythrocytes become apoptotic (eryptotic) and are cleared each day, we engineered a strategy to bind antigen to erythrocytes *in situ* with high affinity and specificity after intravenous injection, conjugating a glycophorin-A-binding peptide that we discovered by phage display to the antigen. Using a transgenic T cell model in the mouse sensitive to the model antigen ovalbumin, we demonstrate that erythrocyte-binding antigen bioconjugate (ERY1-OVA) induces extensive $CD8^+$ T cell cross-priming, leading to an apoptotic and exhausted fate. Furthermore, mice tolerized with ERY1-OVA induced far fewer OVA-specific cytotoxic IFN-γ^+ $CD8^+$ T cell responses compared with OVA-tolerized mice following antigen challenge with a potent adjuvant. Tolerization with ERY1-OVA also promoted growth of OVA-expressing transplanted cells in mice immunized against OVA, indicating functional inhibition of CTL generation.

Erythrocyte binding also attenuated humoral immune reactions. The E. coli protein asparaginase (ASNase) is used to treat acute lymphoblastic leukemia, yet immune recognition of the microbial protein limits its efficacy and safety. ERY1-ASNase induced at least 4 orders of magnitude lower humoral immune response after multiple dosing in mice than the wild-type ASNase. Moreover, pre-tolerization with ERY1-ASNase ameliorated immunity to subsequent treatment, resulting in 6000-fold lower antibody titers than in the absence of pre-tolerization.

Thus, we report a novel biomolecular approach to hijack the body's mechanisms in maintenance of tolerance to apoptotic erythrocytes to induce deletional tolerance to an exogenous erythrocyte-binding antigen to create a technology for antigen-specific tolerogenic vaccination. Prophylactic and therapeutic induction of antigen-specific immune tolerance has potential value in protein replacement therapies, cell transplant therapies, and prevention and treatment of autoimmunity. For example, in protein replacement therapy, a patient usually expresses a mutant form of the protein of interest (for example, factor VIII in hemophilia A), or expresses no protein at all, and as such the patient has not developed immunological tolerance to the replacement form and frequently develops immunity versus the replaced protein (approx. 30% incidence in factor VIII). As another example, in type 1 diabetes, the patient developed humoral and more importantly cellular autoimmunity versus pancreatic islet beta cell antigens. Thus, there exists a pressing need for antigen-specific tolerogenic vaccine technology to deliver such antigens (e.g., factor VIII prophylactically to induce pre-treatment tolerance; islet antigens therapeutically to re-introduce tolerance after early presentation of the disease).

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Thus, we report a novel biomolecular approach to hijack the body's mechanisms in maintenance of tolerance to apoptotic erythrocytes to induce deletional tolerance to an exogenous erythrocyte-binding antigen to create a technology for antigen-specific tolerogenic vaccination.

204 - EFFECT OF DNA DEMETHYLATION ON MYOBLASTS DIFFERENTIATION AND MYOGENESIS

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Skeletal muscle regeneration and hypertrophy are important adaptive responses to both physical activity and pathological stimuli. This research was performed to investigate the action of DNA demethylation on the late phase of muscle differentiation and early stage of hypertrophy.

The epigenetic process involved in myogenesis was studied with the DNA-demethylating agent 5-azacytidine (AZA). We induced muscle differentiation in C2C12 mouse myoblasts in presence of 5μM AZA and growth (GM) or differentiation (DM) medium for 48, 72 and 96 hours. To study a potential AZA hypertrophic effect, we stimulated 72h differentiated myotubes with AZA for 24h. Unstimulated cells were used as control. By Western blot and immunofluorescence analysis, we examined AZA action on myogenic regulatory factors expression, hypertrophic signaling pathway and myotube morphology.

During differentiation, protein levels of myogenic markers, Myf6 and Myosin Heavy Chain (MyHC), were higher in AZA stimulated cells compared to control. Myostatin and p21 analysis revealed morphological changes which reflect a tendency to hypertrophy in myotubes. In AZA stimulated neo formed myotubes, we observed that IGF-1 pathway, kinases p70 S6 and 4E-BP1 were activated. Furthermore, AZA treatment increased MyHC protein content in stimulated neo myotubes.

Our work demonstrates that DNA demethylation could play an important role in promoting the late phase of myogenesis, activating endocellular pathways involved in protein increment and stimulating the hypertrophic process.

205 - EXHAUSTION OF MUSCLE PROGENITOR CELLS DURING AGING & DISEASE: IMPLICATION FOR STEM CELL THERAPY

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Aging is characterized by the progressive erosion of tissue homeostasis and functional reserve in all organ systems. Although controversy remains as to the molecular mechanism(s) underlying the process of aging, accumulated cellular damage, including DNA damage, appears to be a major determinant of lifespan as well as age-related pathologies. Moreover, there is evidence that the accumulation of damage in stem cells renders them defective for self-renewing and regenerating damaged tissues. We have recently demonstrated that a population of muscle progenitor cells(MPCs) isolated from the ERCC1-deficient mouse model of accelerated aging, are defective in their proliferation abilities, differentiation capacity and resistance to oxidative stress. We have observed that intraperitoneal (IP) injections of wild-type (WT)-MPCs into Ercc1 knockout (Ercc1-/-) mice resulted in an improvement in age related pathologies. Although the mechanisms by which the transplantation of WT-MPCs extend the lifespan of these progeria mice is still under investigation, we have obtained evidence that the beneficial effect imparted by the injected cells occur through a paracrine effect that involve angiogenesis.

Duchenne muscular dystrophy (DMD) is a fatal genetic disease characterized by a deficiency in dystrophin and the progressive wasting of the patient's muscles. Current treatments for DMD have centered on the restoration of dystrophin; however, they were fraught with serious limitations. Interestingly, DMD patients lack dystrophin from the time of birth; however, the onset of muscle weakness only becomes apparent at 4-7 years of age, which happens to coincide with the exhaustion of the muscle progenitor cell (MPC) pool. There are several lines of evidence that support this concept including the gradual impairment of the myogenic potential of MPCs isolated from DMD patients during aging which results in a reduction of muscle regeneration in older DMD patients. In contrast to that observed with MPCs isolated from the mdx mice (dystrophin deficient and mild phenotype), we have recently shown a defect in the MPCs isolated from double Knock-Out (dKO) mouse (dystrophin/utrophin deficient and severely affected). We have recently observed that the MPC defect from the dKO mouse model appears to be age dependent and not specific to MPC since other stem cell population also appears to be affected. These results taken together support the concept that the rapid disease progression associated with the dKO model, is related to a defect in the stem cell pool, as observed in DMD patients. We have also obtained preliminary data indicating that the defect in dKO-MPC appears to be related, at least in part, to the notch and NF-kB signaling pathway, negative regulator of myogenesis and muscle growth. Our recent works indicate that stem cell defect in dKO mice, not only leads to muscle abnormalities and weakness but also to other abnormalities of the musculoskeletal system, including bone morphology and healing capacity. We have investigated potential approaches to rescue the defect in dKO MPCs by blocking the notch pathway as well as the inflammatory mediator and negative regulator of muscle growth, NF-kB *in vitro* and potentially rescue the overall histopathology of the dKO mice after MPC implantation *in vivo*. This technology could be very helpful for altering DMD progression through the reduction of stem cell exhaustion and modulation of inflammation.

206 - NOVEL APPROACH TO PLURIPOTENCY AND CARDIOVASCULAR COMMITMENT: THE USE OF RADIO ELECTRIC CONVEYED FIELDS AND HUMAN ADIPOSE-DERIVED STEM CELLS OBTAINED WITH A NON-ENZYMATIC METHOD

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Human adipose-derived stem cells (hASCs) may represent a suitable tool for regenerative therapies due to their simple isolation procedure, and high proliferative capability in culture. We have recently developed a novel non-enzymatic method and device, named Lipogems, to obtain a fat tissue-derived stromal vascular fraction highly enriched in pericytes/mesenchymal stem cells by mild mechanical forces from human lipoaspirates. When compared to enzymatically dissociated cells, Lipogems-derived hASCs exhibited enhanced transcription of vasculogenic genes in response to pro-vasculogenic molecules, suggesting that these cells may be amenable for further optimization of their pluripotency.

We exposed Lipogems-derived hASCs to a Radio Electric Asymmetric Conveyer (REAC), an innovative device asymmetrically conveying radio electric fields, affording both pluripotency optimization in mouse embryonic stem cells, and efficient direct multi-lineage reprogramming in human skin fibroblasts. We show that specific REAC exposure remarkably enhanced the transcription of prodynorphin, GATA4, Nkx2.5, VEGF, HGF, vWF, neurogenin1 and myoD, indicating the commitment towards cardiac, vascular, neuronal and skeletal muscle lineages, as inferred by the overexpression of a program of targeted marker proteins. REAC exposure also finely tuned the expression of pluripotency genes, including Nanog, Sox2, and Oct4. Noteworthy, the REAC induced responses were fashioned at a significantly higher extent in Lipogems-derived than in enzymatically-dissociated hASCs.

In conclusion, the interplay between radio electric asymmetrically conveyed fields and Lipogems-derived hASCs appears to involve the generation of an ideal "milieau" to optimize pluripotency expression from human adult stem cells, paving the way to unprecedented cell therapy perspectives.

207 - CARDIAC STROMAL CELLS AND HEART REGENERATION

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Aims: Bone marrow mesenchymal stromal cell (BMStC) transplantation into the infarcted heart improves left ventricular function and cardiac remodeling. However, it has been suggested that tissue-specific cells may be better for cardiac repair than cells from other sources. The objective of the present work has been the comparison of *in vitro* and *in vivo* properties of adult human cardiac stromal cells (CStC) to those of syngeneic BMStC.

Methods and Results: Although CStC and BMStC exhibited a similar immunophenotype, their gene, microRNA, and protein expression profiles were remarkably different. Biologically, CStC, compared with BMStC, were less competent in acquiring the adipogenic and osteogenic phenotype but more efficiently expressed cardiovascular markers. When injected into the heart, in rat a model of chronic myocardial infarction, CStC persisted longer within the tissue, migrated into the scar, and differentiated into adult cardiomyocytes better than BMStC.

Conclusions: Although CStC and BMStC share a common stromal phenotype, CStC present cardiovascular-associated features and may represent an important cell source for more efficient cardiac repair.

208 - PRETRANSPLANT INFUSION OF DONOR STEM CELLS OPENS GATEWAY TO TOLERANCE ASSOCIATED WITH INDUCTION OF REGULATORY T-CELLS - SINGLE CENTRE EXPERIENCE IN LIVING DONOR RENAL TRANSPLANTATION

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Introduction: Transplantation tolerance is a state in which there is a lack of a destructive immune response by the recipient towards a well-functioning donor organ in the absence of maintenance immunosuppression and with a fully intact immune system. Limited long-term success has been achieved in transplant tolerance using hematopoietic stem cells (HSCs) and/or conditioning regimen. However dataregarding stem cell infusion (SCI) associated with tolerance induction in renal transplantation (RTx) is still sparse. We designed a prospective trial in a cohort of live-donor RTx (LDRT) patients subjected to pre-transplant SCI to test its tolerance promoting effects.

Materials and Methods: Ninety patients (81 males, 9 females) with mean age 32.2 years and donor-recipient HLA match, 2.81 who were subjected to pretransplant donor SCI of HSC and adipose tissue derived mesenchymal stem cells (AD-MSC) under non-myeloablative conditioning of cyclophosphamide, rabbit-

antithymocyte globulin and rituximab with total lymphoid irradiation/ Bortezomib were included in the study. Commonest cause of end stage renal failure was chronic glomerulonephritis in 45.6%, chronic pyelonephritis in 25.6% and hypertensive nephropathy in 10%. Patients with diabetes, unwillingness, HIV, hepatitis C/B were excluded. Mean quantum of SC infused was 250 ml, with mean CD34+ 2.1 x 10^6/kg body-weight (kgBW), mean CD45-/90+ 4.4 x 10^4 /kgBW and CD45-/73+ 0.67 x 10^4/kgBW. There were no untoward effects of SCI/ conditioning. Immune monitoring included donor specific antibodies (DSA) by luminex assay and peripheral regulatory T-cell (pTregs) [$CD4^+CD25^{high}CD127^{neg/low}$] by flow-cytometry. Peripheral blood lymphohematopoietic chimerism was evaluated by fluorescent in-situ hybridization in subset of patients with gender-mismatched donors.Initial maintenance immunosuppression was calcineurin-inhibitor based, to be discontinued with stable graft function and absence of rejection episodes. Graft biopsy was performed after 100 days of immunosuppression withdrawal in willing patients (protocol biopsy) or, for graft dysfunction. Rejections were treated by standard anti-rejection therapy followed by rescue immunosuppression.

Results: SCI was safe and no untoward effect of conditioning was observed. No patient/graft was lost. All immunosuppression except Prednisone has been successfully withdrawn for mean 2 years in all 90 patients. Their mean serum creatinine (SCr) of 1.4 mg/dL and p-Tregs, 3.63% has remained stable after withdrawal. DSA are absent in 32%, present in 54.4% and reports awaited in 13.3% patients. Chimerism was not always associated with clinical tolerance. Protocol biopsies were performed in 45 willing patients and 93.3% were unremarkable. Rescue immunosuppression was started in 6.7% patients after anti-rejection therapy. Their present mean SCr is 1.7 mg/dL.

Conclusions: Pre-transplant SCI leads to stable graft function with safe minimization of immunosuppression in living donor renal transplantation associated with induction of pTregs [$CD4^+CD25^{high}CD127^{neg/low}$]. MSC may serve as novel, safe and effective immunomodulators in clinical transplantation.

209 - MESENCHYMAL STEM CELLS PRECONDITIONED WITH PROINFLAMMATORY CYTOKINES ENHANCE T CELL INHIBITION BY THE DOWNREGULATION OF CD25 ON ACTIVATED T CELLS, EXPRESSION OF IMMUNOSUPPRESSIVE FACTORS AND INCREASE OF REGULATORY T CELLS

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Mesenchymal stem cells (MSC) require sufficient threshold of proinflammatory cytokines to activate their immunosuppressive function *in vivo*. Hence, we aimed to modify human bone marrow derived MSC with proinflammatory cytokines as a strategy to enhance MSC immunosuppression on T cells. To investigate the effect of exogenous proinflammatory cytokines on MSC immunosuppression, MSC were co-cultured with T cells activated by the mitogen phytohemagglutinin (PHA). The exogenous addition of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), IL-2, IL-12p70 and IL-17A to these co-cultures antagonised MSC-mediated immunosuppression of T cells. However, when MSC were preconditioned with the following cytokines for 5 days, they displayed greater immunosuppressive properties on T cell proliferation by 14.8%, 22.2%, 17.1%, 23.2%, 17.2% and 27.7%, respectively, compared to unmodified-MSC (UT:MSC). Only the IFN-γ modified-MSC (MSC-γ) showed upregulation of the T cell negative co-stimulatory molecule programme-death ligand-1 (PD-L1). The neutralisation of PD-L1 however failed to restore T cell proliferative responses suggesting a partial but non-exclusive role of PD-L1 in MSC-γ immunosuppression. IL-6 gene expression significantly increased in the TNF-α and IL-1β-modified MSC. MSC-γ and TNF-α-modified MSC upregulated prostaglandin-2 gene expression relative to UT:MSC and significantly induced the indoleamine 2,3-dioxygenase gene expression in MSC. The gene expression of transforming growth factor beta-1 (TGF-β1) was unaffected following cytokine modification of MSC. Both the UT:MSC and the cytokine modified MSC showed no detectable levels of IL-10 following 5 days of culture. Furthermore, MSC-γ showed greatest reduction in the T cell activation marker CD25 on $CD3^+$ T cells while the IL-17 preconditioned MSC (MSC-17) reduced CD25 levels comparable to UT:MSC. These MSC show a greater reduction of CD25 on $CD8^+$ T cells than in the $CD4^+$T cells. In addition, a similar increase in proportion of $CD3^+CD4^+$ $Foxp3^+CD127^{low}CD25^{hi}$ regulatory T cells (Tregs) was observed in T cells co-cultured with UT:MSC and MSC-17 compared to the PHA activated T cell control. A 2-fold increase in the proportion of Tregs was evident in the MSC-γ-T cell co-culture compared to UT:MSC and MSC-17. In conclusion, preconditioning MSC with proinflammatory cytokines enhances the therapeutic efficacy of MSC as immunosuppressive agents via the downregulation of CD25 on activated T cells, expression of inhibitory factors and increase of Tregs. The mechanisms of enhanced MSC-mediated immunosuppressive effect on T cells were dependent on the proinflammatory cytokines used for MSC modification.

210 - LOCALIZED IMMUNE MODULATION WITH FINGOLIMOD FOR ALLOGENEIC ISLET TRANSPLANTATION INTO A BIOHYBRID DEVICE

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Replacement of beta-cell function via intrahepatic islet transplantation restores metabolic control in patients with type 1 diabetes. The need for systemic immunosuppression and its associated untoward side effects currently limit the indication of islet transplantation to the most severe cases of instable diabetes. Tissue engineering is opening new opportunities for the development of more efficient biological treatments for diabetes. We have previously reported that transplantation of syngeneic islets corrects diabetes into prevascularized, subcutaneous biohybrid devices (BHD). To overcome the need for chronic systemic immunosuppression, we sought to evaluate the effects of localized immuno-suppression (LIS) on allogeneic islet allograft survival into BHD.

Allogeneic Wistar Furth ($RT1^u$) rat islets were implanted into a pre-vascularized BHD with infusion port in chemically-diabetic Lewis rats ($RT1^l$) on day 0. Induction with IP anti-lymphocyte serum (ALS days -3) was followed by daily oral administration of mycophenolic acid (MPA 20 mg/kg/day) for 3 weeks alone or combined with chronic fingolimod (15 ug/kg/day) delivered directly to the BHD's lumen for the duration of the follow-up starting on day 0. Control animals received no treatment.

Control animals (n=5) rejected islet allografts with a median of 9 (7-11) days. The induction protocol (ALS+MPA) led to graft survivals of 29 (19-40) days (n=5, p=0.002 vs. control). The combinatorial treatment based on induction protocol plus chronic local fingolimod (n=9) significantly prolonged allograft survival to 36 (26->67) days and long-term function in two animals (22%; >42 and >67 days, respectively) (p<0.0001 vs. controls). Notably, LIS reduced donor-specific antigens responses of lymphocytes but did not alter third party responses in mixed lymphocyte reactions and in antiCD3/CD28 stimulation assay. Also, cellular composition (CD4, CD8, B cells, Macrophages) and activation markers of the lymph nodes draining the devices, but not in distal nodes, were altered in animals receiving LIS.

Our study demonstrates that prolonged islet allograft survival can be achieved after transplantation into a BHD by the means of local immunosuppression using doses that are ~100 times lower than needed systemically. Our approach may represent an appealing therapeutic strategy to avoid the toxicity of systemic immunosuppression for beta-cell replacement therapies in insulin-requiring diabetes.

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211 - CRITICAL ROLE OF ALLOGRAFT INFILTRATING ROR GAMMA T POSITIVE INNATE IMMUNE CELLS AND HEMATOPOIETIC PROGENITOR CELLS

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Using a minimally invasive *in vivo* imaging system to serially monitor fluorescence emitting T cell subtypes infiltrating islet allografts, we have now probed for the presence of graft infiltrating $CD4^+$ Th17 cells, characterized by the expression of the nuclear transcription factor RORγt. Since Th17 cells are critical to the pathogenesis of various autoimmune disorders, we suspected that they might also play an essential role in allograft rejection. However, rather than around the time of allograft rejection (Mean Survival Time, MST=12 days), peak infiltration by $ROR\gamma t^+$ cells unexpectedly occurs within the first 3 days post allogeneic islet or heart transplantation. Additionally, the phenotype of the predominant graft infiltrating $ROR\gamma t^+$ cells are neither $CD4^+$ Th17 cells, nor TCR γδ T cells, iNKT cells, nor recently identified innate lymphoid cells (ILCs) subtypes, but a previously uncharacterized subtype of hematopoietic progenitor cells that are lineage negative (Lin^-) and $ROR\gamma t^+$. We subsequently demonstrate using both genetic and pharmacological manipulations that that early infiltrating unconventional $Lin^-ROR\gamma t^+$ cells may powerfully promote allograft rejection. This effect is likely due to the ability of $ROR\gamma t^+$ cells to govern local inflammation. Strikingly, the phenotype of graft infiltrating $ROR\gamma t^+$ cells is amenable to pharmacological manipulation with alpha 1 anti-trypsin (AAT). Either treatment with AAT or use of RORγt deficient mice prolongs engraftment of islet allografts. Our data indicates an important extra-medullary role for $Lin^-ROR\gamma t^+$ hematopoietic progenitor cells in response tolocal injury induced by inflammation and an important role for RORγt gene expression in the response to allografts. $Lin^-ROR\gamma t^+$hematopoietic progenitor cells may provide a novel, unrecognized target for treatment of allograft rejection.

212 - HUMAN ENGINEERED MICRO-PANCREATA (EMPS) SECRETE SIGNIFICANT AND REGULATED LEVELS OF INSULIN FOR VERY LONG PERIODS IN VITRO AND CAN RESCUE HYPERGLYCEMIC MICE

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Islet transplantation that aims to restore the insulin-producing capacity of the patient has become an attractive approach for the treatment of severe diabetes, ye, perhaps the most challenging problem facing clinical islet implantation is the inexorable loss of islet function in the first 5 years post-implantation leading to only 10% of recipients being insulin-free. This loss of function is not completely understood but believed to be caused by a combination of insufficient implanted β-cell mass, allograft rejection, recurrent autoimmunity, incompatibility of the islet implantation site, and immunosuppressive regiments that are toxic to the islet grafts. Currently, a large number of isolated human islets are transplanted in severe cases of diabetes through the portal vein of the patient, resulting in partial integration of the islets within the liver. This procedure suffers from high percentage of islet lost, in part, due to lack of a proper microenvironment that is bound to be required for islet viability and function. Therefore, there is a need to develop *in vitro* natural three-dimensional structures that mimic the islet tissue microenvironment prior to transplantation. We here describe the preparation of engineered micro-pancreata (EMPs) that are made up of acellular organ-derived micro-scaffolds seeded with human intact or enzymatically dissociated islets. We show that EMPs secrete quantities of insulin per cell similar to fresh human islets for more than three months *in vitro* in a glucose-regulated manner. Notably, EMPs respond, even after long periods in culture, by secreting increased amounts of insulin when exposed to Exendin-4, Forskolin, Tolbutamide, Glucagon-like peptide-1 (GLP-1) and Gastric inhibitory polypeptide (GIP). Quantitatively the amount of insulin and Pdx-1 gene copies per cell are similar to those transcribed by fresh normal human islets. We also report that when implanted subcutaneously onto Streptozotocin-treated hyperglycemic NOD-SCID the EMPs become vascularized and can rescue the mice in a dose response manner, with a total of 40 human Ieq on EMPs being sufficient for obtaining normoglycemia. Specificity of action of the EMPS was established by removal of the EMPs after 35 days and demonstrating reversal back to the hyperglycemic state. EMPs could thus form the bases for both an assay for "*in vivo*"-like human b-cell function in the laboratory and as a future treatment modality for cell transplantation in diabetes.

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213 - OXYGEN GENERATING BIOMATERIAL MITIGATES HYPOXIA INDUCED APOPTOSIS ON PANCREATIC ISLETS

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Introduction: Cellular transplantation of tissue engineering constructs is an attractive approach for the replacement or repair of damage tissue. Nevertheless, the success of engineered tissues has been hindered by poor oxygenation upon transplantation, due to the inevitable delay in the vascularization process. Inadequate oxygen delivery of cellular constructs leads to a necrotic core of cells and a dysfunctional, pro-inflammatory environment. This is particularly challenging in constructs comprising highly metabolically active cells, such as pancreatic islets. To mitigate this damage, we have developed an oxygen generating biomaterial capable of in situ oxygenation of cellular implants[1]. Herein, we report on the effect of this oxygen generating biomaterial on alleviating hypoxia-induced pancreatic islets apoptosis.

Methods: Oxygen generating disks (CaO_2-PDMS) were made as previously reported[1]. Rat pancreatic islets were co-incubated with a blank PDMS disk (control) or with CaO_2-PDMS disk (treated) at normoxic (20%) or hypoxic (1%) oxygen tensions and incubated for short (8 hr) and long-term (24 hr) assessment. Analysis of the HIF-1α target genes using RT-PCR was performed, as well as protein assessment, western blotting, and live dead imaging.

Results: Islets cultured in hypoxic conditions for long-term incubation periods show stabilization and accumulation of HIF-1α protein in control groups, compared to treated, as seen by western blotting. Results for the expression of HIF-1α target genes in hypoxic controls show an up-regulation of glycolytic enzymes such as, phosphoglycerate kinase 1, *pgk1*, Lactate dehydrogenase A, *ldhA*, Glucose transporter 1, *GLUT1*, as well as the DNA damage protein, *Ddit-4,* when compared to the treated hypoxic group in both the short and long-term incubations. This up-regulation represents an increase in oxidative stress, a shift to anaerobic metabolism, and induced apoptosis in response to DNA damage. For pro-angiogenic markers, the expected up-regulation of the vascular endothelial growth factor A, *VEGFA,* in the hypoxic controls compared to the treated was observed in the short-term (8 hr). Furthermore, there was a down-regulation of pro-apoptotic genes BCL2-antagonist/killer 1, *BAK*, and BCL-2 associated X protein, *BAX,* in the hypoxic controls compared to treated, suggesting a shift to BNIP3 mediated apoptosis, which resembles more a necrotic pathway of cell death. Live/dead imaging of hypoxic controls presented fragmented islets with exten-

sive dead cells. In contrast, treated hypoxic group showed large, morphologically intact islets comparable to normoxic controls.

Conclusions: Herein, we have illustrated the ability of our oxygen generator to mitigate the effects of hypoxia and HIF-1α involvement on pancreatic islet viability. Thus, this platform can be advantageous to improve early graft loss due to limited oxygen supply for cellular based tissue engineered constructs.

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214 - TRANSPLANTATION OF PANCREATIC ISLETS IN THE OMENTAL POUCH USING A RESORBABLE PLASMA-THROMBIN GEL: PRELIMINARY RESULTS IN RODENTS AND NONHUMAN PRIMATES

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Transplantation of pancreatic islets is a viable therapeutic option for the treatment of unstable diabetes. Currently, islets are transplanted into the portal venous system through their embolization into the liver sinusoids resulting in loss of a substantial islet mass to inflammation and hypoxia. The need for developing alternative, extra-hepatic implantation sites for islets has been recognized. Implantation of islets into omental pouches is appealing because the omentum is well vascularized and has portal blood drainage.

We tested the function of islets transplanted in the omental pouch site in a plasma clot induced with the addition of clinical grade recombinant thrombin. Human islets transplanted in the omental pouch of streptozotocin (STZ)-induced diabetic athymic mice reverted diabetes, maintained sustained normoglycemia and normal clearance during glucose tolerance test. Resection of the graft-bearing omental pouches resulted in prompt return to hyperglycemia. Histopathology of the explanted grafts displayed well-preserved islets with insulin and glucagon signals.

We also performed a pilot allogeneic islet transplant in a STZ-induced diabetic cynomolgus monkey under thymoglobin (10mg/kg IV on days -1, 0, 2 and 4), anti-CTLA4Ig (Belatacept; 20mg/kg IV on days 0, 4, 14, 28, 56, 75 and monthly thereafter at 10mg/kg) and Sirolimus (from day 2 targeting trough levels of 8-12ng/ml). After transplantation, basal c-peptide levels became detectable paralleling reduction in insulin requirements over time. Preliminary histopathological assessment of the graft 49-days after transplant showed well preserved islets with immunoreactivity for endocrine markers.

Our preliminary data suggest that the use of a clinical-grade resorbable plasma-thrombin gel in an omental pouch allows engraftment of transplanted islets.

Diabetes Research Institute Foundation

215 - ASSEMBLY OF FUNCTIONAL TRANSPLANTABLE LIVER GRAFTS

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About thirty million people in the US undergo a liver disorder for different causes and about 28,000 deaths are registered annually in the US due to liver disease. To date the only definitive treatment is liver transplantation, however, a major reason of liver related deaths is the limited donor pool. In the last decade, the paradigm in organ transplantation has shifted away from immune suppressive regiments toward tolerance induction and the need to expand the donor pool through organ engineering. This project aims to use discarded donor livers as natural scaffolds to constitute complete functional livers grafts to be transplantable with success by employing techniques from tissue engineering and cell/organ transplantation.

We have developed optimized protocols for whole rat liver decellularization and designed critical criteria for organ-specific matrix quality based on ECM structure and components, DNA content and proteomics studies. Moreover, we have generated reproducible systems/protocols for recellularization with freshly isolated hepatocytes. Ultimately reconstruction of liver grafts *in vitro* also requires the addition of liver non-parenchymal cells, thus, we have designed complete vasculature and bile duct recellularization protocols. In order to evaluate and optimize different cell seeding protocols of vasculature (portal and central vein) and bile duct recellularization, we have designed different systems for histological and imaging evaluation based on micro CT and MRI technology. This system allows us to perform serial evaluations of the recellularization protocols for further optimizations. We found that up to 80-90% of the vessels in the whole liver were adequately recellularized with microvascular endothelial cells and about 60-80% of the bile ducts were adequately recellularized with bile duct cells. Moreover, endothelial and hepatic function was evaluated over time (endothelial: LDL metabolism and tPA responsive secretion; hepatic: albumin synthesis, urea secretion and total bile salts secretion) after complete recellularization of liver grafts and demonstrated that the newly engineered liver grafts remain functional. The development of methodologies for liver graft assembly has the potential to become a novel platform for

future sophisticated organ engineering techniques that incorporates several different cell types and may ultimately lead to development of entire organs *in vitro* for transplantation drug development and biological studies.

216 - CHIMERIC ENGINEERED BIO-ORGAN FOR BETA CELL REPLACEMENT IN TYPE 1 DIABETES

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Background: β cell replacement in type 1 diabetes can be achieved by isolated pancreatic islet transplantation. The lack of engraftment is a major limitation to a broad application in diabetic patients. In fact ~50% of the transplanted islets die shortly after transplantation. Decellularization of organs is an innovative approach in tissue engineering, it provides an acellular 3D biologic scaffold that can be re-endothelialized and seeded with selected cell population (chimeric bio-organ).

Aim: to generate a chimeric bio-organ composed of decellularized kidney seeded with pancreatic islets for beta cell function replacement in diabetic animal models. The hypothesis is that *ex vivo* controlled infusion and re-cellularization with pancreatic islets could improve engraftment and promote islet survival in the recipient limiting the negative events related to direct islet infusion in the liver.

Methods: Rat kidney was explanted preserving the vascular access to allow whole organ perfusion decellularization. A model of rat renal scaffold (bio-scaffold) was set up by a quick process of decellularization based on continuousperfusion with Triton X-100 0.1% and Sodium Dodecyl Sulphate–SDS- 0.1% for 4 hours. Once established the decellularization procedure, 4 bio-scaffolds were analyzed: 1 was formalin-fixed and evaluated for residual cell material by haematoxylin/eosin (HE) and DAPI staining; 1 was transplanted in recipient rats to test surgical feasibility and organ reperfusion; 1 was infused with pancreatic islets and the last one with single cell suspension of the same islet preparation after trypsin digestion. Decellularized kidney seeded with islets or dispersed islet cells was formalin-fixed after infusion and analyzed for islet cell engraftment by HE and insulin staining.

Results: The decellularization protocol has been optimized to repeatedly produce scaffolds without cellular material. Bio-scaffold structures retained the intricate vascular networks, showed preserved cortical ECM microstructure and no evidence of residual nuclei or intact cells. Syngeneic orthotopic transplantation of decellularized rat kidney showed mechanical resistance to blood flow pressure and functional perfusion. Delivery of beta cells (islets or dispersed islet cells) through arterial pathway was feasible and did not alter organ structure; cell flow was maintained along decellularized vascular network and islet cells spread in the kidney parenchyma and preferentially localized at the level of the glomeruli.

Conclusions: Our data in rat show that i) acellular whole kidney scaffolds can be obtained by perfusion decellularization; ii) decellularized kidney maintains matrix architecture and vasculature after re-implant in a syngeneic recipient; iii) decellularized kidney can be seeded with pancreatic islets after renal artery cannulation. Experiments are on going to re-endothelialize the bio-scaffold before islet infusion. Chimeric bio-organ obtained with this approach will likely normalize glycemia in diabetic animal models.

217 - A NEW GENERATION OF IMPLANTABLE CONSTRUCTS: DEVELOPING IMPLANTS THAT MAKE OF THE ENDOGENOUS RESPONSES AND OF THE HOST BODY RESPECTIVELY THE EFFECTORS AND THE BIOREACTOR OF TISSUE REGENERATION

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Tissue engineering (TE) makes combined use of cells and biomaterials, as scaffolds, in order to restore tissue structure and function mostly thorugh the specific commitment of the implanted cells. Similarly to regenerative medicine and to therapies of immune-related disorders, TE holds many expectations from the use of mesenchymal stem cells (MSCs). For their properties of self-renewal, multipotency, immune system modulation and support for tissue repair and angiogenesis, MSCs are a major current focus of many academic, industrial and clinical interests.

Although MSCs can differentiate into various mesenchymal lineages and even trans-differentiate in culture, when successfully implanted *in vivo*, the new tissue is never of donor origin nor is justified by the incidence of differentiation of grafted MSCs. Within all human organs and tissues from which they are derived, MSCs are perivascular progenitors and regulate tissue homeostasis, repair and regeneration, leading to recruitment of macrophages, endothelial precursors and host pluripotent cells[1].

We recently showed that ectopic implants, in mice, of osteo-conductive scaffolds seeded with bone marrow (BM)-MCSs leaded to endochondral bone formation only when previously enriched in culture for early progenitors. These MSCs triggered regeneration by secreting bioactive

molecules and so modulating the host microenvironment rather than directly differentiating into the injured tissue[2]. Furthermore, like the ability to be enrolled in a specific commitment, the secretion of factors by MSC was shown to be dependent on cell progenitor state, and this activity is likely to be the most important for MSCs therapeutic potential. However, although it is known that MSCs orchestrate regeneration acting as "drugstores", by secreting a mix of factors that trigger endogenous cascades[3], TE is still based on cells potentially differentiating into the injured tissue and on scaffolds engineered for this goal. Contrarily, engineering products based on biomaterials, cells or their derivates that might bring the microenvironment modulations necessary to induce the host specific tissue regeneration *in vivo*, is poorly unexplored. Using mass spectrometry-based proteomics, we quantitatively identified the factors specifically secreted by early progenitor BM-MSCs. By deciphering the milieu of secreted proteins and by systems biology analysis we also identified the biological responses that MSCs can induce *in vivo*[2].

This strategy is currently under further development, in order to understand how to rule TE toward a new generation of implantable constructs not necessarily involving the engraftment of the implanted cells or not even using cells, but ideally being applied as pharmaceutical devices that, if implanted locally within a damaged tissue area, will trigger host regenerative processes, and use the endogenous cells as the effectors and the host body as the bioreactor.

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218 - ADIPOSE DERIVED STROMAL CELL SHEET WITH ARTIFICIAL DERMIS ACCELERATES SKIN WOUND HEALING

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Impaired skin wound healing caused by reduced blood supply is a major complication especially in diabetic patients. Although artificial dermis is currently utilized for the treatment of ulcers with full-thickness skin defects in diabetic patients, it is difficult to cure because of blood flow obstruction.

Previous studies have shown that adipose derived stromal cells (ADSCs) possess angiogenic property, and ADSCs have been transplanted to wounded skin. However, it is hard to retain the transplanted ADSCs on the site and the outcome is limited. To resolve this problem, we take advantage of cell sheet technology using temperature responsive culture dishes and the efficacy of ADSC sheet was evaluated using rat full-thickness skin defect in head.

First, inguinal fat tissue from a normal rat was taken and extracted ADSCs (rADSCs) . Colony forming, osteogenic and adipogenic differentiation potential of the rADSCs were investigated *in vitro*. One thousand of rADSCs were seeded onto 10 cm dishes and cultured in growth, osteogenic, and adipogenic differentiation medium, respectively. rADSCs exhibited colony forming, osteogenic, and adipogenic capacities, suggesting that rADSCs used in this study possessed multipotent mesenchymal stromal cells-like properties. rADSCs from normal rats were seeded onto temperature responsive culture dishes to fabricate cell sheets.

Full-thickness skin defect was made on a normal rat calvariae, and periosteum was removed. The defect in the calvariae of rat was covered with artificial dermises (Pernac(R)) with or without transplantation of rADSC sheets. Two weeks after the transplantation, rats were sacrificed and the wound regions including the calvariae were excised and histologically investigated. Newly formed blood vessels were observed in the middle of wounds both in rats with or without the transplantation of the ADSC cell sheet. However, the density of blood vessels was 1.9-fold higher in the transplanted rat compared that of control rat.

These results suggest that rADSC sheet transplantation combined with artificial dermis accelerates the vascularization in full-thickness skin defect model, and is useful for the skin wound treatment of diabetic patients.

219 - STEM CELLS AND MUSCULAR DYSTROPHIES

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Problem: Muscular dystrophies are a heterogeneous group of inherited disorders presenting a large clinical variability regarding age of onset, patterns of skeletal muscle involvement, heart damage, rate of progression and mode of inheritance. Attempts to repair muscle damage in Duchenne muscular dystrophy (DMD), the most severe case, are facing several problems and no therapy is available for this disease as for all muscular dystrophies.

Background: Cell therapy is one promising approach to correct genetic diseases by contributing to tissue regeneration; stem cells can be isolated from a healthy donor or, when possible from the same patient. In the first case cells will be transplanted under a regime of immune suppression while in the second case, cells will have to be genetically corrected before transplantation in the same patient from which they were derived. The recent identification of different types of multi-potent

stem cells, some of which are suitable for protocols of cell therapy, has disclosed new perspectives in the treatment of genetic diseases.

Hypothesis: Our previous work indicated that CD133+ stem cells, a recently identified population of progenitor cells, produce functional improvement upon intra-arterial injection in a mouse model of muscular dystrophy. Thus it could be possible to focus upon this type of stem cell for autologous transplantation in DMD animal models.

Research: Recently, transplantation of engineered dystrophic canine muscle-derived CD133+ cells has given promising results in Golden Retriever Muscular Dystrophy (GRMD) dogs, the most reliable animal model that shows a form of dystrophy very similar to DMD (and even more severe in most cases).

We isolated CD133+ stem cells from muscle biopsies of GRMD dogs, we then expanded them characterized the CD133+ cells by FACS analysis.

The issue was then to use a specific-designed lentiviral vector capable of eliminating the mRNA segment from exon 6 to 8 in canine dystrophin gene. We tested different concentration of lentiviral vectors and verified the restoration of dystrophin transcript in culture by RT-PCR.

Two dystrophic dogs were treated with serial intrarterial injections of autologous transduced cells.

The transplanted animals were analysed at different times; most of the biopsies in all muscles were morphologically less affected than those of untreated dogs.

Functional improvement of treated dogs was also assessed by a 15 min timed running test and by a combined clinical grading score. The untreated littermates became slower over the treatment time, whereas two treated dogs ran faster after treatment.

Observation: Because of these results, we plan a pilot clinical trial, based on intra-muscular and intra-arterial transplantation of autologous engineered muscle derived CD133+ cells. Efficacy and possible adverse effects will be evaluated to test whether this approach may represent a first step towards an efficacious therapy for muscular dystrophy.

221 - CELLULAR CARDIOMYOPLASTY BASED ON MUSCLE DERIVED STEM CELLS

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Cellular cardiomyoplasty, which involves the transplantation of exogenous cells into the heart, is a promising approach to repair injured myocardium and improve cardiac function. We have isolated a population of muscle-derived stem cells (MDSCs) from the skeletal muscle of both mice and human, that when compared with myoblasts, display a significantly improved capacity for regeneration in a model of acute myocardial infarction (MI). The transplanted MDSCs survive significantly better than skeletal myoblasts, and through a paracrine effect, reduce myocardial fibrosis, promote angiogenesis, and ameliorate left ventricular (LV) remodeling. We have reported that the improved survival of MDSCs in acute MI is related, at least in part, to their high expression of cellular antioxidants which give the MDSCs the unique ability to resist oxidative and inflammatory stress, conditions likely experienced by these cells after their implantation into infarcted myocardium. Although these findings suggest that both murine and human MDSCs represent a therapeutic cell source for MI patients, *important limitations,* such as the *poor delivery approach* (intracardiac injection), as well as the low *cardiomyogenic potential of MDSCs,* have limited the cardiac regenerative potential repair of MDSCs. In fact, we have been utilizing MDSCs for the repair of a variety of tissues, and the MDSCs were always delivered directly into the injured hearts. The use of the innovative FGF2-coacervate as a novel delivery approach represents a new area of research that can further promote the cardiac regenerative potential of MDSCs. In fact, using this innovative coacervate approach, we have shown that FGF-2-coacervate was able to enhance cardiac repair and regeneration by promoting angiogenesis. Therefore, we will present recent work from our laboratory where we combine the new FGF2 coacervate technology with MDSCs to further improve cardiac repair when compared to the intracardiac injection of MDSCs. We will also present recent work from our group where the cell sheet technology will be combined with MDSCs to further improve the regenerative potential of MDSCs in the hearts. Finally, we will present recent work where the viral transduction of MDSCs to express Wnt-11, a molecule required for cardiogenesis , enhance the cardiomyogenic differentiation of the MDSCs *in vitro,* and cardiac repair *in vivo,* when injected directly into the injured hearts. This new technology will not only increase our understanding of the basic biology of muscle-derived progenitor cell populations with enhanced cardiomyogenic potential for cardiac repair, but also facilitate the development of new delivery technologies based on biomimetic coacervate to improve cardiac regeneration and repair induced by cellular cardiomyoplasty using muscle progenitor cells.

222 - LOCO-REGIONAL DETECTION AND STIMULATION OF TRANSPLANTED LIVER CELLS BY PARTICLE-BASED MIRNA DEPLETION

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Introduction: Liver cell transplantation (LCT) is a promising treatment option for children with inborn metabolic liver disease. However, both detection and proliferation stimulation of transplanted liver cells is challenging.

Aim of this study was the development of a functionalized iron oxide particle for detection of transplanted liver cells by MRI and simultaneous loco-regional stimulation by targeting of specific microRNAs (miRNAs).

Methods: Silica-based micron-sized iron oxide particles (sMPIOs) were used as carrier particles. Locked nucleic acid (LNA) antisense oligonucleotides specific for hsa-let-7g or rno-let-7g were covalently bound on the surface of the particles. For *in vitro* studies, primary human hepatocytes were labeled with LNA-sMPIO and miRNA depletion was investigated using qRT-PCR and Northern Blot. Protein expression of Cyclin D1 and c-Myc was investigated by western blot. Light and laser scanning microscopy were used to verify the intracellular localization of the particles. For *in vivo* proof of concept, male rat hepatocytes labeled with LNA-sMPIOs were transplanted to female Wistar rats via intrasplenic injection. 3.0 Tesla MRI was performed 7 days following transplantation. Cell engraftment was analyzed by Prussian Blue staining and Fish-Typing for the Y-chromosome.

Results: *in vitro* incubation with LNA-sMPIOs enabled hepatocyte labeling within 4 hours. Particles were located near the cell nucleus, while not appearing to be encapsulated from cellular membranes. Labeling of human hepatocytes with LNA-sMPIOs lead to a decrease of endogenous let-7g levels by more than 85%. Knockdown efficiency was similar to controls with transfected cells. Cyclin D1 and c-Myc, which are both targets of let-7g, were significantly upregulated upon LNA-sMPIO internalization. *in vivo*, LNA-sMPIO labeled liver cells were detectable as punctual signal decrease of the liver and could be correlated with the presence of Prussian blue positive donor cells.

Discussion: Our results demonstrate the feasibility of particle-based miRNA depletion. Moreover, we show functional effects on the protein expression of labeled cells. *in vivo*, the micron-sized iron core enabled non-invasive detection of transplanted cells. Our concept could be therefore suitable for LCT as well as for further cellular therapies where visualization and therapeutic modification is necessary.

223 - STUDY OF AUTOIMMUNE DIABETES IN MICE USING NON-INVASIVE, LONGITUDINAL LIVE IMAGING IN THE ANTERIOR CHAMBER OF THE EYE

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The islets of Langerhans are endocrine cell clusters that represent ~1% of the pancreatic tissue where they are scattered present and therefore difficult to image noninvasively. Several imaging techniques can be used to study islet biology, including Bioluminiscence, MRI and PET-scan, but lack of cellular resolution. Fluorescent confocal microscopy can be used to obtain high definition imaging data *in vitro*, ex vivo and *in vivo*. We have previously reported that islet cell physiology and immune rejection can be studied using the anterior chamber of the eye (ACE) *in vivo* by confocal fluorescent microscopy. Here we sought to evaluate whether islet autoimmunity could be reproduced in this model to study longitudinally the progression of the autoimmune process.

Progression of autoimmunity was assessed on NOD.SCID islets transplanted into spontaneously diabetic female NOD or NOD.SCID mice receiving recent-onset NOD mouse splenocytes. Antigen-specific immunity was studied transplanting RIP-OVA islets into C57BL/6-Rag1-/- mice followed by adoptive transfer of C57BL/6-GFP-OT-I cells. Animals underwent repeated imaging sessions to acquire data on the same islets over time. Several parameters were assessed, including islet granularity, volume, cellular infiltrate that was monitored using immune cells expressing fluorescent proteins.

Diabetes recurred with a median of 10d (range 5-12) and 8d (5-13) in the ACE (n=6) and kidney capsule (KDN; n=12), respectively. Adoptive transfer of splenocytes induced diabetes within 30-35 days. Antigen-specific OT-I CD8 T-cells accumulated as early as 5 days post-transfer destroying RIP-OVA islets in the ACE. Longitudinal assessment of individual islet volume and granularity in the ACE demonstrated islet swelling starting 1 wk before onset of overt hyperglycemia followed by relatively quick volume reduction within a week. Live time-lapse studies with intraocular cytolabeling and dye injections allowed assessing infiltrating B and T cell behaviors and cell death in the target tissue with single-cell. Preliminary assessment of islet grafts explanted after diabetes onset from ACE and KDN showed infiltrating lymphocyte populations similar to native pancreas by immunostaining.

Collectively, our data suggest that islet transplantation into the ACE represents a valuable model to study islet immunity, allowing longitudinal *in vivo* noninvasive imaging on the very same islets with cellular resolution to characterize the effector phase kinetics of the infiltrating cells on the site of immune attack.

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224 - CIRCULATING MIR-375 LEVELS AFTER ISLET TRANSPLANTATION IN HUMANS: A BIOMARKER OF ONGOING BETA CELL INJURY

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Background: Circulating level of the pancreatic islet enriched miR-375 was recently described as a biomarker of beta cell death[1]. We tested if circulating levels of miR-375 reflect beta cell destruction in patients with type 1 diabetes who received human pancreatic islets.

Materials and Methods: A total of 22 human islet infusion were studied under different immunosuppression regimen: alphaCD25/Rapa/FK506 (n=6), ATG/MMF/FK506 (n=13) and none (auto trasplantation; n=3). MiR-375 circulating levels were evaluated by quantitative PCR (TaqMan Assay and/or droplet digital PCR).

Results: Human pancreatic islet expressed high level of miR-375. After transplantation serum miR-375 increased up to 200-fold during the first 12 hours, and then dropped to moderately elevated levels after 24 hours. This change was present both in allogenic and autologous islet transplantation. Circulating miR-375 levels were again increased at 96 hours post islet-infusion. This second peak was preceded by the rise of C-reactive protein and cross-linked fibrin degradation products, and it was followed by the rise of cell damage markers (AST, ALT and LDH). We compared miR-375 levels in patients with and without CXCR1/2 inhibitor therapy, a treatment that preserves <beta> cell mass by dampening inflammatory damage (Citro A. et al J. Clin. Invest. 2012;122:3647). All patients showed similarly increased circulating miR-375 levels during the 24 hr period that followed the infusion. However, the elevations of miR-375 levels that were observed at 48 and 96 hours post-islet infusion in control patients were not observed in patients treated with CXCR1/2inihibitors (p=0.048 and 0.024, respectively). Concordantly, CXCR1/2 inhibitor therapy preserved increased beta cell function during the time.

Conclusions: The results suggest that circulating miR-375 levels is a useful non-invasive indicator of ongoing beta cell injury after islet transplantation.

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225 - IMAGING AND TARGETING PANCREATIC ISLETS WITH APTAMERS

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The development of technologies for pancreatic islet imaging has become a field of intense study to respond to the high demands in diabetes research and clinical practice. In Type 1 Diabetes (T1D) a selective destruction of islet β-cells precedes clinical manifestation and ultimately leads to the disease. The β-cells mass may change in relation to therapeutic treatments and during the course of the disease. Currently, β-cells mass can be measured only indirectly with functional tests, andimaging of native or transplanted islets remains challenging.Antibodies, peptides and other forms of nanoparticles have been explored as imaging reagents, but they present several limitations: non-penetrating structures, non specific binding, toxicity, immunogenicity, production costs. Aptamers are an emerging class of molecules with unique characteristics that make them extremely appealing for islets imaging. Aptamers are oligonucleotide chains with a high affinity to their target, they are currently tested in clinical trial and commercialized. They can be developed with an unsupervised process called Cell-SELEX, that allows the parallel selection of hundreds of aptamers whose specificity for a given cell type (i.e. islet cells) is not based on a particular receptor but, rather, on all the surface markers that make the target unique. They are obtained by selection from a large random sequence pool, from which they are "evolved" to have a high binding affinity to their target. Given the small size and the ease of production in large scale, they represent extraordinary tools for imaging, proving superior to antibodies, and may prove useful for *in vivo* imaging and targeting strategies. We developed islet-specific aptamers with modified nuclease-resistant RNA structures useful for *in vitro* and*in vivo* applications. The selection approach was based on isolated living murine islets.A library of 10^{20} random aptamers was first depleted of non-specific aptamers using pancreatic exocrine tissue and positive selection was performed by incubation with islets. After removal of non-specific aptamers, islets-bound aptamers were isolated and amplified. The library obtained was used for the following cycle of selection, 12 cycles were performed. Aptamer libraries from the different cycles have been sequenced by the illumina high throughput sequencing and studied with bioinformatics tools for identification. We tested the resistance of aptamers to RNAse digestion and degradation. We demonstrated that our islet-specific aptamers are able

to bind avidly to islet cells with a fluorescent staining approach in tissue sections.The aptamer staining colocalized in insulin-producing beta cells. We observed no specific binding to irrelevant tissues. We plan to scale up the production of the selected aptamers, combine the aptamers with contrast moieties and test them for *in vivo* imaging of islet cells. Additionally, these new class of reagents will be used for the delivery of therapeutic short hairpin silencing RNAs (shRNAs) with the purpose to limit cytokines induced cell death and promote the proliferation of the residual β cell mass at onset in T1D mice models.

226 - GENE EXPRESSION PROFILING AND SECRETOME ANALYSIS DIFFERENTIATE ADULT-DERIVED HUMAN LIVER STEM/PROGENITOR CELLS AND HUMAN HEPATIC STELLATE CELLS

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Adult-derived human liver stem/progenitor cells (ADHLSC) are isolated after primary culture of liver parenchymal fraction. [1] The cells are currently developed as potential alternative to hepatocyte for liver cell based therapies. ADHLSC are of fibroblastic morphology and exhibit a hepato-mesenchymal phenotype. [1-3] Hepatic stellate cells (HSC) isolated from the liver non-parenchymal fraction present a comparable morphology as ADHLSC and are described as liver stem/progenitor cells. [4] In the current work, we strived to extensively compare both cell populations and to propose tools demonstrating their singularity.

ADHLSC and HSC were isolated from the liver of four different donors, expanded *in vitro* and followed from passage 5 until passage 11. Cell characterization was performed using immunocytochemistry, flow cytometry, and gene microarray analyses. The secretion profile of the cells was evaluated using Elisa and multiplex Luminex assays.

Both cell types expressed α-smooth muscle actin, vimentin, fibronectin, CD73 and CD90, in accordance with their mesenchymal origin. Microarray analysis revealed significant differences in gene expression profiles. HSC present high expression levels of neuronal markers as well as cytokeratins. Such differences were confirmed using immunocytochemistry. Both cell types also display distinct secretion profiles as ADHLSC highly secreted cytokines of therapeutic and immuno-modulatory importance, like HGF, interferon gamma and IL-10. Finally, ADHLSC and HSC behave differentially when incubated with specific growth factors involved in hepatogenic differentiation.

Our study demonstrates that, even sharing several phenotypic characteristics, ADHLSC and HSC are distinct liver fibroblastic cell populations as they exhibit significant different intrinsic gene expression and secretion profiles.

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227 - HYPOXIC PRECONDITIONING POTENTIATES HEPATOTROPHIC, ANTIAPOPTOTIC, AND PROSURVIVAL EFFECTS OF MESENCHYMAL STEM CELLS ON CO-CULTURED HUMAN HEPATOCYTES BY A REACTIVE OXYGEN SPECIES DEPENDENT, NON-PARACRINE MECHANISM

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Background/Objectives: Homo-cultured human hepatocytes undergo de-differentiation and loss of liver-specific metabolic function*in vitro*, whereas mesenchymal stem cells (MSCs) preserve the morphology and functionality of hepatocytes in heterotypic co-culture[1]. AsMSCs normally reside in a hypoxic niche[2], we hypothesised that hypoxic preconditioning (HPc) could optimise the co-culturehepatotrophic effect of adipose-derived stem cells (ADSCs), a subtype of MSCs, and HPc-induced potentiative effect depended on intra-ADSCs activity of reactive oxygen species (ROS), a pivotal signalling factor implicated in oxidative stress[3].

Methods: Human ADSCs with or without theaddition of 10-mM N-acetylcysteine (NAC), an antioxidant against ROS, were subjected to 2% O_2 HPc, with 20% O_2

normoxia-preconditioned (NPc) cells as control, for 24 hours. Intra-ADSCs ROS activity was measured using flow cytometry with chloromethyl 2',7'-dichlorodihydrofluorescein diacetate, and normalised to percentage of mean fluorescence intensity of control cells. Fresh human hepatocytes were seeded onto media-refreshed ADSCs monolayers ata ratio of 2.5:1and co-cultured for 7 days, with hepatocyte mono-culture as control. Indirect hepatocyte co-culture in ADSCs-conditioned media was also performed to determine the role ofADSCs-derived paracrine factors in the co-culture hepatotrophic effect. Albumin, caspase-cleaved cytokeratin 18(CCK18), and cytokeratin 18 (CK18) in culture supernatant were measured using enzyme-linked immunosorbent assays to determine the effects of ADSCs co-culture on liver-specific synthetic metabolism, caspase-mediated apoptosis, and total death of co-cultured hepatocytes, respectively; all data were shown as mean ± SD, and normalised to one million seeded viable hepatocytes.

Results: HPc significantly increased intra-ADSCs ROS activity (190.0 ± 22.2%, $p<0.05$),while 10-mM NAC reduced ROS activity in HPc-ADSCs (109.6 ± 28.8%, $p<0.05$). HPc-ADSCs co-cultureimproved albumin synthesis up to day 7 (HPc *vs* NPc *vs* control, 5.4 ± 0.3 *vs* 4.7 ± 0.2 *vs* 1.4 ± 0.2 μg, $p<0.01$), and also reduced the release of soluble CCK18 (17.3 ± 2.6 *vs* 30.4 ± 3.4 *vs* 42.0 ± 2.0 U, $p<0.01$) and CK18 (91.3 ± 13.2 *vs* 179.0 ± 14.0 *vs* 364.8 ± 15.6 U, $p<0.01$) from hepatocytes up to day 4. ROS inhibition reducedhepatotrophic (albumin, 4.5 ± 0.4 μg, $p<0.01$), antiapoptotic (CCK18, 30.5 ± 2.4 U, $p<0.01$), and prosurvival effects (CK18, 176.0 ± 13.2 U, $p<0.01$) of HPc-ADSCsco-culture. ADSCs indirect co-culture had minimal hepatotrophic (1.4 ± 0.1 *vs* 1.2 ± 0.1 *vs* 1.4 ± 0.2 μg, $p>0.05$), antiapoptotic (44.4 ± 3.3 *vs* 46.4 ± 5.3 *vs* 42.0 ± 2.0 U, $p>0.05$), and prosurvival effects (368.4 ± 13.2 *vs* 353.9 ± 21.2 *vs* 364.8 ± 15.6 U, $p>0.05$) on hepatocytes.

Conclusions: HPc-induced oxidative stress potentiatesthe hepatotrophic, antiapoptotic, and prosurvival effects of MSCs co-culture on hepatocytes by a non-paracrine mechanism dependent on intra-MSCs ROS activity.

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228 - AMNIOTIC MEMBRANE-DERIVED CELLS PRODUCE SPECIFIC SOLUBLE FACTOR(S) WHICH REDUCE LUNG FIBROSIS AND PRESERVE PULMONARY FUNCTION IN BLEOMYCIN-INJURED MICE

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We and others have recently provided evidence that the beneficial effects exerted by placenta-derived cells after transplantation in fibrosis disease animal models, might be due to secretion of paracrine-acting factor(s) that promote endogenous tissue repair.

In this study, we validated this hypothesis by injecting conditioned medium (CM) from cells of the mesenchymal region of human amniotic membrane (AMTCs) into mice with bleomycin-induced lung fibrosis. We also explored whether the paracrine effector(s) produced by AMTCs are specific to these cells and not produced by other cell populations, and which mechanisms underlie the anti-fibrotic effects of AMTC-derived CM (AMTC-CM).

CMs used in this study were generated from 5-day *in vitro* cultures of either AMTCs or other cell types (human skin fibroblasts, human peripheral blood mononuclear cells and Jurkat cells).

Fifteen minutes after bleomycin instillation, mice were intra-thoracically injected with CMs or were left untreated. After 14, 21 and 28 days, lung fibrosis was evaluated by histological analysis, by a semi-quantitative scoring system, while lung collagen was determined through a spectrophotometric technique. Lungs were also analyzed for levels of pro-inflammatory and pro-fibrotic cytokines and chemokines. Moreover, arterial blood gas analysis was performed to evaluate the effects of CMs on bleomycin-induced pulmonary dysfunctions.

Among the CM types used, AMTC-CM was the only one able to reduce lung fibrosis and ameliorate the mice impaired health status after bleomycin instillation. Indeed, AMTC-CM-treated mice: *i)* showed a mortality rate lower than in mice treated with the other CMs; *ii)* did not suffer weight loss; *iii)* displayed a reduction in pulmonary fibrosis and lung collagen content up to 28 days post delivery, and *iv)* consistent with the presence of less severe fibrotic lesions, blood gas analyses showed that AMTC-CM-treated mice had better lung function compared to animals treated with the other CMs, with minor levels of P_{CO2} and lower HCO_3^- blood concentration. In addition,

14 days after bleomycin, AMTC-CM-treated mice showed lower lung content of pro-inflammatory cytokines/chemokines such as IL-6, TNF-α, MIP-1α, MCP-1. Meanwhile, at both day 14 and 28, they showed lower levels of the pro-fibrotic factor TGF-β.

This study show that AMTC-CM displays specific anti-fibrotic properties and that may act both by reducing TGF-β levels in lung tissues through early abrogation of the pro-inflammatory cytokine network, and also by acting through a TGF-β independent mechanism, inhibiting the actions of IL-6 and MCP-1, which display direct pro-fibrotic activities. These results strongly suggest that a cell free treatment could represent a potential therapeutic strategy for pathological conditions where a reduction of inflammatory and fibrotic processes could favour reparative/regenerative actions of the endogenous cells.

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229 - HETEROGENEITY AND THE ROLE OF AGING IN THE ACTIVATION OF ISL1+ ENDOGENOUS CARDIOVASCULAR PROGENITOR CELL CLONES ISOLATED FROM HUMAN NEONATES AND ADULTS

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Introduction: Paracrine effects are largely responsible for the improvements in cardiac function that have been achieved with stem cell-based therapies. Insulin-like growth factor-1 (IGF-1) and SDF-1 have been well-documented for their ability to promote cardiovascular cell growth, migration and regeneration. The beneficial effects of these growth factors when administered without a cell type capable of cardiovascular regeneration are transient. Successful stem cell-based therapy will thus require an understanding of how growth factors promote cellular expansion in order to optimize their function towards maximal cardiac regeneration.

Hypothesis: Signaling is impaired in cardiovascular progenitor cells isolated from the aged adult population, rendering them less responsive to growth factors that stimulate migration to the site of injury.

Methods: Our laboratory isolated 264 clones of human neonatal and adult cardiovascular progenitor cells (CPCs) from patients undergoing cardiothoracic surgery, under IRB approval. These clones represent a unique resource for studying functional efficacy and early activation events in endogenous CPCs. Forty seven clones were selected for surface phenotype characterization using flow cytometry. Real time PCR was used to further distinguish the clones during differentiation into cardiac myocytes based on the expression of Isl1, MESP-1, c-kit and TropT. Differences in AKT cell signaling were identified in CPC clones in response to SDF-1 and IGF-1 by Western blotting. Invasion assays were also performed.

Results: Cardiovascular progenitor cell clones can produce both IGF-1 and SDF-1, factors that play a key role in cardiovascular repair. Functional differences were identified in the response of CPC populations to various growth factors. The response of Isl1+ c-kit+ co-expressing neonatal and adult CPCs to IGF-1 and SDF-1 was compared. Cardiomyocytes from neonates responded to IGF-1 treatment with a nearly two-fold increase in AKT phosphorylation while cells from the adult showed no response. Flow cytometry revealed that IGF-1 receptor levels were present on both neonatal and adult CPCs. Neonatal clones could be further distinguished by their differential response to SDF-1. The adult cells were not stimulated to invade in response to SDF-1 and there was no evidence of AKT activation, although the receptors were present on the cells as identified by flow cytometry.

Conclusions: Cardiovascular progenitor cells residing within the heart co-express Isl1 and c-kit. Neonatal CPCs produce and are activated by IGF-1 and SDF-1 and exist as a heterogeneous population. Age impacts cardiovascular progenitor cell signaling and may account for the inability of cardiovascular progenitor cells to mobilize to the site of injury and repair the heart in older adults.

230 - TISSUE REPLACEMENT OF INJURED HEPATIC PARENCHYMA IN A FIBROTIC/ CIRRHOTIC RAT LIVER ENVIRONMENT BY TRANSPLANTED STEM/PROGENITOR CELLS AND HEPATOCYTES

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Hepatic cell therapy could provide a valuable strategy to generate new functional tissue in the diseased liver. We therefore investigated the repopulation capacity of undifferentiated epithelial stem/progenitor cells and mature hepatocytes transplanted into livers with advanced fibrosis/cirrhosis.

The livers had ongoing fibrogenesis and massive hepatocellular damage, indicated by increased levels of fibrogenic markers (e.g., α-SMA, procollagen, TIMP-1, MMP-2) and decreased levels of hepatocyte-specific mRNA transcripts (e.g., ASGPR, CYP3A1, G6Pase mRNA), induced by thioacetamide (TAA) administration in the DPPIV– F344 rats. Prolonged TAA administration caused extensive stellate and stem/progenitor cell

activation and bile duct proliferation in the fibrotic septa. At 3 months after starting TAA administration, fetal liver stem/progenitor cells derived from DPPIV+ F344 rats were transplanted in conjunction with partial hepatectomy (PH). During continued TAA administration, transplanted stem/progenitor cells differentiated into hepatocytic and biliary cells, formed large DPPIV+ cell clusters, and replaced whole fibrotic nodules. At 4 months after cell transplantation, ~40% repopulation was achieved by DPPIV+ cell clusters that encompassed entire fibrotic lobules. Importantly, after cell transplantation of stem/progenitor cells without PH, undifferentiated cells engrafted and formed small clusters within days after cell transplantation, leading to 24% liver replacement after 4 months. Stem/progenitor cells are 2-3 times smaller than hepatocytes, which allowed us to infuse much higher numbers of cells, and comparative studies with hepatocytes showed a clear advantage of stem/progenitor cells over mature hepatocytes. However, transplanted hepatocytes were also capable of engrafting and replaced up to ~10% fibrotic liver tissue at 4 months after cell infusion.

To investigate whether transplantation of stem/progenitor cells can reverse fibrosis, advanced fibrosis/cirrhosis was induced in DPPIV-rats. At 3 months, cells were transplanted into TAA-treated rats, followed by continued TAA administration for 2 months, which was then discontinued for 1 month. Stem/progenitor cell-transplanted rats showed down-regulated expression of fibrosis-related genes (α-SMA, Col1a1, TIMP-1), indicating reduced fribrogenesis after cell transplantation.

In summary, using experimental conditions that reflect circumstances similar to human fibrosis/cirrhosis, we demonstrated that transplanted progenitor cells can efficiently engraft, proliferate after their engraftment, differentiate into hepatic cell lineages, and restore injured hepatic parenchyma. In this model, we also demonstrated the replacement of hepatocytic mass with mature hepatocytes. However, an even greater level of repopulation was achieved with transplanted stem/progenitor cells.

231 - PANCREATIC ISLET ENGRAFTMENT IS IMPROVED BY SPIEGELMER-BASED BLOCKADE OF CXCL12/SDF-1

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Background: Blocking proinflammatory mediators is a successful approach to improve the engraftment after islet transplantation. Spiegelmers are biostable mirror-image aptamers that can bind and inhibit target molecules; they represent a non-immunogenic alternative to antibodies. We evaluated whether Spiegelmer-based blockade of CXCL12/SDF-1 is able to favour islet engraftment in murine models.

Methods/Materials: CXCL12/SDF-1 blockade by Spiegelmer NOX-A12 was tested in a syngeneic marginal mass mouse islet transplantation model in which 200 C57BL/6 pancreatic islets are transplanted into liver of diabetic C57BL/6 mice. We also tested all the compounds in diabetes induced by the treatment of multiple low doses of streptozotocin (MLD-STZ), a mouse model in which a beta-cell damage is induced by inflammation.

Results: CXCL12/SDF-1 blockage by NOX-A12 treatment for 15 days was able to significantly improve islet engraftment. The median time to gain normoglycaemia was 4 ± 3 days and 26 ± 34 days for NOX-A12 (n=10) and vehicle (n=10) treated mice, respectively (p=0.004). Multivariate Cox Regression analysis confirmed NOX-A12 treatment as a significant favourable factor for the engraftment after the correction for pre-transplant glycaemia (HR: 6.4; 95% CI: 1.5-25.4; p=0.009). Concordantly, NOX-A12 treatment was able to protect islets by the inflammation-mediated damage in the MLD-STZ model: the median diabetes-free time was 20 ± 2 days and 33 ± 6.9 days for vehicle (n=12) and NOX-A12 (n=12) treated mice, respectively (HR: 0.296; 95% CI: 0.092-0.945; p=0.04).

Conclusions: CXCL12/SDF-1 blockage by the Spiegelmer NOX-A12 resulted in an efficient strategy to improve islet engraftment

232 - HUMAN AMNIOTIC EPITHELIAL CELL TRANSPLANTATION IMPROVES SURVIVAL IN A MOUSE MODEL OF ACUTE LIVER FAILURE

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Background: Acute liver failure (ALF) can occur after acute viral hepatitis, acute-on-chronic liver disease, toxin exposure and/or major liver surgery, and is characterized by massive acute tissue damage that leads to impaired hepatic function. In the most severe cases, liver transplantation is the only useful therapy. Hepatocyte transplantation in acute liver failure is used to support liver functions and enhance regeneration of native liver. A severe shortage of useful hepatocytes limits a wider application of this cellular therapy. Stem cell sources are being investigated for the production of cells for transplants. Human amniotic epithelial (hAE) cells have been shown to have characteristics similar to pluripotent stem cells, have the ability to differentiate into cells from all three germ layers, are not tumorigenic and when transplanted into the liver of mice, express human liver genes including CYP450, phase II, hepatic transporter and nuclear hormone receptor genes at levels observed in normal

adult human liver. In addition, hAE have anti-inflammatory and immunomodulatory effects that may play beneficial role in ALF progression.

Aim: We evaluated the possible beneficial effects of hAE cell transplants in ALF-induced liver failure.

Materials and Methods: Twenty eight mice received D-galactosamine (Dgal; 5 g/kg) intraperitoneally. Six hours later, half of the mice were directly injected with $2x10^6$ hAE cells in the spleen, whereas the remaining animals were injected with saline (ctrl). Seven animals in each group were sacrificed at day 2 and remaining surviving animals at day 14. Livers and blood were collected to evaluate histopathological, biochemical and gene expression parameters.

Results: Animals that received d-gal developed severe liver failure and all untreated or saline treated animals died within an average of 2.2 days with significant increases in AST, ALT and TGF-beta levels, and liver pathology consistent with ALF. All d-gal-intoxicated animals that received hAE cell transplants survived and appeared healthy until scheduled sacrifice at day 14. In separate experiments with animals sacrificed 48 hrs post d-gal treatment, transplant of hAE cells significantly decreased serum AST, ALT, and TGF-beta levels and decreased hepatic expression of Interleukin 1-beta, and Tumor Necrosis Factor alpha, and increased IL-10 expression.

Conclusions: Human AE cell transplantation improvedsurvival in mice in a Dgal-induced ALF model. These results suggest that human AE stem cell transplantation may be a useful cellular therapy for ALF and have motivated translation to GMP isolation and banking of hAE at our Institutet for the cellular therapy in liver diseases. Supported in part by the PKU foundation.

233 - SAFETY AND FEASIBILITY OF AUTOLOGUS MESENCHYMAL STEM CELL IMPLANTATION IN TWELVE DOGS WITH SUSPECTED DEGENERATIVE MYELOPATHY

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Canine degenerative myelopathy (DM) is an adult onset neurodegenerative disease that occurs in many breeds. The histopathology is classified as a multisystem central-peripheral axonopathy. Similarities between DM in dog and neurodegenerative diseases such amyotrophic lateral sclerosis (ALS) were discovered. In both cases etiology is unknown although overproduction of antibodies and immune complexes, inflammation and apoptosis, lack of growth factors, cytotoxicity and oxidative stress are possible factors. The use of mesenchymal stem cells (MSCs) in patients with ALS or DM is justified by their high degree of plasticity, the ability to release growth factors and modulate the immune system. This study aims to evaluatethe safety and feasibility of the intrathecal and intravenous administration of autologous MSCs in dogs with suspected DM.

Twelve dogs with chronic and progressive upper motor neuron clinical signs of the pelvic limbs were included in the study. In all the patients radiographs, magnetic resonance imaging (MRI; VetGrande, 0.25T, Esaote, Genova, Italy) and cerebrospinal fluid (CSF) analysis were negative. DNA exam for superoxide dismutase 1 (SOD-1) mutation was positive. Autologous bone marrow MSCs were isolated from each dog, cultured and expanded. In each dog 1x10(6) MSCs was injected intrathecaly in cisterna magna, 2x10(6) between L5-L6 space and 0.5x10(6) MSCs/kg intravenously. Follow up of six months included monthly clinical evaluation, complete blood and biochemistry work up and neurological evaluation. In all dogs MRI was repeated. Evaluation of immunitary response on blood samples taken before and after 4 and 24h from MSCs administration was accomplished by means of differentials leukocytes counts and lymphocyte subset using flow cytometry (FC) after peripheral blood centrifugation and staining.

MSCs consistently (>98%) expressed their classical surface markers and were negative for lymphocytes and hematopoietic cells. None of the dogs had an injection-related adverse effects. Blood workup and MRI did not reveal changes compared to previous exams nor onset of new neurological pathologies. Neurological evaluation evidenced static clinical signs in two cases. Two dogs died by natural causes and two dogs were euthanized after 6 and 9 months due to owner's request. Three of them underwent autopsy and DM was confirmed by histology. Six dogs had mild improvement of the hind limb ataxia and are still alive.

Implantation of MSCs in dogs with DM is a feasible and a relatively safe procedure. Clinical relevance of the immunological effects is yet unclear. The results of this study, together with the fact that neurological status and the course of DM is variable among affected dogs, encourage the selection of a greater, statistically more relevant, group of dogs for similar evaluation. Such enlarged study may also increase the possibility to apply results obtained from the canine model in human medicine.

300 - REGULATORY PATHWAYS OF INFLAMMATORY CYTOKINES

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Macrophages are key orchestrators of chronic inflammation. They respond to microenvironmental signals

with polarized genetic and functional programmes. M1 macrophages which are classically activated by microbial products and interferon-γ, are potent effector cells which kill microorganisms and tumors. In contrast, M2 cells, tune inflammation and adaptive immunity; promote cell proliferation by producing growth factors and products of the arginase pathway (ornithine and polyamines); scavenge debris by expressing scavenger receptors; promote angiogenesis, tissue remodeling and repair. M1 and M2 cells represent simplified extremes of a continuum of functional states. Available information suggests that TAM are a prototypic M2 population. M2 polarization of phagocytes sets these cells in a tissue remodeling and repair mode and orchestrate the smouldering and polarized chronic inflammation associated to established neoplasia. Recent studies have begun to address the central issue of the relationship between genetic events causing cancer and activation of protumor, smoldering, non resolving tumor-promoting inflammation. New vistas have emerged on molecules associated with M2 or M2-like polarization and its orchestration. Macrophage polarization has emerged as a key determinant of resolution of inflammation.

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301 - TISSUE REMODELING: LEARNING FROM TUMOR MICROENVIRONMENT

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The clinical inefficiency of cancer immunotherapy is in part due to the presence of an immunosuppressive network that favors tumor progression; in fact, by producing soluble molecules such as cytokines, interleukins and growth factors, tumors induce an alternative hematopoiesis that modifies the normal myeloid cell differentiation, pushing proliferation and expansion of cells with immunosuppressive function called myeloid-derived suppressor cells (MDSCs). The MDSC presence and frequency in blood of tumor patients is often reported as a prognostic marker that correlates with the clinical outcome and response to therapy. These cells use distinctive and redundant pathways to suppress the proliferation and function of antigen-stimulated T lymphocytes. Although MDSCs are heterogeneous, it appears that three main immunosuppressive cell subsets can be identified: granulocytic, monocytic and more immature cells, which might be able to originate the other two subpopulations. Our knowledge about mechanisms used by MDSC to restrain adaptive and innate immunity, the possibility to generate MDSCs by *in vitro* culture of bone marrow precursors, the definition of transcription factors and microRNAs regulating their *in vivo* expansion and maturation allow to hypothesize and design novel strategies for MDSC use as regulators of disorders characterized by excessive or uncontrolled stimulation of the immune response, such as transplant rejection and autoimmune diseases.

303 - ISLET TX AS PARADIGM

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A non-specific immune response mediated predominantly by innate inflammatory processes related to mechanics and site, and pre-existing and transplant-induced auto- and allo-specific cellular immune responses (possibly promoted by the initial inflammation) play a major role in the loss of islets and islet function transplanted in the liver. Although significantly improved by the implementation of the immunosuppression, our capacity of achieving long-lasting insulin independence in patients with T1D undergoing portal vein islet transplantation remains limited. This indicates that the detrimental impact of innate and adaptive immune responses is not fully contained by the current regimen of generalized immunosuppression. Prolong intrahepatic islet survival, by increasing the potency of such regimen is not practicable, due to the likelihood of enhancing susceptibility to cancer and infections, and the toxicity that some of these drugs may have towards kidney functions and transplanted islets. Rather, it is intuitive that alternative strategies aimed at selectively inhibiting undesired islet-specific or non specific immune responses represent an ideal step towards a better management (*i.e.,* weaning/withdrawal of generalized immune suppression) and outcome (*i.e.,* long-lasting insulin independence) of islet transplanted T1D patients. Thromboembolic and necroinflammatory events occurring in the liver early after portal vein islet transplantation are thought to reduce the total islet mass by up to 75%. The magnitude of such loss represents a major factor necessitating the extremely large number of islets needed to achieve normoglycemia. A better understanding and control of these events - including their likely support to effector immune responses – is required if we are to develop ways to prevent them, improve intrahepatic islet engraftment, and achieve long-term tolerance.

305 – PORCINE ISLET XENOTRANSPLANTATION: PROGRESS AND PERSPECTIVES

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Type 1 diabetes (T1D) continues to be a major source of morbidity and mortality. Human islet allografts restore normoglycemia and insulin independence, protect from severe hypoglycemia, and slow progression of microvascular complications in immunosuppressed type 1 diabetic recipients. These data highlight the potential of cell-based diabetes therapy.

Prolonged diabetes reversal for more than six months has now also been achieved after porcine islet xenotransplantation in non-human primates (NHP) by five groups involving the use of various tissue sources (adult, neonatal, and embryonic pig islet tissue; wild-type and transgenic), implantation sites (portal vein, omental pouch, subcutaneous tissue), immunotherapeutic protocols (immunosuppression, encapsulation), and animal models (streptozotocin-induced and surgical diabetes; cynomolgus and rhesus monkeys).

New insights suggest that the risk-benefit ratio of pig-to-human islet xenotransplants could actually be more favorable than that of human-to-human islet allotransplants. *First*, unlimited and on-demand availability of pig islets will boost access to islet transplants without waiting time. *Second*, the quality of islet products from healthy, young, living and designated pathogen-free pigs will be predictably high and not compromised – as with human islet products – by co-morbidity, brain death, age, ischemia, and disease transmission. *Third*, because the porcine islet amyloid polypeptide (IAPP) is considerably less amyloidogenic than human IAPP, transplanted pig islets will not be subject to IAPP-induced, non-immune-mediated graft loss. Fourth, pig islet transplants are less likely to be recognized by MHC-restricted, autoreactive, $CD8^+$ memory T cells, thereby making posttransplant autoimmune recurrence less likely. *Fifth*, preemptive negative vaccination with donor antigen can be exploited to mitigate anti-xenodonor immunity. *Sixth*, pig islet grafts will be a treatment option for highly allosensitized patients who appear to be at no increased risk of xenosensitization compared to non-sensitized patients. Finally, genetic modification of source pigs will present opportunities for minimizing recipient immunosuppression not available to recipients of human islet allografts.

Some of the requirements for clinical translation of pig islet xenotransplantation have been met. *First*, the regulatory framework established by the FDA and the recommendations made by the Int'l Xenotransplant Association and the World Health Organization provide a safe and suitable framework for conducting clinical trials of investigational porcine islet products in T1D. *Second*, a surveillance and safety program has been developed to detect, measure, manage, report, and respond to infectious diseases caused by known infectious agents and, possibly, previously unknown or unexpected pathogens in individual recipients of pig tissues. *Third*, suitable, designated pathogen-free, wild-type source pigs have been generated for planned pilot clinical trials. *Fourth*, significant progress has been made in manufacturing clinical-grade pig islet products for use in T1D recipients. However, for clinical trials of pig islet xenotransplantation to be undertaken with high expectation of benefit, safe and effective rejection prophylaxis strategies remain to be developed in NHP that do not interfere with islet xenograft function.

306 - ISLET CELL TRANSPLANTATION: IDENTIFYING NEW SOURCES, IMPLANT SITES AND EFFECTIVE STRATEGIES FOR IMMUNOPROTECTION

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Islet transplantation for the treatment of Type I diabetes has been proven effective, with many patients no longer needing insulin administration, regaining normal blood glucose levels and retaining islet function for years after the transplant.

Currently, islets are harvested from deceased donors, they are implanted in the liver, and their survival is dependent on the use of powerful immunosuppressive agents.

Therefore, there are still many unresolved issues that require the definition of improved treatment strategies. Among these, the identification of alternative sources of islets, the use of a safer implant site and the use of strategies that will allow for islet survival with reduced/no immunosuppression.

This presentation will address these three issues and focus on the identification of novel beta cell sources in the hapatopancreatic biliary tree and in the exocrine pancreas, on the use of the omental pouch as a safer site for islet transplantation and on the potential positive impact of strategies that aim at the induction of immunoprotection/tolerance by MDSC (Myeloid-derived suppressor cells).

Additional topics will include the definition of effective strategies to image and target beta cells *in vivo* as well as the use of encapsulation to promote islet survival and protect islets from immune rejection.

307 - CASE OF SUCCESSFUL ENDOSCOPIC PANCREATIC ISLETS AUTO-TRANSPLANTATION INTO GASTRIC SUB-MUCOSA IN PATIENT WITH CHRONIC PANCREATITIS – PRELIMINARY REPORT

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Chronic pancreatitis (CP) is a severe illness which may cause significant deterioration of patients well-being because of constant pain. Pancreatic cancer and diabetes are co-morbities which often follow CP. Pancreatic resection following pancreatic islets autotransplantation might be a successful therapeutic option for selected group of those patients. Unfortunately liver cirrhosis, portal vein thrombosis or hepatic virus infection (B or C) make standard transplantation into portal vein impossible. Alternative implementation sites might be an option for those patients. Successful islets transplantation into gastric submucosa in pre-clinical study on pigs[1,2] makes gastric submucosa promising option for future.

Aim of this study was to report a successful resection of the pancreas and endoscopic islets autotransplantation into gastric sub-mucosa in man.

Patient and Methods: A 53-year old man with alcohol related chronic pancreatitis for 15 years was operated on 14th of march 2013. *In anamnesis:* 3 months earlier patient was diagnosed with type 2 diabetes – only dietary treatment. Suffering with chronic pain treated with opioid analgesics, 16-years ago underwent antrectomy due topeptic ulcer perforation, also HBS-Ag positive and diagnosed with thrombosis of right portal vein. Patient underwent pancreatectomy and splenectomy modo Child. Pancreas was immediately flushed with 1000 ml of UW solution through splenic artery and vein and sent to isolation facility. Islets were isolated, Cobe procedure was not performed. Endoscopic gastric sub-mucosa transplantation was performed 6 hours later. Levels of fasting c-peptide was measured prior to procedure and 1, 2, 3, 4, 5, 7, 14 and 30 days post autotransplantation. C-peptide stimulation (CPS)test was performed prior to procedure and one month post autotransplantation. Fasting glicemia and oral glucose tolerance test (OGTT) was performed prior and 7 and 30 days post transplantation. Control gastroscopy and endoultrasonography (EUS) were performed within first week post-trasnplantation.

Results: *Isolation:* 250 000 IEq (unpurified) was isolated – 3700 IEq/ kg of patients' body weight. Pellets had 6 ml and was suspended in 60 ml of ringer solution. *Transplantation:* 18 injection was performed -3 to 5 ml of islets suspension was introduced into submucosa of gastric trunk and fundus. No complication during procedure was observed. *Post-operative period*: Patient did not require any insulin nor oral hypoglicemics post operation. In early post-operative patient underwent pneumonia and pulmonary embolism successfully treated with antibiotics and Low Molecular Weight Heparin. POST-procedure gastroscopy did not revealed any signs of inflammation or ulceration. EUS did not revealed any fluid collections within gastric wall. PRE-procedure fasting c-peptide was 1.23 ng/ml. POST-procedure C-peptide was (in 1, 2, 3, 4, 5, 7, 14, 30 days): 1.11, 1.27, 0.55, 0.94, 1.08, 0.55, 0.69, 1.2, 1.09 ng/ml, respectively. PRE-procedure CPS-test in 0, 5, 10, 15, 30, 60, 120 min was: 1.22, 1.06, 1.35, 1.71, 4.39, 9.63,5.07 ng/ml, respectively. POST-procedure CPS-test was: 1.09, 1.13, 1.31, 3.9, 5.22, 3.82, 1.93 ng/ml, respectively. PRE-procedure OGTT in 0, 5, 10, 15, 30, 60, 120 min was: 85, 91, 98, 98, 167, 210, 76 mg/dl respectively. POST-procedure OGTT was: 96, 116, 127, 191, 224, 153, 68 mg/dl.

Conclusions: Preliminary results of endoscopic gastric sub-mucosa islets autotransplantation showsthat it might be an alternative option for islets autotransplantation in case of patients with contraindication for transplantation into portal vein.

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308 - ECTOPIC HEPATOCYTE TRANSPLANTATION INTO THE PERI-HEPATIC AND MESENTERIC LYMPH NODES: PRELIMINARY DATA IN A PRE-CLINICAL LARGE ANIMAL MODEL

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Introduction: Liver transplantation continues to be the only effective therapy for end-stage-liver disease

(ESLD). Unfortunately, over 30% of the patients with ESLD initially considered as potential transplant candidates are unsuitable for liver transplantation due to additional medical conditions. This population lacks an effective therapeutic option that would address both the control of their portal hypertension (PH) and the subsequent enhancement of their functional hepatocellular mass (HCM). This protocol was developed to create a reliable site for hepatocyte transplantation in patients receiving transjugular intrahepatic portosystemic shunt (TIPS) as part of their minimally invasive therapy for PH, since TIPS can induce subsequent hepatic atrophy and progressive liver failure.

Methods: A group of 5 Landrace pigs (~70 kg) underwent a left hepatectomy (LH). The right portal vein was subsequently ligated (RPVL) in the recipients (n=3) immediately after the initial LH. Hepatocytes were obtained from the left liver lobe and were transplanted into the lymph nodes (LN) by direct injection. Three perihepatic (PH) and over 10 mesenteric (MT) LN were infused with the same technique. These animals were followed up for 30 days. A subsequent group of animals (n=2) had a full portacaval shunt (PCS) after the initial LH. The main portal vein was fully ligated at the hepatic hylum and reimplanted in the lower inferior vena cava as previously described. The first animal was followed up for 30 days and the second animal for 60 days.

Results: All the animals survived the operative procedures, but displayed a transient weight loss with reversible mild to moderate hepatocellular damage. The RPVL group (n=3) had moderate signs of hepatic encephalopathy (HE), treated with Lactulose. The PCS group (n=2) had moderate to severe signs of HE. Liver function tests (LFTs) showed a progressive pattern of liver damage when the 2 groups were compared. The PCS group showed a higher yield of hepatic engraftment in LN and a higher proliferation index when compared to the RPVL group. The final hepatocellular mass obtained in the ectopic sites was higher in the PCS group followed up for 60 days.

Conclusions: Ectopic hepatocyte engraftment in both the PH and MT lymph nodes can be successfully obtained in an autologous pre-clinical large animal model when a partial hepatectomy followed by liver devascularization procedures are combined. The degree of liver injury has a direct impact in the subsequent level of cell engraftment and proliferation. Ectopic liver mass can be macroscopically identified within the LN while displaying normal parenchymal architecture. In spite having a full PCS, the animals showed signs of progressive liver regeneration, contrary to the progressive liver atrophy previously seen in dogs and primates. Ectopic hepatocyte engraftment into the LN followed the same pattern as previously described in mice models[1, 2].

We would like to thank Dr. Roberto Gramignoli, Dr. Stephen C. Strom and Dr. Hongzhi Xu for the isolation and preparation of swine liver cells.

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309 - AN ISOLATED VENOUS SAC AS A SUITABLE PLACE FOR PANCREATIC ISLET TRANSPLANTATION.

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Background: Cell therapy with transplantation of pancreatic islets is of significant interest for type-1 diabetes mellitus. Although transplanted islets survive after intraportal injection, limited engraftment efficiency and eventual loss of transplanted islets constitutes a major limitation, and alternative approaches are required. We evaluated the feasibility of minimal islet mass transplantation into the isolated venous sac with the potential to correct hyperglycemia in diabetic rats.

Methods: Pancreatic islets were isolated from adult Lewis rats and transplanted into either isolated venous sac or intraportaly in diabetic syngeneic rats. The engraftment, survival and function of transplanted islets was determined by histological analysis of tissues, as well as by glycemic control in animals.

Results: Islets transplanted in isolated venous sac restored euglycemia in diabetic rats, in contrast animals with intraportal transplantation of islets remained diabetic. Morphological studies revealed

that islets transplanted in isolated venous sac had normal morphology, and maintained expression of insulin. Removal of islet graft-bearing venous segments in diabetic rats led to recurrence of hyperglycemia.

Conclusions: We demonstrated that minimal mass of pancreatic islets can be successfully engrafted into the isolated venous sac and restore euglicemia in STZ diabetic rats. Transplantation of pancreatic islets into the isolated venous sac would prove a major step forward, can be clinically applicable and eventually can solve problems encountered with the intrahepatic islet transplantation.

310 - THE RELEVANCE OF SITE ON IMMUNE RESPONSES AFTER ISLET TRANSPLANTATION: BONE MARROW (BM) VS LIVER

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Background: Using a syngeneic mouse model of islet transplantation (Tx) we have demonstrated that islet infusion in the BM is safe and more efficient in restoring normoglycaemia than liver.

Aim: The aim of our work is to evaluate whether immune responses after islet infusion are site-dependent (BM vs Liver).

Methods and Results: A MHC-full-mismatched mouse model (C57BL/6 islets in alloxan-induced Balb/c mice) was used to evaluate alloimmune response. The time of rejection in the absence of immunosuppression was 6 ± 0.82 and 6 ± 0.35 for Liver-Tx (n=8) and BM-Tx (n=9), respectively (p=0.896). The time of rejection in the presence of immunosuppression (MMF+FK-506) was 10 ± 1.22 and 8 ± 2.45 for Liver-Tx (n=5) and BM-Tx (n=7), respectively (p=0.3). A more stringent MHC-full-mismatched mouse model (Balb/c islets in alloxan-induced C57BL/6 mice) was used to characterize leucocytes infiltration at site of islet infusion at 0, 1, 3, 5, 7, 10, 14 days after Tx by flow cytometry analysis. $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were preferentially recruited at 7-10 days after Tx in the BM with islets in comparison to the controlateral sham operated. A single-antigen-mismatched mouse model (RIP-LCMV-GP+/- C57BL/6 islets in alloxan-induced C57BL/6 mice) was used to evaluate single-antigen islet specific response. To evaluate the ability of islet-specific response to reject already engrafted islets, RIP-LCMV-GP+/- C57BL/6 islets were transplanted in liver (n=12) or BM (n=8) of alloxan-induced RIP-LCMV-GP-/- C57BL/6 diabetic mice. Post transplant LCMV infection induced GP-specific immune response leading to β-cells destruction within 10-14 daysboth of islet engrafted in liver (n=10) or BM (n=5). To evaluate the ability of GP- specific memory response to reject islet, C57BL/6 diabetic micewere transplanted with RIP-LCMV-GP+/- C57BL/6 islets in liver (n=2) or BM (n=3) 30 days after LCMV infection. Islets were rejected within 5-7 days after Tx without any difference related to the site of infusion.

Conclusions: BM does not represent a disadvantageous microenvironment in comparison to liver in term of immune response, suggesting that could be used as alternative site for type 1 diabetes therapy.

311 - CONTROLLED LOCAL DELIVERY OF PRO-ANGIOGENIC GROWTH FACTORS IMPROVE ISLET TRANSPLANTATION IN THE SUBCUTANEOUS AND THE FAT PAD SITES IN MICE

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Islet transplantation in the liver through infusion into the portal vein is the current protocol for clinical trials. According to the CITR 2013 despite promising results, most patients lose insulin-independence and graft function in variable times after transplantation. Among the causes is early graft loss due to immunological, anatomical, physiological and metabolic limitations of the transplant site. Several alternative sites for islet transplantation have been proposed in order to minimize early inflammatory reactions, promote vascularization and easy-accessibility, mimic physiological insulin release and protect from immune responses. Among these is the subcutaneous (SC) site which has easy accessibility but trials in this site have been disappointing due to poor oxygen tension and blood supply and lack of neovascularization. Another alternative site is the omental pouch which can be well represented (being too small in mice) by the epididymal fat pad (EFP) that is well vascularized with good arterial supply, portal drainage and can accommodate large volumes including unpurified islets.

We have engineered the SC and the EFP sites for islet transplantation to promote angiogenesis with the goal of decreasing early graft loss and improving islet engraftment. We have utilized novel fibrin matrices as biodegradable scaffolds for local controlled release of pro-angiogenic growth factors through proteolitically cleavable recombinant fibronectin proteins that can bind both cells involved in tissue re-vascularization and growth factors. We chose to use degradable matrices to prevent fibrotic reactions that have been observed for permanent scaffolds. We show that in the SC site, 60% of mice transplanted with a marginal mass of 1,000 IEQ syngeneic islets within engineered matrices reverse diabetes at 40 days post-transplant (average reversal time 38 days, n=5) versus 20% of mice transplanted with islets alone after 100 days (undefined average reversal time, n=5). In the SC site, islet engraftment in engineered matrices is associated with neogenesis of blood vascular networks by day 7 and full vascularization by day 21. In the EFP site, 60% of mice transplanted with a marginal mass of 250 IEQ syngeneic islets within engineered matrices (average reversal time 24 days, n=10) and 10% of mice transplanted with islets alone reverse diabetes after

40 days (average reversal time: 85.5, n=8). In EFP grafts, islet density in grafts retrieved after 100 days and density of blood vessels is higher in the engineered matrices group ($p<0.05$). Also, proportion of beta and alpha cells in grafted islets is comparable to the one found in native pancreas. Our findings suggest that by promoting early re-vascularization of islet grafts we can promote engraftment and long-term function in the SC and EFP sites, which are clinically relevant sites.

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312 - INTRA BM ISLET TRANSPLANTATION: MODULATION OF MICROENVIRONMENT TO IMPROVE ENGRAFTMENT

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Background: Bone marrow (BM) has been recently proposed as an alternative site for islet transplantation (tx). The BM, for its structure and anatomical position, offers the possibility to modulate microenvironment by local interventions.

Aim: The aim of our study was to investigate whether local irradiation and/or mesenchymal stem cell (MSC) co-tx in mouse are able to improve islet engraftment and prevent rejection in BM.

Methods: A model of BM local irradiation was set up: we established irradiation conditions (932 rad), verified selective cell depletion and chose the time point for tx (3 days after treatment). MSCs from bone marrow (BM-MSCs) or pancreatic tissue (pMSCs) were isolated and characterized for biological and immunomodulatory properties to choose the best candidate for *in vivo* co-tx. Gain of normoglycaemia and time to rejection were evaluated in a fully MHC mismatched model of intra BM islet tx (400 C57BL/6 IEQ in BALB/c). Islets with or without 300,000 syngeneic MSCs were alternatively infused into irradiated or control femur.

Results: Islet tx into locally irradiated BM had better outcome compared to not irradiated recipients in terms of capacity to gain normoglycaemia (100% vs 55% in irradiated vs not irradiated mice, $p=0.069$). Glycaemia in the first two weeks after tx was significantly lower in the group of irradiated mice ($p=0.047$) while time of rejection was not different among the two groups. Pancreatic MSCs showed morphology, phenotype and plasticity comparable to BM-MSC and strong immunomodulatory properties *in vitro*, resulting the best candidate for co-tx. Preliminary results showed that co-tx of pMSCs and islets in irradiated recipients improved the probability to gain normoglycaemia (100% vs 33% in islets+pMSCs vs islet alone group, respectively) and delays the time of rejection (10.2 ± 4.3 vs 4.0 ± 0 days in islets+pMSCs vs islet alone group, respectively).

Conclusions: Local irradiation of the site of implant and co-tx of pancreas-derived MSCs and pancreatic islets in the BM are promising strategies which can be combined together for the modulation of islet engraftment and survival.

313 - MICROENCAPSULATION OF PANCREATIC ISLETS THROUGH CONFORMAL COATING OR THROUGH STANDARD ALGINATE CAPSULES FOR TRANSPLANTATION IN ABSENCE OF SYSTEMIC IMMUNOSUPPRESSION IN MICE

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Islet transplantation (IT) may cure type-1 diabetes (T1D) but current protocols require life-long systemic immunosuppression (SI) to prevent graft rejection. SI is the main cause of adverse events associated with IT. In order to increase safety of the IT procedure and to treat young patients, an alternative and safer anti-rejection therapy needs to be implemented. Immunoisolation of pancreatic islets (PI) with biocompatible hydrogels through cell encapsulation allows transplantation in absence of SI in rodents but has failed in pre-clinical and clinical settings. Reasons for these failures are unknown. We hypothesize that the large size of conventional microcapsules along with *in vivo* instability of encapsulation materials are among the possible causes of failure. The large size of microcapsule (600-1000 μm) diameter reduces diffusion of oxygen and nutrients to enclosed cells and causes delay in insulin secretion. Also, large microcapsule size increases IT volume thus limiting the choices of transplant site to the peritoneal cavity, which is not ideal due to the non-optimal kinetics of insulin absorption and lack of vascularization. In addition to microcapsule size, conventional encapsulation materials like alginate are subjected to *in vivo* remodeling, including swelling and change in permeability and permselectivity to molecules that are involved in triggering immunological responses.

To address this we have developed and optimized a method to conformally coat islets by shrink-wrapping them with polyethylene glycol (PEG) hydrogels that minimizes capsule size and graft volume while controlling coating perm-selectivity and *in vivo* stability. We have shown that rodent islets can be completely coated

with a thin (10-20μm) layer of hydrogel that does not affect islet viability and function *in vitro* (as assessed by live/dead and ROS staining, GSIR index and delta, perifusion up to 14 days) and *in vivo* in syngeneic murine models of islet transplantation (700 IEQ/mouse, kidney capsule). We have also shown that conformal coatings are immunoisolating and prevent rejection of fully MHC-mismatched pancreatic islets in mice in a bioengineered epididymal fat pad (EFP) site (1000 IEQ/mouse) without SI (100% protection from allorejection >100 days PTX vs. 100% rejection of naked islets at 9-12 days, n=2). In addition to this we have optimized conventional alginate microencapsulation by minimizing capsule size (450-550μm in diameter) and by using ultra-pure clinical grade materials that are highly biocompatible. Optimized microcapsules transplanted in a bioengineered EFP site reverse diabetes at day 1 PTX and maintain glucose homeostasis. We are currently comparing the safety and efficacy of conformal coating encapsulation with optimized alginate microencapsulation *in vitro* and *in vivo* in rodent models of islet allotransplantation.

Our findings suggest that conformal coating allows complete immunoisolation of allografts and it might allow cell transplantation in absence of SI. Future work will focus on determining whether conformal coating encapsulation is superior to alginate microencapsulation for islet transplantation in absence of SI.

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314 - IN VITRO IMMUNOGENICITY OF ALGINATE MICROENCAPSULATED HUMAN HEPATOCYTES

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Background: Transplantation of alginate microencapsulated human hepatocytes without the use of immunosuppession is an attractive approach for the management of acute liver failure. The biocompatibility of the alginate microbeads is an important issue for efficacy following transplantation into the peritoneum. The microbeads themselves or antigen shedding through microcapsule pores, may initiate a host immune response from the encapsulated cells and subsequently lead to an inflammatory reaction and cell death.

Aim: To investigate the alloimmune response toward clinical grade microencapsulated human hepatocyte *in vitro*.

Materials and Methods: Microbeads were produced using an encapsulator (250μm nozzle) with sterile grade, highly purified sodium alginate (PRONOVA™ SLG20: low viscosity, high guluronic acid). Empty and human hepatocyte ($3.5x10^6$ cells/ml alginate; n=4) microbeads were polymerised in 1.2% calcium chloride. Peripheral blood mononuclear cells (PBMCs) were obtained from four healthy adults. PBMCs were either cultured alone (monoculture) or co-cultured with empty or hepatocyte alginate microbeads. The ratio of PBMCs to hepatocytes was 1:5. Microbead morphology was examined before and after co-culture with PBMCs. The frequency of activation of T-lymphocyte, B cells, NK cells and monocytes was analysed 24h post co-culture by flow cytometry using antibodies to CD3, CD4, CD8, CD25, CD14, CD40L, CD38, CD56, CD19, and CD54.

Results: Empty and hepatocyte microbeads were of uniform shape and size (diameter: 500 ± 100μm). After 24hr in co-culture with PBMCs, both empty and hepatocyte microbeads were intact and maintained their uniform shape with no PBMCs adherent to their surface. There was no significant difference in the level of activation markers expression on PBMCs co-cultured with empty or hepatocyte microbeads compared to PBMCs in monoculture. Interestingly, human hepatocyte microbeads decreased the frequency of $CD14^+CD25^+$ activated monocyte on PBMCs (mean 2.66 ± SEM 0.80) in co-cultures compared to PBMCs monocultures (45.65 ± 10.98).

Conclusions: This study provides evidence that clinical grade human hepatocyte microbead biocompatible with the human peripheral immune system *in vitro*.

315 - PROLONGED EUGLYCEMIA FOLLOWING INTRAPERITONEAL TRANSPLANTATION OF ENCAPSULATED PORCINE ISLETS

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The limitations of current islet transplantation including the scarcity of organ donors, consistency of human islet isolations and the negative complications of chronic immunosuppression have stimulated researchers to explore both xenoislet transplantation and encapsulation to protect transplanted islets from the immune response. Our research group's focus has been the development of a novel, robust, and scalable method of isolating and purifying piglet islets coupled with a stable alginate based microcapsule. **The aim of this study was to demonstrate survival and function of these encapsulated porcine islets transplanted in diabetic immunocompromised and immmunocompetent mice**.

Six to eight-week old Athymic Nude (Nude) and C57BL/6 mice (n=20 per group, 10M/10 F) were rendered diabetic with a single IV dose of streptozotocin (150 mg/kg). Diabetes was confirmed after 3 consecutive days of hyperglycemia and mice were maintained on insulin (Lantus, 1-2u/day SC) until islet transplantation. Control, non-transplanted mice (Nude and C57BL/6) were maintained on insulin for 28 days. Pancreatic tissue from preweaned Yorkshire piglets (22 ± 0.4 days old) was cultured (37°C/5%CO_2) after partial enzymatic digestion. After 7 days of culture, islets were then encapsulated in 3% alginate (capsule diameter 487 ± 2.7um). Groups of diabetic mice were then transplanted with 3,000 encapsulated porcine islets into the peritoneal cavity. Mice were monitored daily for general health, 3 times a week for non-fasting blood glucose levels and weekly for body weight. Islets were evaluated post isolation and after explantation using islet enumeration (IEQ), viability (Newport green/PI) and function (GSIR, Stimulation Index (SI)=insulin released in 28mM glucose over insulin released in 2.8mM glucose). No immunosuppression was administered to any mice.

Prior to transplantation, capillary non-fasting blood glucose in non-transplanted and control diabetic mice averaged 551.7 ± 24mg/dL in Nude and 504.5 ± 43 in C57BL/6 mice (mean ± sem). All transplanted mice became euglycemic after islet transplantation. Average blood glucose levels were at 30 days (167.2 ± 23mg/dL, Nude; 141 ± 24mg/dL, C57BL/6), at 60 days (Nude 139.0 ± 25mg/dL, Nude; 126 ± 12mg/dL, C57BL/6) and at 90 days (122.9 ± 8 mg/dL, C57BL/6) post transplant.

Explanted encapsulated islets collected at the conclusion of the study were viable (76.5 ± 3% Newport Green/PI) and functional as demonstrated by GSIR SI=1.95 ± 0.4; (n=10) within the alginate capsules.

These studies demonstrate that encapsulated piglet islets, isolated using a novel protocol and encapsulated in alginate microcapsules, can survive and function after intraperitoneal transplant in both Aythmic Nude and fully immunocompentent C57BL/6 diabetic mice. This data is critical to design primate studies and ultimately clinical trials using this technology.

316 - ENDOTHELIAL PROGENITOR CELL (EPC) CO-TRANSPLANTATION ENHANCES THE ENGRAFTMENT OF PANCREATIC ISLETS AND MAY INVOLVE CONNEXIN 36 (CX36) INTERACTIONS

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Islet transplantation is limited by apoptosis of insulin-secreting β-cells and suboptimal revascularization post-transplant. EPCs have the potential to promote islet engraftment, but their mechanism of interaction with β-cells is unknown. Cx36 is expressed on β-cell sand may be involved in intercellular communication within islets. The interaction between islets and EPCs was investigated *in vitro*, and theability of EPCs to improve islet transplantation was tested *in vivo* in a syngeneic marginal mass mouse model. C57B6 islets were isolated by Liberase® digestion and density purification. Bone marrow-derived EPCs were enriched by culture in defined mediumand confirmed to express endothelial markers (CD31 and E-selectin), bind lectin and uptake acetylated low-density lipoprotein by flow cytometry. To investigate soluble mechanisms, islets were cultured with EPC-conditioned medium and tested for glucose-stimulated insulin release. Secreted insulin was detected by ELISA and corrected for total protein. Stimulation indices were calculated as secretion at 20mM glucose divided by basal secretion (at 3mM). EPC-conditioned islets had an increased basal insulin release (0.7 ± 0.22 ng/min/mg total protein; n=7) compared to controls (0.2 ± 0.04 ng/min/mg; n=6; $p<0.05$). However, they had reduced ability to upregulate insulin release in high glucose (stimulation index = 1.3 ± 0.5 compared to 4.2 ± 0.9 for controls; $p<0.05$). Effects on gene expression were analysed by PCR using Taqman® primers (normalizing to the house-keeping gene *B2m*) following non-contact co-cultures where islets were cultured above EPC monolayers. Co-cultured islets down-regulated the expression of Cx36 (0.4 ± 0.05 fold relative to control islets; n=18; $p<0.05$). There was no difference in expression of the Insulin gene between groups. These data suggest that Cx36 expression is modulated during intercellular communication between β-cells and EPCs, resulting in dysregulated insulin *release*. Diabetes was induced in C57B6 mice by streptozotocin (200mg/kg) and a marginal mass of islets (200) was transplanted under the kidney capsule with or without d7 EPCs ($1x10^6$ cells). Blood glucose levels (BGL) were monitored for 28d with cure defined by two consecutive BGL <11.1mM. Graft function was assessed in cured mice by intraperitoneal glucose tolerance test (IPGTT) with 2g/kg glucose. Mice co-transplanted with EPCs had an improved cure rate (83% at d14; n=12) compared to mice receiving islets alone (20%; n=10; $p=0.002$). There was no significant difference in IPGTT.

Conclusions: The interaction of islets with EPCs causes the modulation of Cx36 *in vitro*and the improvement of islet engraftment during pancreatic islet transplantation. This may represent the adoption of a 'survival' islet phenotype where baseline insulin secretion is maintained during vascular remodeling – albeit in a dysregulated fashion.

317 - CO-INFUSION OF INSULIN-SECRETING ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS AND HEMATOPOIETIC STEM CELLS – NOVEL APPROACH TO MANAGEMENT OF TYPE 1 DIABETES MELLITUS

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Introduction: Type 1 diabetes mellitus (T1DM) results from a cell-mediated autoimmune attack against pancreatic beta cells confirmed with positive antibodies against glutamic acid decarboxylase (GAD). Among them 30% of patients develop end-organ failure. Stem cell therapy (SCT) has promising results in regeneration of injured tissues/cells as well as in correcting immune dysregulation. We present our experience of co-infusion of autologous and allogeneic adipose tissue derived insulin secreting mesenchymal stem cells (IS-AD-MSC) along with hematopoietic stem cells (HSC) in a cohort of patients with T1DM.

Materials and Methods: This was an Institutional Review Board approved prospective non-randomized open-labeled clinical trial after informed consent of 20 patients (15 males and 5 females) with T1DM for SCT. Their mean disease duration was 9 ± 5.51 years. Mean age was 19.95 ± 8.35 years with mean weight of 49.9 ± 14 kg. Our study includes GAD antibody positive, T1DM patients with history of diabetic ketoacidosis. Associated findings were keratoconus, parathyroid adenoma producing hyperparathyroidism, attention deficit disorder, depression, mitral valve prolapse, nephritic syndrome and non-autoimmune hypothyroidism each in 1 patient and autoimmune thyroiditis was noted in 2 patients who were diagnosed as polyglandular autoimmune syndrome type-3 during the treatment. They were monitored for blood sugar levels, serum c-peptide, GAD antibodies and glycosylated hemoglobin (Hb1Ac) at 3 monthly intervals post-therapy. Patients/donors were subjected to adipose tissue resection for *in vitro* generation of IS-AD-MSC and bone marrow (BM) aspiration for generation of HSC. Generated SCs were infused in liver through portal circulation since liver is the most tolerogenic organ, in thymus to generate central tolerance and subcutaneous tissue of abdomen since that is the most immunologically privileged site. SC infusion was performed via femoral catheterization under local anesthesia. Exogenous insulin administration was made on sliding-scale with an objective of maintaining fasting blood sugar (FBS) <150 mg/dL and post-prandial blood sugar (PPBS) < 200 mg/dL. Associated findings were managed independently in all patients.

Results: Total mean quantum of SC infused was 99.5 ± 22.5 mL with mean nucleated cell count of 2.38 × $10^4/\mu$L and mean CD34+ 0.57%. Mean CD45-/90+ and CD45-/73+ were 47.22% and 24.66% respectively. All the generated cells expressed transcription factors ISL-1, PAX-6 and IPF-1. No untoward effect of SCT was noted. Variable and sustained improvement in mean FBS, PPBS, HbA1C and serum C-peptide was noted in all patients over a mean follow-up of 43.94 ± 19.8 months. Mean GAD antibody has decreased from 525.15 to 120.15 after treatment. Mean insulin requirement decreased from 60.89 IU/day to 39.76 IU/day. There was no significant difference in acquiring insulinopenic stage between allogeneic and autologous infusion. There was absence of ketoacidotic episodes in all of them after SCT. All of them are feeling good with weight gain and improved energy levels.

Conclusions: Co-infusion of IS-AD-MSC with BM derived HSC offers a safe and viable therapy for T1DM.

318 - CO-INFUSION OF INSULIN SECRETING AND HEMATOPOIETIC STEM CELLS WITH RENAL TRANSPLANTATION OFFERS BETTER GLYCEMIC CONTROL AND PROTECTION OF THE RENAL ALLOGRAFT FROM IMMUNE INJURY IN DIABETES MELLITUS WITH END STAGE RENAL DISEASE – AN INITIAL EXPERIENCE

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Introduction: Diabetes mellitus (DM) is a common cause of end stage renal disease (ESRD). Various factors contribute to wide fluctuations in blood glucose levels and exogenous insulin requirement in these patients even after renal transplantation (RT). Therapeutic options for such patients are simultaneous kidney-pancreas transplants or stem cell therapy (SCT) with RT. We present an initial experience of SCT preceding RT in diabetic nephropathy leading to ESRD.

Material and methods: Five patients (4 males, 1 female, mean age 39 ± 11 years) suffering from DM since 12.4 ± 4.7 years and ESRD since 7.8 ± 1.64 months, underwent living donor RT (LDRT) following co-infusion of *in vitro* generated insulin-secreting cells differentiated from donor adipose tissue derived mesenchymal stem cells (ADMSC) and bone marrow (BM)-derived hematopoietic SC (HSC) into subcutaneous tissue, portal and thymic circulation. Pre-infusion conditioning was done with Bortezomib, 1.3 mg/m^2 body surface area with methylprednisone 125 mg, on day 1, 4, 8 and 11, and rab-

bit anti-thymocyte globulin, 1.5 mg/kg BW on day 12. SC were infused on 14th day and RT was performed on any day from 16th to 19th day after favourable immune response (lymphocyte cross match).

Results: Total quantum infused was 86 ± 16 ml, out of which 2 ml were infused in thymus, 54 ml in portal and 30 ml subcutaneously. Total nucleated cell count was 5.05 ± 0.83 x 10^6/kgBW, HSC CD34+, 2.85 ± 1.43x 10^4/kgBW, ADMSC CD 90+/73+, 0.82 ± 0.26 x 10^4/kgBW and insulin-making cells 1.48 ± 0.28 x 10^4/kgBW. SC infusion was uneventful.

Over a follow-up of 7.97 ± 4.91 months, their pre-transplant weight of 56 ± 16 kg is sustained at 59.6 ± 15.2 kg, fasting and post-prandial blood sugar of 213 ± 26mg/dL and 295 ± 30mg/dL respectively sustained at 94 ± 5.2mg/dL and 145 ± 21 mg/dL respectively, and HbA1c of 9.1 ± 0.54 % is reduced to 6.7 ± 0.40% with sustained insulin requirement of 25 ± 10 IU/day which was 75 ± 29 IU/day before ESRD and 32 ± 12 IU/day pre-transplant. They all have stable graft function with serum creatinine, 0.96 ± 0.05 mg/dL and zero rejection on Tacrolimus, 0.05 mg /kg and prednisone, 10 mg /day.

Conclusion: This initial single centre experience of co-infusion of insulin secreting and hematopoietic stem cells subcutaneously and in portal and thymic circulation followed by renal transplantation shows better glycemic control and protection of the renal allograft from immune injury in diabetes mellitus with end stage renal disease. SCT with RT will open up safe and effective avenues for diabetic nephropathy patients.

319 - HUMAN HEPATOCYTE TRANSPLANTATION

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Liver transplantation is the accepted method of treatment for end stage liver disease. The improvements in patient and graft survival have mainly resulted from the developments in immunosuppressive drug therapy. Advances in surgical techniques, now allow the use of auxiliary liver transplantation in the management of patients with acute liver failure and certain liver based metabolic defects such as Crigler-Najjar syndrome, urea cycle defects, and familial hypercholesterolaemia. The success of auxiliary transplantation in humans has supported the observation in animal experiments that relatively small amounts of liver tissue can correct the underlying metabolic defect. This has led to the interest in using human hepatocytes for cell transplantation in the management of various liver-based metabolic conditions and acute liver failure.

There are a number of potential advantages of hepatocyte transplantation if the technique can prove successful. It avoids the risks and undertaking of major surgery and as the native liver is still in place can help bridge a patient to whole organ transplant or allow liver regeneration in the case of acute liver failure. There is the possibility of better utilisation of donor organs which remain in short supply, particularly if methods can be developed to isolate good quality hepatocytes from marginal donor livers, currently rejected for clinical transplantation. Hepatocyte transplantation has been used as a new treatment for acute liver failure and metabolic liver diseases such as Crigler-Najjar syndrome type I , glycogen storage disease type 1a , and urea cycle defects for long-term correction of the underlying metabolic deficiency.

Considerable progress has been made in bringing hepatocyte transplantation to the bedside. However, there are a number of areas for improvement and development. In terms of the livers currently available to isolate hepatocytes, fatty livers are those most commonly rejected for clinical transplantation and represent an important potential source for hepatocytes. Thus improvement of the outcome of isolation and purification of cells from fatty livers is key part of future research plans.

There is no doubt that stem cells offer the potential to overcome the current limitations of both supply of hepatocytes and the extent of repopulation of the liver after transplantation. Human foetal hepatocytes, presumably with greater numbers of stem cells, were administered intraperitoneally to patients with fulminant hepatic failure in 1994. Our group has since used alginate micro encapsulated human hepatocytes in the management of acute liver failure in new born babies with success in avoiding transplantation or as bridge to transplantation.

Sources of stem cells for therapy could be foetal hepatocytes, cord blood, embryonic, and bone marrow. This is the focus of research world-wide on stem cell biology and there is no doubt that there are many hurdles to cross before any clinical application. If these are overcome then stem cells could differentiate into all types of liver cells, be easier to cryopreserve and thaw with good function, have proliferative capacity *in vitro* and *in vivo*, may be less immunogenic and could be used for *in vitro* gene therapy. Autotransplantation of stem cells would avoid the need of immunosuppression and its attendant problems and this could be combined with specific gene replacement eg. ornithine transcarbamylase, bilirubin glucuronyltransferase.

In summary the experience gained so far in the handling of hepatocytes and hepatocyte transplantation gives a good basis for the application of the stem cell technologies now being developed.

320 - HEPATOCYTE TRANSPLANTATION AND REGENERATION IN THE TREATMENT OF LIVER DISEASE

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Hepatocyte transplantation holds promise as an alternative to organ transplantation for the treatment of liver disease. Clinical trials of hepatocyte transplantation have demon-

strated the long-term safety of the procedure, but only partial and short-term correction of metabolic disorders has been achieved. In the laboratory, host conditioning with low-dose liver-directed radiation facilitates repopulation of the native liver by transplanted hepatocytes and can lead to complete correction of models of hereditary metabolic deficiencies. Since the ability to diagnose and treat cell transplant rejection is limited, monitoring of peripheral blood donor specific CD154+ T cell activity for rejection risk following hepatocyte transplantation may allow optimization of immune suppression to produce better long-term disease correction. With respect to the most common indication for liver transplantation, decompensated end-stage cirrhosis, we have discovered that chronic injury stably reprograms the critical balance of transcription factors and that diseased cells can be returned to normal function by re-expressing a subset of critical transcription factors, a process similar to the type of reprogramming that induces somatic cells to become pluripotent or to change their cell lineage. Forced re-expression of HNF4α re-induces expression of the other hepatocyte-expressed transcription factors, immediately restores the phenotype of diseased hepatocytes *in vitro*, and rapidly reverses terminal liver failure *in vivo* by phenotypically correcting diseased hepatoctyes, not by stimulating their replacement by new hepatocytes or stem cells.

321 - THE EX-VIVO ASSEMBLY AND TRANSPLANTATION OF A LGR6+ EPITHELIAL STEM CELL-DERIVED HYBRID GRAFT USING COMMON ACELLULAR MATRICES TO AUGMENT WOUND HEALING AND NASCENT HAIR GROWTH IN TISSUES DEVOID OF ADNEXAL STRUCTURES

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Background: Previous research shows that the migration of the Leucine-rich repeat-containing G-protein coupled Receptor epithelial stem cell (LGR5+ and LGR6+ ESC) into wounds augments healing and permits nascent hair growth in areas devoid of these cells following local full thickness soft tissue trauma. Here, we assess the LGR-expressing cell's potential as a hybrid graft in full thickness soft tissue wounds with the goal of developing an easily assembled biological dressing to provide patients with their own focal epithelial stem cell population, capable of growing functional, hair producing skin.

Methods: GFP expressing LGR6+ ESCs were isolated from C57BL/6 (UBC-GFP) mice using FACS gated on CD34, CD73, and LGR6. These cells were then seeded on a variety of acellular matrices. Viability and attachment was assessed using confocal, scanning electron microscopy and bioluminescence. LRG6+ ESC seeded matrices were placed into acute 3rd degree burn wound beds of Nu/Nu mice and monitored for viability, wound healing, and growth factor expression. Bioluminescent imaging supported LGR6+ ESC confluence within the graft as well as transplant viability. Healing rates were documented and compared between mice receiving the LGR6+ ESC enriched grafts vs. control. On days 0, 5, 10 and 15, grafted areas were harvested and assessed for gross, microscopic and molecular healing using digital imaging, immunofluorescence, RT-PCR, and angiogenic proteome array analysis.

Results: LGR6+ seeded matrices show significantly enhanced gross wound healing and up regulation of key WNT, EGF, VEGF and angiogenesis mRNA transcripts and peptides, when compared to matrix-only and negative controls. Additionally, those full thickness wound beds receiving the LGR6+ ESC hybrid grafts were capable of growing nascent hair from the wound bed within a Nu/Nu murine model.

Conclusions: Here, we suggest a novel role for an LGR6+ ESC-enriched hybrid graft for the development of a fully transplantable living matrix for use in tissue engineering and reconstructive transplantation. Furthermore, with the epithelium being a major source of alloantigenicity, this system can be used to develop a series of chimeric epithelial expansion foci for engraftment within more complex composite tissue allografts in order to promote tolerance.

322 - MESENCHYMAL STROMAL CELLS (MSCS) PRIMED WITH PACLITAXEL AS TOOL FOR CARRYING AND DELIVERING THE DRUG IN CANCER THERAPY

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Mesenchymal stromal cells (MSCs) can be easily isolated from bone marrow and adipose tissue and cultured and expanded *in vitro*. Their migratory capacity and tropism to solid tumors (both primary and metastatic) have increased the interest in their use as a "carrier" to transport molecules for cancer therapy. Many methodologies have been described to create "engineered" MSCs capable of secreting therapeutic cytokines, pro-drugs or inhibitory factors.

As previously demonstrated, MSCs derived from human bone marrow, if exposed to high doses of Doxorobicin, are able to inhibit, without any manipulation, the proliferation of hematopoietic stem cells (HSCs). We therefore assessed whether human mesenchymal stromal cells (hMSCs) *in vitro* loaded with Paclitaxel (PTX), were able to release the drug in sufficient quantity to inhibit the proliferation of tumor cells. The incorporation of the drug into hMSCs was analyzed by FACS and confocal microscopy, using PTX FITC-labeled probe, and the release of PTX in the cellular culture medium (CM) has been demonstrated by liquid chromatography HPLC. The hMSCs loaded with PTX (hMSCsPTX) show some degree of chemo-apoptosis, but 80% of the hMSCsPTX are inhibited to proliferate maintaining their viability and capacity to release PTX in the CM in a time-dependent manner. The ultrastructural analysis of hMSCsPTX with transmission electron microscope (TEM) showed that the treatment with PTX does not induce morphological alterations. Leukemic cells are attracted and bound by MSCs as demonstrated by the formation of "rosettes" (aggregates of hMSCs surrounded by a "crown" of leukemia cells MOLT-4) in *in vitro* co-cultures. The morphology of rosettes analyzed with TEM and SEM (scanning electron microscopy) revealed the presence of cytoplasmic and nuclear damage in MOLT-4 adherent to hMSCsPTX.

The anti-tumor effects of hMCSsPTX were demonstrated by *in vitro* experiments: CM obtained from hMSCsPTX inhibit the proliferation of MOLT-4 and other human tumor lines (DU-145 prostate cancer, glioblastoma T98G). Furthermore, *in vivo* experiments in nude mice have demonstrated that hMSCsPTX co-injected subcutaneously with tumor cells (MOLT-4, DU145 and U87MG glioblastoma), inhibit the early stages of tumor proliferation; if hMSCsPTX were injected into preformed tumoral nodules, they reduced the capacity of engraftment and tumor vasculature. We demonstrated that also mature stromal cells, as human skin derived fibroblasts (hSDFs), had the same properties of hMSCs. *In vitro* experiments showed that hSDFs loaded with PTX (hSDFsPTX) released the drug in a time-dependent manner and their CM inhibited tumor growth *in vitro*.

Our data demonstrate that hMSCs, without the need of any genetic manipulation, can be used as "drug carrier" opening their application for new complementary anticancer therapeutic approach.

323 - MESENCHYMAL STEM CELLS AS DELIVERY VEHICLE OF PORPHYRIN LOADED NANOPARTICLES: EFFECTIVE PHOTOINDUCED IN VITRO KILLING OF OSTEOSARCOMA

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Osteosarcoma (OS) is the most common primary tumor of human bone and the most frequent bone sarcoma in children and adolescents[1]. Current treatments consist of multiple modalities, traditionally including amputation or limb sparing surgery as well as chemotherapy. The estimated 5-year survival is 65%. Unfortunately, most of the current drugs are infused systemically and cause harsh side effects. The poor outcome in the efficacy of therapy is due in part to an inability to deliver the drugs to the infiltrative tumor cells. Therefore, significant efforts need to be undertaken to develop new delivering strategies. One approach is to dispense therapeutic agents using mesenchymal stem cells (MSC) which have the unique ability to home and engraft in the tumor stroma. The therapeutic potential of MSC is in fact not only linked to a broad spectrum of biological activities such as anti-inflammatory, immunomodulative and tissue reparative activities thanks to expression of genes encoding a large variety of important growth factors and cytokines. They also could represent an ideal vehicle for targeted drug delivery, since they can be loaded with therapeutic agents, while maintaining their ability to migrate to sites of disease[2].

In this study we evaluated the efficacy of a combined strategy in which MSC are used as a delivery vehicle for photodynamic therapy (PDT), an approach that applies light and molecular oxygen in combination with a photosensitizing agent[3], to selectively eliminate cells.

For our purposes, we used biocompatible multi-functional core-shell poly(methylmethacrylate) (PMMA)-based core-shell nanoparticles (NPs) loaded with meso-tetrakis(4-sulfonatophenyl) porphyrin (TPPS). In addition, NPs are fluorescently labeled by incorporation of fluorescein (FNPs) in the inner hydrophobic core, while the external shell is decorated with amino groups and a number of ammonium salts, able to electrostatically bind TPPS (TPPS-NPs). These molecules are well known to exhibit a high photo-activity and to generate ROS after excitation with 405nm emission wavelength source. ROS species represent the cytotoxic agents responsible of cell death. The very stable and light-activable TPPS-NPs are then efficiently loaded into MSC.

Our results demonstrate that TPPS-FNPs are efficiently taken up by MSC at a concentration of 45μg/ml without evident sign of toxicity, as shown by cytofluorimetric analyses and different proliferation/cytotoxicity assays. Then, through laser confocal microscopy and time lapse imaging of TPPS-FNPs-MSC co-cultured with OS cells *in vitro*, we tested the ability of this system to induce cell death when stimulated with laser light. In order to roughly mimic *in vivo* conditions, we carried out the co-culture experiment with a 1:5 (MSC *vs* OS cells) ratio, demonstrating the high effectiveness of our system even in this environment. TPPS-FNPs loaded MSC can

in fact induce controlled and massive cells death of themselves and of OS cells in a short time frame right after stimulation with laser light. Collectively these encouraging preliminary data indicate that our bio-system could represent an efficient targeted delivery strategy in killing human OS cells. Our results proponea novel yet therapeutic option for the treatment of bone sarcomas and other tumors, such as breast cancer and gliomas, all of which currently require different therapeutic approaches to overcome recurring and drug treatment failures.

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324 - ISOLATION AND CHARACTERIZATION OF MULTIPOTENT CELLS FROM HUMAN FETAL DERMIS

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The use of human fetal cells may have interesting applications in Regenerative Medicine. Particularly, the fetal tissue is expected to contain a larger number of "immature" cells than the adult tissue, without posing the ethical dilemmas of the embryonic tissue. We report that cells from human fetal dermis, termed multipotent fetal dermal cells (MFDCs), can be isolated with high efficiency by using a non-enzymatic, cell outgrowth method. The employed isolation method is rapid and simple, does not require a purification step, and it is easily reproducible. Moreover, it yields a quite homogeneous cell population which could be expanded up to 28 passages under standard culture conditions. The expanded cell population have features of mesenchymal stromal cells. MFDCs were plastic-adherent, had fibroblastic appearance, were >90% CD90, CD105, and CD73-positive, and negative for hematopoietic markers CD34, CD14, CD45, and for HLA-DR. Moreover, MFDCs exhibited osteo-, adipo- and chondrogenic differentiation capacities. As MFDCs proliferate extensively, with no loss of the multilineage differentiation potential up to passage 25, they may be an ideal source for cell therapy to repair damaged tissues and organs. In addition, MFDCs were not recognized as targets by lymphocyte T *in vitro*, thus supporting their feasibility for allogenic transplantation. Moreover, the expansion protocol did not affect the normal phenotype and karyotype of the cells evaluated by classic cytogenetic analysis. When compared with adult dermal cells, fetal cells displayed several advantages, including a greater cellular yield after isolation, the ability to proliferate longer, and the retention of differentiation potential. Interestingly, MFDCs exhibited the pluripotency marker SSEA4 (90.56 ± 3.15% fetal vs. 10.5 ± 8.5% adult), and co-expressed mesenchymal and epithelial markers (>80% $CD90^+/CK18^+$ cells), suggesting a broad potency and a predisposition toward the epithelial differentiation of fetal dermal cells. MFDCs differentiated into epithelial-like cells after DMSO treatment, and were also able to form capillary structures in an angiogenesis assay.

In conclusion,the most interesting aspect of our study is the fact that multipotent cells can be successful isolated from small fetal skin biopsies and maintained in culture for long periods, with retention of multipotency, stability and low immunogenicity, thus generating large quantities of cells and supporting their feasibility in clinical settings. Possible applications of MFDCs could concern tissue engineering for bone reconstruction. If we could obtain bone tissue starting from a small skin biopsy this would dramatically facilitate the use of tissue engineering methods. Given the promising results, the future perspective is to translate the concept of MFDCs as cells of therapeutic interest into experimental models of tissue regeneration.

325 - ISOLATION AND CHARACTERIZATION OF HUMAN ROTATOR CUFF TENDON STEM CELLS

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Rotator cuff (RC) tendons are often prone to lesions and raptures, as 30 to 50% of the population over fifty suffers of partial- and full-thickness RC tears. Several approaches have been developed over the years, including the use of growth factors, bone morphogenetic proteins and, more recently, stem cells. Among adult stem cells, bone marrow mesenchymal stromal cells (BMSCs) are

by far the most studied, although tendon-derived progenitor cells (TSPCs) have been found in several animal species, including humans[1,2]. However, the isolation of a cell population with stem cells characteristics from the human rotator cuff has yet to be reported.

Methods: Human tendon samples (from the *supraspinatus* and the *long head of the biceps*) were collected during rotator cuff tendon surgeries from 26 patients, washed with PBS, cut into small pieces, and digested with collagenase type I and dispase. After centrifugation, cell pellets were resuspended in appropriate culture medium and plated. Adherent cells were cultured, phenotypically characterized, and then compared to human bone marrow stem cells (BMSC), as an example of adult stem cells, and human dermal fibroblasts, as normal proliferating cells with no stem cell properties.

Results: Two new adult stem cell populations (from the *supraspinatus* and from the *long head of the biceps* tendons) were isolated, characterized, and cultured *in vitro*. Cells showed adult stem cell characteristics, *i.e.* they were self-renewing *in vitro*, clonogenic, and multipotent, as they could be induced to differentiate into different cell types, namely osteoblasts, adipocytes and skeletal muscle cells.

Conclusions: This work demonstrated that human rotator cuff tendon stem cells (HRCSC) and human long head of the biceps tendon stem cells (HLHBSC) can be isolated and possess a high regenerative potential, which is comparable to that of BMSC[4]. Moreover, comparative analysis of the sphingolipid pattern of isolated cells with that of BMSC and fibroblasts revealed the possibility of using this class of lipids as new possible markers of the cell differentiation status.

Clinical Relevance: Rotator cuff and long head of the biceps tendons contain a stem cell population that can proliferate *in vitro* and could constitute an easily accessible stem cell source to develop novel therapies for tendon regeneration.

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326 - HUMAN IPS CELLS A SOURCE OF INSULIN-PRODUCING CELLS

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Background and aims: New sources of insulin-secreting cells are strongly required for the cure of type 1 diabetes. Recent successes in differentiating human embryonic stem cells (ESC), in combination with the discovery that it's possible to derive human induced pluripotent stem cells (hiPSC) from somatic cells, have raised the possibility that a sufficient amount of patient-specific insulin-secreting islet-like cells might be derived from patients cells through cell reprogramming and differentiation.

Methods: We performed the differentiation of hiPSC, derived from the reprogramming of both fetal and adult fibroblasts, in insulin-producing cells, optimizing some protocols already established for ESC. The expression of marker genes of pancreas differentiation was measured through real-time PCR analysis (Taqman) and expressed as fold changes (FC) compared to undifferentiated hiPSC. Proteic expression was confirmed by cytofluorimetric analysis. HiPSC differentiated *in vitro* at two different stages (posterior foregut and endocrine cells) were transplanted under the kidney capsule of NOD/SCID mice. One, four, eight and twelve weeks after transplantation the secretion of human C-peptide after oral glucose administration was measured and an immunohistochemical analysis of grafts was performed.

Results: With this protocol we were able to obtain the down-regulation of the pluripotency genes Oct4 and Nanog and the up-regulation of the definitive endoderm genes Sox17 and Foxa2 (28.3 ± 11.8 and 6.5 ± 3.6 FC) and of the pancreatic endoderm genes Pdx1, Ngn3 and Nkx2.2 (7883 ± 153.1, 90 ± 6.8 and 17.6 ± 10.5 FC). At the end of the differentiation process the production of insulin mRNA was highly increased (65484 ± 977 FC) and 5 ± 2.9% cells resulted insulin-positive; terminally differentiated cells also produce C-peptide *in vitro* (1.7 ± 0.1 ng/mL). In mice transplanted with differentiated hiPSC secretion of human C-peptide after glucose stimulus was observed at 1 and 2 months after transplantation (C-peptide mean release at 30 minutes: 0.52 ± 0.1ng/mL). Hystochemical analysis of the grafts showed the presence of pancreatic (Pdx1, ChgB, Syp positive) but also pluripotent cells (Sox2, Oct4, Ki67 positive).

Conclusions: *in vitro* results show that hiPSC, following the stages of pancreatic organogenesis, differentiate in insulin producing cells. Furthermore, *in vivo* study suggests that some differentiated cells engraft and survive in the recipient mice, but highlight the necessity to look for new markers in order to select and transplant only the differentiated pancreatic cells, avoiding the contamination of pluripotent cells with tumorigenic potential.

327 - DIFFERENTIATION OF EMBRYONIC STEM CELLS UNDER MODULATED OXYGEN CONDITIONS INCREASE STAGE 4 NKX6.1+ CELLS AND IN VIVO MATURATION TO BETA CELLS

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Pluripotent stem cells (PSC) hold promise for cell replacement therapy and investigation of embryonic development. However, efficient differentiation to desired cell types remains a major obstacle. Most PSC differentiation is performed in high, non-physiological O_2, but cells during embryonic development are exposed to much lower O_2. Here we report a wide-ranging study showing that physiological O_2 markedly influences differentiation to insulin-producing cells. We differentiated CyT49 human embryonic stem cells (hESC) under different, well-characterized pO_2 environments, controlling cellular oxygen exposure through adhesion culture on highly O_2-permeable silicone rubber membranes,using a modification of the 5-stage protocol reported by ViaCyte, Inc (San Diego, CA) (D'Amour 2006 Nature Biotech). Each stage was examined at multiple controlled high and low oxygen levels, and O_2 conditions were identified that increased the fraction of the appropriate intermediate cell type by flow cytometry or increased expression of appropriate genetic markers by real-time PCR. The best differentiation was produced by an oxygen-modulated protocol. Differentiation under 5% O_2 from hESC to definitive endoderm (stage 1), primitive gut tube (stage 2), and to posterior foregut (stage 3), then under 20% O_2 to pancreatic endoderm (stage 4) and insulin-producing cells (stage 5) gave rise to a cell population that was 43% positive for NKX6.1, after stage 4, and was 10% positive for both c-peptide and NKX6.1 after stage 5. In comparison, differentiation of cells at normoxic oxygen (20% O_2) gave rise to a population that is 33% positive for NKX6.1 after stage 4 but 3% positive for both c-peptide and NKX6.1 after stage 5. Both normoxic and the modulated oxygen differentiations produced cells that passively secreted c-peptide into the medium but were not glucose responsive. Pancreatic endoderm markers NKX6.1 and PDX1were increased by a factor of two and four respectively for the controlled-hypoxia (5% stage 1-3, 20% stage 4-5) when compared to the normoxic condition (20% stage 1-5). After differentiation to pancreatic endoderm (stage 4) under the modulated oxygen condition or normoxia, 1 million were implanted under the kidney capsule of SCID/beige mice to allow maturation into functional beta cells. Human c-peptide was detected in serum of 2/8 animals containing oxygen-modulated grafts (one at 12 weeks, the other at 20 weeks post implantation) and 0/8 of animals with normoxic grafts 60 min after stimulation with glucose. Grafts from the same 2 mice transplanted with modulated oxygen differentiation cells had cells positively immunostained for insulin and for a cocktail of non-beta cell hormones. Mice from both groups had cells positive for non-beta cell hormones but no insulin. Based on these results, O_2 combined with directed differentiation protocols is a potentially straightforward method that could be applied to future hESC therapy protocols for improved differentiation and maturation to beta cells.

328 - RESVERATROL PROMOTES MYOGENESIS AND HYPERTROPHY IN MURINE MYOBLASTS

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Nutrigenomics elucidate the ability of bioactive food components to influence gene expression, protein synthesis, degradation and post-translational modifications.

Resveratrol (RSV), natural polyphenol found in grapes and in other fruits, has a plethora of health benefits in a variety of human diseases: cardio- and neuroprotection, immune regulation, cancer chemoprevention, DNA repair, activation of Sirtuins (SIRT 1), prevention of mitochondrial disorder, avoidance of obesity-related disease. Inskeletal muscle, RSV acts on protein catabolism and muscle function, conferring resistance against oxidative stress, injury and cell death, but itsaction mechanismsand protein targets in myogenesis process are not completely known. Myogenesis is a dynamic multistep process regulated by Myogenic Regulator Factors (MRFs), responsible of the commitment of myogenic cell into skeletal muscle: mononucleated undifferentiated myoblasts break free from cell cycle, elongate and fuse to form multinucleated myotubes. Skeletal muscle hypertrophy can be definite as a result of an increase in the size of pre-existing skeletal muscle fibers accompanied by enhanced protein synthesis, regulated by Insulin Like Growth Factor 1 (IGF-1) and the PI3-K/AKT signaling pathways.

Aim of this work was the study of RSV effects on cell cycle regulation, differentiation process and hypertrophy in C2C12 murine cells.

To study proliferation, cells were incubated in growth medium with/without RSV (0.1 or 25 μM) for 24, 48, 72 hours. To examine differentiation, at 70% confluence, cells were maintained in growth medium or transferred in differentiation medium both with/without RSV (0.1 or 25 μM) for 24, 48, 72, 96 hours. After 96 hours of differentiation, hypertrophy genesis in neo-formed myotubes was analyzed.

Datashowed that RSV could regulate cell cycle exit and induce C2C12 muscle differentiation. Furthermore, RSV might control Myogenic Regulatory Factors expression and muscle-specific proteins synthesis. In late differentiation, we evaluated the positive effects of RSV on hypertrophy: RSV increased AMPK, IGF-1 and ERK 1/2 proteins content and induced hypertrophic morphological changes in neo-formed myotubes modulating cytoskeletal proteins expression.

RSV might control cell cycle to promote myogenesis and hypertrophy *in vitro*, opening a novel field of application of RSV in clinical conditions characterized by chronic functional and morphological muscle impairment.

329 - PERIBILIARY GLANDS STEM CELLS AND PANCREATIC DUCT GLANDS COMMITTED PROGENITORS: STEMNESS, BETA CELL DIFFERENTIATION POTENTIAL AND CELL-SURFACE MARKERS FOR PROSPECTIVE ISOLATION

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The existence and the phenotypic traits of stem/progenitor cells in the postnatal pancreas are actively debated. This is hindering investigations aimed at determining whether such populations might be involved in pathological processes in type 1 and type 2 diabetes. Moreover, ways to isolate stem/progenitor cells from cadaveric donors or surgical resections could pave new paths for regenerative strategies for diabetes. We investigated the presence of cells with stemness signatures in the pancreas and biliary tree of cadaveric donors. We performed *in situ* immunofluorescence for markers of pluripotency, proliferation, early and late pancreatic commitment, and endocrine maturation. We isolated cell cultures under conditions designed for stem cells: Kubota Medium (KM) and plastic adherence. We assessed the *in vitro* differentiation potential of the isolated cells in a serum-free hormonally defined medium (HDM-P) and extracellular matrix components tailored for an islet fate. We tested the *in vivo* differentiation potential into immunocompromised $Rag^{-/-}$/$Ilrg2r^{-/-}$ mice chemically rendered diabetic with streptozotocin. The most primitive stem cell populations were found in peribiliary glands of the hepato-pancreatic common duct: these cells express biomarkers for both liver and pancreas, including markers of pluripotency, endodermal lineage, and early hepatic and pancreatic commitment (NANOG+, OCT4+, SOX2+/-, SALL4+, SOX17+, PDX1+, SOX9+ and NKX6-1+ subpopulations, NGN3-). Populations with signatures of committed progenitors (SOX17-, PDX1+, NGN3+ and SOX9+ subpopulations) were present in PDGs. Populations at different stages of development were discriminated by combinations of the surface markers EpCAM, SSEA4 and Syndecan-1. Cell cultures were isolated in KM and differentiated to a mature fate in HDM-P. The cells matured into glucose-regulatable, insulin-producing cells both in culture and after transplantation *in vivo*. The net findings were that peribiliary glands (PBG) of the biliary tree bear stem cells of endodermal nature and pancreatic duct glands (PDG) harbor pancreatic progenitors. Cells are organized in proximal-to-distal maturational lineages: PBG cells near the duodenum express at high levels markers of pluripotency and early hepato-pancreatic commitment; committed progenitors in PDGs display a loss of pluripotency and hepatic markers and increased expression of pancreatic endocrine maturational markers. Biliary tree stem cells may precur committed progenitors within the pancreas and islet cells.

PBG cells can be instructed to lineage restrict to a specific adult fate under defined culture conditions or if transplanted *in vivo*. Combinantions of the cell-surface markers EpCAM, SSEA4 and Syndecan-1 can be used to distinguish the lineage stage and represent a novel tool for the immunoselection of stem and progenitor cells for the beta cell fate.

330 - HUMAN FETAL HEPATOCYTES CULTURED IN HIGH DENSITY DISPLAY MATURE HEPATOCYTIC FUNCTIONS

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Human hepatocyte transplantation entered clinical practice more than 10 years ago aiming to treat inborn errors of liver metabolism, and also fulminant liver failure with favorable results. However, a major challenge of hepatocyte transplantation is the limited supply of donor organs to isolate good quality cells. Hepatocytes for transplantation are obtained from steatotic livers, or those undergoing a long cold ischemia time, and also from surgical leftovers. We recently reported a phase I-II clinical study where freshly isolated human fetal liver cells (FLCs) were transplanted to bridge patients with chronic liver disease to solid organ transplantation, with encouraging and safe clinical results. The functional characterization of FLCs was conducted through the establishment of primary cultures, and by using

adult hepatocytes and fetal liver MSC-like precursors as positive and negative control cells, respectively.On the basis of morphological and functional evaluations, we distinguished two groups of FLCstermed early-gestation (from 16 to 19 week-gestation) and late-gestation FLCs (from 20 to 22 week-gestation). The early-gestation FLC cultures were populated by various clusters of proliferating cells, while contained few hepatocytes. Consequently, they displayed poor liver-specific functions. On the contrary, the late-gestation FLC cultures contained cells that did not divide *in vitro*, but were functionally competent and similar to mature hepatocytes. Cultured 22-week-gestation fetal hepatocytes in particular, secreted albumin, synthesized urea and showed cytochrome P450 activity, isoform CYP3A4, and glucose-6-phosphatase enzymes at levels comparable to that of their adult counterparts. Treatment with dexamethasone in combination with oncostatin M induced a further maturation of fetal hepatocytes, which acquired additional functions, i.e., the ability to store glycogen and perform uptake of the vital stain indocyanine green (ICG). We also observed that the hepatic functionality was strongly dependent on culture conditions, with a low density culture system leading to rapid loss of the hepatocytic phenotype. In two weeks, these cultures became populated by spindle-like cells that were expanded up to 35 passages, and displayed a predominant mesenchymal phenotype after three subcultivations (approximately 75% $CD90^+$, $CD105^+$, and $CD73^+$). On the basis of these observations, we conclude that late second trimester human fetal hepatocytes might be a valid alternative to adult hepatocytes in liver cell-based therapies, thus overcoming the difficulty of obtaining functional cells from unused livers for transplantation. Moreover, the high frequency of cells with features of precursors isolated from FLC cultures might improve the drawbacks of obtaining intrahepatic stem/precursors for clinical purposes, due to the low frequencies of cell precursors within the organ. Since FLCs can be long-term cryopreserved in the presence of 10% DMSO, without significant loss of viability and functions (e.g., plating efficiency and albumin secretion evaluated up to 1 year storage in liquid nitrogen), our results also suggest a novel approach for FLC transplantation by introducing the use of thawed cells, which will ultimately increase the number of available cells for transplantation.

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331 - BETAIN SUPPLEMENT ENHANCES SKELETAL MUSCLE DIFFERENTIATION IN MURINE MYOBLASTS VIA IGF-1 SIGNALING ACTIVATION

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Betaine (BET) is a component of many food, including spinach and wheat. It is an essential osmolyte and a source of methyl groups. Recent studies have hypothesized that BET might play a role on athleticperformance. However, BET effects on skeletal muscle differentiation and hypertrophy remain not completely known.

In this study, we examined BET action on neo myotubes maturation and myogenesis, using C2C12 murine myoblastic cells. By dose-response study, we determined that 10 mM BET was the dose able to stimulate morphological changes and hypertrophic process in neo myotubes. Using RT^2-PCR array strategy to identify the expression profile of genes, encoding proteins involved in IGF-1 pathway, we found that 10 mM BET could promote IGF-1 receptor (IGF-1 R) expression. Western blot and immunofluorescence analysis pointed out that, in neo myotubes, 10 mM BET improved not only IGF-1 signaling, but also the synthesis of Myosin Heavy Chain (MyHC), the major skeletal muscle marker. Moreover, 10 mM BET increased neo myotubes length.

In addition, we investigated BET role on myoblasts proliferation and differentiation. During proliferation, BET did not modify C2C12 proliferative rate, but promotedmyogenic induction, enhancing MyoD protein content and cellular elongation. During differentiation, BET raised muscle-specific markers and IGF-1 R protein levels.

These findings provide the first evidence that BET could promote muscle differentiation and myotubes size by IGF-1 pathway activation, indicating that BET might represent a possible new drug/integrator strategy, not only in sport performance but also in clinical conditions characterized by muscle function injury.

400 - CLINICAL EXPERIENCE WITH IMMUNOSUPPRESSIVE DRUGS

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Organ transplantation has proven to be an effective therapeutic for a large variety of disease states, but the chronic immunosuppression required for allograft survival increases the risk for infection and neoplasia. In the past 50 years, a wealth of experimental data has accumulated relating to strategies to preserve function and prolong graft survival. These strategies operate by inducing peripheral or central tolerance to the allograft, with protocols based on regulatory T cell induction as the most promising ones. However, as these protocols move into the clinic, there is recognition that little is

known as to their efficacy when confronted with the human immune system: pre-existing memory T cells and "heterologous immunity" in Ag-experienced humans but not in immunologically naive rodents, infections and early activation of innate immune response and the related inflammation-induced cytokine milieu that inhibit Treg activity while augmenting the T effector response, all pose significant barriers to tolerance induction.

A better understanding of cellular and molecular mechanisms by which memory T cells and innate immunity modulate transplantation tolerance and detailed immunological studies of the rare "spontaneous tolerant" patients may lead to development of combined strategies that target and modulate the immune system at multiple levels.

Since the first successful renal transplantation in Boston in 1954, more than a million such procedures have been performed worldwide. By strikingly minimizing the incidence of acute rejection, immunosuppressive drugs have led to overall improvements in allograft and patient survivals. However, the improved short-term survival rates have come at a cost: these drugs generally need to be given for the entire life, induce many indirect and direct side-effects and pose an increased risk of life-threatening complications, infections and malignancies. Furthermore these therapies have had little effect on the inexorable loss of transplanted organs because of chronic allograft rejection.

Recent trends in long-term survival rates have indicated a progressive improvement of renal allograft half-lives, but this has been only observed in patients who never had an acute rejection episode. These data emphasize the critical role of the recipient's alloimmune response as a major determinant of transplant outcome and highlight the need to develop novel strategies to induce immunologic donor-specific tolerance defined as a lack of a destructive immune response towards the graft in the presence of generalized immune competence. Ideally, tolerance should also translate into a lack of chronic rejection and late graft loss.

401 - METABOLIC EFFECTS OF IMMUNOSUPPRESSIVE AGENTS

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Cellular and organ transplantation have the highest rate of success when autologous or from a twin living donor with the notable exception of transplanting cells/organs in presence of autoimmunity. Autoimmunity recurrence make it always necessary the utilization of immunosuppressive agents. Endocrine organs are particularly susceptible to elicit antigens evoking auto-antibodies. Type 1 diabetes, Hashimoto thyroditis, Addison disease, celiac disease are all characterized by organ-specific autoimmunity.

Most immunosuppressive drugs have potent metabolic effects aside the immune-modulation effect. Therefore in endocrine diseases, the action of immunosuppressive drugs must be carefully balanced to avoid negative effects on the graft and the whole recipient as the development of *de novo* diabetes. The metabolic effects of immunosuppressive drugs will be presently reviewed systematically highlighting metabolic methodology required for correct assessment and follow up of the metabolic outcome of the graft.

Genetic and epigenetic factors of donor and recipient which may determine the final outcome of the graft will be reviewed. The panel of drugs and food integrators available to counteract metabolic effects of immune-suppression will be also reviewed.

402 - ANTI-INFLAMMATORY NUTRITION; IMPLICATIONS FOR CELL TRANSPLANTATIONS AND REGENERATIVE MEDICINE

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It is becoming evident that inflammation plays an important role not only in obesity, diabetes and other chronic degenerative conditions, but also in immune-mediated events that could condition long term graft survival, autoimmunity and tolerance induction strategies. The understanding of the molecular mechanisms behind the control of the inflammatory process is only beginning to be understood, but natural components of the diet can affect molecular targets and modulate inflammatory responses with important implications for providing an attractive and cost-effective alternative to more traditional pharmacologic interventions. This presentation will outline linkages between diet, hormones and genetic factors affecting inflammation, and how nutrition could play a role for preventing chronic low-level inflammation and subsequent immune-mediated events that could condition the success of cellular terapies and regenerative medicine clinical applications.

403 - EYE REPORTS ON THE STATUS OF THE ENDOCRINE PANCREAS

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The islets of Langerhans constitute the endocrine pancreas and are responsible for maintenance of blood glucose homeostasis. They are deeply embedded in the exocrine pancreas and therefore their accessibility for functional studies is limited. To understand regulation of function and survival and assess the clinical outcome of individual treatment strategies for diabetes, a monitoring system continuously reporting on the endocrine pancreas is needed. We describe the application of a natural body window that successfully reports on the properties of *in situ* pancreatic islets. As proof of principle "reporter islets" were transplanted into the anterior chamber of the eye of leptin-deficient mice. The "reporter islets" displayed obesity-induced growth and vascularization patterns that were reversed by leptin treatment. Hence "reporter

islets" serve as optically accessible indicators of islet function in the pancreas, and reflect the efficiency of specific treatment regimens regulating islet plasticity *in vivo*.

404 - WHAT HAVE WE LEARNED FROM POST-MORTEM STUDIES IN GRAFTED PATIENTS WITH HUNTINGTON'S DISEASE?

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Cell therapy offers the possibility of replacing degenerated neurons thereby improving the symptoms of neurodegenerative diseases such as Huntington's disease (HD). However, clinical benefits in patients with HD, if any, have been transient and modest. Grafts have been demonstrated to survive well at 18 months in one patient with HD, but graft survival was markedly attenuated by 10 years in two other patients from this transplantation cohort. It is critical to delineate the causes of graft degeneration if such therapies will be utilized in patients with a goal of achieving meaningful clinical benefit. Similar challenges may also accrue to future stem cell therapies. I will discuss the potential causes of suboptimal long-term graft survival in HD patients, which we suggest include allograft immunoreactivity, microglial

405 - DEFINED STEM CELL THERAPEUTICS FOR NEURODEGENERATIVE CONDITIONS. A PROMISING APPROACH

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Transplantation of NSCs to the injured CNS improves neuronal survival and functional outcome in short-medium term experiments are fundamental features already well demonstrated. However, longer term outcomes pose serious problems.Pluripotent embryonic stem cells have the potential to generate a wide variety of cell types but exhibit an increased tumorigenic potential and lack target specificity when used for cell transplantation in the injured nervous system. The use of neural progenitor cells (NPCs) that have already undergone linage restriction reduces concerns about aberrant (non-neuroglial) phenotypes in the fully differentiated daughter cells, although their ability to generate a variety of surviving neuronal phenotypes is less certain. A successful transplantation of NPCs in acute neurodegeneration requires their survival in an unfavourable environment characterized by complex conditions of ischemia-like syndrome and neuroinflammation. Adult NSCs and ES cells accumulate at spinal cord injury (SCI) site, improve functional recovery, and die within 3 weeks. In view of such results we aimed at isolating ischemia-resistant neural precursors (PM-NPCs), their transplantation favours axonal regeneration, formation of a rich neuropile at injury site, and permanent recovery of function in models of spinal cord injury and Parkinson's disease. We have recently been paying attention also to autologous adult stem cell sources. The differentiation potential and the ease of their isolation have made the multipotent mesenchymal stem cells very important for the development of a vast range of clinical applications in regenerative medicine and many laboratory and clinical settings have focused their attention on their use and development. Here we report our findings on human adipose tissue-derived stem cells (hADSCs) obtained from micro-fractured Lipogems-derived adipose tissue. The use of such a device allows the successful establishment of hADSCs colonies even without liberase H1 treatment, and after cryopreservation at -80°C. Differently it is difficult to obtain hADSCs from cryopreserved lipoaspirate. The cell cycle analysis showed that 75% of cells are in G0/G1phase and 21% in S+G2/M, and only a marginal 0.2% apoptosis. No Chromosomal abnormalities. These hADSCs from either fresh or frozen lipogems preparations are bearer of typical mesenchymal markers at values above 90%, and express embryonic markers such as NANOG and OCT4 and neural markers such as nestin, neurod1, pax 6 and musashi. The superficial epitopes are maintained even when hADSCs were grown in culture after storage at -80°C. Their driven osteogenic and adipogenic differentiation *in vitro* yields hADSCs with finer intracellular micro-organelles and fat deposits are more numerous and smaller in size. We shall also present how these lipogems-derived hADSCs can transdifferentiate *in vivo* in specific lineages determined by site of transplantation and condition of application.

406 - LIVER-DIRECTED CELL TRANSPLANTATION IMPROVED SERUM AND BRAIN PHENYLALANINE AND NEUROTRANSMITTER IMBALANCES IN A PHENYLKETONURIA (PKU) MURINE MODEL

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Introduction: PKU (OMIM 261600) is one of the most common inborn errors of metabolism (IEM) with an inci-

dence of 1:16,000 in the USA. The disorder is characterized by deficiency of the phenylalanine hydroxylase (PAH) enzyme, the rate limiting step in phenylalanine (Phe) catabolism. Importantly, Phe is a precursor of tyrosine, which is catabolized to form dopamine (DA). Chronic elevation of Phe in the body, and most importantly the brain, is responsible for PKU pathophysiology (mental retardation, seizures, growth delay). A special Phe-restricted diet can improve Phe imbalance and prevent most severe phenotypes with good compliance, however some patients still endure frequent hospitalizations and persistent cognitive defects. Partial correction of another IEM mouse model by hepatic transplantation of allogeneic hepatocytes[1,2] and human amnion epithelial cells (hAEC)[3,4] despite low functional engraftment provided proof-of-principle for these studies.

Methods: Neonatal PAH-/- mice were given 3 direct hepatic injections of $1x10^6$ syngeneic mouse hepatocytes (HTx) or hAEC (hAEC-Tx) during the first 10 days of life (DOL). Some animals given early HTx were also given a single splenic-directed HTx ($2x10^6$ cells) after 21 DOL. Animals were maintained on normal mouse diet and were sacrificed at 1 or 3 months for analysis.

Results: Phe was severely elevated above wild type in the sera and brains of PAH-/- mice. HTx resulted in a 25% reduction in Phe levels in the sera of PAH-/- females while males were not improved. Human AEC-Tx also reduced Phe 25% in females despite fewer total cells being transplanted. Phe in the sera of untreated female PKU mice was >35% higher than untreated males, which masked post-Tx male results. Importantly, amino acids in the brain showed multiple improvements following transplant, and no sex difference was apparent. Phe levels in PKU-HTx mice were reduced up to 75% while PKU-hAE-Tx mice were normalized. Additional brain amino acids, including those that can act as neurotransmitters, were normalized in PAH-/- animals after HTx. DA metabolites (DOPAC, 3-MT, HVA) and DA turnover (DOPAC+HVA/DA) were disrupted in PAH-/- brains, though DA and tyrosine were unaffected. HTx normalized 3-MT/DA, suggesting DA release was improved. DOPAC was improved ~35% and DA turnover was improved ~25%, some to a level similar to wild type, though statistical significance was not achieved.

Conclusions: HTx and hAE-Tx were able to significantly reduce brain Phe in PAH-/- animals and in the sera of PAH-/- females. The discrepancy between male and female mouse sera Phe is likely unique to the model, as this has not been reported in patients. Importantly, in addition to reduction of brain Phe levels following transplant, multiple other amino acids and neurotransmitters were normalized after HTx. Metabolites along the dopamine pathway, dopamine turnover, and dopamine release were also improved. Therefore, cell therapies may be a viable option for PKU.

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407 - SETTING-UP OF A CHIMERIC ANIMAL MODEL TO VERIFY THE ROLE OF BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELLS IN THE DEVELOPMENT OF AGE-RELATED MACULAR DEGENERATION (AMD)

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Age-related macular degeneration (AMD) is a debilitating disease that causes vision loss and represents the leading cause of blindness in industrialized countries among adults older than 60 years. Most cases of severe vision loss in AMD result from the development of choroidal neovascularization (CNV), which occurs in approximately 10% of patients with AMD. The abnormal new blood vessels grow from the choroidal capillaries through Bruch's membrane and into the spaces beneath the retinal pigment epithelium and the retina.

The mechanisms that cause CNV are still unclear, but it is known that once the process is triggered, the lesion grows rapidly. In humans, the therapeutic benefit of intravitreal injections of agents that target vascular endothelial growth factor (VEGF) indicates that angiogenesis (the process that involves the migration, proliferation, and remodeling of endothelial cells derived from preexisting vessels) is one of the processes that sustain the rapid formation of the new capillaries in CNV. A parallel role of endothelial progenitor cells (EPCs) recruited to the process from bone marrow (so called vasculogenesis) cannot however be presently ruled out.

To verify this issue, bone marrow transplantation was performed in 20 C57BL mice using a GFP transgenic strain as a donor and a co-isogenic strain as the recipient. Choroidal neovascolarization was then induced by laser treatment aimed to obtain a major retinal damage (disrupture of Bruch's membrane), a procedure that gave rise to choroidal neovascularization approximately 15 days after treatment.

Choroidal neovascularization was confirmed *in vivo* by simultaneous Fluorescein Angiography and Optical Coherence Tomography (OCT) (Spectralis™ device from Heidelberg Engineering). After this procedure, the

animals were perfused with TRITC-labeled dextrans to highlight retinal vascularization and sacrificed. The eyes were then removed, the retina extracted and treated appropriately to obtain retinal whole-mounts. Under fluorescence the perfused retinal vascular tree could be evaluated and EPCs were detectable along the vessels being GFP+. Staining to confirm EPCs phenotype was performed and quantification of EPCs involvement in neovascularization was performed by analysis of digital pictures. Altogether our results confirmed this chimeric mouse as a valid model of AMD of potential use to test the effect of novel drugs on the onset and progression of this sight-threatening disease.

408 - MESENCHYMAL STROMAL CELLS ISOLATED FROM GREEN FLUORESCENT PROTEIN TRANSGENIC PIGS: A NEW LARGE ANIMAL MODEL FOR TRANSLATIONAL STUDIES

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Large animals, pig in particular, have emerged as an important tool for development of surgical techniques, advancement of xenotransplantation, creation of important disease models, and preclinical testing of novel cell therapies. Large animal models play a crucial role to investigate the biological and functional properties of Mesenchymal Stem Cells (MSCs) as novel cellular therapy.

Transgenic swine has been generated through genetic engineering and somatic cell nuclear transfer that will express EGFP (Enanched Green Fluorescente Protein) at high level in somatic cells in order to have a specific source of marked MSCs.

Porcine MSCs (pMSCs) were isolated from 6 EGFP-transgenic pigs, expanded *ex vivo*, characterized by flow cytometry and assessed for their differentiation capacities. EGFP-enhanced pMSCs resulted positive for CD90, CD29 and CD105. The proliferative capacity was evaluated in terms of population doubling from passage P1 to P4 and compared to hMSCs. We also evaluated the proliferation of porcine and human PBMCs stimulated with phytohemagglutinin in the presence of autologus and allogeneic pMSCs or hMSCs[1-3].

EGFP-enhanced pMSCs were traced through the identification of the EGFP by direct visualisation under blue light or by immunohystochemistry. We evaluated also the level of interleukin-6 in colture supernatants collected at the end of PHA proliferation assay in the autologus and allogeneic porcine settings, both porcine settings showed a strong increase in pIL-6 levels after EGFP-enhanched pMSC addition whereas IL-10 and TGF-beta were undetectable[4].

We have demonstrated that the methods for harvesting, culturing and *ex vivo* expanding hMSCs can be successfully reproduced in EGFP transgenic pigs. EGFP-enhanched pMSCs and hMSCs have similarities in terms of morphology, immunophenotype and differentiation potential. We also documented that EGFP-enhanched pMSCs have the capacity to inhibit the proliferation of pPBMCs in both the autologus and allogeneic settings. Our study provides evidence that green fluorescent pigs represent a very interesting animal model with a lot of potential applications in the field of cellular transplantation.

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409 - DEVELOPMENT OF AN AUXILIARY PARTIAL LIVER TRANSPLANTATION MODEL IN RATS FOR FUNCTIONAL EVALUATION OF ENGINEERED LIVER GRAFTS IN VIVO

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There is an ever-growing demand for transplantable livers to replace acute and chronically damaged tissues. This demand cannot be met by the current availability of donor organs. Efforts to provide an alternative source have led to the development of organ engineering, a discipline that combines cell biology, tissue engineering and cell/organ transplantation.

Over the last several years, techniques to decellularize organs have been developed. We have developed a cadaveric liver decellularization protocol to create a whole-liver scaffold for engineering hepatic grafts in rats. We also demonstrated that adult hepatocytes, endothelial and bile duct cells can be seeded into these scaffolds, remaining viable and providing essential liver functions for up to 10 days. However, the implantations of engineered livers into recipients of rats have been limited to just a few hours due to the inadequate implantation sites and coagulation problems as naked collagen from the graft can be exposed to the blood stream and platelets.

To solve these problems, we have developed a novel auxiliary partial liver transplantation (APLT) model in rats and an anticoagulant coating method for engineered liver scaffolds. In order to establish an animal model for liver repopulation to be used for transplantation of engineered liver grafts, we used FK506-immune suppressed Nagase analbuminemic rats as recipients. Prior to APLT, the recipient animal was injected with retrorsine and underwent a reduction of portal blood flow at the time of APLT, to create an environment where there was a selective growth advantage to transplanted grafts. The auxiliary partial graft was obtained by resection of the donor median and left lateral lobes, and was heterotopically transplanted into the recipient. Portal-Portal anastomosis and infrahepatic-infrahepatic vena cava anastomosis were performed in an end-to-side manner and bile duct was implanted into the duodenum of the recipient. Graft survival was evaluated over time (up to 28 days) by graft weight, histological evaluation of proliferative markers and serum albumin levels in analbuminemic rats. FK506-based immunosuppression protocol effectively control graft rejection. Transplanted grafts revealed regenerative potential as evaluated by increase of liver mass weight of the donor graft and Ki67 staining analysis. Serum albumin levels were maintained for the duration of the study. Additionally an anticoagulant coating for decellularized liver scaffolds was performed using N-hydroxylsuccinimide functionalized polyethylene glycol (PEG-NHS), which is used to inhibit platelet activation and deposition by blocking surface protein-platelet interactions. PEG-NHS coating in decellularized liver scaffolds reduced significantly (>40%) platelet deposition mainly in major vessels and allowed continued blood perfusion when compare to bared scaffold. Thus, it can be concluded that we have developed a novel APLT model in rats and we have established protocols for the future evaluation of engineered liver grafts with anti-thrombotic activity.

410 - INTRA-ARTERIAL DELIVERY OF MYOBLASTS TO SKELETAL MUSCLES IN PRIMATES

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Background: The intramuscular transplantation of myogenic cells presents several constraints and the intravascular route seems to be a better strategy of cell delivery. However, so far this route seems to work only with special cells such as mesoangioblasts and CD133+ cells. Experiments of intravascular delivery of myoblasts in mice produced limited results, but mice are not a good reference for human extrapolation given important differences in the myoblast transplantation biology. The close phylogenetic relationship between humans and macaques provides the best anatomical, cell culture, antigenic and immunological similarities for preclinical research in this field. Thus, we decide to test the intra-arterial (IA) allotransplantation of myoblasts in macaques.

Methods: Experiments were conducted in adult cynomolgus monkeys. β-galactosidase (β-Gal)-labeled myoblasts were proliferated *in vitro*, resuspended in heparinized saline and injected slowly in one femoral artery. Regions of muscles both in the leg ipsilateral to the injection and other limbs were damaged with a 27G needle (100 parallel penetrations per cm^2) or by electroporation. Monkeys were submitted to euthanasia 1 hour or 1 day (n = 3) and 3-4 weeks (n = 3) post-transplantation. Monkeys were immunosuppressed with tacrolimus in the long-term follow-up. Samples were taken in different skeletal muscles and organs (lungs, heart, brain, cerebellum, gut, liver and spleen). Samples were frozen in liquid nitrogen and sections were made in a cryostat to be analyzed by histology.

Results: Most myoblasts were retained mainly in the capillaries of the skeletal muscles of the leg ipsilateral to the injection. Some small accumulations were observed in arterioles. No histological evidences of micro-infarcts were observed. Scarce β-Gal+ cells were observed in the lungs 1 hour after transplantation. No β-Gal+ cells were observed in other organs or other muscles. β-Gal+ myofibers were observed 3-4 weeks after transplantation in muscle biopsies of the ipsilateral leg to the injection, in the regions damaged at the time of the cell injection.

Conclusions: Myoblasts can be safely delivered by the femoral artery in primates. Most of these myoblasts are retained in capillaries between myofibers. Myoblast retention in capillaries might be due to a mechanism of plugging, as is frequent in the case of leukocytes, rather than to specific homing. These myoblasts are capable to fuse with regenerating myofibers. Further experiments are needed to know the potentiality of the IA delivery of myoblasts in humans.

411 - HEPATOCYTE TRANSPLANTATION COMBINED WITH PARTIAL HEPATIC RESECTION PRECONDITIONING FOR CRIGLER-NAJJAR TYPE I: A CLINICAL PROGRESS REPORT

Carl Jorns[1], Antal Nemeth[3], Greg Nowak[1], Helen Zemack[1], Lisa-Mari Mörk[1], Helen Johansson[1], Roberto Gramignoli[2], Björn Fischler[3], Stephen Strom[2], Ewa Ellis[1], Bo-Göran Ericzon[1]

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Background: Crigler-Najjar (CN) syndrome type I is a rare disorder of bilirubin metabolism caused by a deficiency of uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1). Patients are at risk of developing fatal brain injury due to unconjugated hyperbilirubinemia throughout life. Treatment consists of blood exchange transfusions in the neonatal period and 10-12h phototherapy daily thereafter. Most patients undergo orthotopic liver transplantation as phototherapy becomes less effective after puberty and constitutes a significant impairment in quality of life.

Case report: A 13-year-old boy and an 11-year-old girl with CN syndrome type I were evaluated at our center. Clinical diagnosis had been confirmed in both patients by lack of conjugated bilirubin in bile and identification of a mutation in the *UGT1A1* gene causing loss of function. Both patients required 8-12h of phototherapy to maintain serum bilirubin at 390 to 450 μmol per liter. After ethical committee approval and informed consent, patients were accepted to the waiting list for hepatocyte transplantation. Hepatocytes were isolated under good manufacturing practices from liver tissue obtained from deceased organ donors not accepted for whole organ transplantation or from split or size reduced liver transplantations. Fresh ABO compatible hepatocytes were infused by a portal catheter introduced through the umbilical or a mesenteric vein. Immediately before placement of the catheters patients underwent liver resection of segments 2 and 3 to induce liver regeneration and proliferation of transplanted hepatocytes. Immunosuppression consisted of basiliximab induction, tacrolimus and steroid pulse followed by tapering. The girl received 5.3 x 10^9 viable hepatocytes at one transplantation event and the boy received two infusions from two different donors three months apart with 2.2 and 9 x 10^9 viable hepatocytes. In both patients serum bilirubin levels increased initially after the first procedure up to 530 μmol per liter. Thereafter serum bilirubin decreased continuously in both patients to 50% of pre-transplant levels for more than 6 months. The boy experienced a sudden increase of serum bilirubin to pre-transplant levels 6 months after the first infusion associated with a scabies infection. Despite intensified phototherapy serum bilirubin did not improve. Due to the risk of encephalopathy we decided together with the family to list him for orthotopic liver transplantation. The girl still remains on significantly decreased serum bilirubin levels and is on the waiting list for further hepatocyte infusions.

Conclusion: These studies confirm that hepatocyte transplantation can be a useful treatment for Crigler-Najjar syndrome type I. Preconditioning patients with hepatectomy prior to cell transplantation is safe, however additional patients will need to be evaluated before conclusions can be made concerning the efficacy of this procedure as compared to traditional hepatocyte transplants.

412 - TRANSPLANTATION OF A REGENERATIVE AND CADAVER TRACHEA – NOVEL TECHNOLOGY IN MANAGEMENT OF SUBTOTAL TRACHEAL STENOSIS: LONG TERM RESULTS AND LESSONS LEARNED

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Background: Surgical treatment of total stenoses of a trachea remains a difficult problem of thoracic surgery. Interventions available in an arsenal not always can considerably help the patient with incurable stenosis of a trachea. Transplantation of trachea appeared to be saving procedure for this group of patients.

Aims: To study long term results of 2 cases of tracheal transplantation (TT) performed in patients with subtotal tracheal stenosis.

Materials and Methods: 37 y.o. male patient with tracheostomy, subtotal stenosis of trachea and stridor had undergone transplantation of trachea with revisualization in 2006. Patient was discharged with free breathing, without tracheostoma and fever. In 3 years after procedure he had undergone stenting on lower part of donor trachea because of compression from outside probably by donor thyreoid gland. In 4 years after TTR patient breathing well. His immunosupression regimen include cyclosporine A 200 mg/d, methylprednisolone 2 mg/d, mofetyl mycofelonate 2 g/d. He had evaluated quality of life as good. In 7 y after TT he is still with tracheal stent, receiving same immunosupression, but lower dose of MMF. And bronchoscopy revealed tracheal esophageal fistula without clinical signs on level of upper end of stent.

25 y.o. female with subtotal stenosis of trachea, stridor and "Polyflex" stent had undergone transplantation of trachea received by methods of regenerative medicine in 2010. The early postoperative period was accompanied by the moderate respiratory insufficiency, hemoptysis, expressed bronchial secretion. Sinus tachycardia and high levels of red blood and white blood cells, C-reactive protein and procalcitotnin have been connected with introduction of growth factors. By the moment of discharge breath was free, a gleam of trachea on all extent was satisfactory, in transplant wall we revealed squamous cell ephitelium. Patient does not require immunosupression. In 6 m. we observed progressive deterioration of breathing and revealed stenosis of tracheal transplant. We need to perform tracheoplasty with T-tube. Twice we try to detubate patient but in short period restenosis occurred. So patient continue having T-tube in trachea.

Conclusions: In some patients with critical subtotal stenosis resistant to accepted management tracheal transplantation may be curative option with satisfactory short-term results. Long term results occur challenges either to patients or doctors and need a lot of efforts to control specific conditions.

413 - NEW INDICATIONS FOR ISLET AUTOTRANSPLANTATION IN HUMAN

Gianpaolo Balzano[2], Paola Maffi[1], Rita Nano[1], Alessandro Zerbi[2], Massimo Venturini[2], Raffaella Melzi[1], Alessia Mercalli[1], Paola Magistretti[2], Marina Scavini[1], Renato Castoldi[2], Massimo Carvello[2], Marco Braga[2], Antonio Del Maschio[2], Antonio Secchi[2], Carlo Staudacher[2], Lorenzo Piemonti[1]

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Background Data: Islet autotransplantation (IAT) is performed to improve glycemic control after extended pancreatectomy, almost exclusively in patients with chronic pancreatitis. Limited experience is available for other indications or in patients with pancreatic malignancy.

Methods: In addition to chronic pancreatitis, indications for IAT were: grade C pancreatic fistula (treated with completion or left pancreatectomy, as indicated); total pancreatectomy as an alternative to high-risk anastomosis during pancreaticoduodenectomy; distal pancreatectomy for benign/borderline neoplasm of pancreatic body-neck. Malignancy was not an exclusion criterion. Metabolic and oncologic follow-up is presented.

Results: From November 2008 to June 2012, 41 patients were candidates to IAT (accounting for 7.5% of all pancreatic resections). Seven out of 41 did not received transplantation for either inadequate islet mass (4 pts), patient instability (2 pts), contamination of islet culture (1 pt). IAT-related complications occurred in 8 pts (23.5%): 4 bleeding, 3 portal thromboses (1 complete, 2 partial), 1 sepsis. Median follow-up was 546 days. Fifteen out of 34 patients (44%) reached insulin independence, 16 patients (47%) had partial graft function, 2 patients (6%) had primary graft non-function and 1 patient (3%) had early graft loss. Seventeen IAT recipients had malignancy (pancreatic or periampullary adenocarcinoma in 14). Two of them had already liver metastases at surgery, thirteen were disease-free at last follow-up and none of two patients with tumour recurrence developed metastases in the transplantation site.

Conclusions: Though larger data are needed to definitely exclude the risk of disease dissemination, the present study suggest that IAT indications can be extended to selected patients with neoplasm.

414 - CLINICAL PROTOCOL FOR LIVER PARTIAL RESECTION BEFORE LIVER CELL TRANSPLANTATION – VIDEO PRESENTATION

Greg Nowak[1], Carl Jorns[1], Antal Nemeth[3], Helen Zemack[1], Lisa-Mari Nilsson[1], Helen Johansson[1], Roberto Gramignoli[2], Björn Fischler[3], Stephen Strom[2], Ewa Ellis[1], Bo-Göran Ericzon[1]

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There is general consensus that some form of liver preconditioning will be required to improve engraftment and proliferation of hepatocytes post-transplant. Partial liver resection is a widely used technique to induce hepatocyte proliferation in studies with animals. Our group established a clinical protocol for partial surgical liver resection prior to liver cell transplantation. The protocol was used on two pediatric patients and the clinical results are presented in a separate abstract. The aim of this presentation is to show a video of the techniques used with a focus on the surgical techniques and patient safety issues. In both patients, resection of the left lateral liver lobe (segments II and III) was performed in using CUSA for parenchymal dissection. At the end of the operation, and before closing the abdominal wall, the umbilical vein or a mesenteric vein was cannulated. Positioning of the catheter in the right branch of the portal vein was verified radiologically by contrast injection. After closing the abdominal wall, the catheter was used for intraportal infusion of liver cells. The same catheter was used to monitor portal blood pressure and infusion of cells was adjusted accordingly. Patients did not experience any complications related to the surgery and in one patient the catheter was re-used for a second intraportal infusion of hepatocytes twelve weeks later. Although, the effect of resection on cell engraftment and proliferation still needs to be evaluated, the protocol is safe and easily performed by an experienced liver surgeon with a low risk of postoperative complications.

415 - AUTOLOGOUS TRANSPLANTATION OF BONE MARROW MONONUCLEAR CELLS IN PATIENTS WITH DECOMPENSATED CIRRHOSIS. PHASE I CLINICAL TRIAL

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Background: Liver cirrhosis is characterized by distortion of the hepatic architecture and the formation of regenerative nodules. Liver transplantation is one of the few available therapies for such patients. It has been shown recently, that hat bone marrow cell infusion repairs liver fibrosis in the cirrhotic liver.

Methods: This study was performed to determine the safety and tolerability of intrahepatic transplantation of autologous bone marrow mononuclear cells into five patients with liver insufficiency. The bone marrow mononuclear cells were isolated and infused into liver via hepatic artery. At different time points after the transplantation, the liver function and prothrombin time (PT) were evaluated, and the survival rate and symptoms of the patients were recorded.

Results: No complications or specific side effects related to the procedure were observed; all patients showed improvements in serum albumin, bilirubin and ALT after one month from the cell infusion. Conclusion: Our study has shown both the safety and feasibility of this type of liver cell therapy and may be a bridge to liver transplantation.

416 - ANIMAL MODELS FOR EVALUATING ENGRAFTMENT OF HUMAN IPSC-DERIVED HEPATOCYTE-LIKE CELLS (IHEP)

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Background: Hepatocyte transplantation is being evaluated as a temporary or permanent substitute for liver transplantation. However, application of this therapeutic modality has been hindered by the scarcity of donor hepatocytes. Toward the goal of generating a renewable substitute for primary human hepatocytes, we and others have generated induced pluripotent cells (iPSC) from human skin fibroblasts and differentiated these to cells with many hepatocyte characteristics (iHep). Here we have evaluated the engraftment, proliferation and function of iHeps in recipient rodent models.

Methods: Three types of small animal models were used: (1) immune-deficient FAH (fumeryl acetoacetate hydrogenase) FRG mice, where selective repopulation by engrafted cells is accomplished by withdrawal of the drug 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclo hexaedione (NTBC), which is needed for protection of host hepatocytes from toxic metabolites; (2) immune-deficient mice transgenic for mutant human alpha 1-antitrypsin (PiZ-SCID), where selective expansion of engrafted cells is accomplished by injection with an adenovector expressing hepatocyte growth factor (ad-HGF); and (3) Tacrolimus-immune suppressed UGT1A1-deficient jaundiced Gunn rats (model of Crigler-Najjar syndrome type-1, CN1), where selective repopulation by engrafted cells is accomplished by X-irradiation of a single liver lobe 24hr before transplantation, followed by ad-HGF injection.

Results: In FRG mice, iHep cell engraftment was identified by FAH and human serum albumin (HSA) immunostaining, and showed engrafted iHeps only as single cells or 2-cell clusters at 3 months. In PiZ-SCID recipient mice, iHep cell engraftment was identified by HSA staining and by human DNA qPCR, and showed 7-15% replacement of host hepatocytes. In the irradiated lobe of the Gunn rat liver, repopulation by iHep cells was 2.5 to 7.5% by morphometric analysis, with a similar repopulation level confirmed by human DNA qPCR. Serum bilirubin levels declined to 30-61% of pre-transplantation levels in 6 months and bilirubin glucuronides were excreted in bile. Despite considerable levels of engraftment in these models serum levels of HSA did not exceed 250 ng/ml.

Conclusions: Human iHep cells engraft and proliferate in the liver of rodent models, but the extent of repopulation is lower than that with primary hepatocytes, and varies markedly in different types of animal models. Nevertheless, iHep transplantation can provide a therapeutically relevant degree of amelioration of the metabolic defect in the Gunn rat model of CN1.

417 - COMBINATION USE OF AUTOLOGOUS PERIODONTAL LIGAMENT-DERIVED MULTIPOTENT MESENCHYMAL STROMAL CELL SHEETS AND BETA-TRICALCIUM PHOSPHATE GRANULES REGENERATED PERIODONTAL TISSUES IN A CLINICAL TRIAL

Takanori Iwata[1,2], Kaoru Washio[1], Toshiyuki Yoshida[1], Kohei Tatsumi[1], Azusa Yamada[1], Yuka Tsumanuma[1], Tomohiro Ando[2], Isao Ishikawa[1], Masayuki Yamato[1], Teruo Okano[1]

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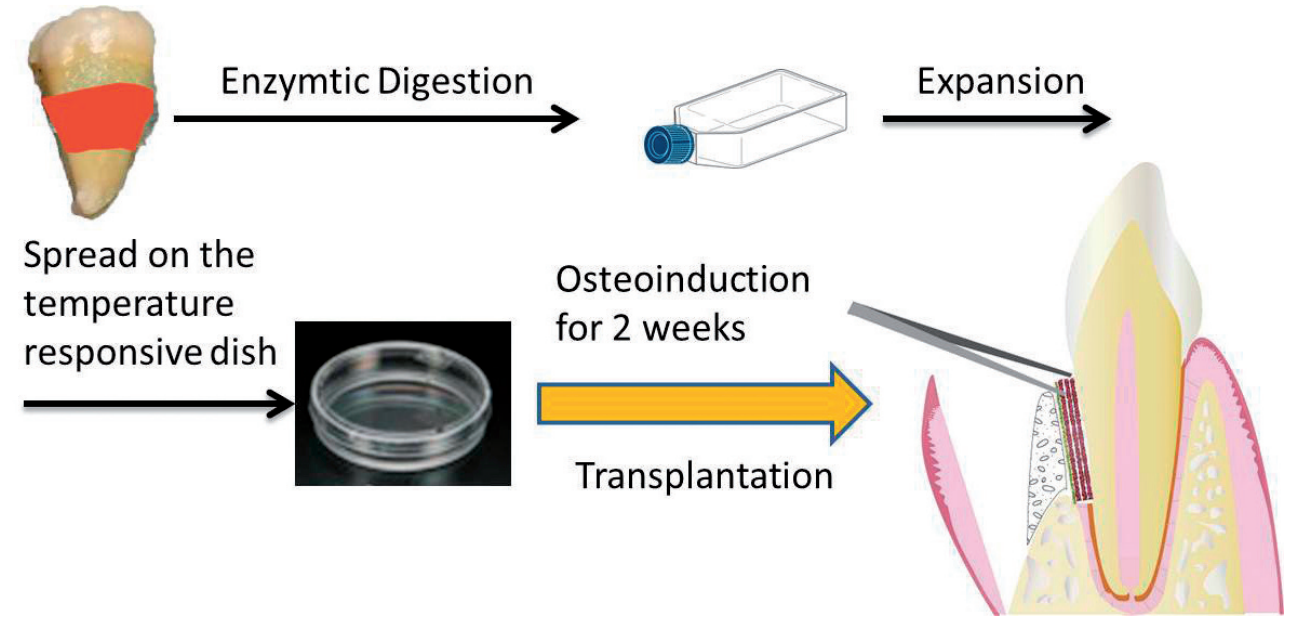
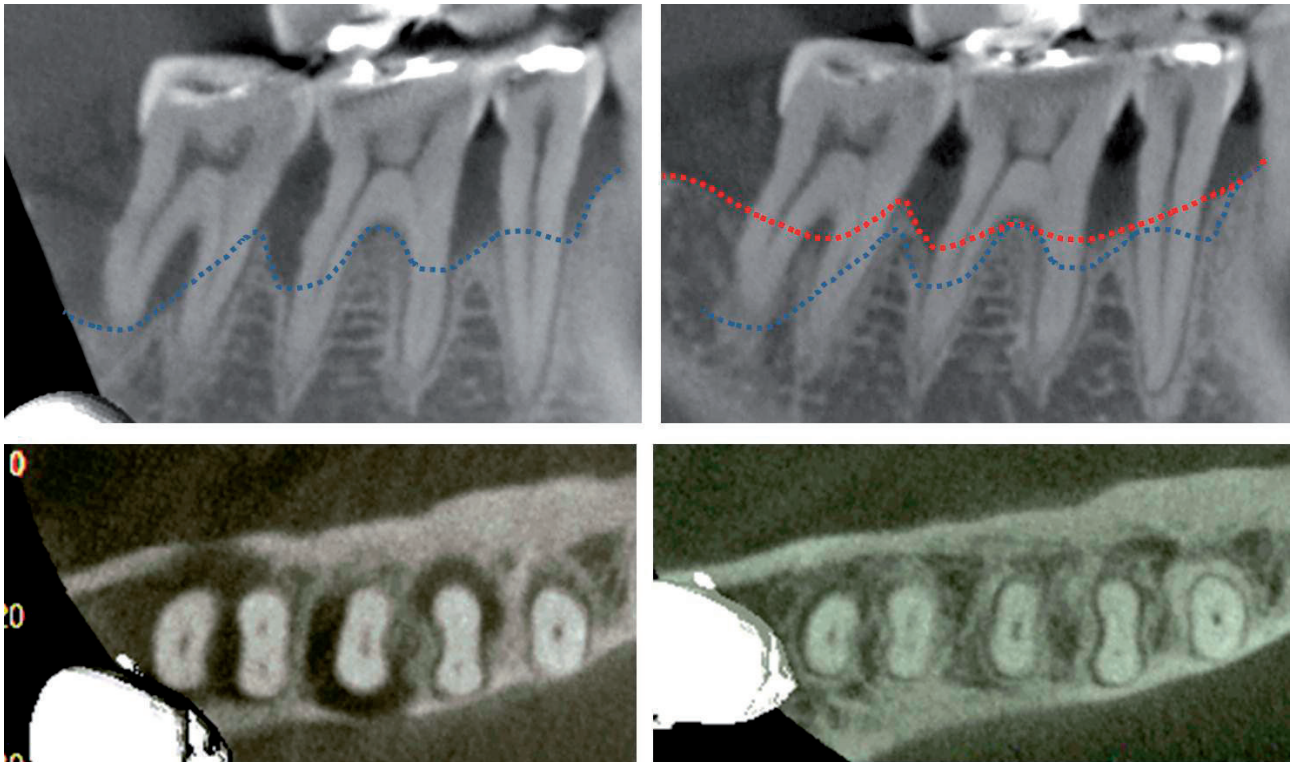
Introduction: Previous studies demonstrated that autologous transplantation of periodontal ligament-derived multipotent mesenchymal stromal cell (PDL-MSC) sheets combined with β-tricalcium phosphate (β-TCP) regenerated the true periodontal tissue in canine models[1, 2]. Based on these translational researches, we started the clinical trial named "Autologous transplantation of periodontal ligament cell sheets for periodontal reconstruction", after the approval of the Ministry of Health, Labor and Welfare in Japan. In this presentation, current status of this clinical trial will be reported.





Experimental Methods: PDL tissue obtained from patient's wisdom tooth was digested andseeded on temperature-responsive culture dishes (35 mm in diameter, UpCell®, Cell Seed, Tokyo, Japan) at a cell density of 4×10^4 cells/dish and cultured in an osteoinductive medium for 14 days. Three-layered autologous PDL-MSC sheets wereplaced on the denuded root surface, and infrabony defect was filled with β-TCP granules (Osferion®, G1, Olympus Terumo Biomaterials, Tokyo, Japan) (Figure 1). Clinical and radiographic measurements were made at the baseline and the 6-month post-surgery.

Results and Discussion: One case of radiograhic images was presented (Figure 2). 53-year-old female patient had infrabony defects in the lower right molars. Left panels showed the infrabony defects before the transplantation. Note osseous defects existed in all teeth. Right panels showed the radiographic images 6-month after the transplantation. The pocket depth was decreased from 9 mm to 2 mm and the bone height was increased 5.9 mm at the distal side of 2nd molar. Clinical attachment level was also gained (12 mm to 5 mm).

Conclusions: We have experienced 4 cases of autologous PDL cell sheets transplantation, and no adverse reaction has been observed. In the finished 4 cases, the reduction of pocket probing depth (3.75 ± 2.22 mm) and clinical attachment gain (3.25 ± 2.87 mm) were observed. PDL cell sheets/β-TCP treated sites also had greater linear bone gain (2.52 ± 2.42 mm). Although this clinical trial is still conducting, the safeness and efficacy of our clinical trial will be proven in the near future.

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420 – EXOSOMES/MICROVESICLES AS PARACRINE MEDIATORS OF STEM CELL ACTION

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Multipotent mesenchymal stromal cells (MSCs) were shown to favour the recovery of acute and chronic kidney injury in several experimental models. However, only few MSCs permanently engraft the kidney and differentiate into renal resident cells, and the beneficial effect observed is ascribed to the release of paracrine factors. Recently, it has been found that exosomes/microvesicles released from MSCs retain most of their properties. These extracellular vesicles (EVs) contain selected patterns of proteins, mRNA, long-non coding RNA and microRNA characteristic of MSCs. The transfer of these functional transcripts to injured cells changes their phenotype and activates self-regenerative programs. Therefore, EVs released from stem cells, by acting as a vehicle of information, emerge as integral components of the paracrine network of factors involved in stem cell action.

421 - CHARACTERIZATION OF METABOLIC ACTIVITIES FROM ISOLATED FETAL HUMAN HEPATOCYTES

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Introduction: Hepatocyte transplantation (HTx) is an alternative to liver transplantation for certain liver diseases. Clinical HTx programs are hampered by the shortage of hepatocytes for this therapy. Fetal hepatocytes have been proposed as an alternative source of cells for transplants, however, little information is available concerning the metabolic activities of fetal cells as compared to adults.

Aim: The viability and metabolic activity of fetal hepatocytes isolated from donors of different gestational ages were assessed and compared to the values obtained from pediatric and adult hepatocytes.

Methods: Hepatocytes were isolated from 252 fetal liver tissues of 11-24 weeks gestational age and 200 "adult" hepatocyte preparations (age range 3 months-85 years). Cell viability, recovery and apoptosis were evaluated immediately post-isolation. When possible, data on 11 different measurements of hepatocyte function including ATP content, plating efficiency basal and induced Cytochrome P450 (CYP) activities, phase II conjugation and ammonia metabolism were collected and analyzed.

Results: Cells isolated from fetal tissues had a significant higher viability compared to adult hepatocytes (91 ± 7% vs 78 ± 12%, mean ± SD; $p<0.0001$), and a 2-fold higher level of apoptosis. Specific hepatic functions were measured on approximately 2/3rds of the preparations. ATP content was significantly higher in fetal hepatocytes compared to adult cell preparations (328 ± 123 vs 32 ± 38 LCU/min/10^3 hepatocytes; $p<0.0001$). Up to 500-fold differences were observed between different gestational and post-natal ages in functional assays. In fetal hepatocytes, a robust induction (up to 30-fold) of CYP enzymes was observed after three days of exposure to specific CYP inducers. The fetal form of CYP3A (3A7) was expressed at low level at early gestational ages, and its activity increased during fetal development, reaching the maximum value in hepatocytes isolated from postnatal infant tissues (<3 years in age). A continuous increase was observed in CYP3A4 and 1A activity from fetal to adult donors. Phase II metabolism, measured as the conjugation of Resorufin by hepatocytes, was easily measured in fetal cells immediately after isolation; however, this activity did not increase in culture as is usually observed in mature hepatocytes. Ammonia metabolism was null or low immediately after isolation in fetal hepatocytes, but increased to approximately adult levels if cells were cultured for 3-5 days (123 ± 101 nmol/min/mg).

Conclusions: Viability and metabolic functions were measured from almost 500 hepatocyte preparations, from fetal, infant and adult human liver tissues. Data is presented on the normal ranges for distinct hepatic functions from attachment to ATP content and ammonia metabolism, to phase I and II drug metabolism in each age group. The inducibility of CYP enzymes and the rapid maturation of ammonia metabolism suggest that fetal hepatocytes could be useful for clinical hepatocyte transplantation.

422 - HIGH DOSES OF EVEROLIMUS AS INDUCTION TREATMENT IMPROVE GRAFT FUNCTION IN ISLET ALLOTRANSPLANTATION

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Although largely improved in the last years, graft function is still variable in human islet transplantation. Early islet inflammatory reaction in the site of islet implant was suggested to reduce islet engraftment and to impair early and long term graft function. MTOR inhibitors seem to have anti-inflammatory effects.

Human islet transplantation was performed in 8 patients affected by type 1 diabetes mellitus . 7 patients were affected by brittle diabetes, 1 patient had been previously transplanted with kidney pancreas transplantation and had lost pancreas for rejection. Recipient body weight was 67kg (range 49-86). Pre transplant c-peptide was <0.5ng/ml. Patients were transplanted with 9.700 +/-3.200 IE/kg b.w.

Immunosuppression consists on an induction treatment with polyclonal ALG (1mg/kg/day for 5 days) in the case of the first transplant, 20mg Basiliximab in the case of islet after kidney recipient or in the case of a second islet transplant (in 4 patients). 3mg of Everolimus was added to the induction treatment 12 hours and 1 hour before islet transplantation. Maintenance immunosuppression treatment consists of micofenolate and tacrolimus according to standard protocol.

No primary non function was observed. 6 patients out 8 are still insulin independence after the last islet transplantation (36, 28, 27, 24, 3, 1 months follow up). In onepatient a recurrence of autoimmune reactivity was observed with the loss of engrafted beta cell mass; a second patient refused a second islet transplantation and lost graft function 1 year after transplantation.

In this pilot study, in a small cohort of patients a refinement of immunosuppression induction treatment improved the overall islet graft function.

423 - STEM CELL THERAPY IN PRIMARY GLOMERULAR DISEASES- AN EARLY SINGLE CENTRE EXPERIENCE

Himanshu Patel[1], AV Vanikar[2,3], UG Thakkar[2,3], SD Dave[2,3], RD Patel[2], KV Kanodia[2], KS Suthar[2], VB Kute[1], HL Trivedi[2,3]

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Introduction: Stem cell therapy (SCT) has now moved from bench to bedside. We present early experience of co-infusion of adipose tissue derived mesenchymal stem cells (AD-MSC) and bone marrow (BM) derived hematopoietic SC (HSC) in patients with primary glomerular diseases unresponsive to conventional therapy.

Materials and Methods: This is a prospective open-labeled single armed trial of co-infusion of ADMSC and HSC in patients with primary glomerular diseases after obtaining their Institutional Review Board approved written informed consent. Twelve patients (7 males, 5 females) were subjected to SCT between January,'09 to January,'13. Inclusion criteria were biopsy proven reversible primary glomerular lesions, steroid resistance, poor response to cytotoxic drugs and any gender/age. Exclusion criteria were non-glomerular/systemic diseases, infections with HIV, hepatitis B/C and co-morbid cardiac conditions. Primary study end points were clinical response and decrease in urinary protein leak, and secondary end points were complete recovery proved on protocol biopsy after 1 year of successful clinical remission.

All patients received autologous AD-MSC + BM-HSC infused in equal amount in bilateral renal arteries and thymic circulation under C-arm guidance, after in-vitro expansion for 8-10 days in self-designed media comprised of Dulbecco's modified eagle's media, growth+ nutrient factors and antibiotics. Conditioning was given with Bortezomib, 1.3 mg/m^2 body surface area, on days-1,4, 7, 10 with intravenous methylprednisone, 125 mg, and rabbit anti-thymocyte globulin, 365 mg/m^2 on day-9. BM was stimulated using granulocyte colony stimulating factor, 300 micrograms twice a day on days 5 and 6. Subcutaneous fat (10 grams) was resected from anterior abdominal wall on day-1 and BM (100 ml) was aspirated from posterior superior iliac crest on day-7. SC infusion was carried out on day-11. They received Prednisone, 10 mg/day post-infusion.

Results: Mean patient age was 21.92 years. Five patients had primary focal segmental glomerulosclerosis (FSGS), 3 had membranoproliferative glomerulonephritis; 2, IgA nephropathy with mesangial proliferative glomerulonephritis (MePGN); 1 had IgM nephropathy (IgMN) with MePGN and 1 had idiopathic MePGN. Their mean 24 hours proteinuria at presentation was 9.92 gm, serum creatinine (SCr), 1.28 mg/dl and serum albumin, 2.11 gm/dL.

Mean volume of infused SC was 109.42 ml for renal arteries, and 2 ml for thymic infusion. Mean CD34+ %, CD90+% and CD73+% counts were 0.32, 34.65 and 13.33 respectively.

No adverse effects were noted.

Over mean follow-up of 34.6 months, there was reduction in mean 24 hrs proteinuria to 3.29 gms with maintained stable mean SCr of 1.1 mg/dl and increased

mean serum albumin to 3.59 gm/dL. Seven patients are under complete remission on Prednisone, 5 mg/day and 5 are in partial remission under cover of Prednisone, 5-10 mg/day and Cyclosporin, 2.5 mg/kgBW/day. Follow-up biopsies in patients under complete clinical remission in 2 with FSGS and 1 with IgMN revealed reversal of lesions.

Conclusions: Co-infusion of AD-MSC with HSC is a viable and safe therapeutic option with promising results in achieving remission of primary glomerular diseases not amenable to conventional therapy.

424 - RESULTS FROM 55 CASES OF ROBOTIC AND OPEN PANCREATECTOMY WITH AUTO ISLET TRANSPLANTATION SHOW THAT TP/AIT MAINTAINS METABOLIC FUNCTION, REDUCES PAIN AND IMPROVES QUALITY OF LIFE IN PATIENTS WITH SEVERE SYSTEMIC DISEASE

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55 (35F/20M) patients, underwent total pancreatectomy with autologous-islet transplant (AIT) for the treatment of unremitting pancreatitis at University of Arizona Medical Center between August 2009 and April 2013. The majority (62%) of patients presented with severe systemic disease as defined by the American Society of Anesthesiologists (ASA) Physical Status Classification (ASA) 3, 36% were ASA 2 and 2% ASA 1. Average patient age at time of transplant was 42 (range 20-66). Etiology of CP was idiopathic for 73% of patients, 16% suffered from hereditary pancreatitis and 11% were alcohol induced (all male). Years between 1st CP diagnosis and TP/AIT ranged from 1-36 (mean 7, median 9). Prior to AIT, 52 patients (95%) had previous surgical or endoscopic interventions, including 80% who presented with previous abdominal surgeries. 15% had undergone a Whipple procedure, and 56% had prior and often multiple ERCPs with stent placement. 46 (84%) patients underwent total pancreatectomy; 6 of these surgeries were performed robotically. 8 patients underwent completion and 1 patient underwent partial pancreatectomy. Preoperatively, 53 patients presented with normal HbA1c (< 6.4%), range 4.6-7.3 (mean/median 5.6); however, preoperative continuous glucose monitoring (CGM) reflected abnormalities in 91% of patients despite normal HbA1c. There was a positive correlation between CGM results and HbA1c ($p<0.01$). Transplanted islet equivalents by body weight (IEQ/kg) ranged 10-17,770 (mean 3,227). Following AIT 19% of patients were insulin-independent (range 1-24 months); these patients received > 3,000 IE/kg. 27% of patients required ≤ 9 units insulin per day, 23% 10-25 and 31% ≥ 25; measure of insulin units per day includes a combined total of the amount of long-acting (Lantus), short-acting (Humalog) and additional insulin taken for CHO correction per meal per day by patients. All patients had preoperative pain and were on opioid analgesics; 71% were pain-free and no longer required analgesics at 12 months post-operatively. Patients reported an improved quality of life (QoL) in all eight SF-36 subscales post-transplant compared with their pre-transplant scores. QoL with regards to pain improved significantly at all time-points (Pre-to-1 month, $p=0.0004$; pre-to-6 months, $p\leq0.0001$; 1-to-6 months, $p=0.0002$). Emotional health quality of life was significantly increased after surgery compared with pre-transplant scores (p=0.0001; p=0.0002). General health quality of life improved significantly with respect to all time points (Pre-to-1 month, $p\leq0.0001$; pre-to-6 months, $p\leq0.0001$; 1-to-6 months, $p=0.0023$). SF-36 physical and mental component subscores increased significantly following TP/AIT. The increase in the PCS was significant between all time-points analyzed (Pre-to-1 month, $p=0.0407$; pre-to-6 months, $p\leq0.0001$; 1-to-6 months, $p\leq0.0001$). Patients reported decreased pain scores in all 3 McGill measures post-transplant compared with their pre-transplant scores. Scores on the Pain Rating Index decreased significantly from pre- to both one and 6 months post-transplant ($p\leq0.0001$; $p\leq0.0001$). Present Pain Intensity scores decreased significantly from pre- to both one and six months post-transplant ($p\leq0.0001$; $p=0.0002$). Sample size for 12-24 months was comparable but too small to analyze at this time. These results demonstrate that following TP/AIT, patients maintain metabolic function and experience improved quality of life.

425 - ISLET OXYGEN CONSUMPTION RATE PREDICTS CLINICAL ISLET ALLO-TRANSPLANT INSULIN INDEPENDENCE FOR FIRST TRANSPLANTS

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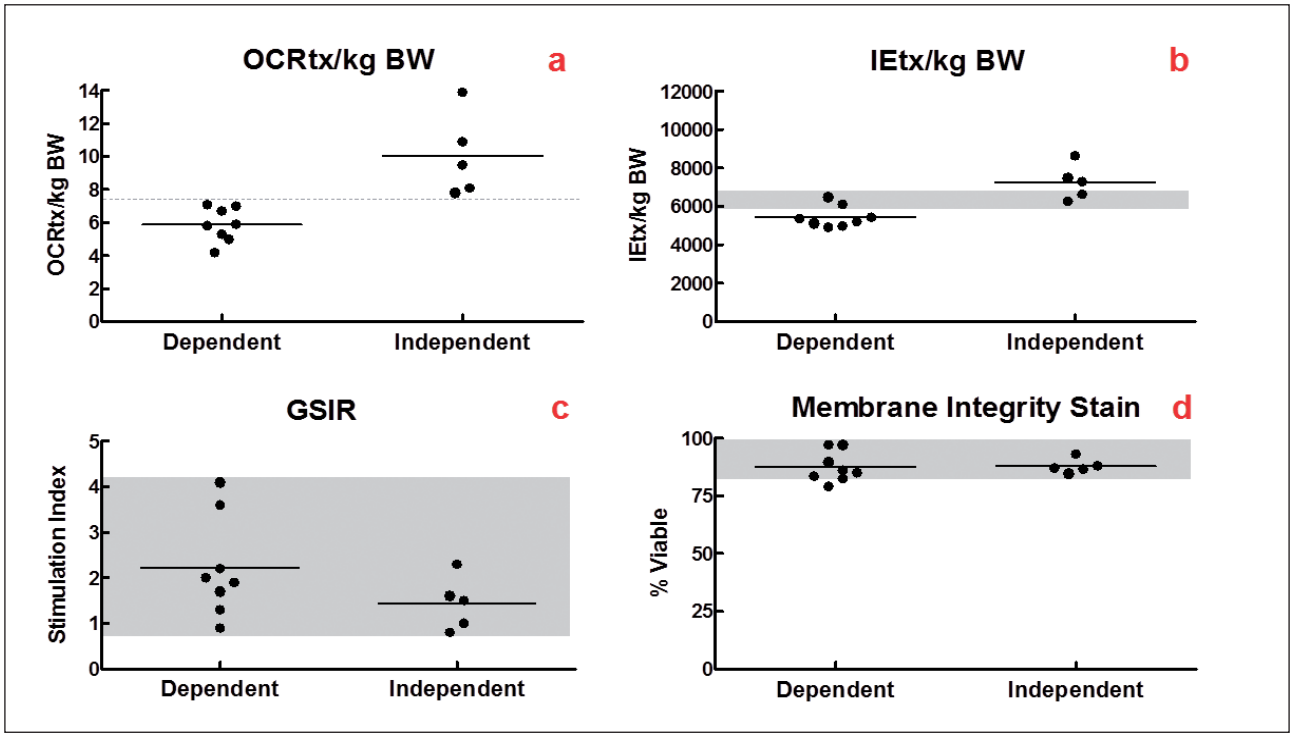
Human islet allo-transplantation (ITx) is in phase III clinical registration trials in the US and standard of care in several other countries. Current clinical islet product release criteria include viability based on cell membrane integrity (>70%), glucose stimulated insulin release (GSIR; >1 stimulation index, SI), and islet equivalent (IE) dose based on counts (IE per kilogram body weight; >5,000). However, only a fraction of patients transplanted with islets that meet or exceed these release criteria become insulin independent. There is a need for more reliable assays that are predictive of clinical transplant outcome (CTO). Measurements of islet oxygen

consumption rate (OCR) have been reported as highly predictive of transplant outcome in small and large animal models as well as clinical islet auto-transplantation. In this paper we report on the assessment of clinical islet allograft preparations using OCRtx/kg BW (a measure of transplanted viable IE – the product of total IE transplanted and islet viability measured by OCR/DNA) and current product release assays in a series of 13 first transplant recipients. The predictive capability of each assay was examined using receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) was determined. Successful graft function was defined as 100% insulin independence within 45 days post-transplant. All transplanted preparations met, or exceeded, current product release criteria. However, only 38% of transplanted recipients were insulin independent within 45 days. The results showed that OCRtx/kg BW was the measure most predictive of CTO (AUC: 1.000; Figure 1a). IE dose was also highly predictive (AUC: 0.975; Figure 1b) while GSIR and membrane integrity stains were not (AUC: 0.825, 0.625 respectively; Figure 1c,d). Interestingly, preparations with higher GSIR were less likely to reverse diabetes. In conclusion, OCRtx/kg BW can predict CTO with high specificity and sensitivity and is a useful tool for evaluating islet preparations prior to clinical ITx.



500 – STEM CELL BASED THERAPIES FOR CANCER

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Successful treatment of tumors remains one of the greatest challenges in oncology. The recognition that different stem cell types can integrate appropriately throughout the mammalian brain following transplantation has unveiled new possibilities for their use in neural transplantation. Our laboratory has shown that both human mesenchymal and neural stem cell types home to sites of cerebral pathology and thus can be armed with therapeutic transgenes, a strategy that can be used to inhibit tumor growth by targeting angiogenesis or selectively induce apoptosis in proliferating tumor cells in the brain. Using our recently established malignant, invasive and resection models of highly malignant human brain tumors that mimic clinical settings and synthetic extracellular matrix (sECM) encapsulated therapeutic stem cells, we show that secreted therapeutic proteins are continuously delivered by encapsulated stem cells, target both the primary and the invasive tumor deposits and have profound anti-tumor effects. Our studies demonstrate the efficacy of encapsulated therapeutic stem cells in clinically relevant mouse tumor models and will have implications for developing effective cancer therapies. These studies demonstrate the strength of employing engineered stem cells and real time imaging of multiple events in preclinical-therapeutic tumor models and form the basis for developing novel cell based therapies for cancer.

501 - IMPROVING MESENCHYMAL PROGENITORS TRANSPLANTATION IN CELLULAR THERAPIES

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While multipotent mesenchymal stromal cells (MSC) have been introduced into pre-clinical and clinical studies since the far sixties, their full potentials to regenerate tissues have been partially clarified. We retain several aspects shall be considered and improved to generate optimized regenerative potentials by MSC in humans. In this paper, starting from *in vivo* detection of MSC, we will describe technologies and models that in our hands allow: (1) to ex-vivo isolate and characterize MSC for clinical applications, (2) to assess their *in vitro* potency to regenerate tissues, (3) to create pre-clinical *in vivo* rational for their clinical implementation and, (4) to establish protocols for their delivery. The attempt to standardize these aspects for a specific clinical indication is an essential prerequisite to better define and challenge MSC biological properties. This applies particularly in case of multi-centric tissue regeneration studies, as is currently taking place in the REBORNE consortium sponsored by the FP7.

502 - DEMYSTIFYING MESENCHYMAL STEM CELLS

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What is known worldwide by the biological misnomer "Mesenchymal Stem Cells" is in essence what was know for many years before as the Bone Marrow Stromal Cells, long postulated to include a genuine stem cell for the different tissues that together comprise the skeleton. The concept of a stem cell for skeletal tissues has recently found direct experimental proof in humans and mice, and was significantly developed beyond the orginal formulation. It is now apparent that skeletal stem cells (MSCs) are perivascular cells of skeletal origin, provide the unique niche for hematopoiesis and hematopoietic stem cells, and are bona fide stem cells inasmuch as capable not only of multipotency (as classically known) but of self-renewal *in vivo*. Over the years, the original concept has been impressivley distorted without the distortions ever being backed by the necesary experimental evidence. The isolation of human pluripotent embryonic cells in culture initially casted on the field the ungrounded suggestion that "MSCs" could be an adult version of pluripotent cells, a notion now dispelled by experimental evidence. The suggestion had two implications: 1) that MSCs could be "doctored" to differentiation ex vivo, like pluripotent cells can, and 2) that they could be used within a true regenerative paradigm to restore the anatomy and function of non-skeletal and even non-mesodermal tissues (such as heart or brain). This view later gave way to the alternative view that restoration of such tissues could be ascribed not to a genuine regenerative event, but to vaguely defined "trophic" "antiinflammatory", immune modulatory effects. While none of these has either been conclusively proven as a biological function of MSCs, or shown to be clinically relevant, over 300 studies around the planet attempt to verify such effects in clinical studies that lack either the rationale or the statistical power needed. Most of these studies are driven, directly or indirectly, by commercial interests, which are slowly replacing, in the field of MSCs, science as the source of science itself and of medical advances. The implications of misread biology and medicine are manifold, and do include the flourishing of a new kind of stem cell quackery that is rapidly spreading worldwide and undermining drug regulation. Meanwhile, major progress in understanding the biology of "MSCs" are being made, which would suggest novel but strikingly different directions for medical developments, which remain disregarded as a result of the heavily commercial climate established in the field.

503 - MESENCHYMAL STEM CELLS TO IMPROVE ORGAN TRANSPLANTATION OUTCOMES: INITIAL CLINICAL RESULTS

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The widespread application of organ transplantation to the large population of patients with end-stage organ failure who are in dire need of its benefits is currently being hampered by a number of challenges. Amongst them are the shortage of organs for transplantation and the need for life-long immunosuppression with the associated untoward side effects. The field of regenerative medicine is rapidly evolving, thus creating exciting opportunities toward the development of novel therapeutic protocols aimed at ameliorating, reducing, modifying, correcting and curing medical conditions. Mesenchymal stromal (stem) cells (MSC) are appealing for inclusion in organ transplantation protocols because of the increasing body of evidence in support of their beneficial properties both in tissue repair and for the modulation of immunity. We will review the encouraging results of the recent clinical trials on the use of MSC to improve organ transplantation outcomes.

504 - EFFECTS OF SPECIFIC INHIBITION OF THE IMMUNOPROTEASOME ON FULLY MHC MISMATCHED ISLET GRAFTS IN DIABETIC MICE

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The immunoproteasome plays an important role in innate and adaptive immune responses. Introduction of immunoproteasome inhibitors has proven promising for the treatment of autoimmune disorders and sensitized transplant recipients.

The novel cell-permeable epoxyketone immunoproteasome inhibitor ONX 0914 (formerly PR-957; Onyx Pharmaceuticals, Inc.) selectively targets the immunoproteasome subunits b1i (low molecular mass polypeptide [LMP] b5i (LMP7) in human and murine cells, resulting in blockade of proinflammatory cytokine production in lymphocytes, and inhibition of IL-17–producing T cells under Th17-polarizing cytokines *in vitro*. Furthermore, ONX 0914 treatment was shown to attenuate autoimmune disease progression in experimental models of arthritis and colitis.

We sought to evaluate the effects of ONX 0914 in experimental models of fully MHC-mismatched islet transplantation under the kidney capsule in diabetic mice. We used a model of pure islet allorejection in chemically-induced diabetic C57BL/6 mice (H2b) receiving DBA/2 (H2d) islets, and a model where islet immune destruction to allo- and auto-immunity in spontaneously diabetic NOD female mice ($H2g^7$). Non-fasting glycemia was measured on peripheral whole blood. Treatment of C57BL/6 mice consisted of ONX 0914 given (i.v. for 9-12 days, then s.c.) for 10-20mg/kg on days -1, 0, 1, 3 and q.o.d. until day 50. Treatment of NOD mice consisted of ONX 0914 (10mg/kg), rapamycin (0.2mg/kg daily on 0-to-28, then q.o.d.) alone or in combination. Control animals received saline or no treatment.

in vitro stimulation of islets with IFN-gamma resulted in remarkable increase of MHC class I expression that could be profoundly prevented by treatment with ONX 0914. In the pure allogeneic transplant combination (DBA/2àC57BL/6), ONX 0914 treatment resulted in prolonged islet allograft survival to a median of 55 (range: 10-81; n=7) days compared to 15 (range: 7->100) days in controls. In the NOD mice, median graft survival was 15 days (range: 7-15; n=9); 12 days (range: 8-12; n=6) in ONX0914; and 13 days (range: 11-13; n=4) in rapamycin. Significant synergy was observed with combinatorial treatment ONX 0914+rapamycin resulting in median survival of 44 days (range: 13-44; n=5; $p<0.0001$).

Collectively, our data suggests that ONX 0914 treatment can reduce immunogenicity of islet cells via reduction of MHC-I expression upon inflammation exposure. Also, ONX 0914 treatment alone prolongs islet allograft survival in C57BL/6 mice, but is not effective in NOD recipients that have an underlying autoimmune process. However, ONX 0914 synergizes with rapamycin therapy resulting in significant prolongation of islet function in the autoimmunity + rejection model. Immunoproteasome inhibition may represent a viable approach in organ transplantation.

505 - PRE-TRANSPLANT INFUSION OF DONOR STEM CELLS PROTECTS THE GRAFT FROM ANTIBODY MEDIATED IMMUNE INJURY- SINGLE CENTRE EXPERIENCE IN LIVING DONOR RENAL TRANSPLANTATION

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Introduction: Transplantation is a well-accepted therapeutic modalityfor end organ failure. However it has limitations of requirement of life-long immunosuppression to prevent rejection of the grafted organ in spite of which rejection is not fully controlled. Over and above, it increases morbidity and mortality due to infections/ malignancies associated with immunosuppressive medications. Stem cells (SC) are known to have immunomodulatory role in organ transplantation. We report a cohort of living donor renal transplant patients who were subjected to pre-transplant stem cell infusion (SCI) under non-myeloablative conditioning with an aim of minimizing immunosuppression with minimum/ no immune injury.

Material and Methods: Totally 138 patients with mean age 32 years and mean HLA-match 2.6, were subjected to pre-transplant SCI consisting of donor peripheral blood stem cells, hematopoietic stem cells (HSC) from cultured bone marrow; and donor adipose tissue-derived mesenchymal stem cells (ad-MSC) in a subset of patients, under principal nonmyeloablative conditioning of irradiation to delete stimulated T and B-cell clones and to create

space in marrow for grafting of SC. Transplantation was carried out with favourable cross-match under calcineurin inhibitor based low dose immunosuppression with mycofenolate and prednisone. Post-transplant immune monitoring included serum creatinine (SCr) with routine hematology, biochemical parameters and peripheral blood lymphohematopoietic chimerism by fluorescent in-situ hybridization in subset of patients with gender-mismatched donors. Immunosuppression minimization was performed with stable graft function, and normal protocol biopsies in patients who gave their consent. Their peripheral T-regulatory cells (pTregs) [$CD4^+CD25^{high}CD127^{low/neg}$] were measured by flow-cytometry and HLA antibodies by luminex assay. Rescue immunosuppression was started with rise of SCr/biopsy-proven rejection.

Results: Mean quantum of SC infused was 273 ml, with mean CD34+ 2.02 x 10^6/kg body-weight (KgBW), mean CD45-/90+ 4.3 x 10^4/kgBW and CD45-/73+ 0.7 x 10^4/kgBW. There were no untoward effects of SCI/ conditioning. All patients had stable graft function; however there was presence of donor-specific antibodies (DSA) in one set of patients and absence of DSA in other set. Chimerism was not observed consistently. Hence they were further subdivided into 2 groups based on presence/ absence of DSA; group-1consisted of 90 patients (81 males, 9 females) with mean age 31.8 years, mean HLA-match, 2.41and presence of DSA, and group-2 of 48 patients (46 males, 2 females) with mean age 32.5 years, mean HLA-match, 2.93 had absence of DSA.

Over a mean follow-up of 6.3 years, mean SCr (mg/dL) was 1.43 in group-1; and 1.4 in group-2 over a mean follow-up of 6.06 years. Totally 36.7% patients in group-1 vs. 56.3% in group-2 were on low dose steroid monotherapy and others were on mycofenolate sodium, 360 mg BD. Out of 51 protocol biopsies in group-1, 90.2% were unremarkable and out of 26 protocol biopsies in group-2, 76.9% were unremarkable. Mean pTregs were 3.3% in group-1 and 3.5% in group-2. There was no patient/ graft loss in either group.

Conclusions: SC help in achieving stable renal allograft function with minimization of immunosuppression in spite of presence of DSA. We believe that SCI has protected the graft from immune injury.

506 - MSCS IMPROVE REFRACTORY CHRONIC GVHD BY INDUCING REGULATORY CD5+ B LYMPHOCYTES

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Background: Refractory chronic graft-versus-host disease(cGVHD) is a significant complication resulting from allogeneic hematopoietic stem cell transplantation. Mesenchymal stromal cells (MSCs) have unique immunomodulatory properties and have shown promising results in the treatment of GVHD.

Method: 23 moderate or severe refractory cGVHD patients that enrolled in study received MSCs treatments as 1×10^6 cells/kg per infusion and 3 doses at 4-week intervals. Clinical manifestations, laboratory data and peripheral lymphocyte subsets were analyzed pre- and post- MSCs treatment.

Result: In this prospective study, we treated refractorycGVHD patients with MSCs and found that none of the MSCs recipients experienced immediate or long-term toxic side effects. At a 12-month follow-up, 20/23 patients showed complete response (CR) or a partial response (PR), 2/23 patients died of fungal pneumonia, and 3 patients died of leukemia relapse.The most dramatic improvements in GVHD symptoms were observed in the skin, oral mucosa, and liver. Clinical improvement was accompanied by an increase in the absolute lymphocyte number; but no significant differences in the frequencies of $CD3^+$ T cells among total lymphocytes, $CD4^+$ T cells among total $CD3^+$ T cells, $CD8^+$ T cells among total $CD3^+$ T cells, or Treg cells among $CD4^+$ T cells were observed between either pretreatment or post-treatment cGVHD patients and non-GVHD patients.In contrast, MSCs treatment decreased the frequency of B lymphocytes(from 14.92 ± 14.10% to 6.40 ± 4.70%, $p<0.01$) in the peripheral blood, but significantly increased the frequency of $CD5^+$ regulatory B cells (from 17.60 ± 13.05% to 28.72 ± 20.24% of total B cells, $p<0.01$). Importantly, $CD5^+$ B cells from cGVHD patients showed increased IL-10 expression after treatment (from 24.90 ± 17.50% to 49.67 ± 22.61% of $CD5^+$B cells, $p<0.01$), and this was associated with reduced inflammatory cytokine production by T cells, but not proliferation of T cells. Our data showed that MSCs perform the immunosuppressive function by enhancing the frequency of $CD5^+$B cells in cGVHD patients through multiple mechanisms, including increasing survival of $CD5^+$ B cells (76.19 ± 8.74% versus 19.94 ± 6.03% without MSCs, $p<0.01$), promoting proliferation of $CD5^+$ B cells (21.78 ± 3.56% versus 3.91 ± 1.11% without MSCs, $p<0.01$), and stimulating the production of the anti-inflammatory cytokine IL-10 by $CD5^+$ B cells (9.53 ± 2.91% versus 1.44 ± 0.56% without MSCs, $p<0.01$). These effects required direct cell-cell interactions and were partially mediated by indoleamine 2, 3-dioxygenase (IDO).

Conclusions: MSCs might exert their immunomodulatory effects on $CD5^+$ regulatory B cells to improve the clinical symptomsof refractory cGVHD. These findings suggest that MSCs represent an effective and novel cell-based therapeutic modality for cGVHD.

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507 - MESENCHYMAL STEM CELL INDUCED IN-VITRO GENERATION OF REGULATORY T-CELLS – FUTURE NOVEL CELLULAR THERAPY FOR TOLERANCE INDUCTION IN TRANSPLANTATION

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Introduction: Induction of antigen-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune regulation. A subpopulation of suppressive CD4+ T cells, termed regulatory T-cells (Tregs) have recently been recognized as key players in transplantation immunobiology. However there is little knowledge about their source and dose required for tolerance induction. Tregs induction and activation is an attractive approach of immunotherapy for many diseases/disorders. However Treg therapy may require *in vitro* expansion since Tregs are present at low frequency in most sites clinically accessible for harvesting. Mesenchymal stem cells (MSC) have promising role in differentiation and preferential activation of Tregs to suppress cytotoxic T-cell activity in transplant recipients. We present *in-vitro* model of induction of donor-specific Tregs using donor adipose tissue derived MSC (AD-MSC) and recipient peripheral blood mononuclear cells (PBMC).

Materials and Methodology: Ten grams subcutaneous adipose tissue was collected from each of 25 renal allograft donors during nephrectomy and cultured in lab using α-minimal essential media. On 10^{th} day, AD-MSC were harvested and cultured in separate culture dishes. PBMC were derived from immunosuppressed recipients and were divided in two parts, one as responder-PBMC (R.PBMC) and other subjected to irradiation served as stimulator-PBMC (S.PBMC). R.PBMC and S.PBMC were layered on AD-MSC for 9 days in culture medium RPMI-1640 supplemented with IL-2 and human albumin. R.PBMC and S.PBMC co-cultured in absence of AD-MSC under similar conditions served as negative controls. Cells were harvested, analysed for viability, suppression assay and phenotypic characterization using FACScan for $CD127^{-/low}CD4^+CD25^{high}$, $CTLA\text{-}4^+$ and infused in thymus of recipients 3 weeks post-transplant.

Results: Mean 3.5 ± 1.3 $x10^3$ AD-MSC were generated and co-cultured with mean 3.6 ± 1.9 $x10^4$ PBMC. Mean $CD127^{-/low}CD4^+CD25^{high}$ were increased from 2.2 ± 2.3 % to 16.6 ± 12.8% and $CTLA\text{-}4^+$ increased from 27.2 ± 11.6 % to 49.8 ± 16.8 % at the end. Interestingly there was rise in $CD4^+CD8^+$ cells from mean 2.7 ± 6.8 % to 29.5 ± 27.6 %. Tregs suppressive capacity was maintained in populations layered on AD-MSC for 12 days while control cells lost all suppressive activity after 3 days. Mean quantum of Tregs infused was 2.9 ± 5.3 x 10^4cells/kg body weight (BW). Tregs infusion was uneventful. Over a mean follow-up of 114.4 ± 48.2 days, all patients are doing well with stable mean serum creatinine of 1.2 mg/dL on immunosuppression of Tacrolimus, 0.05 mg/kgBW/day and Prednisone, 10 mg/day without any immune injury.

Conclusions: We have successfully induced T-regs [$CD127^{-/low}CD4^+CD25^{high}$, $CTLA\text{-}4^+$ and $CD4^+CD8^+$] using donor AD-MSC from immunosuppressed recipient PBMC. This novel model will open up the gateway to tolerance in autoimmunity and organ transplantation.

508 - DIFFERENTIAL EXPRESSION OF P2X7 RECEPTOR AND ECTONUCLEOTIDASES IN B-CELLS UNDER PHYSIOPATHOLOGICAL CONDITIONS: RELEVANCE TO ISLET IMMUNOBIOLOGY

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Under basal conditions extracellular concentrations of ATP are regulated by ATP release through the P2X7 receptor and degradation by ENTPDases (CD39) and ecto-5'-nucleotidase (CD73). ATP may play a dual role in the homeostasis of islet cells. Both CD39 and CD73 are found on cell surfaces where they convert ATP, a major source of energy, into adenosine that is the end product of this degrading activity. High ATP concentrations are released by cells exposed to stress and undergoing death resulting in heightened inflammation, resulting in their demise. Thus, expression of purinergic receptors and ectonucleotidases is dynamic and change under physiopathologic conditions.

We explored the expression profile of P2X7R and ectonucleotidases in Balb/c islets and pancreata from different strains of mice under physiologic and pathologic conditions. Isolated Balb/c mouse islets were exposed *in vitro* to different noxious stimuli for 24 hrs, consisting of either: IFN-γ alone (1000U/ml), proinflammatory cytokine cocktail [CTK; (IL-1β (50U/ml), IFN-γ (100U/ml), TNF-α (2000U/ml)] and high glucose (HG; 25mM). Untreated islets were used as a control (CTR). Viable β-cells (R2D6+) in islets exposed to 24 hrs stress were 45.5% in CTR, 65.7% in IFN-γ, 66.6% in CTK, and 61.3% in HG. R2D6+P2X7R+ cells were 6.8% in CTR islets, and increased to 11.5% in IFN-γ treated islets, 17.8% in CTK-treated islets, while being almost absent under HG (0.6%). R2D6+CD39+ cells were 1.7% in CTR islets, 6.5% and 8.1% in IFN-γ and CTK-treated islets, and 4.6% in HG. R2D6+CD73+ cells were 21.4% in CTR islets, 36% in IFN-γ treated islets, 54.7% in CTK-treated islets and 47.5% under HG conditions. Expression of MHC-I on β-cells was 2% in CTR and 1.6% in HG, while it dramatically increased after exposure to IFN-γ (46.3%) and CTK (17%). In dissociated pancreas of C57BL/6, NOD and NOD.SCID mice β-cells were 80%, 29.9% and 30.2%. R2D6+P2X7R+ cells were 71.5%, 26.2% and 74%. R2D6+CD39+ cells were 8.5%, 46% and 26%. R2D6+CD73+ cells were 8.2%, 5.7% and 26.6%. CD3+ cells were higher in NOD (10.9%) than in C57BL/6 (15.3%); as expected, no CD3+ cells were present in NOD.SCID pancreas. CD3+P2X7R+ cells were higher in NOD (11.7%) than in C57BL/6 (6.1%) pancreas. CD3+CD39+ cells were more elevated in NOD (23.6%) than in C57BL/6 (3.3%), and CD3+CD73+ similar (55.4% and 66%, respectively).

Collectively, P2X7R and ectonucleotidases expression increase in β-cells under stress conditions and may contribute to the amplification of microenvironment inflammation during the development of autoimmune diabetes and after islet transplantation. Modulation of this pathway could be appealing to preserve functional β-cell mass in diabetes.

509 – REGENERATIVE MEDICINE FROM BENCH TO BEDSITE: OVERCOMINGTHE CHALLENGES TO RESTORE VISION

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Limbal cultures restore the corneal epithelium in patients with ocular burns. We investigate biological parameters instrumental for their clinical success.We report a long-term multicenter prospective study on 152 patients, carrying severe burn-dependent corneal destruction, treated with autologous limbal cells cultured on fibrin and clinical-grade 3T3-J2 feeder cells. Clinical results were statistically evaluated both by parametric and non-parametric methods. Clinical outcomes were scored as full success, partial success and failure in 66.05%, 19.14%, and 14.81% of eyes, respectively. Total number of clonogenic cells, colony size, growth rate and presence of conjunctival cells could not predict clinical results. Instead, clinical data provided conclusive evidence that graft quality and likelihood of a successful outcome rely on an accurate evaluation of the number of stem cells detected before transplantation as holoclones expressing high levels of the p63 transcription factor. No adverse effects related to the feeder-layer has been observed and the regenerated epithelium was completely devoid of any 3T3 contamination. Cultures of limbal stem cells can be safely used to successfully treat massive destruction of the human cornea. We emphasize the importance of a discipline for defining the suitability and the quality of cultured epithelial grafts, which are relevant to the future clinical use of any cultured cell type.

ENGINEERING NOVEL CELLULAR PRODUCTS FOR THE REGENERATION OF THE MUSCULOSKELETAL TISSUES

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The regeneration of musculoskeletal tissues is a very promising scientific area and is getting increasing clinical results in tendon, soft tissues and bone healing.

Musculoskeletal tissues regeneration covers many different surgical specialties like orthopedics, spine surgery, sport medicine, dental implantology, maxillo-facial surgery and neurosurgery.

The tissue regeneration requires a scaffold with or without human stem cells.

The cellular product used can be autologous or allogeneic and minimally or more than minimally manipulated.

In the minimal manipulation case, the cells are isolated from the donor tissue using methods not altering the cells fundamental characteristics. An example of physical method for obtaining mesenchymal stem cells from adipose tissue will be presented.

If a cell is minimally manipulated and the use is considered homologous (same function in donor and recipient) the procedure is considered a cell transplantation.

In the more than minimal manipulation case, like enzymatic isolation and culture expansion, the cells are defined altered and thus GMP production and “drug like” clinical cGCP path is required.

Several products are already available for clinical use in musculoskeletal regeneration and many others are in pipeline.

CELL TRANSPLANT SOCIETY

ABSTRACTS FROM THE 12TH CONGRESS

POSTERS

P-01 – EXPRESSION OF THE CHEMOKINE RECEPTORS CXCR3 AND CCR5 IN NATURAL KILLER CELLS FROM UMBILICAL CORD BLOOD

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Introduction: natural killer cells (NKC) are of great interest for their cytotoxic and immunomodulatory properties, which make them candidates for immunotherapy of cancer and immunodeficiencies. Most NKC from adult peripheral blood (PB) express CD56 and two NKC subpopulations can be detected based on CD56 and CD16 intensity of expression: the CD56+weak/CD16+ NKC (CD56+ NKC), which are prevalent and have cytotoxic functions, and the CD56+strong/CD16-/+ (CD56++ NKC), with immunomodulatory functions. These subpopulations differ on the expression of various molecules, including the chemokine receptors (CKR) which regulate leukocyte migration to various organs and tissues through interaction with their respective ligands. Among CKR we highlight the CXCR3 (CD183) and CCR5 (CD195) that are expressed on memory Th1 lymphocytes and on NKC subpopulations. An increase in the representation of CD56- NKC has also been observed in umbilical cord blood (UCB), however UCB NKC are still poorly featured in terms of phenotypic and functional properties.

Purpose: we characterized CD56-, CD56+ and CD56++ NKC from UCB and compared them with the corresponding populations of healthy adult subjects PB, in particular with regard to the expression of two CKR, the CXCR3 (CD183) and CCR5 (CD195).

Materials and Methods: we studied 15 CB and 15 PB samples. The expression of CKR was evaluated by flow cytometry (FACS Canto v.2, BD), by direct immunostaining of leukocytes in whole blood, recurring to multiple combinations of monoclonal antibodies with different specificities conjugated to different fluorochromes. Analysis was performed recurring to Infinicyt software (Cytognos).

Results: the percentage of NKC (in lymphocytes) from UCB was similar to that observed in PB (media 16.6% in UCB vs. 12,8% in PB). However, UCB had a higher representation of CD56-NKC (media 20,3% in UCB vs. 5,1% in PB) and a smaller representation of CD56+ NKC comparing to PB (media 71.9% vs. 87.7%), while the representation of CD56++NKC was similar (7.8% in UCB and 7.2% in PB). Both in UCB and PB the majority of CD56++ NKC expressed CXCR3 and CCR5, with relatively strong intensity, while only a small part of CD56+ and CD56- NKC expressed these CKR. However, UCB NKC had globally lower expression of CXCR3 and CCR5 than NKC from PB (p=0.003) and this was particularly significant for the CD56++ subpopulation (p<0.001).

Discussion: it is known that infiltration in inflamed tissues by activated memory T lymphocytes is mediated by adhesion molecules and CKR, such as CCR5 and CXCR3. The same receptors have an important role in transplant rejection, being proved that their blockage

with monoclonal antibodies leads to an increased survival of the transplanted tissue. There are virtually no published studies on the expression of UCB NKC CKR. This study shows a reduced expression of CXCR3 and CCR5 in UCB NKC, which may be associated with less antigenic stimulation of these cells, in analogy to what was observed in PB "naive" T lymphocytes which do not express CCR5 and CXCR3.

P-02 – ANALYSIS OF QUALITY INDICATORS IN A CORD BLOOD BANK

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Purpose: to perform an analysis of the quality indicators defined in a private cord blood bank.

Methods: we retrospectively evaluated documental and technical aspects of cord blood (CB) and cord tissue (CT) processing during 2012, namely: Information about the donor (by evaluation of a clinical query, information about delivery, maternal viral markers and informed consent), cord blood unit and cord tissue quality conditions (initial and post-processing total nucleated cell (TNC) and CD34+ number, mesenchymal cell number isolated from CT, cell viability, number of units with bacterial contamination, technical aspects about quality of shipping and processing (temperature and time of shipping, time between DMSO addiction and the beginning of programmed freezing).

Data were introduced in our database (iCrio version 4.3.1) and analysed by Microsoft Excel 2010.

Results: we received 3774 CB and 1355 CT units in the considered period. We did not cryopreserve 183 CB units (positive viral markers in the donor n=6, TNC count < $250x10^6$ n=148, technical problems during collection/defects in primary collection bag n=18, clots/haemolysis n=4, shipping to the processing facility>72 hours n=6, hereditary disease in the newborn n=1) and 36 CT units (positive viral markers in the donor n=2, shipping to the processing facility >48 hours n=18, contamination with resistant bacteria n=16). We observed incomplete information about the donor in 244 processes (no clinical query n= 65, no informed consent n= 83, no information about delivery n= 96) and incomplete labelling in 40 processes. The mean initial TNC was $879,4x10^6$ (min 250-max $3930,8 x10^6$) with a mean TNC recovery rate of 82,7% (mean post-processing TNC 705,1; min 200-max $3269,9x10^6$). The mean CD34+count was $15,7x10^5$ (min 1-max $193,4x10^5$). The mean mesenchymal cell number isolated from 8 cm cord tissue was $15,47x10^4$ (min 0-max $434,78x10^4$). The mean cell viability was 97% in CB units and 91% in CT. We observed a positive microbiological culture in 10% and 32% of the CB and CT units, respectively. E. coli was the most frequent isolated bacteria both in CB and in CT.

The mean shipping time to the processing facility was 27 hours. Dataloggers in each collection kit revealed that 67 CB and 29 CT units were shipped above the predefined temperature range (4-24ºC). The mean time between DMSO addiction and the beginning of programmed freezing was 27 minutes, pointing out that each unit was always maintained at 4ºC after DMSO addiction.

Conclusions: We observed a very high rate of bacterial contamination in our CT units. Based on these results we implemented correction actions to improve our practices. We organized formations for health care professionals to emphasize the importance of disinfection steps during collection as well as to optimize the UCB collected volume. Additionally, we planned GMP formation for all our lab professional to decrease intralaboratorial contamination. Parents were asked to complete and send all missing documents to allow a final clinical validation of the process of CB and CT cryopreservation. The remaining evaluated indicators in CB and CT processing were considered acceptable.

P-03 – ANALYSIS OF APOPTOSIS IN NATURAL KILLER CELLS FROM UMBILICAL CORD BLOOD

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Introduction: Two natural killer cell (NKC) subpopulations may be detected in peripheral adult blood (PB) and umbilical cord blood (UCB) based on CD56 and CD16 intensity of expression: the CD56+weak/CD16+ NKC (CD56+ NKC), which are prevalent and have cytotoxic functions, and the CD56+strong/CD16-/+ (CD56++ NKC), with immunomodulatory functions. Additionally a third CD56-CD16+ NKC (CD56- NKC) subset may be observed, being this population more represented in UCB. In the PB, this population is increased in patients with cancer, patients treated with IL2 and in other immunosuppressive conditions, but its significance remains uncertain. Some authors consider CD56- NKC as an immature/precursor population mainly on the basis that these cells may acquire CD56 after in vitro estimulation with IL-2. We hypothesize that these cells are mainly apoptotic NKC.

Purpose: We studied the expression of apoptosis markers in the CD56-, CD56+ and CD56++ NKC in normal PB and UCB.

Methods: We evaluated the expression of annexin V in NKC subsets from 5 UCB and 5 PB samples. The expression of annexinwas analyzed by flow cytometry (FACS Canto v.2, BD), after direct immunostaining of leukocytes in whole blood with the following combination of monoclonal antibodies: AnnexinV (FITC), CD7 (PE), 7-AAD (PERCP), CD56 (PECy7), CD2 (APC), CD3 (APCH7), CD16 (PB), CD45 (PO). We always run out a positive and negative control for each processed sample. Analysis was performed recurring to Infinicyt software (Cytognos).

Results: We observed a higher percentage of annexinV positive cells in CD56- and CD56+ NKC populations compared with CD56++ NKC, both in PB and in UCB. In particular, the median percentage of annexinV+ cells in PB was 2.4% in CD56++NKC, 6.4% in CD56+ NKC and 11.6% in CD56-NKC (p=0.07; Kruskal Wallis) whereas in UCB these percentages were of 9.9%, 17.7% and 20.3%, respectively (p=0.037; Kruskal Wallis).

Conclusions: Our results show a trend to a higher Annexin V expression in CD56+ and CD56- NKC subsets, which is particularly evident in UCB. This supports our initial hypothesis and seems to be in contradiction with the theory that CD56- NKC in UCB is a more immature subpopulation.

P-04 – HEPATOCYTE AND LIVER NON-PARENCHYMAL CELLS CO-CULTURE: IMPROVEMENT OF FUNCTION AND SURVIVAL OF PRIMARY HUMAN HEPATOCYTES

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Background: Previous studies have shown the positive effect of mesenchymal stem cells (MSCs) on various primary cell types, improving their viability and function *ex vivo*. Liver non-parenchymal cells (NPCs), such as sinusoidal endothelial cells, Kupffer cells and stellate cells can recreate a more physiological niche and help hepatocytes to proliferate and regenerate after injuries. We consequently hypothesized that MSCs and NPCs would improve the viability and function of hepatocytes isolated from unused donor livers and be beneficial for encapsulated hepatocyte transplantation.

Aim: To study the effects of liver NPCs and MSCs co-culture on primary human hepatocytes survival and function.

Materials and Methods: Percoll-purified cryopreserved human hepatocytes were plated onto collagen-coated culture plates with or without 10% umbilical-cord derived MSCs (n=3), or 24hrs before the addition of 10% NPCs (n=6), in Williams' E medium supplemented with 50IUml penicillin, 50μg/ml streptomycin, 0.1μM insulin, 0.1 μM of dexamethasone, 2mM glutamine, 10mM of HEPES and 10% FCS. Supernatants were collected at day 1, 2 and 6 for measurement of cell function (albumin synthesis). Cell attachment and overall survival was performed (SRB test). All data were normalized to 100% of day1 control values. The data were analyzed by two-way ANOVA.

Results: 1 - Preliminary data showed that the MSC addition dramatically improved hepatocyte attachment on all days of study, increasing SRB values in the range of 4 to 8 folds in the different sets. Whilst MSC co-culture did not have any effect on hepatocyte function on day 1 and 2, by day 6 the production of albumin was increased by an average of 7 fold as compared to the control cells.

2 - NPCs, added 24 hours after plating, did not improve cell attachment on any day of our experiment (SRB mean OD for monoculture *vs* co-culture were on day 1: 0.91 *vs* 0.8; day 2: 0.72 *vs* 0.77 and day 6 0.86 *vs* 0.77) but improved albumin synthesis of the co-culture in all days (day 1: 100 *vs* 174 ; day 2: 52.5 *vs* 87.9 and day 6: 113 *vs* 160%, for hepatocytes culture *vs* hepatocyte co-cultured with NPCs, respectively), though these results are currently not statistically significant for the addition of the NPC in the primary hepatocyte culture (n=6, p=0.08).

Conclusions: MSCs and NPCs appear to have a beneficial effect on primary human hepatocytes. The scales and kinetics of their respective effects are very different, which probably indicates distinctive mechanism of actions. Further analysis should shed some light on their potential contribution to the function of primary hepatocytes for use in cell transplantation.

P-05 – ATTACHMENT CAPACITY OF ADIPOCYTE TISSUE MESENCHYMAL STEM CELLS IN SUTURE FILAMENTS: A NEW TOOL FOR THE TREATMENT OF ENTEROCUTANEOUS FISTULA

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Background and Aims: Enterocutaneous fistulas (EF) are difficult to resolve and surgical failure is frequent. Mesenchymal stem cells (MSCs) are able to self renew, has high proliferative capacity, and can differentiate into several lineages. They also have immunomodulatory capacity. Adipocyte tissue (AT) is an easy source as lipoaspiration is a common procedure. Enterocutaneous fistula

(EF) treatment with AT-MSCs was yet performed but some time, the fistula did not close completely. Perhaps if the fistulas were closed with MSCs added to suture filaments with fibrin glue it would be closed totally. The aim of this study is to analyze if the MSCs could attached in the suture filament in order to be used for EF treatment.

Materials and Methods: AT was obtained from lipoaspirate procedures. AT was submitted to collagenase digestion. Cells were cultured in DMEM low glucose medium, with FBS during 3 days. At the 4th passages, cells were characterized by flow cytometry, confocal microscopy, differentiated to mesodermal lineages to confirm MSCs. The experiments were performed with 4-0 Vicryl. MSCs, 10^6 cells, were fixed in the 4-0 Vicryl by adding-fibrin glue. Samples were analyzed by confocal (CM) and scanning electron microscopy (SEM). The animal experiments were performed on male Wistar rats divided into 3 groups: Control Group (CG) included 5 animals undergoing fistula formation alone. Injection Group (IG) consisted of 8 animals receiving 10^6 AT-MSC injected around the suture line. Suture Group (SG) consisted of 9 animals in which suture was performed using 4-0 Vicryl with 10^6 MSC attached in the filament with fibrin glue. The cecum was accessed through a standard 7 mm stab incision on the lower left side of the abdomen. Upon exposure, a 5 mm enterotomy was performed and sutured to the abdominal wall in order to produce the fistula. To ensure normal closure of the fistula the opening in the cecum wall was fixed to the internal surface of the skin, without maturation, using four separate 4-0 Vicryl stitches. The fistulas were photographed on the day of operation and on the 3rd, 6th, 9th, 12th, 15th, 17th, 19th and 21st day, in which they were anesthetized and sacrificed. Measure of the size of the fistula was performed using ImageJ software. Statistic comparison between the groups was performed by ANOVA.

Results: CM and SEM results demonstrated that the cells were able to attach to the suture filaments. The animal experiments showed that the average size reduction of the fistula area at the 21th day was 46.54% in the CG, 71.80% in the IG and 90.34% in the SG ($p<0.05$), demonstrating that MSCs were effective in promoting healing of an EF both as an injection or attached to the suture material.

Conclusions: MSCs were able to attach to the suture filaments. When EF were sutured with filaments containing MSCs they showed better recovery and healing. AT-MSCs adhered to suture filament might be a new and effective approach for EF treatment.

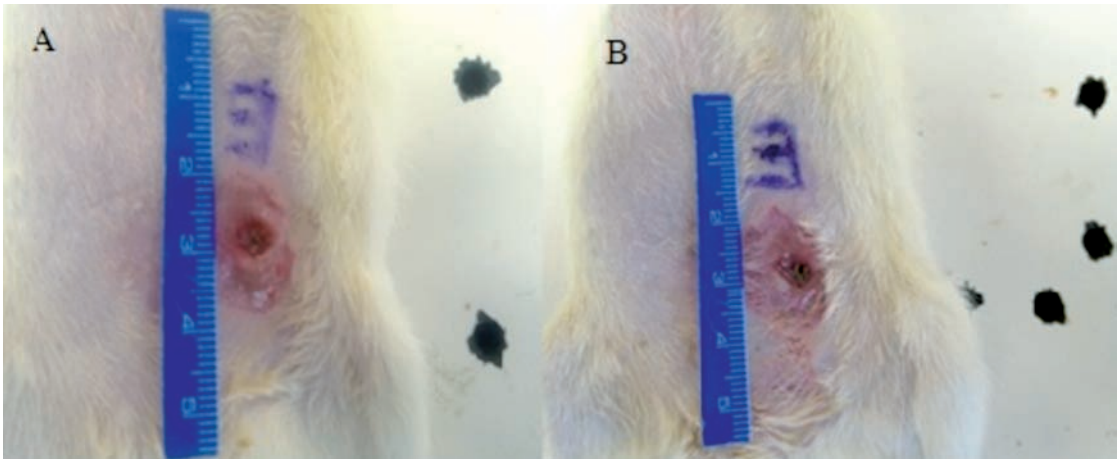

Figure 1. Control group Animal 3. *A,* 1st day of fistula formation. Note the infected region well. *B,* 21 days. The healing of the fistula did not occur. The region is well infected.

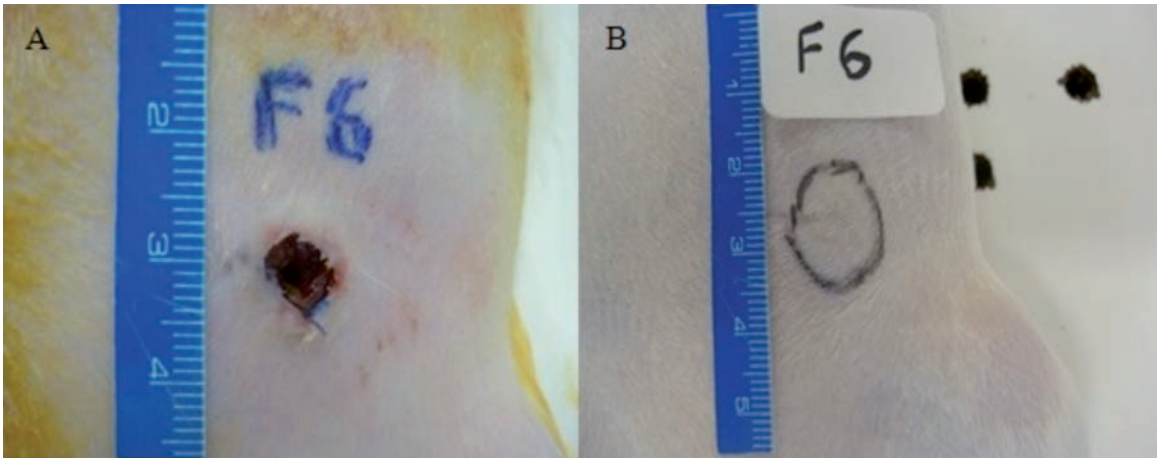

Figure 2. Suture Group. Animal 9. *A,* 1st day of fistula formation. Little infection locals. *B,* 21 days. The healing of the fistula was total. The circle shows the area where the leak was.

P-06 – CAN HUMAN LIPOASPIRATE BE CONSIDERED AN AUTOLOGOUS INJECTABLE SCAFFOLD TO REPAIR CARTILAGE DEFECTS?

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In the present study we aimed to achieve a chondrogenic differentiation of stem cells, not extracted from tissues but just in their own natural anatomical site (adipose tissue obtained as lipoaspirate) thinking that resident stem cells will transform the adipose tissue surrounding them into a fibrous cartilage at the beginning that will become hyaline cartilage under mechanical stress.

Today chondropathies treatment and prevention of osteoarthritis sees inta-articular administration of hyaluronic acid, treatment with PRP, bone marrow stimulation techniques (subchondral drilling, abrasion, microfracture), osteochondral grafting (mosaicplasty), autologous chondrocyte implantation (ACI) and matrix assisted autologous chondrocyte implantation (MACI) with autologous chondrocytes cultured on collagen membranes prior to re- implantation[1]. None of these techniques produces long lasting articular cartilage and alternative cell sources become necessary. Mesenchymal stem cells are giving promising results for cartilage repair[2] but in all these studies cells need to be isolated from the originating tissue and expanded and differentiated in vitro prior to transplantation into the damaged cartilage or into materials used as scaffold to deliver cells into the defect area.

With the aim to overcome excessive manipulation with enzymatic digestion, economic costs derived from a long period of cell expansion and differentiation and side effects derived from the scaffolds used for cell based cartilage repair we have tested the possibility to use intra-articular injection of human lipoaspirate thinking that adipose tissue works as natural scaffold for MSCs just entrapped in its stromal vascular portion and will be induced to differentiate to chondrocytes transforming adipose tissue into cartilage with or without the help of growth factors.

We have compared in vitro 3D floating cultures of lipoaspirates obtained with the techniques most frequently used from plastic surgeons (lipoaspirate, Coleman, PureGraft, Celution, Lipogems) treated or not for three weeks with the combination of growth factors that we have found to be chondrogenic on hMSCs[3]. Morphological, histological, histochemical, immuno-histological, mechanical, biochemical and molecular characterization evidenced that the treatment with the combined growth factors, induces the formation of a tissue completely different from the starting one and from that treated 3 weeks without chondrogenic growth factors. Comparing the 5 lipoaspirates, obtained with the 5 techniques above, the untreated lipoaspirate and that obtained with the Lipogems[4] technique showed the most significant changes in cellular organization, in mechanical properties, in chondrogenic specific proteins synthesis and in their mRNA expression. Our study is going on to evaluate with a bioreactor (BoseCorp., ElectroForce Systems Group, MI, USA) if the obtained tissue will further modify its structure when treated with mechanical load similar to physiological conditions.

If our preliminary data will be confirmed, in future orthopaedics will treat cartilage and osteoarthritis using lipoaspirate from the own patient.

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P-07 – THE IMPACT OF HUMAN SERUM, HUMAN SERUM ALBUMIN AND TEMPERATURE ON COLLAGENASE AND NEUTRAL PROTEASE ACTIVITIES

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An optimized ratio of collagenases and neutral protease activities are required to successfully release human islets from pancreatic extracellular matrix. During the tissue digestion process, free islets can be damaged with extended exposure to the exogeneous proteases used for digestion or the endogeneous proteases released from acinar cells. Clinical human islet isolations typically utilize cold human serum albumin containing buffer to collect the digestion solution to reduce protease activity and minimize the chances for islet damage. This report looks at some factors that inhibit collagenase and neutral protease activities used in tissue dissociation.

Collagenase and neutral protease activity were assessed by using FITC labeled type I calf collagen fibrils and FITC labeled bovine serum albumin, respectively. Purified Class 1 Collagenase (C1) from *C. histolyticum;* purified neutral protease from *B. polymyxa* (BP Protease), or purified neutral protease from *B. thermoproteolyticus* (Thermolysin) were diluted in Tris buffer at pH

7.5 or in the same buffer containing human serum or human serum albumin (HSA). Collagen degradation activity (CDA) or neutral protease activity (NPA) was measured at 35, 30, and 26°C.

Neither human serum at 10% nor HSA at 12.5% inhibited the C1 CDA but reducing the temperature to 30°C decreased the CDA by about 60% relative to those results obtained at 35°C (the temperature the CDA assay is routinely performed). Human serum reduced the BP Protease and Thermolysin activity. In contrast, HSA alone had little effect on neutral protease activity, other than at concentrations > 5% where it likely acts as a competitive substrate. The neutral protease activity of either protease was minimally affected by changes in temperature. Thermolysin activity was reduced by < 5% and BP Protease activity was reduced by about 20% at 26°C relative to 35°C.

These results indicate the low concentration of HSA typically used in human islet isolations (0.625%) does not impact the enzyme activities of collagenases or neutral proteases that may damage free islets. Two groups have reported on the value of using fetal bovine serum or human serum in the media for islet cell culture relative to human serum albumin alone[1,2]. This is likely as a result of protease inhibitors in serum inactivating exogeneous and endogenous proteases. While temperature does reduce the activity of tissue dissociating enzymes, these results suggest islets could benefit from being collected into serum immediately from the digestion circuit.

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P-08 – PRMT3 PLAY A KEY ROLE IN MPA-INDUCED BETA CELL DEATH THROUGH BINDING TO RHOGDI-A

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Introduction: MPA-induced beta cell toxicity is one of limiting factors for islet graft survival. However, the signal transduction mechanisms underlying MPA-induced beta cell toxicity have not been fully elucidated. Previously, we showed that MPA-induced apoptosis in pancreatic beta cell proceed through RhoGDI-α down-regulation linked to Rac1 activation. In the present study, we investigated the factor affecting the RhoGDI-α in MPA-induced β-cell apoptosis.

Methods: RhoGDI-α related protein was found by using the Yeast Two Hybrid (Y2H) analysis. Y2H screening of RhoGDI-α was performed in yeast PBN204 strain containing three reporters (*URA3*, *lacZ*, and *ADE2*)that are under the control of different *GAL* promoters. INS-1E cells, pancreatic β-cell line, were treated with MPA for 12hr, 24hr and 36hr. Functional screening was determined by using small interference RNA (siRNA)-mediated knockdown PRMT3 gene in INS-1E cell line.

Results: Using the Y2H analysis, we have identified 83 real positives. Among them, we found that Protein arginine N-methyltransferase 3 (PRMT3) was interacted with RhoGDI-α in INS-1E cells. PRMT3 was significantly decreased during MPA-induced apoptosis. PRMT3 silencing with siRNA induced MPA-induced cell death and altered Rac1 activity.

Conclusion: PRMT3 and RhoGDI-α were found to interact in pancreatic beta cell. Furthermore, MPA was found to regulate the interaction between RhoGDI-α and Rac1 in insulin-secreting cells by down-regulating the expression of PRMT3. We suggest that control of the interaction between PRMT3 and RhoGDI-α could be used to prevent MPA-induced beta cell death.

P-09 – THE ROLE OF THIOREDOXIN 1 IN THE MYCOPHENOLIC ACID INDUCED APOPTOSIS OF INSULIN PRODUCING CELLS

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Introduction: Mycophenolic acid (MPA) is a potent, noncompetitive, reversible inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH), a key enzyme in the purine *de novo* synthetic pathway of guanosine nucleotides, and is being widely used to prevent rejection after solid organ and islet transplantation. However, MPA can induce cellular toxicity and impair cellular function in β-cells. To address this issue, we used various methods, including an Illumina microarray, to examine which genes are regulated during pancreatic β-cell death following MPA treatment.

Methods: Pancreatic β-cell line, INS-1E cell, and isolated rat islets were treated with MPA for 12 h, 24 h and 36h. Microarray was performed according to the Macro-

gen rat BeadChip technical manual using Illumina RatRaf-12 Expression BeadChip. Functional screening was determined by using small interference RNA (siRNA)-mediated knockdown and over-expression of Trx1 gene in INS-1E cell line. The peroxide-sensitive fluorescent probe 2, 7-dichlorodihydrofluorescein diacetate (DCF-DA) was used to assess the generation of intracellular reactive oxygen species (ROS).

Results: We found that thousands of genes, especially *txn1*, were significantly altered during MPA-induced apoptosis. MPA reducesnot only Trx1 gene expression but also Trx1 protein expression in the INS-1E cell line and isolated rat islets. Over-expression of Trx1 restores cell viability, prevents MPA-induced apoptosis via the MAPK pathway and suppresses ROS generation in response toMPA. However, knockdown of Trx1by siRNA increased MPA-induced cell death, p-JNK and Caspase-3 activation. It was also observed that ROS levels were increased by MPA treatment.

Conclusions: MPA significantly provoked the apoptosis of insulin-secreting cells via Trx1 down-regulation, and that the expressional down regulation of Trx1 by MPA was associated with elevated ROS levels. Our findings suggest that the control of Trx1 downregulation in response to MPA is critical for successful islet transplantation.

P-10 – IMPACT OF CO-CULTURE WITH ISCHEMIC PRECONDITIONED HEPATOCYTE ON INSULIN SECRETING FUNCTION AND SURVIVAL OF ISLETS

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Introduction: The mechanisms of early graft failure following intraportal islet transplantation remain unclear. Hepatic ischemia and insufficient neovascularization of the transplanted islet were considered as one of the barriers against long-term survival of islet. On the other hand, hepatocyte is well known as its regenerative power. Also, hepatocytes have protective mechanism from ischemia. On the basis of the ideas of the effect of hepatocyte under ischemia, we hypothesized that ischemic hepatocytes beneficially effect on islet cells co-cultured with hepatocytes themselves. Thus, we designed this study cell line model co-cultured RIN-5F (insulin secreting cell line) with Hep-G2 (hepatoma cell line) and primary islets and hepatocyte model isolated from SD rat using hypoxic condition.

Methods: Hep-G2 cells and primary isolated hepatocyte were incubated in hypoxic chamber under the 0 % and 1 % O_2 hypoxic condition. The RIN-5F cells were co-cultured with each Hep-G2 incubated under different 1 % O_2 hypoxic and the islets were co-cultured with 0 % O_2 hypoxic treated hepatocyte.

Results: Insulin secretion of RIN-5F and islets were increased by ischemic-preconditioned Hep-G2 and hepatocytes. No change of cell viability of hepatocytes means that ischemic-preconditioning was not damaged to cell viability. IL-6, STAT3 pathway which known as a beneficial effect on the hepatocyte after ischemic injury also increased by hypoxic preconditioning. The IGF, HGF, TGF-alpha and TGF-beta of hepatocytes were also increased by reperfusion of ischemic-precondition. Insulin secretion and survival related gene expressions of islets were increased by co-culture with ischemic-preconditioned hepatocyte.

Conclusions: These results suggested that ischemia-preconditioning of liver or hepatocyte might enable well-function of insulin secretion and long-term survival in the environment as intraportal islet transplantation.

P-11 – HEPATOGENIC DIFFERENTIATION OF MURINE MESENCHYMAL STEM CELLS AS THERAPEUTIC STRATEGY IN CHRONIC LIVER DISEASE

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Background: Mesenchymal stem cells possess the ability to differentiate into hepatocyte-like cells in vitro. Thus, they open the possibility for use as therapeutic alternative instead of hepatocyte or even whole organ transplantation in different chronic (metabolic) liver diseases such as e.g. alpha-1-antitrypsin deficiency. This study aimed to examine functional parameters of murine mesenchymal stem cells after hepatogenic differentiation in order to develop a pre-clinical assessment of their potential for clinical application in chronic liver disease models.

Methods: Mesenchymal stem cells were isolated from murine adipose tissue of immunodeficient Pfp/Rag2$^{-/-}$ mice and were cultured in differentiation media after reaching 90% confluency. After 0, 7, 14 and 21 days, hepatogenic features like glycogen storage (PAS-staining), urea synthesis and the enzyme activity of ethoxyresorufin-*O*-deethylase (EROD) were measured. Periportal markers like phosphoenolpyruvatcarboxykinase (PCK1) and perivenous markers like glutamine synthase were stained immunocytochemically.

Results: The procedure of differentiation induced morphological changes from a fibroblastoid structure of undifferentiated cells into a polygonal shape of differentiated cells. Glycogen deposits were found from day 7 of differentiation further increasing until day 21. After 14 days of differentiation, functional markers like urea synthesis and EROD enzyme activity were significantly increased. Sub-populations of cells stained either positive for PCK1 or GS or both as identified by immunocytochemistry.

Conclusions: Hepatogenic differentiated murine stem cells feature specific hepatocyte functions already after 14 and 21 days of differentiation. Thus, they may serve as an alternative therapeutic agent instead of hepatocyte and/or whole organ transplantation in chronic liver diseases. Transplantation experiments in a mouse model of chronic alpha-1-antitrypsin deficiency will provide more insight soon.

P-12 – STABLE FEEDER- AND XENO-FREE SURFACES FOR LONG-TERM GROWTH OF UNDIFFERENTIATED HUMAN EMBRYONIC STEM CELLS

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Stem cells hold enormous potential for application in regenerative medicine and tissue engineering. Human embryonic stem cells (hESC) are difficult to culture while maintaining an undifferentiated state. Currently used techniques of culture on mouse embryonic fibroblast feeder layers or on a layer of Matrigel introduce xenogeneic proteins of unknown composition. Approaches for producing feeder-free culture surfaces composed of well defined human components are in their infancy. Most rely on physical adsorption of proteins, providing limited long-term stability of the protein layer. Silicone rubber (SiR) membranes were used as the culture substrate because reports show controlled oxygen at reduced levels can aid directed differentiation. However, hESC do not attach and grow on uncoated SiR. We describe a compositionally defined matrix that supports hESC expansion over five+ passages, consisting of a cross-linked hydrogel with carboxylic functional groups, produced byinitiated Chemical Vapor Deposition (iCVD) and deposited on SiR. This is the first report of iCVD coatings used for hESC growth. This allows us to coat SiR without modifying its oxygen permeability; iCVD allows easy copolymerization with monomers bearing useful functional groups such as carboxylic groups that can be easily functionalized with peptides and proteins; iCVD-prepared hydrogels can have a tunable cross-linking density that makes them stable under sterilization conditions and long term storage; and iCVD allows easy tuning of the functional group density on the surface directly relating to the density of peptides or proteins bonded on the surface. Changes in the vapor feed ratio of the monomers used in iCVD allowed variation of the density of -COOH groups on the surface. The -COOH groups were functionalized by reaction with $–NH_2$ groups of proteins to covalently bond proteins. The formation of amidic (-CONH) groups was detected by FT-IR. Surface density of bound protein (fibronectin) increased with increasing surface density of COOH groups. The density of protein influenced cell attachment and proliferation. Cell attachment was monitored by DAPI staining, and pluripotency was monitored by the transcription factor OCT4. For intermediate densities of COOH groups, cells formed a uniform confluent monolayer. The majority of the seeded cells expressed OCT4. When the density of COOH groups was the highest, the cells formed agglomerates instead of a uniform monolayer. When the density of the COOH groups was the lowest, the cells formed a uniform monolayer but not a confluent surface. The loss of confluency and monolayer formation resulted in spontaneous differentiation. Overmore than 5 passages, hES cells seeded on the coated surfaces displayed a normal karyotype, retained pluripotency, and formed teratomas after implantation into SCID/Beige mice. The stable, xeno-free synthetic approach for hESC culture described here is important for future scale-up of hESC production.

P-13 – DECELLULARIZED BOVINE CARDIAC TISSUE AS A NATURALLY DERIVED MATRIX FOR MESENCHYMAL STEM CELL CULTURE AND DIFFERENTIATION

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Introduction: Cardiovascular diseases lead to the decrease in both number and regenerative capacity of cardiomyocytes over time. Cardiac tissue engineering aims to repair, regenerate or replace damaged tissue by cell transplantation, by *in vitro* engineering of cardiac tissue and by using biomaterials designed for cardiac repair through vascularization or cell recruitment[1]. Scaffolds have been instrumental in promoting cell adhesion, growth, proliferation, and in providing a mechanical and instructive support. Decellularization technology offers new possibilities to make use of specialized tissues and organs for scaffold development[1]. Here, we have evaluated the efficiency of decellularized bovine cardiac extracellular matrix (bc-ECM) as a natural scaffold for mesenchymal stem cell (MSC) culture and differentiation.

Methods: Bovine heart tissue was decellularized using several protocols involving physical (freezing/thawing), acidic (deoxycholic acid, peracetic acid) and detergent (SDS/Triton X-100) treatments, besides lyophilization and sterilization. To determine cellular remnants we performed DNA content analyses. SEM and histological analyses were carried out to evaluate decellularization. bc-ECM was seeded with bone marrow MSCs (between P2-P5) obtained from Wistar rat femurs. MSCs were cultured under standard expansion and cardiomyogenic differentiation conditions. Viability of attached cells was determined by MTT assay. Immunohistochemistry (IHC) was employed to follow the level of differentiation.

Results and Discussion: SEM analyses demonstrated that decellularized bc-ECM had macroporous structure allowing MSCs to attach and proliferate within the scaffold. DNA content analyses revealed a significant decrease in the DNA level of decellularized bc-ECM, compared to

that of the native bc-ECM. Acellularity was also confirmed by histochemistry. MTT assay demonstrated the viability and proliferation of seeded bone marrow MSCs on decellularized bc-ECM scaffold. IHC showed somewhat increase in the expression of cardiac markers compared to that of MSCs cultured on polystyrene culture plate, both under cardiomyogenic conditions.

Conclusions: Decellularized bc-ECM supports the attachment and growth of mesenchymal stem cells, and may have potential as a natural inductive scaffold for use in cardiac repair with a suitable environment for regenerating cells.

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P-14 – BIDIRECTIONAL CELLULAR SUPPORT IN THE COCULTURE OF HEPATOCYTES WITH MESENCHYMAL STEM CELLS IN ACUTE

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Objectives: Human hepatocyte (HC) transplantation has promise as a bridge to organ transplantation or spontaneous recovery in acute liver failure (ALF). Intraperitoneal transplantation of alginate encapsulated HC hepatocytes is an exciting new approach though the function and viability of transplanted heaptocytes is a concern. There has been interest in mesenchymal stem cells (MSC) in ALF therapy as support for coencapsulated HC in addition to their anti-inflammatory/anti-apoptotic properties. We have previously shown trophic effects of MSC on HC in standard coculture. The aim of this study was to investigate this coculture system in the presence of human ALF serum to assess the potential of this cellular therapy in ALF.

Methods: Human HCs were isolated from donor organs and MSC from donated umbilical cord. Cells were plated in monoculture or in direct coculture at a ratio of 6:1 (HC:MSC). After 24 hr, serum from patients with ALF, normal controls or fetal calf serum (FCS) was added in increasing concentrations. 24 hours later, standard culture medium replaced serum following washing. Cytotoxicity was measured using the sulforhodamine B and MTT assays at 24 and 48 hr of culture. Specific HC toxicity was estimated using the soluble K18 M65 cell death assay. Albumin production was measured using ELISA. Experiments were repeated in triplicate.

Results: At 24 and 48 hrs, MSC monoculture demonstrated higher cytotoxicity (>50% reduction in metabolic activity) in ALF serum versus FCS control (p<0.001). HC monoculture maintained metabolic activity and cell survival in ALF serum versus FCS at both time points. Cocultured HC and MSC demonstrated better metabolic activity in ALF serum compared to HC or MSC monoculture (p=0.04). At 48 hrs, specific HC death in coculture versus monoculture was increased by a factor of 2.3 in FCS and by 1.3 in normal serum. This adverse effect was abroagated in ALF serum with no increase in HC death in this culture condition. Albumin production at 48 hrs was greatly increased in cultures which had previously been exposed to human serum versus FCS. This effect was seen best in cocultured HC with albumin production 7.5 times greater following ALF serum versus FCS exposure (p=0.02).

P-Conclusion: ALF serum has toxic effects on MSC but when cocultured with HC, cytotoxicity appears to be reversed, suggesting a possible protective effect of HC on MSC in this context. This is a novel finding as previous work has described the trophic effects of MSC on hepatocytes. This bidirectional cellular support may have an important role in cellular therapy of ALF.

P-15 – OPTIMIZATION OF MOUSE PANCREAS TISSUE DIGESTION USING RECOMBINANT COLLAGENASE CLASS I AND II BLEND AND PROTEASES

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Introduction: Collagenase (COL) are produced by two separate and distinct genes in *Clostridium histolytic*; both genes have been cloned and sequenced, generating the recombinant forms of COL-G and COL-H [Seidita *et al*. Xenotransplantation 18(5):291-292;2011]. Enzyme activity, purity, and formulation strongly influence the outcome of the islet isolation. One of the most critical steps on preparation of enzymes is determination of their activity. We analysed the potency of recombinant COL-G and COL-H blends and neutral proteases or thermolysin in order to obtain the best pancreatic islet extraction. We used in parallel classical pz test and collagen digestion unit (CDU), together with *ex vivo* mouse pancreas dissociation assessment, to evaluate COL-G and COL-H activity and determine the amount of proteases necessary to accelerate the extractive reaction.

Results: The activity of COL-G was lower compared to COL-H when we used Chromophore-Substrate Pz peptide, 4-Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg-OH (Fluka 27667) according to Wunch; in this test, one unit liberates 1μmol of Pz-Pro-Leu from Pz- Pro-Leu-Gly-

Pro-D-Arg in 1 minute at pH 7.1 at 25°C; we have COL-G 0.78 unit/mg and COL-H 4.9 unit/mg. Similarly, using pz Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH according to Grassman (Fluka 27673), COL-G activity was 0.4 unit/mg and COL-H was 1.4 unit/mg. This is likely because the preferred substrate for COL-G is the native collagen, while for COL-H denatured collagens. The Pz activity in the mixture of collagenases G and H depended prevalently from the amount of COL-H present. The blend H/G = 2:1 have an apparent higher activity compared to the blend H/G= 0.5 (1:2). However, *ex vivo* extractive capability of the two formulations showed opposite results: 90% tissue dissociation was obtained with H/G=1:2, and only 40% dissociation with the H/G=2:1 blend. Our data suggest that the pz method is not optimal to determine the activity of recombinant collagenase blends. More similar to the activity *ex vivo* was the CDU method for which we used insoluble collagen (Sigma C-9879); in this test one unit liberates peptides from collagen equivalent in ninhydrin color to 1.0 μmole of leucine in 5 hours at pH 7.4 at 37°C in the presence of calcium ions. In this case it is possible to see the synergy between G and H; the enzyme blend G/H 2:1 have about 900 CDU/mg. The collagen digestion unit of COL-G and COL-H alone were ~500 CDU/mg and and ~300 CDU/mg, respectively. No extractive process occurs when we used the two enzymes individually. Moreover our data confirm the fundamental role of proteases to accelerate islet extraction from pancreas: the optimal dosage of thermolysin was 2 mg/ml; while, for neutral proteases 0.05 unit/ml, added to 4 ml of mix Col G-H (2:1) 1 mg/ml in mouse islets pancreas purification.

Collectively, our preliminary data suggest that enzyme blend formulations based on recombinant collagenases H and G can be generated and may allow for efficient and reproducible dissociation of pancreatic tissue. Optimization of blend formulations may be of assistance in standardizing the human pancreatic islet isolation process.

P-16 – COATING OF PANCREATIC ISLETS WITH REGULATORY T CELLS FOR LOCAL IMMUNOPROTECTION – BIOTIN-PEG-SVA VERSUS BIOTIN-PEG-NHS AS A BINDING MOLECULE

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Introduction: T regulatory cells (Tregs) possess immunosuppressive properties and can serve as an effective therapy in transplantation. Recently, we have shown that Tregs can be attached to the surface of human pancreatic islets using Biotin-Polyethylene- (biotin-PEG-) glycol-N-hydroxylsuccinimide (NHS) molecule providing local immunoprotection[1]. Further optimization of the method can improve coating parameters and the efficiency of this novel immunoprotective approach, which may prolong graft survival. In this study, we compared two molecules to coat pancreatic islets with Tregs in order to increase number of Tregs attached to islets surface without compromising islets viability and function.

Methods: Cell surface of human Tregs and pancreatic islets was modified using our previous approach-biotin-PEG- NHS or new molecule instead- succinimidyl *valeric*acidester (SVA) (1 mg/ml and 0.6 mg/ml was used for Tregs and islets, respectively). Then, islets were incubated for 15 minutes in 37°C with 1mg/ml of streptavidin as islet/Tregs bridging molecule. Subsequently 150 islets were combined with $50x10^6$ Tregs and incubated overnight for binding. To compare coating effectiveness between using NHS or SVA group, Tregs were stained with CellTracker™ CM-DiL dye and visualized using the Olympus FV1000 Laser Scanning Confocal Microscope. The number of Tregs attached to islets and islet surface area were counted on Imaris software. The effect of coating on islets functionality was determined using Glucose-Stimulated Insulin Response (GSIR) assay: naked (control) or Tregs coated islets were exposed to high (28 mM) and then to low (2.8 mM) glucose solution for 1h. The solutions of each glucose concentration were collected, concentration of released by islets insulin in each was determined by Enzyme-Linked Immunosorbent Assay (ELISA).

Results: Coating procedure with biotin-PEG-SVA in comparison to biotin-PEG-NHS allowed attaching 40% more Tregs per 1 μm^2 of islets surface. While viability was comparable, function of the islets after coating using biotin-PEG-SVA molecule was better preserved than with NHS molecule. GSIR was 62% higher for islets coated with biotin-PEG-SVA than biotin-PEG-NHS, and as high as in unmodified islet controls.

Conclusions: Coating of islets with Tregs using biotin-PEG-SVA improves effectiveness of coating with better preservation of the islet function comparing to biotin-PEG- NHS. Improvement in the method of coating pancreatic islets with Tregs can further facilitate the effectiveness of this novel immunoprotective approach and translation into clinical settings.

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P-17 – AUTOLOGOUS ISLET TRANSPLANTATION AFTER TOTAL PANCREATECTOMY FOR CHRONIC PANCREATITS INTO THE LIVER AFFECTED BY PRIMARY SCLEROSING CHOLANGITIS

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Purpose: Intraportal islet autotransplantation is an attractive procedure for patients requiring total pancreatectomy for benign disease. It may improve long-term glucose control or even prevent the development of postsurgical diabetes. A healthy liver has been considered the most optimal site for islet transplantation. We present for the first time successful autoislet transplantation into a liver affected by primary sclerosing cholangitis(PSC).

Methods: Total pancreatectomy and subsequent islet autotransplantation were performed in a 16-year-old man with intractable pain due to chronic pancreatitis. Patient also had a history of ulcerative colitis and PSC with multiple biliary strictures. Wedge hepatic (portal) pressure during the preoperative transjugular liver biopsy was 14 mmHg and biopsy revealed PSC with focal bridging fibrosis. During the procedure, the pancreas was surgically removed and digested, islets were isolated, highly purified, and infused intraportally as 1 ml tissue volume suspended in transplant media containing 70u/kg heparin.

Results: Opening portal pressure prior to islet infusion was as high as 19 mmHg, but did not further increase after completion of islet infusion. Postoperatively, liver function and portal flow were not affected by the islet autoransplant.

At one year follow up, patient had excellent glycemic control with HbA1c 5.9%, c-peptide 0.77 pmol/ml (N=0.3-2.3), and requiring only periodic short-acting insulin despite chronic oral steroid therapy (Budesonide EC 9 mg daily) to control his autoimmune gastritis. Liver function remains unchanged; serum albumin, INR and bilirubin have been within normal limits. His transaminases and alkaline phosphatase are also not significantly changed from baseline prior to procedure.

Conclusions: Pancreatic autoislet can be successfully transplanted into the liver with a hepatobiliary disease related to PSC without affecting liver or graft function. Durability of the procedure may be compromised in the future by the natural course of the liver injury caused by PSC.

P-18 – EFFECTS OF MICRO-ENCAPSULATION ON SURVIVAL AND FUNCTION OF CRYOPRESERVED NEONATAL PORCINE ISLET-LIKE CELL CLUSTERS

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Background: Xenogeneic islet transplantation is promising for overcoming the shortage of human islets. For the success of clinical islets transplantation, it is necessary to develop a long-term storage method that assures the availability of viable and genetically stable stocks of functional islet.

Methods: Neonatal porcine islet-like cluster cells (NPCCs) were microencapsulated in 1.5% alginate. NPCCs were frozen and thawed using three different media containing varying amounts of Ham's F10 medium (F10) and neonatal porcine srum (NPS); Group1 (G1; 100% NPS+2M DMSO), Group2 (G2; F10+2M DMSO), Group3 (G3; F10+10% NPS+2M DMSO). Post-thawing cell viability, and insulin release function were assessed using CCK-8 assay, glucose-stimulated insulin secretion (GSIS), and immunohistochemistry (IHC). We also tested GFP expression in order to verify genetically engineering maintains its functioin during cryopreservation and thawing. Selective permeability of the microcapsules after cryopreservation was confirmed using 20 kDa and 150 KDa fluorescein isothiocyanate-labeled dextran (FITC-dextran).

Results: G2 had a significantly lower survival rate compared wih G1 and G3 (G1: 97.69±1.74%, G2: 72.98±4.46%, G3: 99.69±1.67%) after 3 days of cryopreservation. G2 also had lower GSIS compared with G1 and G3 after 3 days (G1: 3.43±0.44, G2: 0.66±0.13, G3: 4.20±0.52). TUNEL assay showed that necrotic islet cells increased in G2, and insulin staining demonstrated that insulin-positive cells were also slightly decreased in G2. Microcapsules were not affected by the cryopreservation and thawing, and maintained their selective permeability, which allowed 20 kDa FITC-dextran, whereas did not 150 kDa FITC-dextran. GFP expression was deteriorated after thawing in G2.

Discussion: The use of microcapsule and NPS in islet cryopreservation provided further protection against apoptosis, and maintained insulin secretion, selective permeability and stable expression of genetic engineering in encapsulated NPCCs after cryopreservation and thawing.

P-19 – INTRAPORTAL AUTOISLET CELL TRANSPLANTATION (IAT): CHANGES IN PORTAL PRESSURE AND FLOW AND ASSOCIATED CLINICAL OUTCOMES

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IAT tissue volume (TV) delivered into the portal venous system can be associated with acute portal hypertension. We analyzed the relationships between transplanted TV, portal vein (PV) pressure and PV flow, as well as the timing and nature of complications.

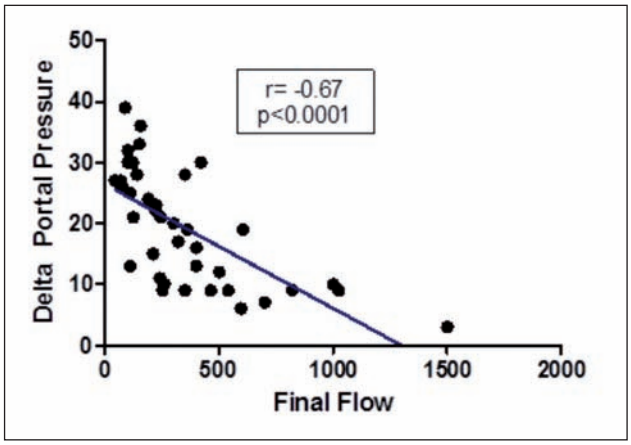
Methods: Between 7/2011 and 11/2012 we studied 39 total pancreatectomy IAT patients who underwent pre- and post-infusion portal pressure and blood flow measurements. TV (ml), islet equivalents/kg, PV pressure and PV flow (pre-infusion, peak post infusion, and post-infusion) were evaluated for the cohort. Patients were evaluated for PV thrombotic complications. The correlations between changes in PV flow, pressure and tissue volume were evaluated.

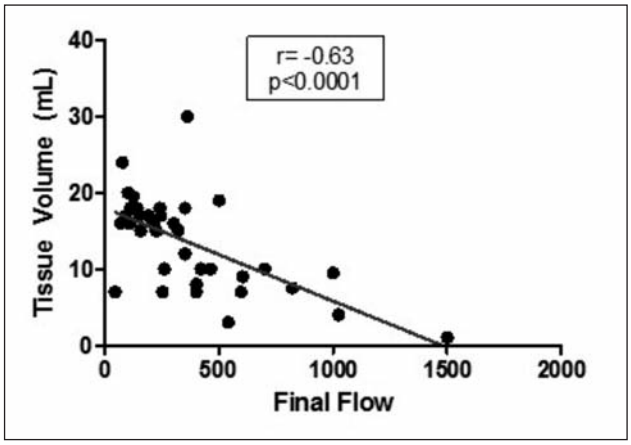
Results: Transplant data for the cohort are shown in Table 1. We found correlation between the delta PV pressure (peak minus baseline) and PV final flow rates (r=-0.67, p<0.0001), as well as TV and PV flow (r=-.063), p<0.0001). 3 patients had transient perterbations in flow; reversal of flow or no flow in the left PV, detected within a week of transplant. However, these resolved within a few weeks. We observed no episodes of main PV thrombosis.

TABLE 1

	Mean	SD	Min	Max
Baseline pressure (cmH_20)	1.6	3.3	-4	13
Baseline flow (L/min)	0.627	0.31	0.14	1.4
Peak Pressure (cmH_20)	20.63	9.87	5	40
Delta Pressure (cmH_20)	19.15	9.45	3	39
Peak Flow (L/min)	0.348	0.35	0.08	1.6
Final Pressure (cmH_20)	18	9	3.5	39
Final Flow (L/min)	0.356	0.31	0.04	1.5
Tissue pellet (ml)	13.65	5.98	1	30

Conclusions: Autoislet cell transplant is associated with sometimes significant acute portal hypertension. PV pressures and TV both correlate with final PV flow. No serious/persistent complications were observed. Real-time PV flow measurements may be more useful than intermittent PV pressure measurements during Tx.





P-20 – CELLULAR INTERNALIZATION OF TAT-HIGH MOBILITY GROUP BOX 1 A FUSION PROTEIN FOR CYTOPROTECTION OF XENOTRANSPLANTED PANCREATIC ISLETS

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Although islet transplantation is an attractive procedure for the therapy of type 1 diabetes, it is usually fails to achieve permanent insulin independent because of early graft loss. When diabetic patient were transplanted islet from 2-3 donor, the transplanted islets are recognized as antigens by host immune system and they trigger the process of recruiting immune cells such as macrophages, fibroblast, and lymphocytes. The activated immune cells release various cytokines, which can induce structural and functional damage to islets. Recently, High-mobility group box 1 (HMGB1) protein is a DNA-binding protein, stabilizes nucleosome formation, and acts as a nuclear factor in a cell. The HMGB1 has 2 homogenous domains (A- and B-box) and acidic tail. The B-box is associated with proinflammatory activity, while the A-box is a specific antagonist by which it inhibits the proinflammatory properties of HMGB1.Recently, HMGB1 is known to be secreted by activated immune cells such as macrophages, dendritic

cells and NK cells in response to infection and inflammatory reactions. Interestingly, it is reported that it is markedly increased during initial events of early graft loss of transplanted islets. Specially, pancreatic islets strongly expressed the HMGB1 protein with higher contents. Therefore, we predicted that the higher content of HMGB1 in pancreatic islets is related to early islet graft loss. To control the activity of HMGB1 protein in islets, we here newly designed the cell-absorbable Tat peptide-conjugated HMGB1 A-box (TAT-HMGB1A) fusion protein. When we treated isolated rat islets with TAT-HMGB1A, it was significantly internalized into islet cells with dose-dependency. Also, compared to control islets, the TAT-HMGB1A-treated islets had normal viability and functionality. Moreover, TAT-HMGB1A treated islets reduced HMGB1 activity. To evaluate that TAT-HMGB1A could attenuate the essential islet mass *in vivo*, we xenotransplanted marginal mass of rat islets into streptozotocin-induced diabetic mice (200 islets equivalent/mouse). Control islets did not normally control recipient's blood glucose levels, whereas TAT-HMGB1A-treated islets reduced recipient's blood glucose levels for more than 1 month. This interesting result might be derived from the inhibition of HMGB1 activity via the internalized TAT-HMGB1A. Therefore, this TAT-HMGB1A delivery could be used for successful pancreatic islet transplantation to cure type 1 diabetes mellitus.

P-21 – NUMB EXPRESSION CONTRIBUTES TO THE MAINTENANCE OF AN UNDIFFERENTIATED STATE IN HUMAN EPIDERMIS

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Background: The epidermis is a stratified epithelium with a stem cell subpopulation in the basal layer that replicates constantly and periodically detaches from the basis, undergoing a differentiation process that involves various developmental signals and regulatory pathways. Over the course of the last ten years, a number of studies have aimed to elucidate the intricate scenario that maintains the epithelial shield during the entire life span.

Objectives: In this study we tried to establish the role of Numb in the skin compartment, and in particular its involvement in stem cell maintenance.

Methods: Numb expression in skin compartment was evaluated by immunofluorescence and immunohistochemistry. Cell separation by adhesion and through FACS fractionation was used to evaluate, by Western blot, Numb expression in primary cells at various differentiative stages. Moreover, we overexpressed Numb in the isolated population enriched for undifferentiated progenitors to establish its involvement in in vitro differentiation.

Results: The presence of Numb in high proliferating epithelial undifferentiated progenitors contributes to the maintenance of an undifferentiated state. This regulation involves the E3 ligases Itch binding. Moreover, the analysis of a cohort of cutaneous carcinomas showed that Numb is highly expressed in squamous cell carcinoma (SCC) and basal cell carcinoma; in both types of carcinoma we observed a direct correlation between the expression of Numb and Ki67.

Conclusions: Our data indicate that Numb is involved in the maintenance of the undifferentiated proliferating stem cell pool in the epithelial basal layer, and its expression could become a new marker in skin cancer.

P-22 – HUMAN BONE MARROW-DERIVED MULTIPOTENT STROMAL CELLS VIABILITY IS DEPRESSED BY UREMIC TOXINS - P-CRESOL AND INDOXYL SULFATE

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Over recent years, multipotent stromal cells, also known as mesenchymal stem cells (MSCs) receive a lot of interest in many medical fields as cellular therapies. In renal disease, patients may profit from their capacity to aid tissue regeneration and modulate the immune system. However, the influence of uremic conditions on MSCs' properties has not yet been elucidated. Meanwhile, it is essential to predict the efficacy of MSCs therapy in renal disease patients. Therefore, we examined the effect of two important protein – bound uremic toxins - p-cresol (PC) and indoxyl sulfate (IS) – on MSCs' viability in vitro.

Human bone marrow-derived MSCs, obtained mainly from patients undergoing hip arthroplasty, were incubated with PC and IS at concentrations corresponding to different stages of chronic kidney disease (0.005 mM - 0.01 mM - 0.25 mM - 0.5 mM of PC and 0.004 mM - 0.04 mM - 0.2 mM - 1.0 mM of IS). After exposure to toxins for 12 hours cells' proliferation rate (BrdU immunoassay) declined significantly in comparsion to untreated controls. PC at 0.25 mM and 0,5 mM caused inhibition of MSCs' proliferation by 17% (p=0.008) and 28% (p=0.008), respectively. IS had a stronger impact on DNA synthesis, causing its decrease by 34% (p=0.008) at 0.2 mM and by 47% (p=0.008) at 1.0 mM. Mitochondrial activity (MTT assay) decreased similarly in dose-dependent and also time-dependent manner. After 48h of incubation PC at the highest concentration of 0.5 mM decreased MSCs' mitochondrial dehydrogenases activity by 38% (p=0.008), whereas IS at concentration of

1.0 mM by 56% (p=0.005). PC at 0.25 mM and IS at 0.2 mM did not induce MSCs' apoptosis (Annexin V test), but significantly damaged cells membranes (LDH activity assay). Taken together, MSCs' viability was significantly downregulated by protein-bound uremic toxins, p-cresol and indoxyl sulfate, in vitro.

Presented data indicate potentially negative influence of uremic conditions on MSCs' functionality. Therefore, their use as autologous therapeutic tools in patients suffering from kidney diseases and the hypothesis that MSCs mobilized from the bone marrow can participate in kidney regeneration may be questionable. Moreover, pathologic complications of chronic kidney disease associated with low mesodermal cells turnover (such as cardiovascular events or osteodystrophies) may be regarded as a consequence of MSCs dysfunction caused by uremic toxins.

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P-23 – HEPATOCYTE TRANSPLANTAION USING AFTER HEPATECTOMY ORGAN OF CARCINOMA

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Introduction: The patient who underwent large hepatectomy sometime easily became liver failure. Recently large hepatectomy is very popular for cholangiocarcinoma (CCC). Recently, we paid attention to this recected liver. This recected liver have a large quantity of normal hepatocytes with carcinoma cell. If completely divited two group using any method, these cell were able to transplant. This transplant became the singenic transplant and thought that it was possible for the hepatocytes transplant that future clinical for liver failure treatment.

Materials and Methods: Immortalization rat hepatocytes having SV40 largeT antigen as the artificial chromosome vector which we already developed in our laboratory. Immortalizis cell means cancer cell. At first marked this cancer cell (use the immortalization hepatocytes which introduced GFP into) and mixed this cell and normal hepatocytes and cultured it and I divided cancer cell group using FACS afterwards and examined it whether the next normalcy hepatocytes were implantable cells.

- G1 Cancer cell group: Artificial chromosome (hepatocytes having SV40 largeT antigen +GFP): 1X106),
- G2 Normal hepatocytes group: normal hepatocytes (Lewis Rat cell separation; 1X106). Mix these both counties at 7:1 (I do each cell) and divide into two groups using FACS.

This hepatocyte (1X105 cell) with solution of 0.5ml and injects it in 25G to the spleen directly in retrosin treated NARs (Non albumin Rat).

And also all groups were collected blood sample on 0,7,21, the 28th day and measured blood albumin value by ELISA.

Conclusions: The normal hepatocytes and cancer cell were able to prove that they were separable by this method. However, it is this experiment problems whether I can isolate it surely and the cell count can extract a large quantity of implantable cells with a small quantity in the real clinical place.

P-24 – HOW THE RADICAL SCAVENGER WORKING FOR HEPATOCYTE TRANSPLANTATION IN RATS?

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Introduction: It has already been established that hepatocyte transplantation (HTx) in animal models, such as both chemically and surgically induced acute liver failure, liver-based metabolic disease, resulted in significant improvement of liver function and survival.

However, the transplant effects are not enough, and it is thought that the transplant cell by the hepatocyte transplant wears the life of the cell by a large quantity of NO by the stoppage of the organization, and an obstruction is angry at the one of the reason.

We investigated the effects of edaravone, a new free radical scavenger, on liver oxidative stress in vitro and in vivo.

Material and Method: The edaravone method, It takes effect in edaravone (3mg/kg. iv.) to a no albumin rat (NARs) just before 24 hours Hepatocyte transplant. The hepatocyte isolated with cell separation method and mixes a 30X106 cell with solution of 0.5ml and injects it in 25G to the spleen directly. (Lewis rat (150-250g) entirely.)

- G1: Control (medium injection)
- G2: Normal hepatocyte transplant
- G3: Normal hepatocyte transplant + edaravone,
- G4: edaravone without Tx

All groups animal were sacrificed by day one and measured Annexin V positive cell (eary apoptosis cell) and Propidium Iodide positive cell (dead cell) using FACS.

And also all groups were collected blood sample on 0, 3, 5, 7, the 14th day and measured blood albumin value by ELISA.

Result: A V positive cell and P I positive cellimproved in both edaravone groups compared with control.

The albumin value accepted increase with the edaravone treated group significantly. At the same time the spleen which transplanted these hepatocytes could observe a hepatocyte entirely, and, transplant two weeks later, hepatocytes increased with the edaravone treated group significantly again.

Considerations: It was proved that the edaravone was effective for a transplant cell in this study, and clinical application would be possible in future, and it was thought that I would be useful in the future.

P-25 – DEXAMETHASONE CONJUGATED POLYAMIDOAMINE DENDRIMER GENERATION 2 AS EFFICIENT PLASMID GENE DELIVERY CARRIER

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Polyamidoamine (PAMAM) dendrimers have been utilized as drug and gene carrier due to their well-defined size and properties. Dexamethasone, a glucocorticoid steroid, translocates into the nucleus when it is bound to its glucocorticoid receptor and has anti-inflammatory effects. In addition, the glucocorticoid receptor dilates the nuclear pore complexes, which is beneficial for DNA delivery into the nucleus. In this study, the dexamethasone (dexa) was conjugated to PAMAM generation 2 (G2) (PAMAM G2-dexa) in order to increase transfection efficiency of PAMAM. In addition, PAMAM G2-dexa has an anti-inflammatory effect. PAMAM G2-dexa was synthesized by one-step reaction using Traut's reagent. The characterization of PAMAM G2-dexa is performed by various methods. In order to determine whether PAMAM G2-dexa formed complexes with pDNA, a gel retardation assay was performed. PAMAM G2-dexa retarded completely plasmid DNA (pDNA) at a 1:3 weight ratio. A heparin competition assay was performed to evaluate the stability of the PAMAM G2-dexa/pDNA complexes. PAMAM-dexa begins to release pDNA in the presence of a thirty-fold weights excess of heparin. The average size and zeta-potential of the PAMAM G2-dexa/pDNA complex were measured at various weight ratios and compared to the PEI/pDNA complex. The cytotoxicity was evaluated by MTT assay in neuro 2A cells. Although the toxicity of PAMAM G2-dexa had slightly higher than PEI, In vitro transfection assay showed that PAMAM G2-dexa had higher gene delivery efficiency at an 1:4 (pDNA:carrier) weight ratio into Neuro2A cells, compared to those of the PAMAM G2 and polyethylenimine (PEI). Heme oxygenase-1 (HO-1) expression, which delivered by PAMAM G2-dexa was measured by the HO-1 ELISA. The delivery efficiency of the HO-1 gene showed that PAMAM G2-dexa had higher than PAMAM G2 and comparable to PEI. Therefore, PAMAM G2-dexa may be an efficient gene carriers with high transfection efficiency and low cytotoxicityand useful for the treatment of ischemic diseases.

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P-26 – ENCAPSULATION OF ISLETS WITH CLODROSOME IN MATRIGEL IN A XENOGRAFT MURINE MODEL

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This study proposes a strategy to generate new immunosuppressive therapy to prevent islet rejection after transplantation in type 1 diabetes. This strategy consists of co-encapsulation of islets and liposomal clodronate to inhibit activation of macrophage and immune cells in the early stage of transplantation. There is need to develop successful strategies for immunosuppression to the transplanted insulin-producing cells in the absence of continuous recipient immunosuppression. Liposomal clodronateis clodronate-encapsulated liposome for depletion of alveolar macrophages and prevention of macrophage activation. In this study, we have co-encapsulated pancreatic islets with clodrosome in the Matrigel (BD bioscience, San Jose, CA) in order to improve the islet survival time since clodrosome deplete phagocytes in the islet transplanted area. Macrophage depletion is important to improve the graft survival time of grafted islets because cellular immune reaction triggers macrophage activation in the early stage of transplantation.

In order to evaluate the releasing profile of liposomal clodronatefrom the Matrigel, we have used Cy5.5 labeled liposome as a drug tracer. Cy5.5 labeled liposomal clodronate releasing profile was observed for 7 days.The releasing profile of liposomal clodronatedemonstrated that liposomal clodronate remained in the Matrigel for over 48 h. Matrigel-encapsulated or unmodified pancreatic islets (2000 IEQ/mouse) were subcutaneously transplanted into Sprague-Dawley (SD) rats. The mean survival time (MST) of Matrigel-encapsulated islets recipients (5.5 ± 0.22 days) was statistically increased when compared to unmodified islets (0 ± 0 day, blood glucose level > 200 mg/dl; All recipients). On the other hand, when a low dose of liposomal clodronate (6.25 mg/kg) was co-encpasulated with islets in the Matrigel, the MST of encapsulated islets (> 60 days) was significantly increased compared to that of native islets (0 day) with liposomal clodronate(Subcutaneously, 6.25 mg/kg). After 60 days of transplantation,

transplanted islets co-encapsulated with liposomal clodronate respond rapidly when exogenous glucose was injected. These results proved that early macrophage depletion is very important to prevent islet rejection after transplantation. Immunohistochemical staining of islet-transplanted area demonstrated that there was strong evidence of insulin-positive cells in islets co-encapsulated with liposomal clodronatein the Matrigel. In addition, immunohistological data indicated that local delivery of a low dose of liposomal clodronate was effective in inhibiting immune cell migration and activation.

In conclusion, this newly developed method for immunosuppression would certainly be an effective strategy for preventing immune activation and for improving the overall survival of grafted islets after transplantation.

P-27 – ALGINATE MICROENCAPSULATED HUMAN HEPATOCYTES: ASSESSMENT OF PHYSICAL INTEGRITY AND EFFECT OF CELL DENSITY ON HEPATOCYTE VIABILITY AND METABOLIC FUNCTION

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Encapsulated human hepatocyte (microbead) transplantation into the peritoneal cavity is an attractive option for the management of acute liver failure. Optimal hepatocyte function and physical integrity of microbeads are essential factors for clinical use.

Aim: To optimise the protocol for production of alginate microbeads and to investigate hepatocyte viability in relation to cell distribution within the microbead.

Methods: Microbeads were produced using an encapsulator (250μm nozzle) with GMP grade materials. Empty and human hepatocyte ($2.5x10^6$cells/ml alginate) microbeads were made using different polymerisation times (10, 15 & 20 min). Physical stability of microbeads was determined using an osmotic stress test. Empty microbeads (EMB) were incubated for 3h in 4 hypotonic and 1 isotonic solutions (transplant medium: CMRL) to establish the osmolarity at which microbeads are stable. Microbeads immediately after production were used as controls. MB size (n=100/sample), MTT assay, and viability (FDA/PI) were evaluated. Hepatocyte functions (urea and albumin synthesis) in human hepatocyte microbeads (HMB) were assessed after maintenance in CMRL for 24h. HMB were produced at 4 densities: 2.0, 2.5, 3.0 and 3.5 million cells/ml alginate. HMB were maintained in culture for 3d. HMB morphology and cell viability were assessed at day1using FDA/PI and 3D confocal microscopy. Cell functions (MTT, urea and albumin) were evaluated at day1 and day3.

Results: Microbeads were of uniform shape and size (mean diameter: EMB, 577±SEM 0.89μm and HMB, 583±0.64μm). There was a significant increase in EMB diameter in all samples incubated in hypotonic solutions compared to control (p<0.001; ANOVA). The 15min polymerisation group tended to resist osmotic shock better than 10 and 20min groups. HMB showed a similar trend, but was not statistically significant between groups. No ruptured microbeads were observed. There was no significant difference in metabolic function between the 3 different polymerisation time groups. Cell density data showed that a density of $3.5x10^6$ cells/ml provided the highest viability compared to the other three groups (p=0.001). 3D reconstruction of confocal microscope images demonstrated that there was no significant difference in cell viability within a microbead (inner half vs outer half) in all 4 groups. MTT, urea and albumin assays also demonstrated no significant differences between the 4 groups on either day1 or day3.

Conclusions: An optimised protocol for production of clinical grade human hepatocyte microbeads with good cell viability, function, and physical integrity has been established.

P-28 – DEVELOPMENT EPITHELIAL CELL SHEETS TRANSPLANTATION AFTER ENDOSCOPIC MUCOSAL RESECTION IN ESOPHAGEAL NEOPLASMS

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Instruction: Recently, cell-based therapies, regenerative medicine, and tissue engineering have been progressing rapidly. We have developed a novel strategy for regenerative medicine to recover tissue functions using temperature-responsive cell culture surfaces[1]. To overcome of conventional methods such as the usage of single-cell suspension injection, we have applied transplantable cell sheets fabricated with temperature-responsive culture surfaces for cell delivery. In the field of gastroenterology, these regenerative medicine and tissue engineering approaches have attempted to prevent postoperative stricture by structurally and functionally reconstructing normal tissues through the promotion of early re-epithelialization after endoscopic large size mucosal resection. Our group previously reported a method of regenerative therapy involving the transplantation of fabricated epithelial cell sheets in an animal model and

demonstrated its human clinical application[2,3]. So far, the endoscopic technique of cell sheet transplantation was not easily procedure, and there were no endoscopic delivery devices to be useful for cell sheets transplantation. Presently, we are developing two types of novel endoscopic device for cell sheets transplantation, and we also show recent our research for esophageal regeneration using cell sheet engineering after circumferential endoscopic large size mucosal resection.We examined allogeneic epidermal cell sheet transplantation using a novel endoscopic delivery device in order to transplant more than one cell sheet at the same time in porcine.

Methods: The novel devices were designed with a computer-aided design system, and the three-dimensional data were transferred to a 3D printer. And then, primary epidermal cells were isolated from the lower abdominal skin of miniature pigs, cultured for 18 days at 37°C on temperature-responsive culture inserts. Transplantable cell sheets were harvested from the inserts by reducing temperature to 20°C. Immediately after creating full circumferential esophageal endoscopic submucosal dissection (ESD), an allogeneic epidermal cell sheet was endoscopically transplanted to the ulcer site. The pigs were endoscopically monitored, and sacrificed 2 weeks after transplantation.

Results: 2-3 pieces of the epidermal cell sheets (20mm in diameter) were successfully transplanted onto the ulcer site after circumferential ESD. In addition, early epithelialization and moderate stricture were observed by a number of transplanted cell sheets.

Conclusions: These endoscopic delivery devices for cell sheet would enable easily transplantation of cell sheets onto the lumen of the esophagus. Additionally, fabricated allogeneic epidermal cell sheets might be useful for prevention of stricture after esophageal ESD as well as autologous oral mucosa epithelial cell sheets in swine model.

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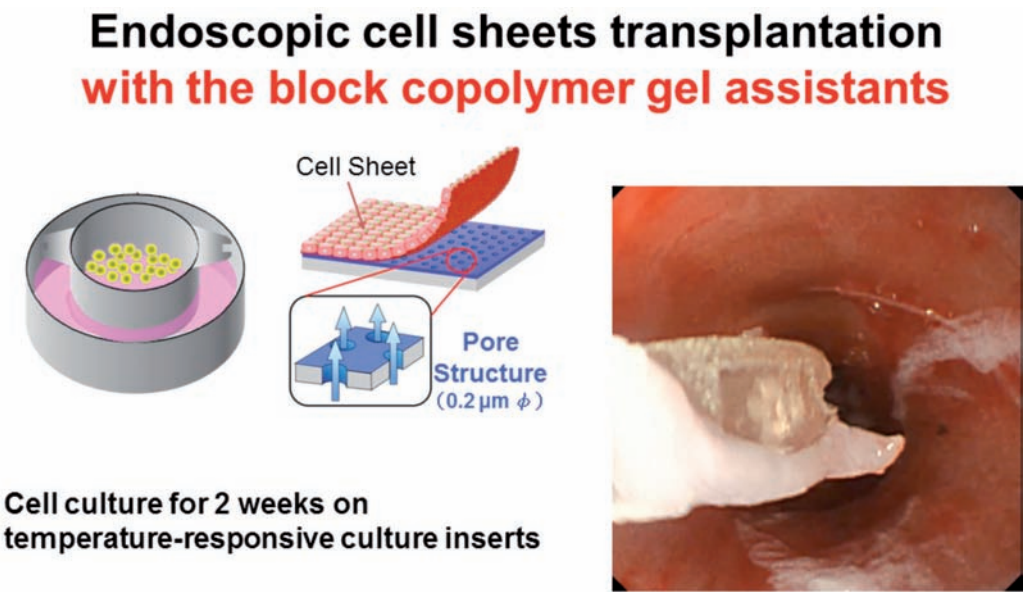

P-29 – CHARACTERIZATION OF LACRIMAL GLAND-DERIVED EPITHELIAL CELLS FROM NORMAL MOUSE

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Purpose: Regeneration of lacrimal gland is necessary to reconstruct severely damaged ocular surface with no wetness. To analyze the function of lacrimal gland in cellular level, lacrimal gland cell line might be useful. Because of no report and no availability of such cell lines, we tried to establish the murine lacrimal gland cell lines spontaneously, and analyze the phenotype of those cells.

Methods: Exorbital lacrimal glands of 3-week old mice (C57B/6) were dissected. The tissues were treated with Collagenase/Hyarulonidase for digestion. Cells were seeded on plastic dishes in culture medium (Cnt07 medium with cholerae toxin). Primary culture were passaged at the time of semi-confluent. RNA was extracted for real time PCR of E-cadherin and Lactoferrin. Immunostaining of antibodies against Cytokeratin 14, Aquaporin 8 and alpha-

SMA was performed. At the time of passage 20, limited dilution of the cells were performed for cloning of the cells.

Results: Long term culture of murine lacrimal gland epithelial cells was established spontaneously in Cnt07 culture medium with cholerae toxin. After clomg by limiting dilution, cell were maintained more than 1 year, which showed E-cadherin positive, cytokeratin 14 positive, alpha-SMA negative phenotype confirmed by immunostaining and real time PCR.

Conclusions: Murine lacrimal gland epithelial cells line was established spontaneously, and coule be used for future research.

P-30 – FABRICATION OF 3-DIMENSIONAL (3-D) FUNCTIONAL HEPATIC STRUCTURES USING CELL SHEET STRATIFICATION TECHNIQUE

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Introduction: Hepatocyte is an attractive cell source for the study of drug screening tests, and liver tissue engineering, because hepatocyte plays various roles *in vivo*. However, hepatocytesin culture are susceptible to loss their functions in the order of days. Furthermore, hepatocytes are known to perform morphological dedifferentiation process under a monolayer culture condition. Therefore, it is necessary to establish novel culture systems that allow hepatocytes to preserve their morphologies and functions for a long period of time *in vitro*. The present study was designed to develop a highly functional 3-dimensional (3-D) hepatic culture systemthat closely mimics the native liver microstructure using hepatocyte sheets and endothelial cell sheetsto preserve hepatic functions.

Methods: In this study, hepatocyte isolated from F344 rats and bovine carotid artery endothelial cells were used. Both cells were separately cultured on temperature responsive culture dishes at 37°C. After a 3-day culture, both cultured cells were harvested as a sheet form by decreasing culture temperature to 20°C for 30 min. A hepatocyte sheet layer (Hep) was sandwiched between two endothelial cell sheet layers (ECs) using a cell sheet stratification manipulator for creating a triple-layered hepatic structure (EC-Hep-EC). Double-layered hepatic structure (EC-Hep) was created by stratifying EC onto a Hep. Cell morphologies and hepatic functions were assessed in EC-Hep-EC, EC-Hep, and conventional monolayer hepatocytes(Hep).

Results: Microstructures of each group were individually investigated by a transmission electron microscope (TEM) at 10 days after cell sheet stratification. A hepatocyte layer in EC-Hep-EC was thicker than those of Hep and EC-Hep. The thickness of hepatocyte layer in EC-Hep-EC at day 10 was nearly identical to that of hepatocytes *in vivo*. In addition, numerous bile canaliculi were observed at inter-hepatocyte in EC-Hep-EC. These ultrastructural observations indicated that hepatocytes in EC-Hep-EC preserved their differentiated phenotype including biliary surfaces. Functional analysis demonstrated that EC-Hep-EC had the highest values in albumin secretions and urea synthesis among the three groups. Cytochrome P450 (CYP) activity related with a drug metabolism was also measured at 10 days. As a result, the activities of CYP3A and CYP1A in EC-Hep-EC were higher than those of the other groups.

Conclusions: The cell sheet stratification technique allowed us to create functional triple-layered hepatic constructs that structurally resembled a microstructure of the liver. The triple-layered hepatic constructs showed the highest liver-specific functionalities compared with those of EC-Hep or Hep. The present study highlighted that cell sheet-based fabrication of 3-D hepatic constructs would be valuable systems for drug screening, liver tissue engineering, and hepatocyte transplantation field.

P-31 – DEVELOPMENT OF AN ECONOMICAL, CONSISTENT AND SCALABLE METHOD OF YOUNG PORCINE ISLET ISOLATION AND TISSUE CULTURE

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Xenotransplantation of porcine pancreatic islets continues to make research advances to clinical trials for patients with insulin-dependent diabetes. Our specific efforts have been focused on a model of using young, pre-weaned porcine pancreases, and gentle enzymatic digestion followed by tissue culture for 8-10 days at 37°C. Unpurified pancreatic digest is maintained in tissue culture in a novel culture media containing specific collagenase and protease inhibitors, specific nutrients to improve islet survival and methods to selectively reduce exocrine content over time in tissue culture.

Using this model we have demonstrated yields of >5000 IE/ gm of pancreas, islet purity of 75-80% and viability >80 % (Newport green/PI) after 10 days of culture, while demonstrating functional response to glucose stimulation. Given the small size of young piglet pancreases, there is a need to use several pancreases to achieve a clinically transplantable volume of viable islets. **The aim of this study was to demonstrate both consistency and scalability of the islet isolation and culture process using the young piglet pancreases model.**

Groups of pancreases from young pre-weaned male Yorkshire piglets (15-22 days) were rapidly procured (<5mins), with cold storage times limited to under 2 hours (OPS solution, Optatio LLC. at 4°C). Pancreases were then digested in a low-dose collagenase enzyme (Sigma Type V or VitaCyte) for 15±2minutes at 37°C. Unpurified pancreatic tissue was cultured in T175 suspension flasks (Corning) at a dose of 0.25g tissue/mL

with culture media changes every 2 days for 8-10 days.

From a series of 10 consecutive isolations, Islet yields averaged 29,088±1959 IE per piglet pancreas with an average viability of 89.3±4.1% (Newport Green/PI) at 8 days post culture. Islet yields ranged from 54,200 – 62,341 IE for islet yields with 15.7% percent variability between isolations and on average 5,529IE/g of pancreas. Islet viability ranged from 84.0-96.2% (2.7% variability between isolations) with average viability of 89.3±0.4%. Purity of islet tissue to exocrine was 74.8±3.9% (5.5% variability between isolations). Islet function using GSIR was equivalent from isolation to isolation with the mean calculated stimulation index of 2.54±0.8 (p<0.05, ANOVA) with a variation of 2.3% between isolations.

In terms of scalability, the isolation and culture process was performed using 12 pancreases at one time with yields of 29,432.2±413 per pancreas with a viability of 87.6±2.3%. This equates to 5,457.2 IE/gm of processed pancreas (p=ns, t-test).

This study demonstrates the consistency of islet isolation using this model, and the feasibility of porcine islets to produce a scalable process to produce volumes of viable islets for future large animal and clinical transplantation trials of this technology.

P-32 – CHARACTERIZATION OF YOUNG PORCINE TISSUE DURING MATURATION IN TISSUE CULTURE USING FLOW CYTOMETRY

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Porcine pancreatic islets are being explored as a source for islet xenotransplantation. Previous Methods of islet isolation from market-weight pigs are labor intensive, inconsistent yields, and islets tend to fragment resulting in suboptimal yields for transplantation. Additionally, porcine islet isolation is highly dependent on effective and complete enzymatic digestion of the islets from exocrine tissue stroma. New methods of islet isolation of young piglet pancreases yield viable function. **The aim of this study was to further characterize young porcine islets, while tracking changes in islet cellular characteristics using flow cytometry and standard islet isolation endpoints.**

Pancreases from pre-weaned Yorkshire pigs (age range 18-22 days) were rapidly recovered and partially digested using low-dose purified collagenase MA/BP protease. Unpurified pancreatic tissue was then maintained in tissue culture (37°C/5%CO_2) in a novel culture media for up to 11 days with media changes every 48hrs. During in vitro maturation, tissue samples were aspectically collected and analyzed for quality by islet yield (IEQ), purity (dithizone) and cell viability (FDA/PI). In vitro function was assessed using a static glucose-stimulated insulin release (GSIR) assay and calculated as the stimulation index (SI=insulin release in 28mM glucose over the insulin release in 2.8mM glucose). Samples were characterized for islet cellular composition by dissociating islets, using Acutase, and analyzing by immunohistochemistry for antibodies targeted for glucagon (α-cells), insulin (βφ-cells), and amylase (acinar tissue), using flow cytometry.

The proportion of dithizone-positive tissue increased during tissue culture ($12.6x10^3$±183IEQ to $33.3x10^3$±136 after 7 days of culture, p<0.05 mean±SEM) Islet function improved during time in culture (SI 1.3±0.1 at day 0, to 2.6±0.2 after 7 days of culture). During islet maturation, using flow cytometry, amylase proportions decreased from 44.0±4% to 20.4±6%, while beta cell (insulin) proportion within the islet increased from 25.4±4% to 49.8±7% after 3 and 7 days in tissue culture (p<0.05). A further increase of beta cells (54.4±8%)along with a decrease in acinar cells (13.7±8%) was observed after 11 days in tissue culture. We observed no significant difference in proportion of glucagon-positive cells during in vitro culture (30.5±6% day 3 vs. 27.6±3% day 7, p=ns).

During maturation in tissue culture, piglet islets increase beta cell proportions while decreasing exocrine portions. Using a novel isolation protocol, including partial enzymatic digestion and novel islet culture media, we observed a selective decrease in the proportion of amylase-positive cells over time in tissue culture. These viable functional piglet islets achieved using this protocol are being considered for future transplant studies.

P-33 – TISSUE ENERGETICS AS A PREDICTOR OF THE EFFECTIVENESS OF PIGLET PANCREAS ORGAN PRESERVATION

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The development of islet xenotransplantation towards clinical trials requires standardized procedures for the validation of tissue quality and stability of the pancreases and islets during shipping. Our ultimate goal is to develop a predictive assay of islet viability and function that can be used as release criteria for standardizing transplantable islet tissue. **The aim of this study was to understand tissue changes during organ preservation and shipment by measuring tissue apoptosis and necrosis levels in pancreases hyperthermically stored.**

Pancreases (n=30) were obtained from euthanized pre-weaned pig (18-25 days, Spring Point Project Facility) using a rapid surgical approach and immediately placed in either cold (4°C) organ preservation solution (OPS) or EuroCollins (EC) solutions. Pancreases were then shipped, in validated shipping boxes (temp log recorded at 2-6°C), by same day courier to University of California Irvine for islet isolation. Pancreas samples were taken and snap frozen in liquid ni-

trogen at time of procurement (t=0), at time of arrival at UCI (t=12 hours), at 18, 28 and 52 hours post-organ preservation. Samples were placed in labeled cryo tubes and snap frozen and stored at until batch assayed for tissue apoptosis and necrosis by the change in ADP/ATP ratio using ApoSENSOR ADP/ATP Ratio Bioluminescence assay kit.

At the time of procurement (t=0) ADP/ATP ratios remained low when pancreases were stored in both OPS (1.61±0.16) and EC (1.53±0.28) with no significant difference between OPS or EC (p=0.25). There were increases in stored tissue energy losses with an increase in ADP/ATP ratio levels for pancreases stored in both OPS and EC during the total 52 hours of cold storage. At 12 hours ADP/ATP ratio levels were 1.88±0.21(OPS) and 4.18±1.92 (EC), at 18 hours ratios levels were 2.65±0.13 (OPS) and 3.33±0.27 (EC), at 28 hours levels were 3.26±0.29 (OPS) and 7.00±0.83(EC), and at 52 hours ratio levels were 4.40±0.37 (OPS) and 7.34±1.59(EC). All results were shown as mean±SEM. When ADP/ATP ratios were compared between OPS and EC during the 52 hours of cold storage levels was shown to be significantly different (p=0.004), with overall lower ratios seen in OPS suggesting improved maintenance of tissue energy stores.

These studies show that both EC and OPS are suitable preservation solutions for pancreases storage and transport. However, the lower ADP/ATP ratio seen throughout the 52 hours of cold storage in piglet pancreases preserved in OPS, when compared to that in EC, suggests the tissue energy and viability is better maintained. Concluding that OPS may protect pancreases against cellular tissue loss more efficiency than pancreases stored in EC. Future experiments include measuring the correlation between ADP/ATP ratios to islet isolation yields, which will be the foundation for the pancreas preservation acceptance criteria.

P-34 – EXOCRINE ACINAR ZYMOGEN GRANULE DENSITY IS AN IMPORTANT DETERMINANT OF SUCCESS IN YOUNG PIG ISLET ISOLATION AND SURVIVAL

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Pancreata from pre-weaned young pigs offer a scalable and consistent source of viable islets for xenotransplantation and have demonstrated low basal and stunted secretory levels of pancreatic amylase, lipase, trypsin and chymotrypsin. High levels of pancreatic enzymes during tissue digestion and in-vitro culture injure the islets isolated during the process, adversely affecting their survival and function. We hypothesize that the exocrine component of pancreata harvested from pre-weaned young pigs is immature and hence islets isolated from these pancreata are protected from injury during isolation and prolonged tissue culture. **The aim of this study is to determine the impact of piglet donor age and weaning status on exocrine maturity.**

Pancreata harvested from 18-22 day old pre-weaned Yorkshire pigs (Group I, n=8), weaned young pigs (45 day old) fed on solid food for 4 weeks (Group II, n=8) and adult pigs (>90 days old), on a solid diet (Group III, n=8) are fixed and histological sections from the head and tail regions are stained with Hematoxylin & Eosin using standard protocols and compared.

Sections were examined under a light microscope to compare acinar size, structure and morphology. Under a fluorescent microscope using laser-excitation (ex/em 488/540 nm), enzyme-containing zymogen granules localized within the apical cytoplasm of the pancreatic acini were observed to be brightly fluorescent while pancreatic ductal cells and endocrine islet cells were not. Periodic Acid-Schiff (PAS) staining performed with diastase digestion in paired mirror sections helped confirm this observation. The intensity of the fluorescent signal emitted by the acinar zymogen granules was also measured. The intensity of the fluorescent signal is directly proportional to the concentration of zymogen granules within the acini, which is a reliable index of exocrine maturity.

Quantitative evaluation of the fluorescent signal intensity demonstrated a 32% lower signal intensity in pre-weaned young pigs (1.50±0.04U) than weaned young pigs (2.21±0.02U) and 46% lower than adult pigs (2.77±0.05U). All values are expressed in U per 100 μm^2 of tissue (Mean±SEM, p<0.05, ANOVA). These results highlight the relative immaturity of the exocrine component of the pancreas in pre-weaned pigs when compared to weaned young and adult pigs.

In summary, the impact of piglet donor age and weaning status on exocrine maturity and the histological differences between pre-weaned and weaned young pigs have been demonstrated.

Future studies will further expand on these results by assessing tissue harvested from neonatal and retired breeder pigs. Specific islet studies will help understand the impact of exocrine maturity on islet function and survival after isolation and tissue culture as well as help elucidate the mechanisms behind the increased success of islet isolation from pre-weaned pigs.

P-35 – CELLULAR INTERNALIZATION OF ANTIOXIDANT TAT-METALLOTHIONEIN FUSION PROTEIN FOR INDUCING HYPOXIC RESISTANCE TO SUBCUTANEOUSLY XENOTRANSPLANTED PANCREATIC ISLETS

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Pancreatic islet transplantation is a promising method for treatment of type 1 diabetes, but transplantation outcomes have been disappointing due to early graft hypoxia and several immune responses which generates oxygen free radi-

cals. It has been reported that metallothionein (MT), a cysteine-rich and low-molecular-weight protein, provide cellular protection from reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide by using scavenging property. However, MT protein is rarely expressed in pancreatic islets, which means that islets are easily damaged by ROS. Therefore, to overcome this issue, we designed cell penetrating Tat peptide-MT fusion protein (Tat-MT) to enhance MT uptake into islets. The main aim of this study was reducing oxidative stress on islet via delivery of Tat-MT. The second goal was to investigate that Tat-MT could show synergistic effect in islet xenotransplantation when accompanied with biocompatible poly(ethylene glycol) (PEG) conjugation onto islet surface and administration of immunosuppressive agent into diabetic recipients. Firstly, we confirmed that Tat-MT fusion protein that was conjugated with fluorescence Alexa 488 dye was effectively internalized into islets by confocal laser microscopy. Even though Tat-MT was delivered into islets, their insulin secretory function was not significantly difference compared with fresh islets. Also, we confirmed that the viability of Tat-MT-delivered islets could be improved after treatment of paraquat, a chemical of ROS donor. To evaluate the scavenging effects of Tat-MT *in vivo* model, we xenotransplanted 350 IEQ Tat-MT-delivered rat islets or untreated rat islets (control group) under the renal capsular membrane of left kidney in the streptozotocin-induced diabetic Balb/c mice. Median survival times (MST) of Tat-MT-delivered islets were positively improved, but not strongly. Collectively, this result demonstrated that Tat-MT internalization into islets can protect xenotransplanted islets from radical species produced from the host's immune reactions and hypoxic state of transplanted islet.

P-36 – DONOR SPECIFIC TRANSFUSION COMBINED WITH ANTI-CD134L AND RAPAMYCIN INHIBITS ACCELERATED REJECTION MEDIATED BY ALLOANTIGEN-PRIMED MEMORY CELLS IN MICE

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Background: Donor-reactive memory cells threaten the survival of transplanted organs, and hamper maintenance of transplant tolerance. In this study, we investigated the inhibitory effect of donor splenocyte transfusion(DST) combined with anti-CD134L and rapamycin on memory cells-mediated accelerated rejection in an allogeneic heart transplantation model in mice.

Methods: Male Balb/c and C57BL/6 mice were used as the donor and recipient. After sensitization with donor skin allografts, splenocytes from the alloantigen-primed C57BL/6 mice were prepared and adoptively transferred (5×10^6) to a naïve recipient followed by heart transplantation (HTx). Thus, we established a memory cells-mediated accelerated rejection model of heart transplantation. Mitomycin C-treated donor splenocytes were transfused to HTx recipients seven days prior to transplantation, and short-term low dose of rapamycin and anti-CD134L were initiated after transplantation. Graft survival and pathology of rejection was examined.

Memory cells proliferation, function and the proportion of regulatory T and B cell populations were also examined.

Results: The mean survival time (MST) of grafts in the control group was 5.3 days, and more than 40 days in the group treated with DST/anti-CD134L/rapamycin. The DST/anti-CD134L/rapamycin regimen prevented lymphocytic infiltration in the grafts, inhibited memory cells proliferation in the spleen, reduced donor specific antibody level, and increased proportion of $CD4^+Foxp3^+$regulatory T cells (Tregs) as well as $CD1d^+CD5^+$regulatoryB cells (Bregs).

Conclusions: Our data suggest that DST combined with rapamycin and anti-CD134L can synergistically inhibit accelerated rejection mediated by memory cells and thus prolong heart allograft survival in mice. Both regulatory T cells and regulatory B cells may play important roles in modulation of memory cells.

P-37 – PRETREATMENT OF DONOR SPLENOCYTE AND GRAFT WITH MITOMYCIN C ATTENUATES REJECTION IN HEART ALLOGRAFT TRANSPLANTATION IN MICE

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Objective: To investigate effect of pretreatment of donor splenocyte and graft with mitomycin C (MMC) on heart allograft survival, and to demonstrate the mechanism of function.

Methods: Donor splenocytes from donor mice were incubated with MMC *in vitro* and were then transfused into recipient mice. The heart allograft was perfused with MMC prior to harvest.

Results: Donor splenocyte transfusion (DST) combined with MMC-graft pretreatment prolonged heart allograft survival from seven to 28.5 days. DST or combination strategy significantly decreased $CD4^+CD25^+$ population in CD4+ T cells from 15% to 7.4% and 8.6% andmeanwhile increased $CD4^+Foxp3^+$ T cells from 15.5% to 18.2% and 18.6%. MMC incubation *in vitro* induced apoptosis of donor splenocytes from 15.5% to 23.2%.

Conclusions: Transfusion of MMC-treated donor splenocytes either alone or combined with graft pretreatment with MMC could significantly prolong heart allograft survival in mice. MMC pretreatment could induce apoptosis of donor cells, increase production of regulatory T cells, and thus achieve donor-specific immunosuppression.

P-38 – DYNAMIC ANALYSIS OF B CELL SUBSETS IN DE NOVO LIVING-RELATED KIDNEY TRANSPLANTATION WITH BASILIXIMAB INDUCTION

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Background: Accumulative evidences have suggested B cell-mediated alloimmune response and regulation play important roles in renal allograft survival and transplant tolerance. Monitoring dynamics of B cell subsets could enhance understanding of B cell immunology in clinical transplantation, and may facilitate optimization of current immunosuppressive regimens.

Patients and Methods: Between June 2011 and June 2012, 16 *de novo* living-related kidney transplant recipients were enrolled in this study. All patients were given basiliximab (20 mg) at day 0 and day 4 as induction therapy. The maintenance therapy included tacrolimus, MMF and steroids. Peripheral blood samples were collected, and phenotype of B cell subsets was examined at day 0, day 1, day 3, day 7, day 14, mon 1, mon 3 and mon 6.

Results: $CD19^+$B cells in peripheral lymphocytes significantly increased by 1.8~2.1 times within one month after transplant, and recovered to baseline level at mon 3. Meanwhile, $CD3^+$T cells decreased by 20% at day 3, maintained at baseline level by day 14, and then increased by 20% at mon 1-mon 6. Notably in B cells, $CD5^+CD19^+$B cells dramatically decreased by 50% (from 25% to 12%) within six months post transplant. In $CD5^+CD19^+$B cells, $CD27^+$ memory B cells decreased at day 1 and day 3, and then increased by 23%-123% from day 7 to mon 6. Memory B cells in lymphocytes increased by 47%-194% within six months after transplantation.

After transplantation, the germinal center (GC) founder cells (IgD^+CD38^{high}) and GC B cell subpopulation (IgD^-CD38^{high}) consistently decreased by 60%-70.3%. IgD^-CD38^- cells apparently decreased by 48.6% at day 1-3, and then maintained at lower level below baseline after slight increase. IgD^+CD38^- and IgD^-CD38^+ B cells slightly decreased by 13.4%-37.4% at day 3, increased at day 7, and then maintained at baseline level from mon 1. Of note, IgD^+CD38^+ B cells significantly increased by 27.8% at day1-3, dropped down to baseline at day 7-14, and stepped up to a higher level at mon 1-6.

Conclusions: B cells in peripheral blood significantly increased at early stage, while $CD5^+CD19^+$B cells consistently decreased after transplantation. Mature circulating B cell subsets dynamically changed especially at early stage after transplant, which might be attributed to induction therapy.

P-39 – CD8+CD28- T CELLS PLAY A ROLE IN MESENCHYMAL STEM CELLS-MEDIATED IMMUNOREGULATION

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Background & Aims: Mesenchymal stem cells (BMMSCs) infusion yields therapeutic effect for a variety of autoimmune diseases, but the underlying mechanisms are still poorly understood. Several groups have reported that $CD8^+CD28^-$ T cells induce transplantation tolerance and displayed suppressive functions in liver, kidney and other organ transplantations. Here, we investigated the effect of MSCs on $CD8^+CD28^-$ T cells in chronic graft-versus-host disease (cGVHD) patients and healthy donors.

Methods: we observed the change of $CD8^+CD28^-$ T cells in cGVHD patients before and after MSCs infusion. In the meantime, we investigated the frequency and regulatory function of $CD8^+CD28^-$ T cells in the presence of MSC.

Results: We found purified $CD8^+$ T cells co-culture with MSCs 3 days could increase the proportion of $CD8^+CD28^-$ T cells (from 12.79±3.67% to 20.85±4.84%, $p<0.01$). In the cGVHD patients tested, the mean percentage of $CD8^+CD28^-$ T cells before and after MSCs infusion was 37.2% and 46.3%. MSCs enhanced the regulatory functions of $CD8^+CD28^-$ T cells through hampering naive $CD4^+$T cells proliferation, decreasing the early active marker CD25,CD69 expressed on $CD4^+$T cells, decreasing the IFN-γ production of activated $CD4^+$T cells, and inducing activated $CD4^+$T cells apoptosis. Mechanistically, we revealed MSCs affect $CD8^+CD28^-$ T cells function partially through up-regulating FasL and IL-10 expression level on them, but not PDL-1, Trail. Moreover, MSCs have no obvious effects on the shift from $CD8^+CD28^+$ T cells to $CD8^+CD28^-$ T cell, but increased the frequency of $CD8^+CD28^-$ T cell-subsets might through secreting IL-6 to decrease $CD8^+CD28^-$ T cells apoptosis.

Conclusions: In summary, this study show that MSCs could affect the regulatory function of CD8+CD28-T regulatory cells and will shed new light on the the MSCs-mediated therapeutic mechanisms.

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P-40 – CELL TRANSPLANTATION MODULATES THE LIVER MICROENVIRONMENT AND DELAYS THE EMERGENCE OF HEPATOCELLULAR CARCINOMA

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Previous investigations have indicated that a growth-constrained tissue microenvironment is able to promote experimental liver carcinogenesis. In the present studies we tested the hypothesis that normal hepatocyte transplantation might modulate a neoplastic-prone tissue microenvironment and exert an effect on the emergence of chemically-induced hepatocellular carcinoma (HCC). Rats were exposed to a protocol for the induction of HCC (a single dose of diethylnitrosamine coupled with a single injection of retrorsine), followed by intraportal transplantation of 8 million normal hepatocytes. The control group was given the carcinogenic protocol and no hepatocyte transplantation. At the end of 1 year, all control animals displayed large hepatic nodules and 4/9 animals had histological evidence of HCC. However, rats receiving the carcinogenic protocol followed by hepatocyte transplantation developed fewer nodular lesions and no HCC. Further analysis indicated that transplanted hepatocytes repopulated the host liver, as expected, and attenuated the expression of markers related to cell senescence. These findings indicate that normal cell transplantation is able to modify a neoplastic-prone liver microenvironment and to delay the emergence of HCC.

P-41 – ENHANCED FUNCTION AND SURVIVAL OF PANCREATIC ISLETS BY THE USE OF A GELATINE-BASED HYDROGEL

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Background: The use of biomaterials to protect pancreatic islets and improve islet function has been increasingly proposed and investigated. Among the emerging materials, hydrogels, as three-dimensional polymeric networks, can be designed as temporary structures with geometry and physical, chemical and mechanical properties adequate for the specific applications.

Aim: The aim of this study was to test *in vitro* a novel cross-linked gelatin-based hydrogel to be used as "bioartifical scaffold" for pancreatic islet transplantation.

Materials and Methods: The synthesis of cross-linked gelatin hydrogels was performed by Michael-type addition between the amino groups of the gelatin and the acrylamide groups of the N, N'-methylene-bisacrylamide (i.e. cross-linking agent), according to a recently patented process (PCT/EP2012/060277). Human and murine islets were seeded onto the hydrogels, freeze-dried or dehydrated. Islet morphology, cell survival and insulin secretion were tested 48 hrs post seeding by immunohystochemical analysis, MTT and glucose challenge test.

Results: Immunohystochemical analysis demonstrated that murine and human islets maintain, into the hydrogel, their native 3D architecture. Cell survival of human islets was 100%, 98%±25%, 82%±5% in the free floating standard culture, cultured onto dehydrated and freeze-dried hydrogels, respectively. Cell survival of murine islets in the same conditions was 100%, 124%±24% and 100%±45%. Insulin secretion index of human islets was 2.7±0.3, 3.2±0.5, 2.9±0.02 in the free floating standard culture, cultured onto dehydrated and freeze-dried hydrogels, respectively. Insulin secretion index of murine islets in the same conditions was 1.4±0.02, 1.3±0.06, 1.4±0.04.

Conclusions: The gelatin-based hydrogel maintained the 3D islet architecture and did not affect islet survival and function during short term culture. This preliminary report is now leading to preclinical study in which the gelatin-based hydrogel is under investigation as bioartificial scaffold in a murine islet transplantation model.

P-42 – EFFECTS OF GREEN TEA CATECHIN, EPIGALLOCATECHIN-3-GALLATE (EGCG) ON ISOLATED HUMAN HEPATOCYTES IN VITRO

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Background: Agents which help maintain metabolic function of isolated human hepatocytes are needed for cell therapy. Green tea (*Camellia sinensis*) has potential powerful anti-oxidant effects mostly due to the presence of catechins. The main constituent epigallocatechin-3-gallate (EGCG) has been shown to have inhibitory effects on cancer cells, including the human hepatoma cell line HepG2 [Singh *et al*, *Biochem. Pharmacol.* 2011;82:1807-21]. However, there is limited data on the effects of EGCG on normal cells including human hepatocytes.

Aim: The *aim* was to study the effects of EGCG on primary human hepatocyte function and activity *in vitro*.

Materials and Methods: Human hepatocytes were isolated from unused donor liver tissues (n=8) using collagenase perfusion digestion [Mitry *et al*, *Cell Transplant*. 2003; 12(1):69-74]. Collagen-coated culture plates were seeded with cells (fresh or thawed) obtained after Percoll® centrifugation. Plates were incubated overnight in a humidified incubator at 37°C, 5% CO_2, then the culture medium was replaced with fresh medium containing EGCG at 6 concentrations ranging from 0-30 μg/ml. Plates were incubated for a further 24h, and the hepatocyte cultures were assessed for cell attachment (SRB assay), overall metabolic activity (MTT assay), synthetic activity (albumin ELISA assay), and ammonia detoxification function (urea colorimetric assay). Total RNA was isolated from the hepatocytes using TRIzol®, followed by reverse transcription into cDNA, and relative quantification real-time PCR (rqRT-PCR) analysis using TaqMan® and Bak1, Bax, Bcl2, MCL1 and rRNA (endogenous) probes. Data are presented as mean±SEM and were analysed using Student *t*-test.

Results: There was a tendency for a decrease in all measured parameters in the hepatocytes with increasing concentrations of EGCG. However, EGCG at all concentrations did not have statistically significant effects on: cell attachment [control (0μg/ml): 0.90±0.28 OD units vs EGCG (30μg/ml): 0.70±0.17 OD units]; cell metabolic activity [0.22±0.04 OD units vs 0.18±0.0.03 OD units]; albumin synthesis [3.66±0.16 μg/mg cell protein vs 1.96±0.63 μg/mg cell protein]; and urea synthesis [112.19±44.33 ng/mg cell protein vs 79.26±26.48 ng/mg cell protein]. rqRT-PCR analysis showed that EGCG had no effect on expression of either pro-survival genes [fold change: Bcl2, -0.36 to 0.35; MCL1, -0.01] and pro-apoptosis genes [Bak1, -0.46 to 0.18; and Bax, -0.49 to 0.04].

Conclusions: This study has shown that EGCG does not have stimulatory effects on human hepatocytes *in vitro* at the concentrations used, and this is not likely to be useful for application in cell therapy.

P-43 – ISOLATION OF ADULT HEPATOCYTES AND PROGENITOR CELLS FROM EXPLANTED DISEASED HUMAN LIVERS AND EVOLUTION AFTER COLD STORAGE

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Background: Mostly, human hepatocytes are isolated from liver-grafts not suitable for transplantation. We are developping cell isolation from the patients own diseased liver for retransplantation after allogeneic organ transplantation. This should confer an immunological advantage and achieve better outcomes with minimization of immunosuppression.

Methods/Materials: To date, isolation of hepatocytes from 14 explanted human livers from patients with different conditions leading to end-stage liver-disease were performed. Progenitor cell isolation was carried out with density gradient centrifugation and magnetic cell separation (MACS) verified by fluorescence activated cell sorting (FACS). Hepatocytes were cultured directly and after cold storage over night in different media (ChillProtec®, ChillProtec® plus, Williams'medium E) for further analysis.

Results: A mean of $138x10^6$ viable hepatocytes with a mean viability of 79.9% were isolated from explanted diseased livers (app. 40g). Density gradient centrifugation led to a mean number of $10.6x10^6$ progenitor cells. FACS analysis showed a mean proportion of 8.5% of EpCAM+ after density gradient centrifugation, while MACS double-staining led to a mean proportion of 40.7% EpCAM+, but extreme loss of cells (mean: $0.4x10^6$). Preliminary results suggest that diseases like primary sclerosing cholangitis or autoimmune hepatitis lead to a larger number of hepatocytes than alcoholic liver disease, while the proportion of progenitor cell populations seems to be greatest in acute liver failure. Cold storage led to a significant loss of viable cells with *WilliamsE* culture medium (-24.5% after 24h, -39.6% after 48h) compared to the other solutions. Cell culture revealed stable cell function after initial cell loss for up to 7 days.

Conclusions: A high number of hepatocytes and progenitor cells can be isolated from human livers with end-stage disease with a very good mean viability of 79.9%. With suitable solutions, cold storage of cells over night is possible with stable cell function in culture for up to 7 days.

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P-44 – OPTIMAL CONDITIONS FOR CREATING RAT PERIODONTAL LIGAMENT-DERIVED MULTIPOTENT MESENCHYMAL STROMAL CELL (RPDL-MSC) SHEETS

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Introduction: We have developed cell sheet engineering using temperature-responsive culture dishes in which intact cells and proteins are transplantable. Previous studies have demonstrated that periodontal ligament-derived multipotent mesenchymal stromal cell (PDL-MSC) is a promising cell source for periodontal regeneration in animal models. In this study, we clarified the appropriate culture conditions for rat PDL-MSC sheets and transplanted into calvarial defects of rats to chase the fate of transplanted cells in vivo.

Materials and Methods: Periodontal tissues were obtained from molars of 4-week-old CAG-EGFP transgenic rats and digested with collagenase and dispase. Single suspension cells were seeded at a low cell density, and cells possessed colony forming ability (CFA) were subcultured for further experiments. Osteogenic and adipogenic potentials of rat PDL-MSCs were confirmed. Cells at passage 3 were seeded onto temperature-responsive dishes. Various dosage of cell seeding density and serum concentration was tested. Rat PDL-MSCs were cultured with or without osteoinductive supplements for 2 to 3 weeks, then, alkaline phosphatase (ALP) activity and the expression of periostin, a specific marker of PDL, were determined.

Results: Rat PDL-MSCs were successfully obtained by collagenase/dispase and possessed CFA and multipotency. The optimal culture condition to create rat PDL-MSC sheets was 20% FBS and the initial seeding density of 20.000 cells per 35 mm temperature-responsive culture dishs. The elevation of ALP activity and the gene expression of periostin was confirmed when cells were cultured with osteoinductive supplements. Transplantation study confirmed that rat PDL-MSCs enhanced bone formation.

Conclusions: PDL-MSC sheets derived from CAG-EGFP transgenic rats were successfully fabricated and transplanted into rats. The transplantation performed in this study may be a model to trace transplanted cells.

P-45 – EFFECTS OF PIPETTING ON HEPATOCYTE VIABILITY

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Hepatocyte transplantation is one of the important medical treatment initiated as a bridge treatment until orthotopic liver transplantation for newborn babies with severe liver dysfunction emerged. An improvement of the quantity and quality of hepatocytes is significantly important to establish hepatocyte transplantation as a medical treatment for severe liver failure. In particular, improvement of hepatocyte viability, one of the important factors to improve the quality of this treatment, can contribute to the treatment outcome enhancement. High hepatocyte viability can support the increasing rates of engraftment and expanding time length of survival of hepatocytes. A decrease in viability is expected during the processing steps of separation, preservation, and infusion. During these procedures, hepatocytes can be influenced by the flow effects in a catheter, syringe, or pipettes. There are many studies on engraftment, preservation, and isolation techniques, but there are few reports on the flow effect on the viability of the hepatocytes. And also, the effect of flow on the hepatocyte viability during passage through a narrow area during the separation or infusion processes has not been evaluated. In this study, we focused on the flow effect of pipette transaction. It is important to investigate the effect of this flow on hepatocyte viability to understand the mechanism of the lost viability during processing. In this study, relationship between hepatocyte viability and pipette transaction was investigated. In this experiment, a conventional collagenase perfusion technique was employed as an isolation technique for hepatocytes. The hepatocytes were extracted from the livers of Sprague–Dawley rats. The isolated hepatocyte dispersion was injected into conventional pipettes or microchannel with a tapered angle and has a narrow inlet or outlet. Flow rate of the dispersion was maintained to control the intensity of the stretch. The viability of the hepatocytes was calculated by trypan blue assay. In our experiments, the viability of hepatocytes decreased during the pipette transaction compared with a low stretching flow effect generated using a dropping pipette with a wide mouth. These results indicate damage inflicted by the pipette transaction on the hepatocytes, and this damage could result during the separation of hepatocyte clusters.

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P-46 – APPLICATION OF RECOMBINANT COLLAGENASES ABIEL FOR FIBROBLAST EXTRACTION FROM HUMAN DERMA

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Background: Collagenases are proteolytic enzymes that are able to cleave peptide bonds in the triple helical collagen molecule. Collagenase is produced by two separate and distinct genes in Clostridium histolyticum. The colG gene codes for type I collagenase, the colH gene codes for type II collagenase. Abiel use these gene sequences to produce collagenases through DNA recombinant technique. These collagenases are produced in E.Coli and purified chromatographically. Abiel, through the use of molecular biology techniques and in vitroand in vivotests, is capable to produce recombinant collagenases type I or "G" and type II or "H" in pure fraction ensuring, as opposed to commercial collagenases obtained through extractive purification technique, more stability, the complete control of the final composition of the products, high batch-to-batch reproducibility and the absence of toxic compounds.

Objective: The aim of this studywasto test the ability of collagenases Abiel to isolate fibroblasts from human dermis, in order to verify if recombinant enzymes to be better in extraction of primary cells in terms of: yield, digestion time, vitality and proliferation; compared to commercial extractive enzymes (Collagenase SigmaCod. C9891). The human fibroblasts were obtained from skin taken from a dead donor and kept in the Tissue Bank Niguarda for processing and preparation of new tissue to be transplanted in burn individuals. We have tested 8different donors, each experiment was conduced in parallel with the standard method used at the Tissue Bank. According to the protocol, the fibroblasts from a skin on the size of 1 cm x 1 cm, were obtained using an amount of Collagenase Sigma 200 CDU/ml= 0.14PZ/ml = 0.35 mg /ml in PBS, with a digestion time of 3h at 37 °C. We have tested the recombinant collagenase under the same conditions of Sigma collagenase both in terms of activity and quantity in milligrams.

Discussion: Digestionsperformed using Abiel Collagenases to 0.14 Pzor 0.35 mg/ml resulted in a higher extraction of fibroblasts, with a lower digestion time(2h, p=0.006 and 1h 30', p=0.004 respectively) compared to Sigma ones (3h).

P-47 – TISSUE SPECIFIC SUICIDE GENE DELIVERY USING A POLYMERIC CARRIER FOR THE TREATMENT OF GLIOBLASTOMA

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Dexamethasone conjugated low molecular weight polyethylenimine (PEI-Dexa) was synthesized and evaluated as a gene carrier to brain cancer cell. Polyethylenimine (PEI) is widely used as a gene carrier. PEI-Dexa was prepared by one step reaction using the Traut's reagent. Hypoxia and glioma dual specific suicide gene expression plasmid (pEpo-NI2-SV-TK) was constructed. In this plasmid, the expression of thymidine kinase (TK) was regulated by hypoxia and glioma specific promoter. To confirm the formation of the DNA/PEI-Dexa complex, a gel retardation assay was performed. The complete retardation of the DNA/PEI-Dexa complexes were observed at and above weight ratio 1:0.5 (pDNA:carrier). Stability of the DNA/PEI-Dexa complex was evaluated by heparin competition assay. In heparin competition assay, the DNA/PEI-Dexa complexes had higher stability than the DNA/PEI25k complexes. The DNA protection ability of PEI-Dexa was evaluated by serum stability assay. The results showed that DNA was protected by PEI-Dexa for more than 60 min. In vitro transfection assay showed that PEI-Dexa had higher transfection efficiency than lipofectamine and the efficiency of PEI-Dexa was comparable to PEI. Due to the pharmacological effect of dexamethasone of PEI-Dexa induced cell death and inhibited proliferation of brain cancer cells more efficiently than lipofectamine and PEI25k. However, in normal cells, PEI-Dexa had lower toxicity than lipofectamine and PEI25k. The anti-tumor effect of PEI-Dexa/pEpo-NI2-SV-TK complexes was also measured in intracranial glioblastoma rat model. At a week after the implantation of tumor cells, the carrier/pEpo-NI2-SV-TK complexes were injected at the same position of tumor cell implantation. As a result, the PEI-Dexa/pEmpty group showed smaller tumor size than the dexamethasone injection group. Furthermore, the PEI-Dexa/pEpo-NI2-SV-TK reduced tumor size further, due to therapeutic effects of the TK gene. TUNEL assay shows that PEI-Dexa/pEpo-NI2-SV-TK group induced more cell death in tumor site than PEI-Dexa/pEmpty complex or dexamethasone only group. Therefore, the results suggest that PEI-Dexa may be useful for glioblastoma gene therapy as a dexamethasone carrier as well as a DNA carrier

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P-48 – COMPARISON OF PANCREATIC ISLET PROTEIN PROFILES BY MASS SPECTROMETRY-BASED ANALYSIS

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Replacement of pancreas islet cells plays promising role in the treatment of type 1 diabetes mellitus. Umbilical cord serum (UCS) has positive effects on insulin production, islets revascularization and recovery of host islets functions. The best way to see the UCS application results on physiology of islets and understanding the molecular mechanisms is to use proteomic strategies, which considered being the important technology in the global analysis of protein expression. Four groups of islets has been used for this study; control islets (without CBS), 50,

100 and 150 ul serum added islets. The isolation and purification of rat pancreatic islets were performed by hand-picking and incubated with and without UCS for 48 hours. 900 pancreatic islets of each group were lysed with lysis buffer. After centrifugation, the supernatant was decanted and protein concentration of the supernatants was determined by the Bradford method [1] as triplicates using BSA as a standard. We have applied proteomic profiling by using two-dimensional gel electrophoresis (2-DE). 75 μg of protein was loaded on each 7-cm linear, pH 3-10 IPG strips (Immobilize pH Gradient) for the first dimension (Isoelectric focusing=IEF). For the second dimension, IPG strips were loaded on SDS-PAGE to have protein profile map for four sample group (Figure 1). Gels were than analyzed and differences were compared by using PDQuest program. The protein profile maps, 3D demonstration and density bar graphs of each group was shown in Figure 2 for 13 differentially expressed spots. Spots were excised from the 2D gels. After in-gel digestion of the protein spots by trypsin, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF-MS) was performed with peptides by using CHCA (α-Cyano-4-hydroxycinnamic acid)matrix. Spectras were recorded by MALDI-TOF mass spectrometer (Micromass, Waters, UK) operated in positive ion reflectron mode for the mass range 500–3000 Da. A pulsed nitrogen gas laser (337 nm) manually hitting the target spots at several positions was used with the 15 kV of accelerating voltage. 8 proteins from 13 spots were identified with peptide mass fingerprint (PMF) so far, such as 78 kDa glucose-regulated protein, Actin cytoplasmic 1, Actin, cytoplasmic 2, UPF0160 protein MYG1, Cytohesin-1, Katanin p60 ATPase-containing subunit A-like 1, Fibrinogen beta chain, Caspase-12 and Synaptosomal-associated protein 47.

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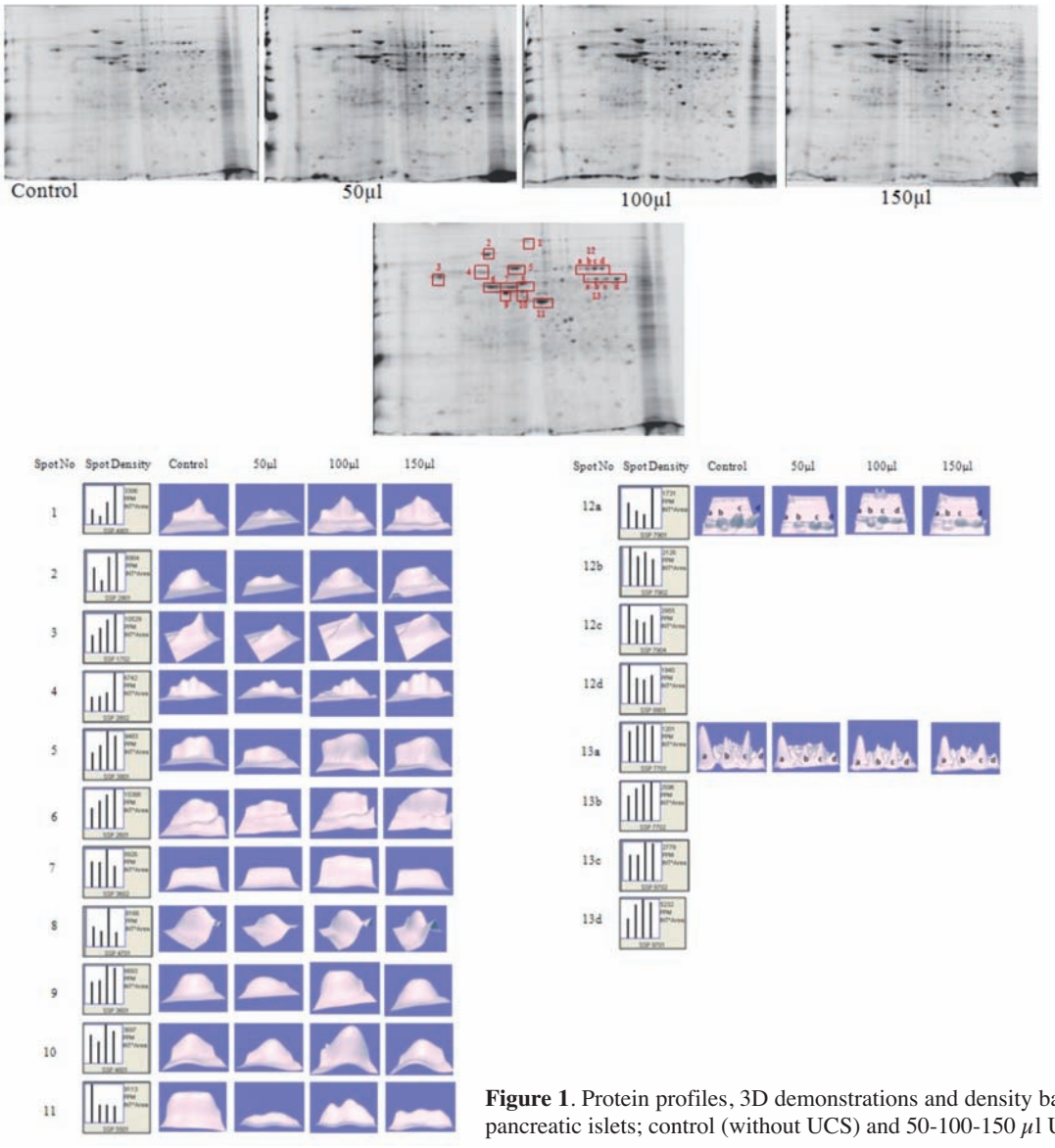

Figure 1. Protein profiles, 3D demonstrations and density bar graphs of pancreatic islets; control (without UCS) and 50-100-150 μl UCS.

Spot No	Protein Name	Access No	Theoretical pI/Mw	Observed pI/Mw	Function
2	78 kDa glucose-regulated protein	P06761 (GRP78_RAT)	5.01 / 70,4	5,3/100,5	Probably plays a role in facilitating the assembly of multimeric protein complexes inside the ER. GRP78 is one of the molecular chaperones that are used in primary quality control for protein folding and maturation in the endoplasmic reticulum (ER). Analysis of ER chaperones showed that the expression of GRP78 and GRP94 are significantly increased in the α-cells of the diabetic 'Atika' mouse compared with wildtype cells (2).
11	Actin, cytoplasmic 1	P60711 (ACTB_RAT)	5,29/42,0	6,8/49,9	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
11	Actin, cytoplasmic 2	P63259 (ACTG_RAT)	5,31/42,1	6,8/49,9	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
11	UPF0160 protein MYG1	Q641W2 (MYG1_RAT)	6,02/43,3	6,8/49,9	-
11	Cytohesin-1	P97694 (CYH1_RAT)	5,48/46,7	6,8/49,9	Promotes guanine-nucleotide exchange on ARF6. Promotes the activation of ARF6 through replacement of GDP with GTP.
11	Katanin p60 ATPase containing subunit A-like 1	Q5XIK7 (KATL1_RAT)	6,67/55,5	6,8/49,9	Regulates microtubule dynamics in Sertoli cells, a process that is essential for spermiogenesis and male fertility. Severs microtubules in an ATP-dependent manner, promoting rapid reorganization of cellular microtubule arrays.
11	Fibrinogen beta chain	P14480 (FIBB_RAT)	7,90/54,8	6,8/49,9	Fibrinogen has a double function: yielding monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation.
11	Caspase-12	Q920D5 (CASPC_ RAT)	5,5/48,3	6,8/49,9	Involved in the activation cascade of caspases responsible for apoptosis execution. Caspase-12 is associated with ER stress in rodents, plays a major role in ER stress–induced apoptosis .
11	Synaptosomal-associated protein 47	Q6P6S0 (SNP47_RAT)	6,49/47,2	6,8/49,9	May play a role in intracellular membrane fusion.

P-49 – THE IMMUNOMODULATORY FACTORS DERIVED FROM AMNIOTIC MESENCHYMAL CELLS ARE ABLE TO MODULATE T HELPER AND T REGULATORY CELLS

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In recent years, mesenchymal stromal cells (MSC) obtained from different sources have generated great interest for their potential application in regenerative medicine, due to their stem cell potential and immunological features. Despite many studies having provided evidence in support of the immunoregulatory effects of MSC, the exact mechanisms underlying these effects remain to be fully elucidated. We have previously demonstrated that cells derived from the mesenchymal layer of the amniotic membrane (hAMTC), when present both in contact and transwell settings, and also conditioned medium derived from culture of these cells, can all inhibit T cell proliferation.

The aim of this study was to evaluate the effects of soluble factors derived from amniotic mesenchymal tissue cells on different subsets of T cells.

Conditioned medium derived from amniotic mesenchymal cells (CM-hAMTC) was obtained by culturing these cells for 5 days in the absence of any activatory stimuli in serum-free conditions. The experiments were carried out using different activation conditions, namely, T cell receptor (TCR) engagement (T cells stimulated with anti-CD3/CD28) and Mixed Lymphocyte Reactions (MLRs).

We demonstrated that conditioned medium derived from amniotic mesenchymal cells reduces the proliferation of both CD4+ and CD8+ lymphocyte subsets. Moreover, our data suggest that CM-hAMTC was able to downregulate both Th1 and Th2 subsets. Interestingly, we showed that CM-hAMTC upregulate the Treg cell population.

Together, these data confirm that soluble factors derived from amniotic membrane cells exert a T cell antiproliferative effect and act specifically on the Treg subsets.

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P-50 – BONE MARROW AS SITE FOR AUTOLOGOUS PANCREATIC ISLET TRANSPLANTATION IN HUMAN

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Background: The bone marrow (BM) is potentially an ideal alternative site for pancreatic islet transplantation, because it offers a protected and extravascular – although well-vascularised – microenvironment.

Methods: Four patients who developed diabetes after total pancreatectomy were candidate to autologous pancreatic islet transplantation. Since they had contraindications for intraportal infusion, islets were infused in the BM of the iliac crest. A needle for BM aspiration (14 G) was inserted into the superior-posterior iliac crest and the islet suspension (1:2.5; tissue: Ringer's Lactate solution) was infused (median volume 8 ml; min-max: 2.5-20 ml). The entire intra BM injection procedure lasted 8-15 min, with no complications recorded.

Results: In all recipients islets engrafted successfully, as shown by measurable post-transplantation circulating C-peptide and by the histopathological evidence of insulin producing cells and/or molecular markers of endocrine tissue on BM biopsies performed during follow-up. Islet function was sustained up to the maximum follow-up of 944 days.

Conclusion: This is the first unequivocal example of successful engraftment of non-hematopoietic tissue in the BM.

P-51 – ELECTROPORATION AS METHOD TO INDUCE MYOFIBER REGENERATION AND INCREASE THE ENGRAFTMENT OF MYOGENIC CELLS IN SKELETAL MUSCLES OF PRIMATES

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Background: In the tiny muscles of mice, best engraftments of intramuscularly (IM) transplanted myogenic cells are obtained after induction of massive myofiber damage triggering profuse myofiber regeneration to recruit the grafted cells. This involves essentially cryodamage or myotoxin injection with high doses of ionizing radiation. There are no effective methods to produce a similar process in the muscles of large mammals such as primates. In this study, we tested the use of intramuscular electroporation for this purpose.

Methods: Tests were performed in skeletal muscle sites of 1 cm^3 of cynomolgus monkeys. Each site was treated by 3 penetrations of a 2-needles electrode (1 cm spacing), applying in each penetration 3 pulses of 5 ms and 400 V/cm with a delay of 200 ms. In some monkeys, transplantation of ß-galactosidase (ß-Gal)-labeled myoblasts was done in electroporated and non-electroporated sites by: (a) matrices of parallel equidistant injections, or (b) simple radial infiltration from only 1-2 needle penetrations in the skin. For transplantation, monkeys were immunosuppressed with tacrolimus. The muscle regions were biopsied at different times post-treatment according to the analysis. Samples were frozen in liquid nitrogen and sections were made in a cryostat to be analyzed by histology.

Results: These parameters of electroporation produced massive myofiber necrosis that was followed by complete muscle regeneration. Myoblast engraftment was substantially increased in electroporated sites compared with non-electroporated sites in both patterns of cell injection ($p<0.01$ in a paired Student's t-test). Using matrices of parallel equidistant injections, the engraftment showed the typical pattern of parallel stripes of ß-Gal+ myofibers matching with the cell injections and the ß-Gal+ area in the grafted region was between 5 % and 11 % (μ = 7.7 % ±2.4 %). The engraftment was diffuse in electroporated sites, and the ß-Gal+ area was between 8.6 % and 38 % (μ = 24.5 % ±12.3 %). Using simple radial infiltration, the injection of the cell suspension was simpler and faster than using matrices of injection. The ß-Gal+ area was between 0.3 and 7.3 mm^2 (μ = 2.4 mm^2 ±2.8 mm^2) in non-electroporated regions and between 8.4 and 18.3 mm^2 (μ = 13 mm^2 ±4 mm^2) in electroporated regions.

Conclusions: Electroporation may be a useful tool to study muscle regeneration in primates and other large mammals, as well as a technique for increasing the engraftment of myogenic cells.

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P-52 – INTRAMUSCULAR TRANSPLANTATION OF MYOGENIC CELLS IN PRIMATES: IMPORTANCE OF NEEDLE SIZE, CELL NUMBER AND INJECTION VOLUME

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Our studies in nonhuman primates and recent clinical trials showed that the allotransplantation of muscle precursor cells needs two conditions: an adequate protocol of cell delivery and an appropriate control of acute rejection. We wanted to progress in defining the technical parameters that may be useful for intramuscular cell transplantation in humans through the use of nonhuman primates for allotransplantation of myoblasts. Myoblasts transduced with the gene coding for ß-galactosidase were injected into the skeletal muscles of 15 cynomolgus monkeys. The following parameters were studied: needle size (27G, 22G and 18G), volume of cell suspension per injection trajectory of about 1 cm (1, 5 or 25 μl) and number of cells per injection trajectory of about 1 cm (10^2, 10^3, 10^4, 10^5 and 10^6 cells). Monkeys were immunosuppressed with tacrolimus. The cell-injected sites were biopsied 1 or 2 months after transplantation. Biopsies were examined histologically to assess the myoblast engraftment and the muscle structure. Our conclusions were: (1) needles should be thin enough to avoid important tissue damage and allow muscle regeneration as sat-

isfactory as possible. Among those tested, 27G should be the choice if the length is consistent with depth of injection. 18G needles produced better engraftment than 22G and 27G needles for a same inter-injection distance, but caused fibrosis. (2) At least 100,000 cells should be delivered per cm of injection trajectory. (3) The smallest volumes of cell suspension per injection should be preferred. In this study, 1 μl per cm of injection trajectory was sufficient, which reduced the amount of fluid injected and the leakage of the cell suspension. In principle, these parameters apply to muscles in which no damage occurred other than the injections.

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P-53 – APPLICATION OF COLLAGEN COATED SILICONE SCAFFOLDS FOR THE THREE-DIMENSIONAL CELL CULTURE OF PRIMARY RAT HEPATOCYTES

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Primary hepatocytes present an appropriate validation system for pharmacological and toxicological studies of agents and compounds in chemical-, pharmaceutical- und cosmetics industries for the evaluation of the potential risk of novel drugs or novel components. Until today, three dimensional culture systems, where the differentiation of the hepatocytes is preserved *in vitro* to estimate the pharmacological effect of agents without systemic impact, are missing.

Aim of the study was to analyse the functionality and growth parameters of 3D collagen coated silicone scaffolds for the culture of primary rat hepatocytes versus the classical 2D cell culture.

Primary rat hepatocytes were cultivated serum free for 72 h on multilayer lattice structure (A= 23.7 cm^2). The development of cell counts, the kind of growth on silicone and the urea production rate versus the 2D culture (A= 8.9 cm^2) on collagen were determined every 24 h.

Based on identical initial cell count the growth rate was about 42%± 5% higher in 3D culture compared to the standard culture. Under serum free conditions the hepatocytes attached to the 3D scaffold, formed cell clusters and organ-like structures. The 3D-culture on the silicone scaffolds displayed no significant different amounts of cytotoxicity after 72h: the percentage of death cells in cultures were 7% ± 3% vs. 9% ± 3%. The urea production rate [μg/10.000 cells] was significant higher in the 3D culture compared to the 2D culture: 24h) 9.1 ± 2.7 vs. 4.7 ±0.2; 48h) 5.7 ± 1.5 vs. 3.7 ±0.05; 72h) 3.5 ±0.5 vs. 2.1 ±0.1.

The results show the appropriateness of collagen coated silicone scaffolds for the 3D-culture of primary rat hepatocytes. The scaffolds support the potential of hepatocytes to growth as organ-like structures. Thus, the collagen coated silicone scaffolds demonstrate an important tool fort he development of three-dimensional hepatocyte cultures sustaining the physiological functions.

P-54 – EFFECT OF SHEAR STRESS ON SURVIVAL OF ISOLATED HEPATOCYTES FOR CELL TRANSPLANTATION

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Shear stress in a catheter during hepatocyte infusion is an important factor to be considered during hepatocyte transplantation because of their biological and physical fragility. Investigations on the effect of shear stress on hepatocytes is important to improve the rate of hepatocyte viability and to establish a routine clinical treatment. The relationship between shear stress and cell viability is still unclear, but there are some empirical clinical research studies on the subject. In this study, the relationship between hepatocyte viability and shear stress was investigated using several types of micro channels with different lengths or heights. Experiments were conducted using a glass micro channel of rectangular cross section. The micro channel with dimensions in height, width, and length of 0.2 × 2 × 50 mm (A), 0.2 × 2 × 100 mm (B), 0.1 × 2 × 50 mm (C), respectively, were employed. A syringe pump supplied a solution of rat hepatocytes into the micro channel with a controlled flow rate. Flow rate ranged from 0.1 to 1 ml/min and 0.02 to 0.22 ml/min for micro channels with heights 0.2 mm and 0.1 mm, respectively. Isolated hepatocytes were suspended in an appropriate medium. Hepatocyte isolation was performed according to the collagenase perfusion method described by Seglen[1]. Hepatocyte viability was observed by a trypan blue test. Viability and total number of hepatocytes were counted before and after the experiment using a hemocytometer. Viability was calculated as a ratio of the total number of viable cells to the total number of cells counted. As a result, a relationship between shear stress and viability in the micro channel was investigated. Figure 1 shows a comparison between hepatocyte viability before and after passage through the micro channel. In this experimental condition, a decline in hepatocyte viability was observed after passage through the micro

channel. This result suggests that hepatocyte viability decreases after passage through the micro channel, which has a high shear stress region near the walls. Future research is important to ascertain a clear relationship between shear stress and viability of cell flow in micro channels, catheters, and needles particularly used for cell delivery during transplantation.

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P-55 – IMPROVED RESISTANCE TO EXERCISE IN MICE TREATED WITH METFORMIN

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Poorly-controlled diabetes induces structural changes leading to muscle weakness, muscle hypotrophy and muscle fibers changes. Skeletal muscle differentiation is a process in which proliferative myoblasts break free from the cell cycleand fuse to form multinucleated myotubes. These events are orchestrated by early myogenic regulator factors (MyoD, Myf-5, myogenin and Myf-6) and late myogenic protein MyHC (myosin heavy chain), through p38 MAP kinase/ERK pathway modulation. Metformin (Met) is a first-line anti diabetic therapy. Our previous data suggested that Met induces ERK pathway activation and MyHC synthesis in *vitro* muscle model (C2C12).

Aim of this work is to confirm *in vitro* and to study *in vivo* in the rodent model the action of Met during myogenesis. In particular we studied muscle proteosynthesis and morphologic characteristics in the late phase of muscle differentiation. Cells were incubated after 72h of differentiation, with 400 μM Met for 4, 8 and 24 hours. We used a positive control with 0.1 nM insulin added to medium and a negative control in which Met and insulin were not added.

MRFs protein contents, evaluated by Western Blot and Immunofluorescence studies, were higher in cells treated with Met. Furthermore, Met treatmentis able to increase cell mass and fusion competence indicating that Met may regulate myogenesis and fibers hypertrophy.

To test those results in vivo, we investigated the action of Met on exercise performance in adult C57BL6 mice. Mice were injected intra abdominally with Met (250 mg/kg) and the control mice with 0.9% saline for 30 days.An endurance performance treadmill running test made at the beginning and at the end of this study revealed that Met treated mice exhibit an enhanced performance respect to the control mice (VO_{2max} ml/kg$^{0.75}$per min: Met 14.41 ± 1.5 respect to control 12.6 ± 2.8).

Ourfindings show a novel therapeutic indication of metformin for musclehypotrophyin chronic wasting diseases.

P-56 – LIVER ORGANOGENESIS FROM HEPATOCYTES IN VIVO

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Introduction: Liver organ engineering is an ultimate goal in the field of liver regenerative medicine. However, the generation of whole liver system has not been fully achieved in vivo. In previous studies, we have demonstrated that the engrafted hepatocytes at ectopic sites retained their growth potential and proliferated in response to the regenerative stimulus derived from partial hepatectomy (PHx). Here we show that hepatocytes continuously proliferate, recruit liver-specific non-parenchymal cells, and finally form an organ-sized large neo-liver under the kidney capsule of mice when recipient mice suffered from chronic liver injury.

Materials and Methods: Donor hepatocytes were isolated from adult mice (8-20 weeks old). Recipient mice, syngeneic to the donor strain, were treated twice with monocrotaline (MCT) at interval of 2 weeks. Two weeks after the last injection of MCT, 1×10^6 isolated hepatocytes were transplanted under the capsule spaces of left kidney. One week later of transplantation, recipient mice were subjected to two-thirds partial hepatectomy (PHx). In some mice, hepatocytes of wild type mice were transplanted into GFP-Tg mice to determine the origin of non-parechymal cells within the engineered liver tissues.

Results: The engineered liver tissue volume remained at stable levels in no-injured control group. In contrast, the engineered liver tissue volume showed progressive and continuous elevation in MCT/PHx group. Although the engineered liver tissues initially consisted of purified

hepatocytes alone, numerous liver-specific non-parenchymal cells (stellate cells, sinusoidal endothelial cells, Kupffer cells) emerged in the neo-livers around days 30-60. Detailed investigation revealed that the non-parenchymal cells were recipient origin. In addition, the neo-livers were observed to have liver cord-like structural arrangements in which single layered hepatocytes were surrounded by endothelial cells, which were closely resemble the architectures found in native livers. We then conducted long-term observation for over a year period and found that the engineered liver tissues were significantly enlarged with tissue volume larger than the chronically injured native liver. The total mass of the injured liver and the neo-liver was almost same as that of the normal mouse liver at a same age, indicating that neo-liver tissue enlarged to compensate for the injured native liver. The large neo-liver contained CK19- and Sox9-positive bile duct-like cells.

Conclusions: The present study demonstrated that liver organogenesis could be achieved by creating small liver tissues initially made only of hepatocytes, followed by hepatocyte proliferation and non-parenchymal cell recruitment. This approach allowed us to create the organ-sized neo-liver which was larger than the injured native liver in vivo.

P-57 – INDUCTION OF FUNCTIONAL HEPATOCYTES FROM FIBROBLASTS BY DEFINED FACTORS

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Differentiated cells are epigenetically plastic as they can be reprogrammed into pluripotent stem cells by nuclear transfer or transcription factor overexpression. Recent studies have revealed that overexpression of lineage-specific transcription factors converts differentiated cells into other lineages without reversion to the stem cell state. Here, we induce mouse mesenchymal fibroblasts directly into functional hepatic (iHep) cells by transduction of three hepatic transcription factors, and inactivation of p19Arf. iHep cells show typical epithelial morphology, express hepatic genes and acquire hepatocyte functions. Importantly, using fumarylacetoacetate hydrolase-deficient mice as a liver injury model, transplanted iHep cells repopulate the liver and rescue the recipients from death by restoring liver functions. Our study thus provides a novel strategy to generate functional hepatocytes for liver engineering and regenerative medicine to treat liver diseases. Recently we are focusing on the generation of human iHep cells.

P-62 – RENAL TRANSPLANTATION IN DIABETIC NEPHROPATHY USING PRE-TRANSPLANT STEM CELL ADMINISTRATION: SINGLE CENTRE EXPERIENCE

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Introduction: End-stage renal disease (ESRD) due to diabetic nephropathy (DN) forms a large pool of renal transplantation (RT) patients. We evaluated the role of stem cell therapy (SCT) in outcome of RT in DN-ESRD patients.

Materials and Methods: This was an open labeled Institutional Review Board approved 2-armed clinical trial conducted between 2002 and 2012. Group-1 included patients receiving SCT group-2 included patients who opted for RT without SCT. Patients with cardiac co-morbid conditions and infections with hepatitis C/B/ HIV were excluded from study. Both groups were compared for survival, graft function in terms of serum creatinine (SCr), rejection episodes and infections.

Group-1 received pretransplant infusion of donor-derived hematopoietic and adipose-tissue derived mesenchymal stem cells (AD-MSC) under non-myeloablative conditioning of total lymphoid irradiation, anti-T and anti-B cell antibodies, and cyclophosphamide.

Group-2 did not receive any conditioning/SCT. Both groups were transplanted after favorable immune response under immunosuppression of Tacrolimus (0.06 mg/kg/day, or cyclosporine 2-3 mg/kg/day) and prednisone (5-10 mg/day). Group-2 received mycofenolate sodium, 360 mg twice a day in addition. Demographics of both groups including donor-recipient HLA matching were comparable.

Results: Group-1 had 48 patients (46 males) with mean age, 47.5 years and group-2 had 27patients (25 males) with mean age, 49.7 years. Mean donor age was 40.2 years in group-1 and 47.2 years in group-2 with 31.2% male donors in the former and 48.1% in the later. Mean patient survival at 1, 5 and 9 years in group-1 was 89.5%, 78.3% and 78.3% respectively vs. 66.2%, 48.1% and 48.1% respectively in controls ($p<0.005$). Death-censored graft survival for the same time intervals was 93.4%, 84.9% and 81.1% respectively in group-1 vs. 95.2% for 1, 5 and 9 years respectively in group-2. Mean SCr (mg/dL) at 1, 5 and 9 years were 1.37, 1.7 and 1.27 in group-1 vs. 1.56, 1.5 at 1 and 5 years in group-2.

Acute Rejections (AR) were noted in 19.1% (n=9) in group-1(grade ≤ag1 at1 av1 ai2) and in 29.6% (n=8) in group-2 out of which 3 had grade ≤ag1 at1 av1 ai2 injury and 5 had more severe injury of grade ≥ ag1 at1 av1 ai2. Chronic rejections (CR) (grade ≤cg1 ct1 cv1 ci1) were noted

in 8.5% (n=4) in group-1 while in group-2, CR were noted in 7.4% (n=2) (grade ≥ cg1 ct1 cv1 ci1). We lost 22.9% (n=11) group-1 patients mainly in 1st year and then in next 2 years, due to HCV (n=4), HBsAg (n=1), bacterial pneumonia (n=2), tuberculosis (n=1), coronary artery disease (CAD) (n=2) and cerebrovascular accident (CVA)(n=1). In group-2 we lost 44.4% (n=12) patients due to HCV (n=2), bacterial pneumonia (n=2), tuberculosis (n=1), fungal infections (n=4), CMV infections (n=1), CAD (n=1) and CVA (n=1).

Conclusions: SCT appears to be a safe and viable therapeutic option with RT for patients suffering from ESRD due to DN as compared to standard transplantation using triple immunosuppression since it leads to improved survival, graft function and has less morbidity.

P-63 – CLINICAL ISLET AUTO-TRANSPLANT OUTCOME IS HIGHLY CORRELATED WITH VIABLE ISLET DOSE AS MEASURED BY OXYGEN CONSUMPTION RATE

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Islet auto-transplant (IAT) is an attractive option for patients with chronic pancreatitis undergoing total pancreatectomy. Having a reliable idea of the expected clinical transplant outcome (CTO) based on islet product characteristics can provide critical guidance for patient management post-transplant. However, such methods are currently unavailable. There is a need for real-time *in vitro* islet characterization methods that can predict CTO. In this paper we evaluate various islet characterization methods for IAT, which is an attractive model for assessing the relationship between characterization assays and CTO as it does not suffer from the presence of confounding factors, such as auto-, allo-, and xeno-immunity or immunosuppressive drug toxicity. Islet autograft products (n=35) with a purity range of 10% to 95% were assessed by the following methods: islet cell membrane integrity stain (FDA/PI), islet equivalent (IE) dose (IE/kg BW), oxygen consumption rate normalized to DNA content (OCR/DNA), OCR/DNA normalized to islet index (IE/number of islets)[1], and viable islet dose (OCRtx/kg BW; as measured by OCR/DNA multiplied by IEtx/kg BW). IAT recipients with fasting blood glucose (BG) <126 mg/dL, 2-hour postprandial BG <180 mg/dL, and HbA1c ≤6.5% without administration of exogenous insulin were considered insulin independent (II). Relationships with outcomes were examined using receiver operating characteristic (ROC) analysis and the AUC was determined. Previously published data from the University of Minnesota[2] has shown that IE/kg BW has a correlation with, but is not highly predictive of CTO [n=59; area under the receiver operating characteristic (ROC) curve (AUC): 0.817; (Figure 1a). In the present transplant series examined both IEtx/kg BW and OCRtx/kg BW were highly predictive of CTO (AUC: 0.944 and 0.964 respectively). However, the range of unpredictability for IEtx/kg BW was greater (Figure 1 b, c). OCRtx/kg BW also helped correctly classify CTO when IEtx/kg BW was high but II was not achieved or IEtx/kg BW was low and II was achieved (Figure 1d). FDA/PI, OCR/DNA, and OCR/DNA/islet index were not predictive of CTO (AUC: 0.492, 0.580, and 0.724). The data presented suggests that OCRtx/kg BW may be useful for the prospective evaluation of the quality of islet preparations prior to clinical transplantation.

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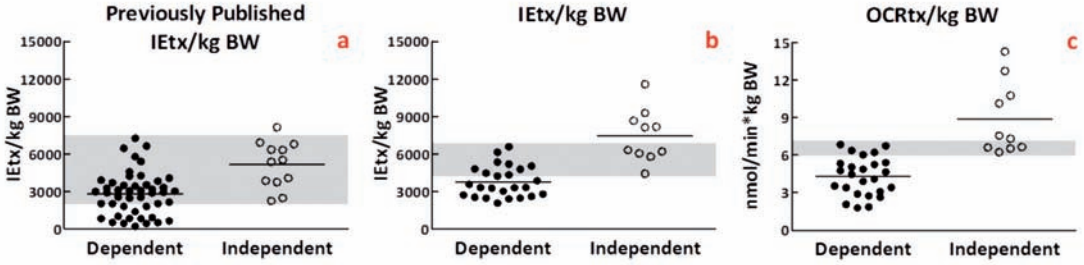

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Case #	IEtx/kg BW	OCR/DNA (nmol/min*mgDNA)	OCRtx/kg BW [(nmol/min)/kg BW]	Insulin Status
25	5,199	76	4.09	Dependent
33	5,314	96	5.06	Dependent
35	6,586	88	6.03	Dependent
32	6,147	97	6.23	Dependent
11	4,414	142	6.53	Independent

P-64 – ITALIAN AIL RESEARCH PROJECT ABOUT CLINICAL SIGNIFICANCE OF ENDOTHELIAL PRECURSOR CELLS IN DIABETIC PATIENTS

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Diabetes is a major challenge in the scientific world, with the ability to become one of the most significant comorbidity in clinical scenarios of the future. The real critical point of diabete are the complications: retinopathy, nephropathy, cardiovascular disease, neuropathy and diabetic foot ulcers. The changes in the structure of blood vessels and nerves can cause ulceration and problems in the lower limbs, especially the foot, in some cases it may be necessary amputation of limbs. It is well known in the literature that the vascular complications of diabetes are related to a reduction of circulating precursor cells (CPC) and endothelial progenitor cells (EPC). These cells are a subset of peripheral blood mononuclear cells capable of differentiating into mature endothelial cells to maintain homeostasis and promote neoangiogenesis. EPC share markers of hemangioblastic (CD34 and CD133) and endothelial (KDR, CD31) lineage. A novel aspect of diabetic neuropathy is its involvement on bone marrow function in terms of EPC impaired release, the so call bone marrow "mobilopathy"; according to this hypothesis, several neuronal nociceptors, as CGRP, are recently demonstrated to be involved in human cardiovascular biology and arterial vasodilation.

In this study we evaluate CD34, CD133, CD45 circulating precursor cells in type 2 diabetes mellitus (T2DM) with foot lesions without or with critical limb ischemia (CLI) and the relationships between circulating nociceptor CGRP with EPC.

We characterized EPC by citofluorimetry and we measured CGRP by ELISA in 8 healthy controls (C) and 62 T2DM with neuropathy (14 N), neuropathic non ischemic (20 N1) and neuroischemic revascularized patients (28 NV) with healed and not healed foot lesions.

In our results CD34+ and CD133+ were reduced in N, N1 and NV versus C, and CD34+ were lower in NV versus N1 (p=0.03). In NV CD34+KDR+ remain elevated in healed versus chronic lesions and in N1 CD133+31+ were elevated in acute lesions. CGRP was reduced in NV and N1 vs C (p<0.004, p<0.04 vs C 26±2 pg/ml). CD34+KDR+ correlated in NV with oximetry (p<0.08) and negatively in N1 with CGRP (p<0.004).

In our study we demonstrate different roles of CPC and EPC in neuropathic and neurovascular type two diabetic patients with foot lesions. Neuropathic patients exhibit a better CD34+ homeostasis capacity to a damage tissue than neuroischemic patients. These data could explain why in diabetes neuropathic foot lesion has a better healing prognosis respect to ischemic foot lesions. Instead neuroischemic patients have an increase of CD34+KDR+ cells only after angioplasty and the level of them remains high in patients with a better healing prognosis. We suppose that CGRP reduction in ischemic patients could induce CD34+ apoptosis or reduced CD34+ bone marrow regeneration. A significative reduction of CD34+KDR+ in chronic diabetic neuroischemic not healed lesions probably explain the frequent restenosis of distal diabetic arteries but we need further investigations.

P-65 – EMBRYONIC STEM CELLS GENE SIGNATURE AND REGULATION IN HUMAN PARATHYROID TUMORAL CELLS

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Parathyroid insufficiency, known as hypoparathyroidism, needs replacement therapy with parathormone (PTH). PTH replacement does not restore the physiologic control of calcium metabolism and might require multiple daily rhPTH injections. Cell-based replacement therapy might be a valid option for hypoparathyroid patients. Up to now, adult stem cells from parathyroid tissues have not been identified due to: a) a parathyroid cell line is not available; b) human normal parathyroids are not available for ethical reasons; c) normal glands from mice and rats are not available due to their small size; d) the only parathyroid cells available for investigation are from parathyroid tumours. Parathyroid tumours are characterized by PTH hypersecretion and hypercalcemia, their initiating and developing processes are largely unknown and curative treatments are missing. Tumoral parathyroid cells showed definitely low proliferation rate related to a qui-

escent status reminiscent of stem cells. Previously we demonstrated that the microRNA clusters C19MC and MIR371-3 at 19q13.4 locus were re-expressed in 67% of parathyroid carcinomas due to C19MC copy number gain extending distal to the MIR371-3 cluster in almost all samples. These two microRNAs clusters have been linked with the signature characteristic for human embryonic stem cells and are silenced in adult normal cells. Thus, we investigated genes of the transcriptional regulatory network governing stem cells pluripotency and self-renewal in a set of 15 parathyroid carcinomas and 30 sporadic typical adenomas. RealTime PCR analysis showed *POU5F1*/*OCT4* mRNA in almost all samples, while the mRNA of the OCT4 target gene *NANOG*was detected in at least 50% of the samples. Similarly, *SOX2* mRNA was detected in 6 out of 15 carcinomas (40%) and 13 out of 30 adenomas (43%). This SOX2 expression pattern was confirmed by western blot, with a nuclear and cytoplasmic localization. Fluorescence activated cell analysis and immunofluorescence showed that SOX2 co-localized with PTH. Immunohistochemistry identified parathyroid epithelial cells with a positive nuclear staining for NANOG that were more abundant in carcinomas (30-70%) compared to normal glands (<10%). Most adenomas expressed NANOG at low levels (1-7%). We further performed experiments aiming to define the regulation of the stemness genes expression in parathyroid tumoral cells. Six hours treatment of dispersed parathyroid adenomatous cells with increasing concentrations (10-20 mM) of lithium chloride (LiCl), determining beta-catenin accumulation, increased both *POU5F1*/ *OCT4* and *NANOG* mRNA expression levels. By contrast, LiCl treatment did not induce significant variation in SOX2 mRNA expression levels. These data identified a genetic embryonic stem cell signature in parathyroid tumours that correlates with more aggressive tumoral features and provides bases for the identification of the adult parathyroid stem cells.

EPIGENETIC REPROGRAMMING OF HUMAN SKIN FIBROBLASTS INTO INSULIN SECRETING CELLS

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The cells of an adult organism acquire their differentiated state through the epigenetic regulation of gene expression that leads to a progressive restriction in their options. Among the different mechanisms involved in lineage specification, DNA methylation plays a major role, therefore the use of a demethylating agent can be used to facilitate the transition of mature cells to a higher plasticity state and thus allow the direct conversion of an adult cell into another differentiated cell type. In our talk we will describe the use of the DNA methyltransferase inhibitor 5-azacytidine (5-aza-CR) to revert fibroblasts from their lineage commitment to a more pluripotent state. We will show that the short exposure to the epigenetic modifier is enough to trigger a transient higher plasticity window in fibroblasts that can then be re-addressed towards the endoderm lineage and pancreatic differentiation. At the end of the treatment, fibroblasts become pancreatic converted cells (PCC) that show an epithelial morphology, produce insulin, release the hormone in response to a physiological glucose challenge in vitro and are able to protect recipient mice against streptozotocin-induced diabetes, restoring normal processing of glucose. The conversion into pancreatic phenotype is obtained without any transgenic modification and avoids a stable pluripotent state which is unphysiological, inherently labile and makes cells prone to alterations. All these aspects, together with the easy accessibility of fibroblasts makes these cells excellent candidates for regenerative medicine and patient specific cell therapy *(the authors performed the experiments with the support of Network Lombardo iPS (NetLiPS) Project ID 30190629 and are members of the COST Action FA1201 Epiconcept: Epigenetics and Periconception environment)*.

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