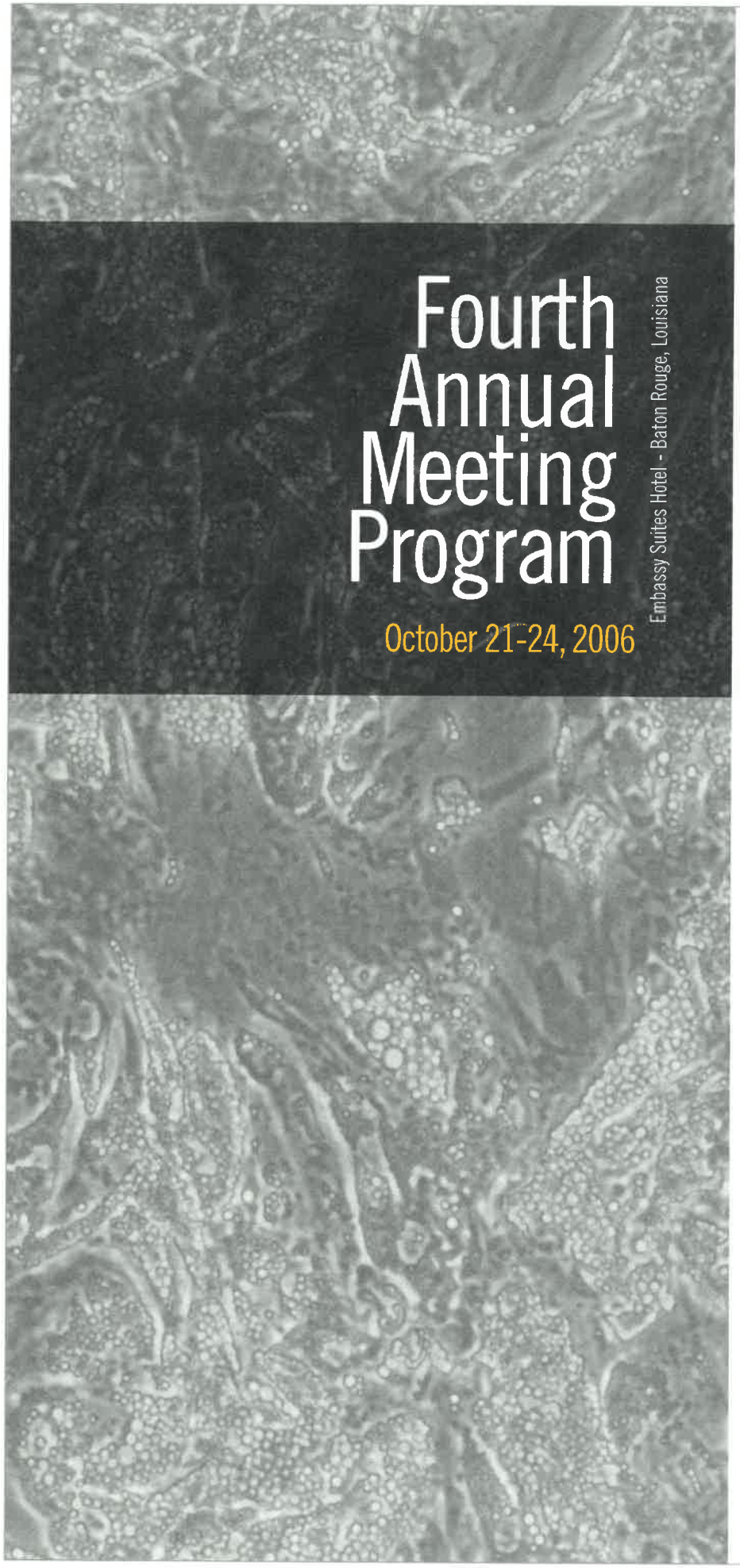

Fourth Annual Meeting Program

October 21-24, 2006

Embassy Suites Hotel - Baton Rouge, Louisiana

IFATS 2006

International Fat Applied Technology Society

ADIPOSE-DERIVED STEM CELLS FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Cytori Therapeutics

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Mission

To improve world health through a better understanding of adipose biology.

Description

The International Fat Applied Technology Society (IFATS), a 501 (c) (3) non-profit organization incorporated in August of 2002, is dedicated to facilitating the development of new technology derived from and directed toward adipose tissue. IFATS is a leading source of information about adipose biology and related technology. The Society identifies new technology and brings together scientists and industry for productive collaborations. Such collaborations will relate to adipose tissue, with far-reaching applications in obesity reduction products and services, fat–derived stromal and cell-based therapies, and fat-applied instrumentation devices which may be directly translated to therapeutic application. IFATS' current scientific areas of interest relate to facilitating the development of treatments for excess body fat, the generation of new fat tissue for reconstruction after cancer or birth-related defects, and the use of adipose tissue as a source of mesenchymal stem cells that have the potential to regenerate and repair many different tissues of the body. IFATS is currently the world's only interdisciplinary fat tissue society. IFATS hope is to serve as the premier global incubator in the field of fat applied and derived technology.

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Pennington Biomedical Research Center, Louisiana State University System

Immediate Past President - Adam Katz

Assistant Professor, Department of Plastic Surgery, University of Virginia

Director, Chronic Wound Care Center

Director, Laboratory of Applied Developmental Plasticity

Scientific Program Chair - Keith March

Indiana University

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Peter Rubin

Adam Katz

Jeff Gimble

Keith March

ADIPOSE-DERIVED STEM CELLS FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Welcome

From This Year's President

It is my great pleasure to welcome you to the 4th Annual IFATS meeting in Baton Rouge, Louisiana. Since its inception in 2002, IFATS has been the world's only interdisciplinary fat tissue society. Each of the past IFATS annual meetings has been the venue for a unique interaction between basic science, clinical, and biotechnology researchers with the common goal of translating the cutting edge discoveries of adipose biology into clinically relevant advances at the bedside. Our society's members represent laboratories and companies from ten countries scattered over four continents. Our goal in the coming year is to continue to extend the scope of IFATS membership, both intellectually and geographically.

This year's meeting will continue the IFATS tradition of collegiality, collaboration, and open discussion. The scientific program this year is focused on adipose-derived stem cells (ASCs) for tissue engineering and regenerative medicine. Keith March, MD, PhD, Director of the Indiana Center for Vascular Biology and Medicine, has done an outstanding job as chair of the Scientific Program Committee. Together, this international body of scientists used a blinded, peer-review process to review and select abstracts/papers. Based on their hard work, we are pleased to highlight over 65 submitted abstracts from our members as both podium talks and posters at the 2006 IFATS Annual Meeting. This excellent science reflects the growth of our field and the promise it holds for the future. In addition, we are honored to host our two keynote speakers, Dr. Michael Longaker (Stanford University) and Dr. Darwin Prockop (Tulane University). Based on the input of our corporate and academic sponsors, we have also scheduled a roundtable forum focusing on the interface between the academic and biotech communities with regard to intellectual property. I, for one, look forward to participating in what I am sure will be a lively and enlightening discussion. Finally, we are fortunate to be joined by a representative of the Center for Biologics Evaluation and Research, FDA, Dr. Richard McFarland, who will bring us up to date on the latest regulatory guidelines. I would also like to encourage everyone to participate in the Society's annual business meeting. We have a number of important issues to address that require the input and insights of our diverse membership.

This year's meeting takes place on the banks of the Mississippi River in historic Baton Rouge, the capital city of Louisiana. The planning committee has made a special effort to provide you with some of the local flavors including our famous Cajun food and music. The Embassy Suite Hotel is conveniently located close proximity to many fine restaurants, shops, theaters, and casinos. To facilitate interactions among attendees, on site luncheons and evening social events have been scheduled at the Pennington Biomedical Research Center and downtown Baton Rouge, courtesy of our generous corporate sponsors. On behalf of this year's sponsors, the Scientific Program Committee and the IFATS board of directors, it is great to have you here at the 4th Annual IFATS Meeting. I hope you enjoy your visit in Baton Rouge as well as the science!

Regards,

Jeffrey M. Gimble, M.D, PhD.
President, IFATS 2006

Oct 16-19

IFATS 2006

International Fat Applied Technology Society

October 21 -24, 2006

Embassy Suites Hotel - Baton Rouge, Louisiana

Saturday, October 21, 2006

3:30 - 7:00 PM	Registration
5:30 - 7:00 PM	Reception / Welcome

Sunday, October 22, 2006

7:00 - 8:00 AM	Breakfast
8:30 AM	Opening Remarks from Organizing Committee *Jeff Gimble, Keith March*
9:00 - 10:30 AM	**Symposium I** Basic Science: ASC Biology Moderators: Robert Considine and Kevin McIntosh
9:00 - 9:15 AM	I-1 Consideration of the Conventional Lineage Specific Markers for Differentiation Potential of Human Adipose Tissue-derived Stem Cells. *Bae, IH, Biotechnology Research Institute, Seoul, Korea*
9:15 - 9:30 AM	I-2 Characterization of Adipose-derived Stem Cells Using Hoechst Staining. *Fujimura, J, Nippon Medical School, Tokyo, Japan*
9:30 - 9:45 AM	I-3 Mapping the Surface Receptor Proteome of Adipose Stromal Cells with Phage Displayed Peptide Libraries. *Kolonin, MG, University of Texas, MD Anderson, Houston, TX*
9:45 - 10:00 AM	I-4 Metabolic Analysis of Adipose-derived Stem Cells and Bone Marrow Mesenchymal Stem Cells using NMR-based Metabolomics. *Fujimura, J, Nippon Medical School, Tokyo, Japan*
10:00 - 10:15 AM	I-5 Regional Anatomic and Age Effects on Cell Function of Human Adipose-derived Stem Cells. *Schipper, B, University of Pittsburgh, Pittsburgh, PA*
10:15 - 10:30 AM	I-6 3-dimensional Suspension Culture of Human Adipose Stromal Cells. *Khurgel, M, University of Virginia, Charlottesville, VA*
10:30 - 10:45 AM	I-7 Comparison of Immunomodulation by Untreated and Cytokine Treated Human Stem Cells from Adipose Tissue and Amniotic Membrane. *Peterbauer, Anja*
10:45 - 11:00 AM	Coffee Break
11:00 - Noon	Business Meeting Society Name (Vote) Society Journal (Vote) Selection of Meeting Organizers and Site for 2008
Noon - 12:30 PM	Lunch
12:30 - 1:30 PM	Poster Session 1
1:30 - 3:00 PM	**Symposium II** Basic Science: ASC in Vasculature Moderators: Louis Casteilla and Keith March
1:30 - 1:45 PM	II-1 A Sub-population of $CD34^-$ Positive Adipose Stromal Cells Occupies a Perivascular Niche and Possesses all the Hallmarks of Pericytes. *Traktuev, D, Indiana University, Indanapolis, IN*
1:45 - 2:00 PM	II-2 Hepatocyte Growth Factor (HGF) Secreted by Cultured Adipose Stem Cells Promotes Proliferation, Migration and Angiogenic Sprout Formation by Endothelial Cells. *Cai, L, Indiana University, Indianapolis, IN*
2:00 - 2:15 PM	II-3 Hepatocyte Growth Factor in Adipose Tissue: Regulation of Angiogenesis and Vascular Growth. *Bell, L, Indiana University, Indianapolis, IN*
2:15 - 2:30 PM	II-4 Chemotaxis and Differentiation of the $CD34^+$/$CD31^-$ Progenitor Cells Toward an Endothelial Cell Phenotype: Modulation by the Stromal-derived Factor-1 Released by Human Adipose Tissue Endothelial Cells. *Sengenes, C, Paul Sabatier University, Toulouse France*
2:30 - 2:45 PM	II-5 Hypoxia in Adipose Tissue Links Obesity to Insulin Resistance. *Ye, J, Pennington Biomedical Research Center, Baton Rouge, LA*
2:45 - 3:00 PM	Coffee Break
3:00 - 4:45 PM	**Symposium III** The Academic Biotech Interface Roundtable Moderators: Jeff Gimble and Adam Katz Welcome Statement *Dr. Richard Rogers, Associate Executive Director of Basic Research, Pennington Biomedical Research Center*
3:00 - 3:15 PM	III-1 Linda Powers, Toucan Capital

ADIPOSE-DERIVED STEM CELLS FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE

3:15 - 3:30 PM	III-2 Dale Peterson, Vertical Group
3:30 - 3:45 PM	III-3 Jon Lovett, Louisiana Fund One
3:45 - 4:00 PM	III-4 Anne Jarrett, PBRC
4:00 - 4:15 PM	III-5 Tony Giordano, LSUHSC-S
4:15 - 4:45 PM	Roundtable Forum
5:15 PM	Buses to PBRC
5:45 - 8:00 PM	Social Gathering at PBRC

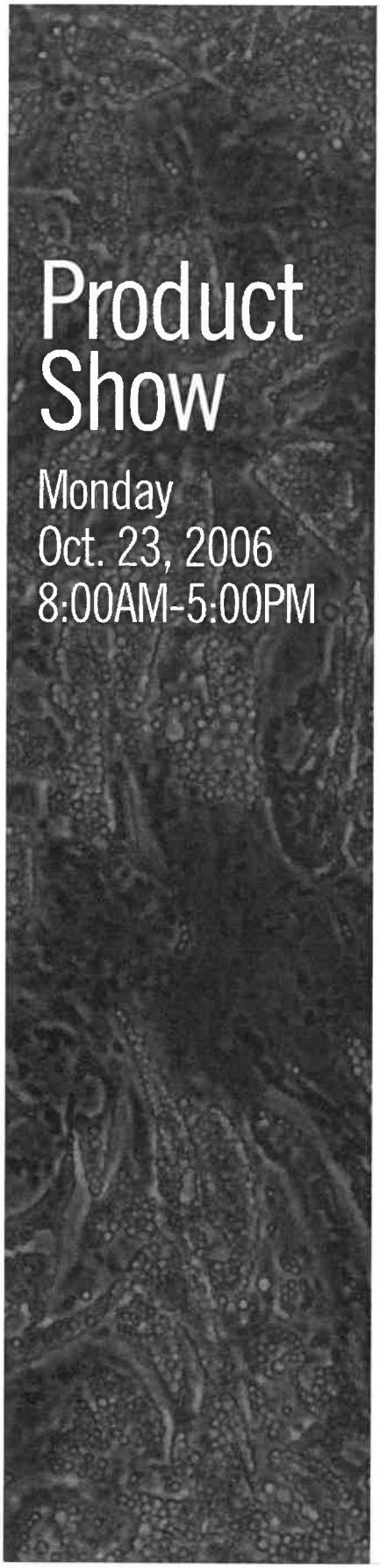
Monday, October 23, 2006

7:00 - 8:00 AM	IFATS Board of Directors Meeting Breakfast Business Meeting (Conference Room)
7:00 - 8:00 AM	Breakfast (Atrium)
8:00 - 5:00 PM	Exhibitors on Site
8:30 - 9:30 AM	Introduction: Marc Hedrick Keynote Address: Michael Longaker MD, Director, Children's Surgical Research, Department of Surgery, Stanford University "Skeletal Tissue Engineering with Adipose-derived Cells"
9:30 - 10:30 AM	**Symposium IV** Translational: Bone Tissue Moderators: Patricia Zuk and John Ransom
9:30 - 9:45 AM	IV-1 Is the Chondrogenic Differentiation Potential of Adipose-derived Stem Cells Influenced by the Site of Adipose Tissue Harvest? *Milazzo, C University of Otago. Dunedin, New Zealand*
9:45 - 10:00 AM	IV-2 Transient Increase of Bone Mineral Density by Tail Vein Injection of Adipose-derived Mesenchymal Stem Cells into OPG/OCIF - Deficient Mice as a Model of Osteoporosis. *Imaizumi, M, Nagoya University, Japan*
10:00 - 10:15 AM	IV-3 Challenge: A One Step-Surgical Procedure for Spinal Fusion using Adipose Stem Cells. *Helder, MN, VU University Medical Center, Amsterdam*
10:15 - 10:30 AM	IV-4 Controlled Disc Degeneratin in the Goat by Varying the Concentration of Chondroitinase ABC. *Helder, MN, VU University Medical Center, Amsterdam*
10:30 - 10:45 AM	IV-5 The Use of the Stromal Vascular Fraction of Adipose Tissue for Cartilage and Bone Regeneration. *Van Milligan, FJ, VU University Medical Center, Amsterdam*
10:45 - 11:00 AM	Coffee Break
11:00 - 12:15 AM	**Symposium V** Translational: Adipose Tissue Augmentation Moderators: Yasuo Kitagawa and Peter Rubin
11:00 - 11:15 AM	V-1 Adipogenesis Within Preadipocyte-seeded and Cross-linked Collagen-chitosan Scaffolds. *Patrick, CW, University of Texas, MD Anderson, Houston, TX*
11:15 - 11:30 AM	V-2 Adipose Tissue Engineering from Human Adipose-derived Stromal Cells: A New Self-assembled 3-D Model Devoid of Exogenous Biomaterial. *Fradette, J, Laval University, Quebec, Canada*
11:30 - 11:45 AM	V-3 Cell-assisted Lipotransfer (CAL) - Supportive use of Adipose-derived Stem Cells (ASCs) for Soft Tissue Augmentation. *Yoshimura, K, University of Tokyo, Tokyo, Japan*
11:45 - Noon	V-4 *De Novo* Adipogenesis at the Site of Injection of Alginate in Combination with Fibroblast Growth Factor-2 and Heparin. *Yamauchi, Y, Nagoya University, Japan*
Noon - 12:15 PM	V-5 Vocal Fold Regeneration by Adipose-derived Mesenchymal Stem Cells from Human Fat Tissue. *Montelatici, E, Università degli Studi, Milan, Italy*
12:15 - 12:45 PM	Lunch
12:45 - 2:00 PM	Poster Session 2
12:15 - 1:45 PM	Press Conference (Conference Room)
2:00 - 2:30 PM	Robert Harman DVM, VetStem "Clinical Liposuction for Collection of Adipose-Derived Stem And Regenerative Cells in the Horse and the Dog."
2:30 - 3:00 PM	Richard McFarland, MD PhD, Associate Director for Policy, Office of Cellular, Tissue, and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration
3:00 - 3:15 PM	Coffee Break
3:15 - 4:15 PM	**Symposium VI** Basic Science: ASC in Adipose Moderators: Anne Boulomie and Beth Floyd
3:15 - 3:30 PM	VI-1 Local Control of the Proliferation of the CD34+/CD31- Progenitor Cells. *Maumus, M, Paul Sabatier University, Toulouse France*

3:30 - 3:45 PM	VI-2 An Organotypic Culture Model of Human Adipose Tissue. *Loiler, S, Pennington Biomedical Research Center, Baton Rouge, LA*
3:45 - 4:00 PM	VI-3 Macrophage-like Behavior of Adipose-derived Stem Cells in Co-culture with Adipocytes and Their Debris. *Kitagawa, Y, Nagoya University, Japan*
4:00 - 4:15 PM	VI-4 A Novel Lipogenic Modulator of Human Adipose Derived Stem Cells: A Human Adenovirus. *Pasarica, M, Pennington Biomedical Research Center, Baton Rouge, LA*
4:15 - 5:00 PM	**Symposium VII** Basic Science: ASC Differentiation Along Cardiovascular Lineage Moderators: Louis Casteilla and Adam Katz
4:15 - 4:30 PM	VII-1 Cardiomyocyte Differentiation Using Adipose-derived Stem Cells *Ogawa, R, Nippon Medical School, Tokyo, Japan*
4:30 - 4:45 PM	VII-2 Cardiomyogenic Differentiation Potential of Human Adipose-derived Stem Cells (ASCs) *Lee, WC, University of Pittsburgh, PA, Pittsburgh, PA*
4:45 - 5:00 PM	VII-3 Novel Patterned Conductive Surfaces for Electrical Stimulation of Adipose-derived Cells for Cardiovascular Differentiation *Marsano, A, Columbia University, New York, NY*
5:45 PM	Buses to Capital City Grill
6:15- 9:00 PM	Dinner at Capital City Grill

Tuesday, October 24, 2006

7:00 - 8:00 AM	Breakfast in Atrium
8:00 - 9:00 AM	Keynote Address "Adult Stem/Progenitor Cells from Bone Marrow. Their Ability to Repair Tissues" *Darwin Prockop MD PhD Professor of Biochemistry; Director, Center for Gene Therapy, Tulane University Health Sciences Center*
9:00 - 9:45 AM	**Symposium VIII** Translational: Neurological Moderators: Kacey Marra, Roy Ogle, and Ramon Llull
9:00 - 9:15 AM	VIII-1 Migration and Differentiation of Mesenchymal Stem Cells from Bone Marrow and Adipose Tissue in Mouse CNS. *Izadpanah, R, Tulane Primate Center, Tulane University, New Orleans, LA*
9:15 - 9:30 AM	VIII-2 Adipose Stromal Cells-secreted Media Protected Hypoxia-ischemia-induced Neonatal Brain Damage. *Du, Y, Indiana University, Indianapolis, IN*
9:30 - 9:45 AM	VIII-3 Neuroprotective Effect of Adipose Tissue-derived Stromal Cells. *Won, CH, Seoul National University, Seoul, Korea*
9:45 - 10:00 AM	VIII-4 Comparative Analyses of Mesenchymal Stem Cells Derived from Bone Marrow and Adipose Tissue. *Bunnell, B, Tulane University, New Orleans, LA*
10:00 - 10:15 AM	Coffee Break
10:15 - 11:30 AM	**Symposium IV** Translational: Wound Healing and Angiogenesis Moderators: Peter Rubin and Keith March
10:00 - 10:15 AM	IX-1 Vascularization During Tissue Injury Repair is Enhanced by Topical Treatment with Adipose Stromal Cells. *Hadad, I, Indiana University, Indianapolis, IN*
10:15 - 10:30 AM	IX-2 Enhanced Diabetic Wound Healing using Human Adipose Stem Cells. *Parker, A, University of Virginia, Charlottesville, VA*
10:30 - 10:45 AM	IX-3 Accerelated healing of full-thickness wound of db/db mouse by murine adipose-derived stromal cells (ASCs). *Gonda, K, University of Tokyo, Japan*
10:45 - 11:00 AM	IX-4 Angiogenic Effect of ASCs in Rat Ischemic Hindlimb Model and In Vivo Tracking of the Cells. *Jeong, JH,*
11:00 - 11:30 AM	Concluding Remarks (Jeff Gimble, Keith March, Organizing Committee)



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Oral Abstracts

Oral Abstracts

SCIENTIFIC SESSION

BASIC SCIENCE: ASC BIOLOGY

I-1 Consideration of the conventional lineage specific markers for differentiation potential of human adipose tissue-derived stem cells

Bae IH, Kim BK, Park S, Kim JH, Kin H, Oh ST[1], Kang WK[1], Oh[1] SN, Park YG[2], Jung J[3], Yoon[3] YD, Kim[3] CG, Do BR
Biotechnology Research Institute, HurimBioCell Inc., Seoul, 157-200,
[1]College of Medicine, The Catholic University of Korea, Seoul, 137-040
[2]Yongdong Severance Hospital, Yonsei University, Seoul, 135-270
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In expectation of regenerative medicine, cases of isolation, expansion, and characterization of human adipose tissue-derived stem cells (ADSCs) have increased. Many researchers have been demonstrated the multi-lineage potential *in vitro* and by transplantation of ADSCs. However, information is not enough to assure clinical application of ADSCs because of the lack of basic data on differentiation ability of ADSCs. Here, we report that expression of several well-known lineage specific differentiation markers is not distinguishable in ADSCs and cells differentiated from them. In the present studies, ADSCs were obtained from the consent volunteer's abdominal fat and their differentiation into cardiomyocytes, myocytes, or adipocytes *in vitro* by conventional methods. The differentiated results were monitored by semi-quantitative RT-PCR and immunocytochemistry using several well-known lineage specific markers. After adipogenic differentiation of ADSCs, distinguishable expression of leptin and PPARγ2 mRNA was observed in differentiated cells. Peculiarly, another adipogenic differentiation marker, C/EBPα, was highly expressed even in undifferentiated ADSCs. Myogenic markers, MyoD1, desmin, and sooth muscle actin, were also continuously expressed before and after myogenic differentiation. In the case of cardiomyogenic differentiation, mRNA expression of cardiac and skeletal muscle specific actins did not change whilst cardiac myosin light chain mRNA showed differential expression after induction. It was recapitulated even in immunocytochemical assays using specific antibodies for sarcomeric myosin heavy chain, sarcomeric alpha actin, and cardiac troponin I. These data indicated that the conventional lineage specific markers are not adequate to distinguish stem/progenitor cells from their differentiated cells. Therefore further verification including new conclusive marker and peculiar methods are required to be useful for monitoring differential potency of stem cells.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC BIOLOGY

I-2 Characterization of adipose-derived stem cells using Hoechst staining

Juri Fujimura[1,2], Rei Ogawa[3,4], Hidemitsu Sugihara[5], Hiroshi Mizuno[3], Hiko Hyakusoku[3], Takashi Shimada[4], Hidenori Suzuki[2]
[1]Department of Pediatric
[2]Department of Pharmacology
[3]Department of Plastic and Reconstructive Surgery
[4]Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan
[5]Tumor Stem Cell Team, Biological research Group, Pharmaceutical Research Laboratories, Research & Development Group, Nippon Kayaku Co. Ltd., Tokyo, Japan

Background: Hoechst staining has been used to purify hematopoietic stem cells. Here we report our efforts to purify and characterize stem cells in the stromal vascular fraction of adipose tissue using Hoechst staining.

Methods: Stromal vascular fractions (SVFs) of adipose tissue and whole bone marrow (BM) were harvested from C57BL/6N mice, as were stem cells. The cells were then stained with Hoechst 33342, analyzed by flow cytometry, and the number of cells in the side population (SP) counted. Moreover, the surface antigens of SP cells were analyzed by flow cytometry using antibodies against CD44, 45, 45R, Sca-1, and c-kit, respectively. The morphologic characteristics of cells in the SP of both BM and SVFs were observed by electron microscopy.

Results: The percentage of SP cells in BM was about 0.05 to 0.1% and that in the SVF was about 1.0 to 3.0%. The cell-surface antigens of BM expressed were CD44(-), CD45(+), CD45R(-), Sca-1(+) and c-kit(+), while those of SVF were CD44(-), CD45(-), CD45R(±), Sca-1(±) and c-kit(-). Upon electron microscopic observation, cells in the SP of SVFs were considered to be remarkably immature (immature cell organelles and a high N/C ratio).

Conclusion: The rate and expression patterns of cell-surface antigens in SP cells derived from BM were consistent with the results of previous reports. However, the same characteristics in SP cells derived from SVFs were clearly different. At present it is not clear whether cells in the SP of SVFs are "adipose-derived stem cells." Indications were that there are 10 to 60 times as many immature cells in adipose tissue as in bone marrow. Moreover, it is possible that the great majority of cells in the SP of SVFs are not hematopoietic stem cells but unique adipose-derived stem cells. Finally, our studies suggest that Hoechst staining may be useful for identifying not only hematopoietic stem cells but also other adult stem cells.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC BIOLOGY

I-3 Mapping the surface receptor proteome of adipose stromal cells with phage displayed peptide libraries

Kolonin M.G., Traktuev D.O., March K.L., Pasqualini R., Arap W., University of Texas, MD Anderson

Cells capable of differentiating into various cell types have been recently discovered in stroma of several adult organs, including white fat. Different populations of such stem cells have unique surface antigen profiles and differentiation potentials, which are still poorly described. Multipotent adipose stromal cells (ASC) that can be obtained in large numbers from white adipose tissue (WAT) show a high proliferative capacity in culture, which makes them promising candidates for use in cell therapy. Development of approaches to profile the cell surface proteomes of stromal cells is necessary for their classification into defined populations directed at specific clinical applications. Here, we explored the cell surface proteome of human ASC by selecting a random peptide phage-displayed library on stromal cells derived from liposuction samples. This combinatorial library screening approach led to the isolation of a panel of peptides that bind to ASC cell surface. We confirmed the ability of six peptides to target functional ASC cell surface receptors by showing that they get internalized into these cells. The peptide ligands isolated based on their binding to ASC can be used as "baits" for the subsequent identification of receptors differentially expressed by subpopulations of adipose stromal cells and for enrichment of liposuction-derived ASC for clinically relevant cell fractions. Further, conjugation of these ligands with reporter tags can be developed as reagents for tracking of ASC populations in culture and intravitally. Finally, our approach will help to define the ASC receptors useful for therapeutic targeting. The ASC-binding ligands may become therapeutically useful as vehicles for directing modulatory drugs to the ASC *in vivo* for the purpose of tissue growth modulation.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC BIOLOGY

I-4 Metabolic analysis of adipose-derived stem cells and bone marrow mesenchymal stem cells using NMR-based metabolomics

Juri Fujimura[1], Rei Ogawa[2], Keiko Hirakawa[3], Kyoko Uekusa[3], Kohtaro Yuta[4], Hiroshi Mizuno[2], Youkichi Ohno[3], Hiko Hyakusoku[2], Yoshitaka Fukunaga[1]
[1] Department of Pediatrics
[2] Department of Plastic and Reconstructive Surgery
[3] NMR Laboratory, Department of Legal Medicine, Nippon Medical School
[4] BioIT Business Development Group, Fujitsu Ltd.

Background: Adipose-derived stem cells (ASCs) had been considered to look like bone marrow derived mesenchymal stem cells (BSCs). However, an increasing number of reports describe ASCs as being different from BSCs. Here we report our study on the differences in the metabolic pathways between both cells using nuclear magnetic resonance (NMR)-based metabolomics

Materials and Methods: 5-week-old Wister rats were used for this study. ASCs were harvested from inguinal fat pads, BSCs from the femur, and fibroblasts from the abdominal dermis. The cells were cultured in DMEM with 10% fetal bovine serum and harvested after 2 passages of the subculture. Then, the cells were subjected to freezing in liquid nitrogen and crushed to extract the aqueous metabolites. ^{1}H-NMR spectra were measured and analyzed by a computer software (Alice2 for metabolome™ and ADMEWORKS/Modelbuilder™).

Results: BSCs, ASCs and fibroblasts were clearly separated into three groups on the principal component analysis (PCA) plot.

Conclusion: ASCs, BSCs and fibroblasts were considered to have different metabolic activities, and NMR-based metabolomics will henceforth be useful for the detection, analysis, and characterization of ASCs.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC BIOLOGY

I-5 Regional anatomic and age effects on cell function of human adipose-derived stem cells

Bret Schipper MD[1], Kacey G. Marra PhD, J. Peter Rubin MD, [1]University of Pittsburgh

Introduction: Adipose tissue has been shown to contain adult stem cells that have therapeutic applications in regenerative medicine. There is evidence that the ability of adipose precursor cells to grow and differentiate varies among fat depots and changes with age. Defining these variations in cell function and molecular mechanisms of adipogenesis will facilitate the development of cell based therapies.

Methods: We compared three age ranges and five different subcutaneous adipose depots. Capacity for differentiation of isolated adipose stem cells (ASCs) with and without a strong PPAR-Gamma agonist was examined in-vitro. Cells were also characterized by lipolytic function, proliferation, and sensitivity to apoptosis. Additionally, PPAR-Gamma protein expression was measured.

Results: We have observed a difference in the apoptotic susceptibility of ASCs from various depots, with the superficial abdominal depot significantly more resistant. We have also showed that a PPAR Gamma agonist aids in the induction of differentiation in cells from all depots and ages. While sensitivity to apoptosis was linked to anatomic depot, differences in cell proliferation were related primarily to age. Free glycerol production as a result of lipolysis has been shown to be highest in the arm depot. The arm depot has been found to be the only depot to consistently express PPAR Gamma 2 with and without a PPAR Gamma agonist. Also the younger patients have PPAR Gamma expression in all depots, where the older patients only have consistent expression in the arm and thigh depots.

Conclusion: There is variability in function of adipose stem cells that have been harvested from different depots. Additionally, we have showed age related changes in function. These data will help select patients and cell harvest sites that best favor tissue engineering therapies.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC BIOLOGY

I-6 3-dimensional suspension culture of human adipose stromal cells: Self-organizing, self-sustaining dynamic cell niches

Moshe Khurgel, Hulan Shang, Anna Parker, Adam Katz
University of Virginia, Department of Plastic Surgery
Laboratory of Applied Developmental Plasticity

Introduction: Converging lines of evidence support the emerging view that mammalian adipose tissue contains progenitor/stem cells (ASCs) with diverse differentiation potential well into adulthood. Related work suggests that stem cells primarily exist and function within the context of a distinct microenvironment, or niche. We hypothesized that cell aggregates represent a favorable environment for studying the properties of adult stem/progenitor cells since 3-D cultures more likely reflect *in vivo* conditions than traditional monolayer cultures. Consequently, we harnessed the propensity of ASCs to spontaneously aggregate to develop a novel culture system in which to study ASCs as they grow and differentiate in a 3-dimensional niche environment.

Methods: Formation of cell aggregates ("mesenchoid bodies") was initiated with varied numbers of ASCs (from 500 to 50,000). using a hanging drop method. ASCs from multiple donors were tested. In some of the experiments the cells were labeled with fluorescent carbocyanine dye (DiI) prior to mesenchoid body (MB) formation. ASC-MBs were cultured for extended periods of time in regular tissue culture multi-well plates or in low attachment labware under different media conditions that included standard DMEM/F12 medium supplemented with 10% FBS, serum-free DMEM/F12 medium, or a serum-free medium that was supplemented with growth factors. Changes in size and shape of MBs were monitored in all conditions throughout all experiments. Some of the MBs in all three media were pulsed with BrdU at different time points to assess the degree and spatial-temporal characteristics of cell proliferation. Some of the MBs were also subsequently cultured in adipogenic, osteogenic, chondrogenic or myogenic differentiation media in suspension or following attachment to tissue culture plastic.

Results: Multiple small clusters/spheroids formed in drops of medium when 500 or 1000 ASCs were used for MB formation. In contrast, large, single MBs of consistent size formed at 95% or higher effectiveness when high numbers of cells were used (2,000, 5,000 and higher). MBs were cultured in suspension, individually or in groups, for more than 2 months without loss of viability. Strikingly, MBs that were cultured in plain, serum-free DMEM/F12 medium *without any* supplements also maintained viable cells for up to several weeks. MBs that were grown in suspension in serum-free, growth factor-supplemented medium exhibited dramatic, polarized growth when compared to those maintained in DMEM/F12+10%FBS. DiI and BrdU labeling studies revealed a subpopulation of cells in MBs that is quiescent and another subpopulation that actively proliferates. The presence of distinct subpopulations of cells was also evident in MBs that were cultured in groups and allowed to attach to and fuse with other MBs. DiI labeling of cells allowed for visualization of dynamic reorganization of cells within fused MBs, whereby the quiescent cell subpopulations migrated towards each other. MBs that were plated in regular lab ware attached to tissue culture

plastic and "spawned" large numbers of adherent progeny cells with multi-lineage differentiation potential, regardless of the initial growth medium. Generation of new adherent multipotential progeny cells was repeatedly achieved after multiple sequential transfers (i.e. passages) of MBs.

Conclusion: Altogether our results 1) describe a novel cell culture paradigm for ASCs, and 2) demonstrate that ASCs can organize into self-sustaining 3-D niches with the potential for extensive renewal and the ability to respond to different environmental stimuli with distinct and dynamic growth and morphogenesis. These findings offer intriguing new directions for future tissue engineering and tissue regeneration strategies using ASCs

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN VASCULATURE

I-7 Comparison of immunomodulation by untreated and cytokine treated human stem cells from adipose tissue and amniotic membrane

Susanne Wolbank[1], Marc Fahrner[1], Sandra Neußl[1,2], Anja Peterbauer[1,2], Simone Hennerbichler[1,2,3], Martijn van Griensven[2], Heinz Redl[2], Christian Gabriel[1]
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Human adult stem cells (SC) isolated from sources like adipose tissue and amnion can be expanded in an undifferentiated state or differentiated along multiple lineages and are regarded to be promising candidates for regenerative medicine and tissue engineering. We have evaluated the *in vitro* immunomodulatory potential of human adipose-derived stem cells in comparison to human amniotic mesenchymal and human amniotic epithelial cells under identical experimental conditions. Peripheral blood mononuclear cell (PBMC) stimulated in mixed lymphocyte reactions (MLR) or in phytohemagglutinin activation (PHA) assays were cocultured with SC at different cell ratios and PBMC proliferation was evaluated. Additionally, the applied SC populations were pre-exposed to cytokines like interferon-γ and tumor necrosis factor-α.

All investigated SC inhibited activated PBMC proliferation in a cell dose-dependent manner in MLR (66-93% inhibition at equal amounts of SC and PBMC) and PHA assays (67-96% inhibition at equal amounts of SC and PBMC). The lowest effective SC to PBMC ratio was 1 to 8. The immunoinhibitory properties were independent of passage number (passage 2-6) but were significantly reduced by prior cryopreservation. Furthermore immunosuppression was not limited by the presence of proinflammatory cytokines. From these *in vitro* data we conclude that all three stem cell types may be considered for future allogeneic transplantation in cell therapy and regenerative medicine.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN VASCULATURE

II-1 A sub-population of CD34-positive adipose stromal cells occupies a perivascular niche and possesses all the hallmarks of pericytes

Dmitry Traktuev, Stephanie Merfeld-Clauss, Jingling Li, Dongni Feng, Brian H. Johnstone, Keith L. March
Indiana University

Introduction: Adipose stromal (stem) cells (ASC) are CD34+ mesenchymal cells that exhibit a high degree of phenotypic plasticity *in vitro*. Isolated ASC accelerate tissue repair and reperfusion of animal models of ischemia. Relatively little is known about the normal biology of these cells with respect to their physiological role in adipose tissues. Accordingly, we examined the *in situ* location of ASC in adipose tissues and discovered that they are tightly associated with the periphery of microvessels. They nature and function of perivascular ASC was determined.

Methods: Frozen sections of human adipose tissue were co-stained with fluorescent antibodies against CD34 and CD144 (an endothelial cell-specific antigen). ASC were isolated from lipoaspirate samples by collagenase digestion and centrifugation and briefly (2 days) cultured on plastic in EGM-2mv medium. Co-expression of CD34 antigen on ASC with markers of endothelial, mesenhymal and pericyte/smooth muscle cells was evaluated by dual color flow cytometry and immunohistochemistry. The profile of cytokines and growth factors secreted by ASC was evaluated utilizing RayBiotech antibody arrays. To evaluate the mitogenic response of ASC to the factors known to secreted by human microvascular endothelial cells (HMVEC), ASC were cultured in EBM-2/5%FBS in presence of 10 ng/ml of purified bFGF, PDGF, or VEGF. Analysis of cellular cord/network formation by ASC alone or with HMVEC was performed by culturing for 4 days in Matrigel with DMEM/10%FBS medium.

Results: Histological analysis of adipose tissues revealed that the CD34+/CD144- ASCs were predominantly associated with the periphery of vascular structures. The perivascular location of ASC suggested that they were mural cells; therefore, the cells were extracted and isolated to determine expression of markers for vascular and mesenchymal cells types. After culturing for 2 days on uncoated plastic, the majority of ASC (up to 95%) express CD34 and are negative for CD45. Analysis of surface and intracellular markers of the CD34+/CD45-/CD144- ASC showed that >95% co-express mesenchymal (CD10, CD13, CD90), pericytic (NG2, CD140a, CD140b, N-cadherin), and smooth muscle (α-SMA, caldesmon and calponin) markers. ASC demonstrated polygonal self-assembly on Matrigel, as did HmVEC. Co-culture of ASC with HmVEC on Matrigel led to cooperative network assembly, with enhanced stability of endothelial networks and preferential localization of ASC on the abluminal side of the cords. Bidirectional paracrine interaction between these cell types was supported by identification of angiogenic (VEGF, HGF, bFGF), inflammatory (IL-6, -8, MCP-1, -2), and mobilization (M-CSF, GM-CSF) factors in media conditioned by CD34+ ASC; as well a robust mitogenic response of ASCs to bFGF, and PDGF-BB factors produced by endothelial cells.

Conclusion: These results demonstrate for the first time that the majority of adipose-derived adherent CD34+ cells are resident pericytes that play a role in vascular stabilization, by structural and functional interaction with endothelial cells.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN VASCULATURE

Il-2 Hepatocyte growth factor (HGF) secreted by cultured adipose stem cells promotes proliferation, migration and angiogenic sprout formation by endothelial cells

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Background: The use of stem cells for promoting repair of injured tissues is a promising new therapeutic approach; however, the exact mechanism of action is not yet fully understood. We previously demonstrated the beneficial effects of adipose stromal (stem) cells (ASCs) in models of acute myocardial infarction (rat) and peripheral vascular disease (mouse). These cells express high levels of many growth factors and cytokines, which we are now selectively

modulating to determine their specific contributions to angiogenesis.

Methods and Results: Human ASCs were transduced with a dual cassette lentiviral construct expressing the green fluorescent protein (GFP) reporter gene and either a small interfering RNA (siRNA) targeting the hepatocyte growth factor (HGF) gene (iHGF) or a non-coding sequence (control). Transduction efficiency was greater than 95% as determined by flow cytometric analysis of GFP^+ cells. Transduction with iHGF selectively reduced HGF levels by >75% ($p<0.01$) without affecting ASC growth or expression of unrelated genes. Growth and metabolic activity of human microvascular endothelial cells (HMVEC) cultured in growth-factor deficient minimal medium(MM) increased 1.7-fold when supplemented with a 1:1 mixture of control conditioned medium (CM)($p<0.01$). This growth stimulation was absent when CM from iHGF-transduced ASCs was applied. The specificity of this effect was confirmed by pre-treating control CM with a neutralizing HGF antibody. Endothelial sprouts formed by HMVECs growing on Cytodex-3 microcarrier beads was 2.03-fold higher when cultured in the CM from control ASC compared to MM ($p < 0.005$). This stimulation was abolished when CM from siHGF-transduced hASC was applied ($p<0.005$ compared to control CM). Furthermore, endothelial progenitor cell (EPC) migration was enhanced by 2-fold when control ASC CM was added to MM ($p<0.01$); whereas, migration was no different than MM with CM in which HGF levels were reduced by either siHGF or an inactivating antibody to HGF.

Conclusions: These results suggest that HGF secreted by ASCs may be an important factor to regulate mature microvascular endothelial cell growth and angiogenic sprout formation. This may partially explain the mechanism of ASC protection against ischemic injury. This novel lenti-iHGF vector will permit testing the contribution of HGF expression by ASC in *in vivo* by directly comparing the efficacy of ASCs transduced with either iHGF or control constructs.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN VASCULATURE

II-3 Hepatocyte growth factor in adipose tissue: Regulation of angiogenesis and vascular growth

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Angiogenesis is important for expansion of adipose tissue, and regulation of vascular growth may be a possible target for therapeutic intervention in obesity. Previous work from our laboratory has established that hepatocyte growth factor (HGF), a potent angiogenic and mitogenic hormone, is produced in human adipose tissue. Further, serum HGF is elevated more than three-fold in obese individuals and isolated adipocytes from obese individuals release significantly more HGF *in vitro* than adipocytes from lean subjects. Based on these novel observations we hypothesize that HGF has a central role in the growth and maintenance of adipose tissue vasculature. In the current study we utilize clonal 3T3-F442A preadipocytes and an *in vivo* angiogenesis model to study the contribution of HGF to vascular growth in developing fat pads.

Results: Cultured 3T3-F442A preadipocytes and mature adipocytes express HGF mRNA (78.4±10.8 relative units and 62.1±6.1 relative units, respectively) and secrete HGF protein (3.8±0.6 ng/mg DNA/48 h and 0.6±0.1 ng/mg DNA/48 h, respectively), as is observed for primary isolated cells from humans. In undifferentiated 3T3-F442A cells, lipoprotein lipase (LpL) mRNA is low (10.3±2.0 relative units) and increases significantly with differentiation *in vitro* (1866.0±132.0 relative units; $p=0.005$; $n=3$). Injection of 3T3-F442A preadipocytes under the skin of BALB/c nude mice results in greater LpL expression over time, confirming that the cells will differentiate into mature adipocytes *in vivo* (**Table 1**). Recruitment of endothelial cells into the differentiating fat pad is evident by the increasing expression of endothelial cell markers TIE1 and PECAM1 (**Table 1**). To determine if HGF can accelerate vascularization and differentiation of fat pads, 3T3-F442A cells were injected under the skin of HGF overexpressing transgenic mice and wild-type littermates. In the presence of greater HGF concentrations in transgenic mice, 3T3-F442A fat pads demonstrate increased endothelial cell mRNA and greater differentiation than cells injected into wild-type littermates at 24 h post-injection (**Table 2**). To examine the effect of loss of HGF activity on fat pad formation, siRNA constructs for silencing HGF

expression in 3T3-F442A cells were tested. Electroporation of 200 nM and 400 nM siRNA resulted in a 55.6% (n=2) and 73.1% (n=2) decrease in HGF mRNA levels, respectively. HGF secretion was also reduced 81.7% with electroporation of 400 nM siRNA compared to control (0.6 ng HGF/mg DNA/48 h vs 0.1 ng HGF/mg DNA/48 h; n=2). Knockdown of HGF expression did not alter differentiation of 3T3-F442A cells *in vitro*. Experiments to test the effect of silencing HGF expression on *in vivo* differentiation and vascular development are currently underway. Finally, adipocytes express the HGF receptor c-Met, and HGF (10 ng/ml; 48 h) stimulates proliferation of 3T3-F442A preadipocytes 145.0±13.8% of control ($p<0.05$; n=6).

Conclusions: 3T3-F442A preadipocytes synthesize and secrete HGF, and are a useful model to study the contribution of HGF to adipose tissue angiogenesis when injected into mice. Ongoing experiments include investigation into whether silencing of HGF expression in 3T3-F442A preadipocytes prior to injection into mice attenuates fat pad neovascularization and adipocyte differentiation. The novel finding that adipocytes express c-Met indicates that HGF may function not only as a paracrine factor regulating blood vessel growth in adipose tissue, but also as an autocrine factor regulating adipocyte function.

Table 1

	Undiff 3T3-F442A	24 hour n=1	4 day n=1	6 day n=1	3 wee n=1	Epididymal Adipose Tissue n=4
TIE1	Undet	0.5	12	32	120	25 ± 11
PECAM1	Undet	11	44	183	1380	184 ± 48
LpL	10	63	46	1017	6408	2309 ± 937

Table 2

	HGF Overexpressors n=2	Wild-type Littermates n=2
TIE1	0.46	0.25
PECAM1	4.66	2.75
LpL	20.42	8.7

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN VASCULATURE

Il-4 Chemotaxis and differentiation of the CD34+/CD31- progenitor cells toward an endothelial cell phenotype: Modulation by the stromal-derived factor-1 released by human adipose tissue endothelial cells.

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Human adipose tissue (AT) contains progenitor cells, defined as CD34+/CD31-, which can differentiate into adipocyte and endothelial cells. In the present work we studied the crosstalk between human AT capillary endothelial cells (CECs) and AT progenitor cells.

Human AT-derived CECs defined as CD34+/CD31+ cells and progenitor cells were isolated as previously described by an immunoselection approach. Experiments were performed on freshly harvested cells or primary culture.

Chemotaxis was assessed by modified Boyden chamber assays. Differentiation of the progenitor cells toward the endothelial cell lineage was determined by the expression of EC specific marker (CD31) as well as by the capacity of the cells to organize into capillary-like structure in matrigel assays.

We showed that AT derived CECs produced and released the chemokine stromal derived factor 1 (SDF-1). AT CECs-conditioned media induced a marked chemotaxis of the AT progenitor cells via the activation the SDF-1 receptor i.e CXCR4, expressed on the $CD34^+/CD31^-$ cells since the migration of the progenitor cells in response to the EC-conditioned media was inhibited by selective CXCR4 antagonist or SDF-1 neutralizing antibody. Moreover co-culture experiments showed that the presence of AT CECs increased the differentiation of AT progenitor cells toward the EC lineage. Moreover the formation of capillary like structures by AT progenitor cells and their expression of the EC marker CD31 were modulated by the SDF-1/CXCR4 pathway. The present study shows that the migration and differentiation of AT progenitor cells are modulated by AT capillary ECs-derived factors. Among them SDF-1 and its receptor CXCR4 play a key role.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN VASCULATURE

II-5 Hypoxia in adipose tissue links obesity to insulin resistance

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Abstract: Increased expression of pro-inflammatory cytokines, reduced expression of adiponectin and decreased response to insulin are dysfunction markers of adipose tissue in obesity. Exact cause of the dysfunction remains to be identified. Our data suggests that hypoxia represents an initiation factor for the dysfunctional. Hypoxia was increased in the adipose tissue with body weight gain and reduced with body weight loss. Hypoxia occurs specifically in adipose tissue as no hypoxia was detected in skeletal muscle in the same condition. In response to hypoxia, insulin–induced glucose uptake and adiponectin expression were reduced, expression of pro-inflammation mediators was increased in adipocytes. In adipose tissue, these changes are companied with macrophage infiltration. In ob/ob mice, hypoxia and the adipose tissue dysfunction were attenuated by VEGF-induced neovascularation, suggesting a role of blood supply in the pathogenesis of hypoxia. Consistently, systemic insulin sensitivity was improved in the ob/ob mice by VEGF administration. These data suggest that hypoxia contributes to adipose tissue dysfunction and insulin resistance in obesity

SCIENTIFIC SESSION

TRANSLATIONAL: BONE TISSUE

IV-1 Is the chondrogenic differentiation potential of adipose-derived stem cells influenced by the site of adipose tissue harvest?

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Adipose-Derived Mesenchymal Stem Cells (ASC) have potential as a source of cells for repairing articular cartilage defects in joints. However, comparisons between ASC and Bone Marrow-Derived Mesenchymal Stem Cells (BSC) have highlighted inconsistencies in the ability of ASC to form hyaline-like cartilage. One crucial aspect of these studies is that the ASC were derived from subcutaneous fat. Few studies have been performed with fat from other

sources and none of these have evaluated the manner in which chondrogenic differentiation potential was affected by the site of harvest. We believe that the stem cell niche may play a decisive role in determining the differentiation ability of ASC. To this end we have compared the chondrogenic potential of ASC extracted from either subcutaneous (SC) fat or the infrapatellar fat pad (IP).

Neocartilage pellets were produced from ASC isolated from each source using a three-phase culture process involving: (I) monolayer culture, (II) pellet formation in serum-free chondrogenic medium, and (III) exposure to 3-dimensional mechanical forces in a rotating cell culture system, also in chondrogenic medium. During this process ASC are induced to differentiate into chondrocytes and secrete matrix-forming proteins that differentially organize into pericellular and territorial matrices. Neocartilage pellets formed by this method and derived from each fat source were evaluated for gross pellet morphology, composition of chondrogenic matrix macromolecules by biochemical analysis, and differential organization of hyaline matrices by immunohistochemical analysis.

Adipose tissue was harvested from 4 sheep by subcutaneous lipectomy and resection of approximately 1g of the infrapatellar fat pad. ASC were isolated by overnight digestion in 0.05% collagenase type II and centrifugation at 300 X g to separate fatty tissue and again at 600 X g to concentrate the ASC pellet. Isolated cells were proliferated in monolayer culture with 10% foetal bovine serum until the first passage reached confluence. Confluent cultures were harvested and cells spun into pellets of $3x10^5$ cells each and cultured in serum-free chondrogenic medium for 1-3 days until pellet integrity was maintained. Pellets were then transferred to a rotating cell culture system that provided low shear force, high mass transfer, and simulated microgravity in a 3-dimensional environment for 14 days. Pellets were evaluated grossly for morphology; frozen and processed for immunohistochemical staining for the phenotypic markers of cartilage, collagen type II and aggrecan, as well as the phenotypic marker of pericellular development, collagen type VI; and sulfated-glycosaminoglycan and total collagen content was measured by dimethylmethylene blue and hydroxyproline assays respectively, and normalized against DNA content.

Results indicate that ASC isolated from the infrapatellar fat pad gave rise to neocartilage pellets with chondrogenic matrix development and organization superior to pellets formed from subcutaneous adipose tissue. Pellets derived from IP ASC were significantly larger than SC ASC-derived pellets with comparable cell density and viability, indicating that IP ASC pellets contained more extracellular matrix. This result was confirmed by biochemical analysis showing greater sulphated-proteoglycan and collagen content per cell in the IP ASC pellets. Immunohistochemical staining confirmed the biochemical assay results and revealed a greater degree of differential organization of chondrogenic phenotype markers, collagen type II, collagen type VI, and aggrecan. The exposure to the environmental forces and interactions with the extracellular matrix proteins found within each niche appears to determine the chondrogenic potential of ASC. Therefore, the source of donor cells for ASC-based regenerative therapies may have very significant impact on the success of cartilage repair procedures and potentially those of other tissue types.

SCIENTIFIC SESSION

TRANSLATIONAL: BONE TISSUE

IV-2 Transient increase of bone mineral density by tail vein injection of adipose-derived mesenchymal stem cells into OPG/OCIF-deficient mice as a model of osteoporosis

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Adipose-derived stem cells (ASCs) in stromal vascular fraction (SVF) from human subcutaneous adipose tissues and mesenchymal stem cells (aMSCs) selectively expanded by culturing SVF cells in low serum medium exhibited high potential of osteogenic differentiation *in vitro*. Both human ASCs and human aMSCs generated bone-like structure by preliminary culturing in collagen gel, osteogenic induction *in vitro* and transplanting them into muscle of SKID/NOD mice. In order to explore the possibility of using ASCs an/or aMSCs for the treatment of osteoporosis, we injected those cells into tail vein of osteoprogerin (OPG)/osteocrastogenesis inhibitory factor (OCIF)-deficient mice.

OPG/OCIF is a factor secreted from stromal cells and interferes the stimulatory signal from osteoblasts through osteoclast differentiation factor (ODF) to receptor activator of NF-kB ligand (RANKL), an important factor for osteoclast differentiation and activation. In OPG/OCIF-deficient mice, a phenotype similar to osteoporosis develops in duration of up to 12 weeks after birth probably due to activated differentiation of osteocrasts. Taking advantage of these mice as a model of human osteoporosis, we tested the effect of tail vein injection of ASCs or aMSCs (>10^6 cells) obtained from subcutaneous adipose tissues of mice with same genetic background. The injection into OPG/OCIF-deficient mice at 12-13 week old when the osteoporosis-like phenotype is evident, the bone mineral density of thighbone measured by a ALOKA DSC-600EX-IIIA increased transiently up to 8 days after injection. When injected into OPG/OCIF-deficient mice at 6 week old and measured further 6 weeks after the injection, however, increase in the bone mineral density was not evident.

Considering that OPG/OCIF is secreted from stromal cells distributed throughout body, we are now examining whether the transient increase of bone mineral density was due to OPG/OCIF secreted from injected ASCs or aMSCs, or due to their differentiation into osteocytes.

SCIENTIFIC SESSION

TRANSLATIONAL: BONE TISSUE

IV-3 Challenge: A one step-surgical procedure for spinal fusion using adipose stem cells

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Background: Adult mesenchymal stem cells (MSC's) are being extensively studied for their applicability in regenerative medicine. Adipose tissue has only recently been identified as a source of MSC's[1,2]. When compared to their well-known bone marrow counterparts, adipose tissue-derived MSC's (ASCs) possess comparable multipotency, but can be harvested with minimal patient discomfort with several orders of magnitude higher yields, thus eliminating the need for culture expansion prior to implantation. This allows one-step surgical procedures where harvesting, differentiation induction, scaffold seeding, and implantation can be performed within several hours and within the operational theatre. General outline (example: spinal fusion).

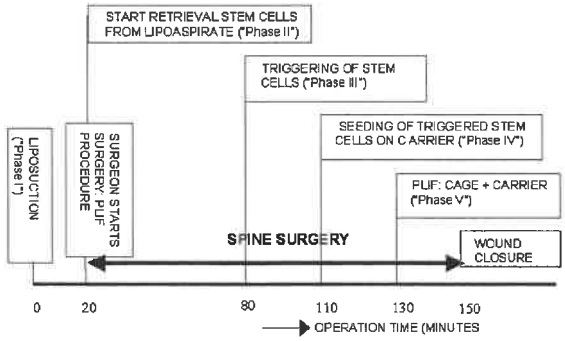

Figure 1 One-step surgical procedure for spinal fusion (see text for details).

Phase I and II: Harvesting of ASC's
Adipose tissue (AT) is obtained by resection or liposuction (vacuum-driven, but not ultra-sound assisted)[3]. The stromal vascular fraction (SVF) can be isolated from the AT using collagenase digestion and centrifugation[1]. The SVF fraction will contain up to 8% of ASC's, based on limiting dilution/CFU-F assays and FACS profiling. ASC's were identified as having a $CD34^+CD105^+CD166^+CD31^-CD45^-$ phenotype[3].

Phase III: Differentiation Induction of ASC's
To fit within the one-step surgical procedure, differentiation induction should be accomplished within a very short time frame. It was shown that a 15-min stimulation with rhBMP-2 followed by prolonged culturing for 4 days in "plain" medium already resulted in upregulation of osteogenic markers, as analyzed by real time-PCR (**Figure 2**). The same principle was demonstrated for chondrogenic induction (not shown)[4].

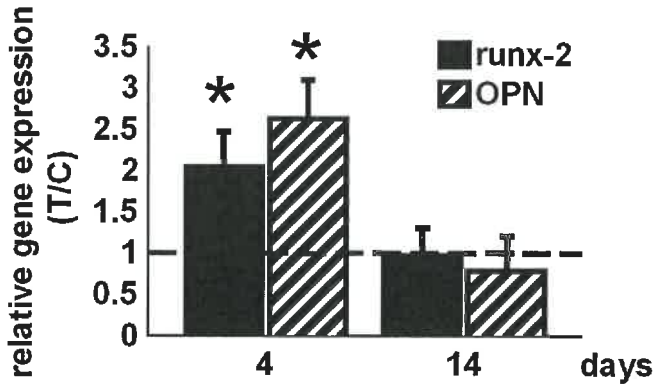

Figure 2 Effect of 15 min of 10 ng/ml BMP-2 treatment, followed by post-incubation for 4 or 14 days without BMP-2, on the relative gene expressions of runx-2 and osteopontin (OPN). Values are mean ± SEM of BMP-2 treated-over-control ratios (T/C, n = 3-8), dashed line, T/C = 1 (no effect). PCR data were normalized for 18S gene expression. * Significant effect of growth factor, $p<0.05$.

Phase IV: Seeding of ASC's on a carrier
ASC's can be seeded on a carrier (below on Biphasic CalciumPhosphate (BCP®) to generate a bio-active filling material. In **Figure 3**, ASC's are shown to distribute evenly throughout the scaffold, and to spread within 30 min on the BCP-surface.

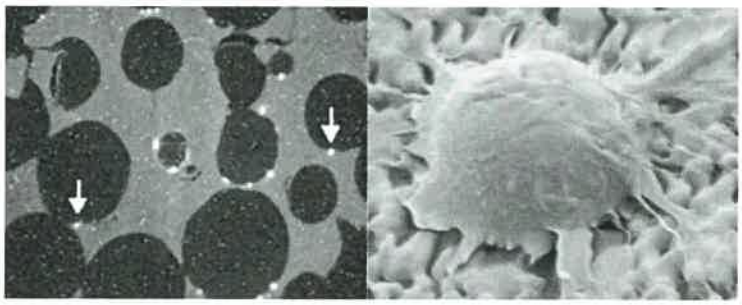

Figure 3 Seeding of ASC's on a carrier.
Left: PKH26-labeled cells (yellow dots; arrows) in the BCP-scaffold (red).
Right: Scanning EM picture of ASC cell 30 min after seeding.

Phase V: Implantation/*in vivo* Follow-up
The cell-seeded scaffold can be used to fill the defect area (here: the cage interior) and the construct **(Figure 4A)** can be implanted at the desired site (here: between two vertebrae to induce bony fusion in a goat spinal fusion model). As seen in **Figure 4B**, both active bone formation (visualized by high alkaline phosphatase activity; block arrows) and remodeling of BCP (visualized by TRAP-staining of osteoclasts; arrows) can be demonstrated at 28 days follow-up. Mineralized bone has already been formed (light green; asterisks).

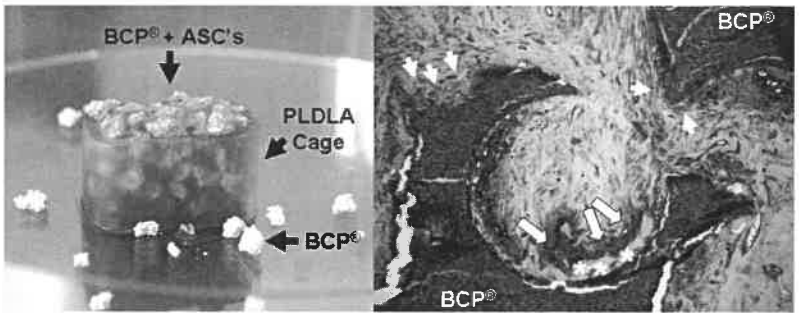

Figure 4
Left: Bioresorbable PLDLA cage filled with cell-seeded BCP granules prior to implantation.
Right: ALP- and TRAP-stained histological section of specimen retrieved after 28 days follow-up (see text for details).

Summary/Conclusions: The one-step surgical procedure as described above appears to be feasible, and can be performed within 2-3 hours. Although the concept has been illustrated for spinal fusion, it can easily be adapted for other purposes, such as stem cell-directed regeneration of degenerated discs, cardiac repair after acute myocardial infarction, regeneration of cartilage defects, and other applications. The concept described here may boost stem cell applications in orthopaedic and other applications considerably.

References:
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SCIENTIFIC SESSION
TRANSLATIONAL: BONE TISSUE

IV-4 Controlled disc degeneration in the goat by varying the concentration of chondroitinase ABC

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Introduction: To evaluate new, regenerative therapies for intervertebral disc degeneration, large animal models are needed. Geometrical and anatomical similarities to human IVDs favour the goat for this purpose.[1] Also, notochordal cells which have a role in disc maintenance, are absent in the mature caprine intervertebral disc.[2] Slowly progressive, reliable and reproducible disc degeneration is essential for a good animal model. Also, to evaluate regenerative treatments, e.g. stem cell therapy, the induced disc degeneration should be mild, without destroying the gross morphology of the disc and adverse events like osteophyte formation and/or endplate fractures.

We have observed symptoms of disc degeneration after the injection of a proteoglycan degrading enzyme (Chondroitinase ABC; CABC) into the nucleus pulposus (NP) of the goat disc.[3] In this experiment the optimal concentration for mild disc degeneration was determined.

Material/Methods: The protocol has been approved by the Animal Ethics Committee of the VU University Medical Center. In each of six female Dutch milk goats (>3.5 years, as determined by their dental status), six lumbar discs were randomly injected with different concentrations of CABC ranging between 0.2 to 0.35U/ml with increments of 0.05 U/ml. Twelve lumbar discs of six non-operated goats, analyzed in previous studies[3], served as controls.

Every two weeks lateral X-rays of the spines were obtained and the loss of disc height was measured. After twelve weeks follow-up the spines were harvested and analyzed using X-ray (disc height), MRI index (area NP * signal intensity NP)[4] and light microscopy.

A histological score was developed for goat disc degeneration. Literature as well as specimens of the control goats and CABC injected discs were reviewed. A histological score developed for rabbits[5] was adapted for goat discs. The annulus fibrosus (AF), the NP and the demarcation between these structures were graded in three different categories (0-2) leading to a maximum score of 6. Slices were stained with Alcian Blue- Periodic Acid Schiff's reagent (AB-PAS).

Results: The injection of PBS only had no significant effect on all analyzed parameters when compared to control levels (**Figure 1**).

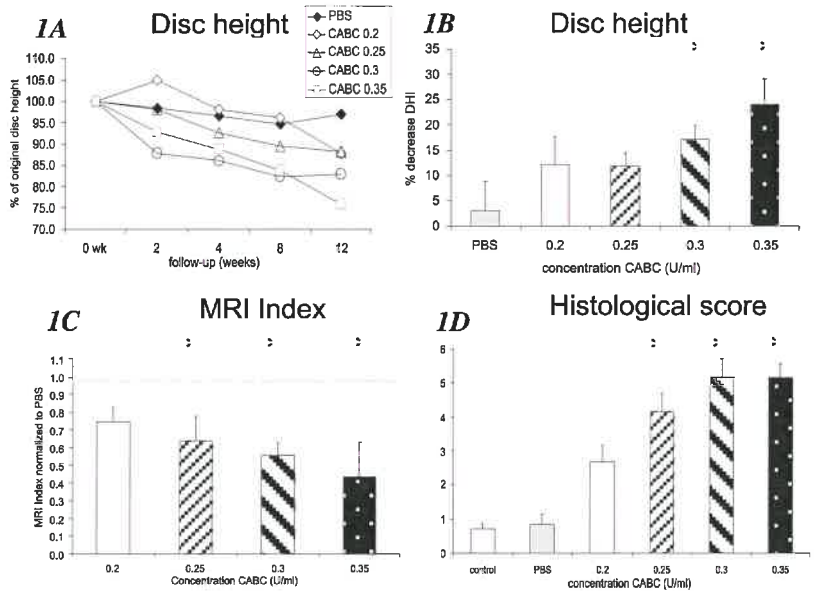

Figure 1 A: The decrease of the disc height, compared to the original height, as a function of time. The highest concentrations are most severely affected. B: Loss of disc height in percentage C: The ratio of the MRI index, normalized to the PBS level, which was used as an internal control. (dotted line)D: Results of the histological score are depicted. The score was increased to 4.2 for the 0.25 U/ml group, higher concentrations lead to severe degeneration (score 5 or more).
* represents significantly higher/lower results compared to PBS values and (in figure 1D) control values ($p<0.05$). Data are presented with standard errors of the mean (SEM).

The disc height decreased during the follow-up time. This effect was strongest for the highest concentration (**See Figure 1A.**) After twelve weeks the disc height had decreased with 12% in the 0.25U/ml CABC group and even more in the higher concentrated levels to a maximum of 24% (0,35U/ml) as depicted in **Figure 1B**. The decrease was significant for the two highest concentrations of CABC.

The MRI index was significantly decreased if concentrations of 0.25U/ml CABC or higher were injected into the NP. This is shown in figure 1C.

The histological score graded three qualities of the goat IVDs: the NP (structure of the extracellular matrix, proteoglycans and collagens present), the AF (convexly or concavely shaped, ruptures, serpentines) and the demarcation between them (clear or unclear). The histological score correlated well with the MRIs (Pearson R= 0.68) and with the macroscopic impression of the discs (data not shown) (Pearson R = 0.63).

All the concentrations did result in a higher histological score in a concentration dependent manner (**Figure 1D**). This was significant for the 0.25U/ml and higher concentrations. Osteophyte formation and endplate fractures were only observed in the 0.3U/ml and 0.35U/ml groups. In the pilot study, the injection of 0.25U/ml CABC only degenerated 50% of the discs (**Table 1**). In this study, the "take-rate" of the discs, injected with 0.25U/ml as well, was 100% (**Table 1**).

	Pilot study	Current study
Number (n)	n = 3	n = 6
Concentration CABC	0.25 U/ml	0.25 U/ml
Take rate	50%	100%
Osteophytes	0/3	0/6
End-plate fractures	0/3	0/6
Loss of disc height	16%	12%
MRI Index	0.60	0.64
Histologic score	5.25	4.20

Table 1 The results of our previous experiment (pilot study) and the current experiment (current study) are compared in this table. The take-rate has increased and the severity of the symptoms is comparable.

Discussion: The histological grading scale was adapted to goat intervertebral discs and correlated well with other parameters of disc degeneration. There was a concentration dependent effect of the injections of CABC into goat intervertebral discs. The 0.25U/ml CABC concentration is most suitable to induce mild disc degeneration. More severe disc degeneration can be induced using higher concentrations of CABC. The take-rate of the disc degeneration increased from 50% to 100% and this can be explained by the increasing experience of the surgeon (RH). The severity of the degeneration was similar to the results of the first experiment **(Table 1)**. Therefore, we concluded that the induced disc degeneration is both reproducible and reliable.

Conclusion: We have developed a reliable large animal model for intervertebral disc degeneration in which the severity of the degeneration can be tightly controlled. Our newly developed histological grading scale correlates well with other parameters of disc degeneration. This model allows reliable and reproducible induction of mild disc degeneration to evaluate new therapeutic strategies e.g. stem cell therapy.

References:

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SCIENTIFIC SESSION

TRANSLATIONAL: BONE TISSUE

IV-5 The use of the stromal vascular fraction of adipose tissue for cartilage and bone regeneration

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The stromal vascular fraction (SVF) of adipose tissue provides an abundant source for adipose tissue derived stem cells (ASCs), which may be used for bone and cartilage regeneration. Currently, ASCs are often being purified and expanded using lengthy and costly *in vitro* culturing procedures. From a clinical point of view it would be ideal if the heterogenous mixture of SVF cells could be directly seeded onto a carrier material, making these lengthy purification and expansion procedures superfluous. This requires that, among the various cell types within the SVF, at least the ASCs adhere to a carrier material, within a short time frame.

We investigate the feasibility of directly seeding SVF cells onto a 70:30 Poly(D,L-lactide-co-caprolactone) scaffold in the context of bone and cartilage tissue engineering, whereby expensive and lengthy *in vitro* expansion steps are avoided. Therefore, we phenotypically characterized the SVF cells and examined which cells of the SVF adhere to the scaffold and in what time frame. In addition, we investigated whether the attached cells can differentiate towards the osteogenic and chondrogenic lineage.

The SVF cells of 3 human donors were seeded onto the scaffolds. Adherence, proliferation and cell phenotype were monitored using DNA assays and fluorescently labeled antibodies against cell-specific membrane markers visualized with confocal microscopy. Osteogenic and chondrogenic differentiation was assessed using RT-PCR, (immuno) histochemistry and confocal microscopy.

SVF cells attached to the scaffold within 10 min. While the SVF contains 34% of ASC-like cells, 72.3% of the adhered cells had an ASC-like phenotype. Only low frequencies of endothelial cells, leucocytes and other cell types were found, indicating that the stem cells preferentially bind to the scaffold. The attached ASC-like cells were capable of differentiating into the chondrogenic and osteogenic pathways.

Our study suggests that the SVF of adipose tissue can be used for bone and cartilage regeneration without expensive and lengthy *in vitro* expansion steps. This opens up the possibilities to perform a cost-effective, patient-friendly surgical procedure in which the SVF is harvested and directly returned to the defect site within a single surgical procedure.

SCIENTIFIC SESSION

TRANSLATIONAL: ADIPOSE TISSUE AUGMENTATION

V-1 Adipogenesis within preadipocyte-seeded and cross-linked collagen-chitosan scaffolds

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Introduction: Current soft tissue defect repair strategies possess critical limitations. The ultimate objective of our research is to generate de novo autologous adipose tissue in the defect site by coupling material science, cell culture technology, and surgical science. To this end, a portfolio of cross-linked collagen: chitosan blends was prepared and assessed *in vitro* and *in vivo*.

Methods: Collagen and chitosan were cross-linked with glutaraldehyde (GA) at concentrations ranging from 0% to 0.5% and were frozen at -20°C, -80°C or -196°C prior to lyophilization. A total of nine polymer configurations were assessed via SEM (microarchitecture) and water binding capacity (hydrogel swelling). Preadipocytes (PAs) were harvested and cultured as previously described (Tissue Eng 8:283, 2002; 5:139, 1999). *in vitro* cytocompatibility of PAs was assessed using two assays: MTT and DAPi.

Two *in vivo* studies were conducted. First, a rat subcutaneous pocket assay was employed to assess PA-seeded scaffolds *in vivo*. Second, scaffolds were placed in silicone domes and approximated to a flow-through artero-venous pedicle (A-V) pair in a rat model (Microsurg 24:378, 2004). The superficial epigastric vessels of the animal were dissected microsurgically and approximated to the constructs. Four factors were examined: (a) the presence or absence of an A-V, (b) the separation distance between the A-V (1 or 2mm), (c) use of an acellular or PA-seeded scaffold, and (d) harvest time (14 or 60 days). For both *in vivo* studies, samples were harvested postmortem at defined time points, then processed histologically and stained for H&E, Oil Red O (lipid), and von Willebrand's Factor VIII (vessels). Quantitative histomorphometry was employed to measure the degree of angiogenesis and adipogenesis.

Results: All polymer combinations yielded flexible, porous scaffolds upon SEM observation. GA concentration had little effect on scaffold morphology or water binding capacity. However, the processing freezing temperature prior to lyophilization affected both. Assays confirmed the viability of PAs throughout the GA cross-linked collagen: chitosan scaffolds. Histological results proved that PA-seeded scaffolds were biocompatible, could induce vascularization, and form adipose tissue. In addition, results support vascularization in the region between the A-V; cell survival in constructs with an A-V; and PA differentiation in seeded constructs with an A-V.

Conclusions: Cross-linked collagen:chitosan scaffolds support adipogenesis with concomitant angiogenesis.

SCIENTIFIC SESSION

TRANSLATIONAL: ADIPOSE TISSUE AUGMENTATION

V-2 Adipose tissue engineering from human adipose-derived stromal cells: a new self-assembled 3-D model devoid of exogenous biomaterial

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Passage 3 cells

Adipose tissue is an accessible and abundant source of mesenchymal stromal cells for autologous soft-tissue reconstruction using tissue-engineering approaches. We extracted human stromal cells from lipoaspirated (LA) and excised fat (LP, lipectomy) to assess their capacity to produce 3-D adipose tissues using a "self-assembly" culture technique. This method consists in coaxing cells to produce their own extracellular matrix following ascorbic acid stimulation *in vitro*. Compared to excised fat, stromal cells from lipoaspirations featured higher yields at extraction (1.8x), slightly higher proliferation rates over 11 passages (P) in culture, as well as increased (1.6x to 2.07x) *in vitro* adipogenic potential at passages 3, 6 and 11 in presence of ascorbic acid, as quantified after Oil RedO (ORO) stainings. The adipose-derived stromal cell populations conserved a high differentiation capacity in P3, P6 as well as P11, suggesting that precursor cells are maintained under our culture conditions. When cultured for 21 to 28 days in presence of ascorbic acid, both LA and LP-derived stromal cells produced manipulable 3-D sheets rich in extracellular matrix components (self-assembly method). By inducing adipogenic differentiation at different stages of matrix formation (namely at day 7, 14 or 21 of culture, passage 3), 3-D adipose sheets were produced (3.5cm² surface area), featuring human adipocytes at early or late stages of differentiation. These adipose sheets were then assembled into thicker adipose tissues (146.5 ± 8.7µm, 3 layers, n=6) by layering multiple cellular sheets. These self-assembled human tissue-engineered adipose tissues secreted increasing levels of leptin and adiponectin over time in culture. Treatment of adipose sheets with the ,β-adrenergic agonist isoproterenol (1µM, 2h) stimulated lipolysis 2.0 fold over basal levels, as determined by quantitation of released glycerol. Taken together, these results confirmed the functionality of

adipocytes within the reconstructed tissues. In summary, we are now able to produce human reconstructed adipose tissues without exogenous biomaterial or scaffolding, from an abundant source of adipose-derived stromal cells. This flexible new tissue-engineered model allows us to study *in vitro*, in a 3-D context, human adipocytes at their various stages of differentiation in regard to their cytokine secretion and metabolic activities under various pharmacological treatments. In the future, it could be anticipated that these reconstructed adipose tissues would be used in a clinical setting as autologous soft-tissues for reconstructive and cosmetic surgery purposes.

SCIENTIFIC SESSION

TRANSLATIONAL: ADIPOSE TISSUE AUGMENTATION

V-3 Cell-Assisted Lipotransfer (CAL): Supportive use of adipose-derived stem cells (ASCs) for soft tissue augmentation

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Objectives: Lipoinjection, a popular option for cosmetic soft tissue augmentation, still has several issues to be resolved such as unpredictable survival rate of transplanted fat and risk of fibrosis or calcification. To overcome these problems, we tried a concurrent transplantation of aspirated fat and ASCs, which we named cell-assisted lipotransfer (CAL). In CAL, ASCs were supportively used to boost the efficacy of autologous lipoinjection, meaning a higher survival rate and persistency of transplanted fat.

Methods: To characterize aspirated fat, aspirated fat and excised fat were histologically compared with light and electron microscope, and ASC yields from both fats were compared. Influences of centrifugation on lipoaspirates were also examined. Aspirated fat was transplanted with (CAL) or without (non-CAL) freshly-isolated SVFs containing ASCs into the back skin of nude mice and the grafted fats were examined in 4 weeks. Based on the experimental results, CAL was clinically done in 31 patients so far; to face in 9 patients and to breasts in 22 patients (including breast reconstruction after mastectomy for 3 patients). Patients have been monitored for 4 to 40 months.

Results: It was found that aspirated fat does not contain large-sized vasculatures. ASC yield from aspirated fat was significantly less than that from excised fat, which means that aspirated fat is relatively ASC-poor compared to excised fat. Thus, a significant amount of ASCs may reside around vasculatures. Centrifugation at 400xg damaged adipocytes, but ASCs were not damaged up to 1200xg. In CAL, ASC-poor aspirated fat was converted to ASC-richer fat by addition of freshly-isolated ASCs. Survived CAL fat was significantly larger than non-CAL fat in an experiment with nude mice, which also showed that ASCs can survive and locate in the interstitial space of fat or differentiate into vascular endothelial cells. In the clinical trial, satisfactory results were obtained so far. Gradual reduction of the augmented soft tissue volume usually seen after lipoinjection was minimal.

Conclusions: CAL is one of ways to increase ASC/adipocyte ratio of transplants. By improving the ASC/adipocyte ratio, it seemed that transplanted fat was prevented from postoperative atrophy. ASCs may not only differentiate into endothelial cells leading to higher survival rate of transplanted fat but also contributing to tissue turnover after transplantation by working as tissue specific progenitors resulting in preservation of tissue volume.

SCIENTIFIC SESSION

TRANSLATIONAL: ADIPOSE TISSUE AUGMENTATION

V-4 *De novo* adipogenesis at the site of injection of alginate in combination with fibroblast growth factor-2 and heparin

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Autografting of fat pads has been an only promising treatment for augmentation of lost soft tissue. However, the outcome of such treatment is often disappointing due to progressive absorption of the graft with time. When the autografts were removed and microscopically examined, necrotic adipocytes were observed and they were replaced by host fibrous tissue. For successful autotransplantation of fat pads, the adipose precursor cells need to be transferred together with mature adipocytes and the microenvironment of their regenerative proliferation needs to be maintained at the site of grafting. Alternatively, if one can successfully prepare the microenvironment for the proliferation of endogenous adipose precursor cells, *de novo* formation of fat can be expected even without fat transplantation.

We have previously shown that migration, proliferation and differentiation of endogenous stem cells can be stimulated at the site of injection of Matrigel (solubilized and reconstructive basement membranes containing laminins, type IV collagens, entactin and parlecan etc. prepared from mouse EHS tumor) in combination with FGF-2 and fat a pad was formed *de novo*. Since Matrigel can be prepared only from mouse tumor, safer substitute for Matrigel has been seeked for clinical application of this observation.

In this study, we show that *de novo* adipogenesis can be induced by injection of alginate in combined with FGF-2 and heparin. When alginate containing FGF-2 and heparin was subcutaneously injected into nude mouse, a fat pad was formed within 4 weeks. Not only a fat pad with large volume was observed, adipose differentiation was confirmed by triglyceride accumulation and by histological demonstration of adipocytes in the pads. In a contrast, injection of FGF-2 or heparin alone exhibited poor adipogenesis. Therefore, application of FGF-2 in combination with heparin and alginate was found to be critical for *de novo* adipogenesis.

SCIENTIFIC SESSION

TRANSLATIONAL: ADIPOSE TISSUE AUGMENTATION

V-5 Vocal fold regeneration by Adipose-derived mesenchymal stem cells from human fat tissue

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Introduction: Regenerative medicine applications making use of human adipose tissue are already in progress, including approaches to treat laryngeal hemiplegia or anatomical defects.

In this regard, investigations on the presence and characteristics of stem cells in the adipose tissue graft could clarify the mechanisms involved in vocal fold repair. With this aim, we analyzed the stem cell population residing in adipose tissue harvested from patients prior to vocal fold lipoinjection in order to understand whether the improvement in glottic competence obtained by lipoinjection, may be due not only to the mechanical support but also to tissue regeneration mediated by adipose-derived stem cells.

Materials and Methods: Adipose tissue was collected from the lower abdomen during the intervention for vocal fold lipoinjection in 9 patients (age 56-67 yrs). Mature adipocytes were separated by centrifugation and the higher density solid phase was collected and digested collagenase A. After neutralization, the cell suspension was centrifuged. The adipocytes floated on the surface and the remaining stromal vascular fraction (SVF) sedimented. SVF cells were plated in low-glucose DMEM+20% Fetal Bovine Serum+penicillin-streptomycin (n=9) (A), EGM-2 (Cambrex) (B). After overnight culture, the non-adherent fraction was removed and the adherent cells were allowed to expand. After expansion for 1 week the cells (A) were maintained in the same culture conditions and cells from (B) were splitted and maintained in EGM-2 (B) or cultured in the presence of high-DMEM+20% FBS (C). After that, $5x10^3$ cells/well for each sample and each conditions were plated in defined media supplemented with specific growth factors to promote cell lineage differentiation (adipogenic, osteogenic, hepatic, endothelial and myogenic). Before and after differentiation the cells were analyzed by flow cytometry, immunofluorescence and RT-PCR. Briefly, for immunofluorescence assay the cells were fixed with absolute acetone/methanol (1:1, -20°C) stained with antibodies against CD90 (Becton Dickinson Co.; 1:25), CD44 (Clone A3D8, Sigma; 1:20), CD105 (Dako; 1:20), HLA-ABC (Immunotech; 1:25), von Willebrand factor (Clone F8/86 Dako; 1:25), VE-cadherin (Chemicon; 1:25), CD146 (Chemicon; 1:100), CK8 (Sigma; 1:100), CK18 (Sigma; 1: 500), alpha feto protein (Sigma; 1:100), HNF-3 beta (Santa Cruz; 1:100), MyoD (Dako; 1:50) and Desmin (Dako; 1:50), Myf5 (Santa Cruz; 1:50), SSEA4 (Chemicon; 1:25), alpha SMA (Sigma, 1:250), NG2 (Immunotech; 1:50). The cells were also analyzed by flow cytometry for the expression of CD90, CD44, CD105 and HLA-ABC. For RT-PCR, total RNA was extracted using Qiagen Kit (Qiagen). Four ng cDNA were used for each PCR assay for adipogenic (PPARgamma2), osteogenic (osteocalcin), chondrogenic (collagen II), myogenic (MyoD, myogenin, MRF4, Myf-5, Desmin), hepatic (alpha feto protein, albumin, CK19, CK18, CYP1B1, CYP2B6), endothelial (VE-cadherin, vWF) and stem cell (Oct-4, Runx-1, Rex-1, Sox-2, hTert, FGF-4, Pax-7, PDX-1, ABCG2) markers.

Results and Discussion: The expanded cells showed a typical mesenchymal stem cell pattern. By immunofluorescence, the cells were found positive for CD90, CD44, CD105, HLA-ABC, CD146 and CK18. They also expressed the embryonic cell antigen SSEA4, thus providing a theoretical basis for their differentiation potential. Moreover, by molecular analysis, the cells expressed several adipogenic, osteogenic, chondrogenic, hepatic and endothelial cell markers. They were also positive for Oct-4, Runx-1 and ABCG-2, which characterize the undifferentiated stem cell state.

Several cell lineages were obtained including adipogenic, osteogenic, endothelial and hepatic confirmed by molecular and specific immunofluorescence assays and preliminary results were obtained for myogenic differentiation.

This comparative study using different culture conditions (A), (B), (C) and our preliminary results, suggest that a cell population with higher growth ability, broader and more dramatic differentiation potential, as compared with DMEM cultured cells, could be isolated. This cell subset has a clear pericyte phenotype ($CD146^+/34^-/45^-$), thus indicating a possible role of this novel cell type in fat-tissue-based cell therapy approaches.

Conclusions: Our data suggest that fat tissue lipoinjection acts both as a mechanical support and as a source of stem cells able to exert regenerative activity in patients who underwent vocal fold repair. In fact, the cells isolated from theat tissue of these patients have different characteristics of multipotent adult stem cells and immunophenotipically resemble the pericyte component of adipose tissue. These cells, under appropriate culture conditions, are able to show multilineage differentiation capacity, including morphologic and phenotypic characteristics of muscular cells. Our preliminary results open new insight in the comprehension of the mechanism underlying fat tissue mediated regeneration and support the clinical use of this new stem cell source.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN ADIPOSE

VI-1 Local control of the proliferation of the CD34+/CD31- progenitor cells

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The excessive development of adipose tissue (AT) is the result of both hypertrophy and hyperplasia of the main cellular component of AT, the adipocytes. Although the hypertrophy phase has been extensively studied, the stimuli involved in hyperplasia remain unclear. The non adipose fraction of AT, the so-called stroma-vascular fraction (SVF) contains capillary endothelial cells (CECs), infiltrated resident macrophages and a CD34+/CD31- cell population that exhibits similar properties although not identical, to those of mesenchymal stem cells. The present study was performed to determine whether the local stimuli such as adipokines, growth factors as well as oxygen tension, could participate to the control of the proliferation of the CD34+/CD31- progenitor cells from human subcutaneous AT.

Mature adipocytes, CECs, progenitor CD34+/CD31- cells as well as macrophages were isolated from human subcutaneous AT by immunoselection as previously described. Freshly harvested adipocytes, CECs and macrophages were cultured for 24 hours and their conditioned media collected. Growth responsiveness of the CD34+/CD31- cells to conditioned media as well as adipokines and endothelial cell growth factors was determined by BrdU incorporation assays on cells cultured for 48 hours under normoxic or hypoxic conditions (1% O^2 atmosphere). In parallel, the receptor expression of adipokines (leptin, adiponectin, lysophosphatidic acid (LPA) and interleukin-6 (IL-6)) and endothelial cell growth factors (vascular endothelial growth factor (VEGF) and apelin) was assessed by real-time PCR analysis.

Adipocyte- and CECs-conditioned media induced the proliferation of AT-derived progenitor cells (2.1 ± 0.5 and 2.7 ± 0.5 fold increase respectively) as well as 48 hours in low oxygen tension (1.5 ± 0.2 fold increase) whereas macrophage conditioned media had no effect on the BrdU incorporation. Neither leptin nor VEGF or adiponectin modified the growth responsiveness whatever the tested concentrations whereas apelin as well as IL-6 and LPA enhanced in a concentration-dependent manner the proliferation of the progenitor cells.

Although additional studies are required to fully characterize the processes involved in the control of the CD34+/CD31- cell proliferation, the present study strongly suggests that local stimuli arising from mature adipocytes and CECs as well as the local oxygen tension modulate the growth capacity of the AT-derived progenitor cells.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN ADIPOSE

VI-2 Rapid immune cytokine suppression induced by adenovirus-36 infection

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Adipose tissue growth is associated with increased in inflammatory cytokines. Due to the association of proinflammatory cytokines with diabetes and other comorbidities, understanding the regulation of adipose tissue

induced proinflammatory response is critical. Ad-36 is a group D human adenovirus that increases adiposity in experimental animal models, shows association with human obesity and induces preadipocyte proliferation and differentiation *in vitro*. We sought to identify the role that Ad-36 infection plays in the *in vitro* and *in vivo* modulation of cytokine expression and obesity development. Human adipose-derived stem cells (hASC) isolated from subcutaneous adipose tissue biopsy of a healthy donor (BMI=25) were infected with either Ad-36 or Ad-2 (moi=3.8). Ad-2 is a non-adipogenic human adenovirus used as a negative control. On day 1, mRNA expression of macrophage migration inhibitory factor (MIF) was significantly higher in Ad-36 infection and significantly lower in Ad-2 infection suggesting that Ad-36 is suppressing the recruitment of macrophages, while Ad-2 is promoting macrophage recruitment **(Table 1)**. By day 2 monocyte chemoattractant protein-1 (MCP-1) and IL-18 mRNA expression were both significantly lower in the Ad-36 infected group, suggesting a coordinated immune suppression is induced by Ad-36 infection in isolated cells of adipocyte lineage. To determine the anti-inflammatory response of Ad-36 in adipose tissue, we infected human adipose tissue explants obtained by lipoaspiration with Ad-36. Similar to the findings in hASC, the Ad-36 infected group showed greater MIF and lower MCP-1 mRNA expression compared to the uninfected control **(Table 1)**. To determine the *in vivo* impact of Ad-36 induced modulation of inflammation, 5-week old male Wistar rats were inoculated intranasally either with media, UV inactivated Ad-36 or replication competent Ad-36. Four days later, serum IL-6 levels were reduced in both virus infected groups suggesting a receptor mediated immune suppression **(Table 1)**. Whereas, IL-18 and MCP-1 levels were significantly reduced in replication competent Ad-36 infection alone, suggesting that active viral replication is required for these effects. Additional studies are underway to identify the tissue source for the effecter molecules *in vivo*. In summary, these data suggest that despite its pro-adipogenic effect, Ad-36 is able to rapidly induce coordinated immune suppression and may play a direct role in modulation of adiposity.

	MCP-1	MIF	IL-18	IL-6
hASC*: Ad-36 infected	42% (p<0.003)	154% (p<0.001)	79% (p<0.007)	N/A
hASC*: Ad-2 infected	136% (p<0.05)	51% (p<0.03)	94% (p=NS)	N/A
hAT explants* Ad-36 infected	70% (p<0.07)	167% (p<0.056)	113% (p=NS)	N/A
Rat serum Ad-36 infected**	52% (p<0.05)	N/A	51% (p<0.005)	22% (p=NS)
Rat serum UV Inactivated Ad-36**	92% (p=NS)	N/A	76% (p<0.054)	28% (p=NS)

Table 1: Percent mRNA* or protein** compared to the respective uninfected control groups. Data are expressed as Mean % change (p value). N/A; Data not available; NS: Statistically not significant.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN ADIPOSE

VI-3 Macrophage-like behavior of adipose-derived stem cells in co-culture with adipocytes and their debris

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Adipose tissue inflammation is recently recognized as an early event of metabolic complications caused by obesity (metabolic syndrome) and it is in part attributed to the proinflammatory actions of adipose-inherent macrophages. This suggestion is based on:1) macrophage numbers and macrophage inflammatory gene expression in adipose tissues are positively correlated with body mass index (BMI) in mice and humans; 2) macrophages are predominant source of TNF-α, interleukin-6 and nitric oxide in adipose tissue of obese mice and humans; 3) increase in macrophage inflammatory gene expression in adipose tissue is coincident with the onset of hyperinsulinemia in mouse diet-induced

obesity. Macrophages are monocytic phagocytes that function in innate immunity and wound healing by sequestering and clearing pathogens, dead cells and cell debris. Macrophage activation consists of biochemical, morphological and functional changes that result in formation of multinucleate giant cells (MGCs) which phagocytes cell debris and can produce proinflammatory cytokines. MGCs have been reported to surround individual adipocytes in adipose tissue of obese mice and humans, suggesting that adipocytes become focal and persistent sites of macrophage activation. Macrophage infiltration and MGC formation are observed in conjunction with adipocyte ("fat") necrosis in various human diseases and following to the failure of autologous adipose implants.

When adipocytes from subcutaneous adipose tissue were incubated while attached to the ceiling surface of T-flasks filled with medium (ceiling culture), fibroblastic cells with multiple potential of mesenchymal differentiation proliferate. When the movement of cells at the ceiling surface were recorded by time-lapse microscopy, macrophage like interaction of the fibroblastic cells with adipocytes was observed. The observations were; 1) fibroblastic cells were detected as early as Day 1 as the cells migrating at the surface of adipocytes; 2) on Day 3, fibroblasts wrapped fat cells and showed adipocytes like changing morphology and proliferating by themselves; 3) most adipocytes from transgenic mice expressing green fluorescent protein did not show a ring-shape under fluorescent microscopy, suggesting the adipocytes had lost intact plasma membranes before Day 3; 4) on Day 4-5, fibroblastic cells migrated to the ceiling surface and collected small lipid droplets released from broken adipocytes; 5) confocal microscopy (after differential fluorescent dye staining), showed the lipid droplets to be outside the cells. A limited population of these lipid droplets were phagocytosed by fibroblasts but the activity was far below that of adipose-inherent macrophages (CD11b-positive) which accounted for 7-10% of SVF from subcutaneous adipose tissue.

We thus observed behavior of ASCs similar to adipose-inherent macrophages but ASCs appeard to be less responsible for adipose tissue inflammation due to their low activity of phagocytosis.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC IN ADIPOSE

VI-4 A novel lipogenic modulator of human adipose-derived stem cells: A human adenovirus

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Adipose tissue has an abundance of multi-potent adult stem cells. Considering the potentially important use of these cells for tissue engineering, identifying factors that modulate their differentiation potential is critical. Human adenovirus type 36 (Ad-36) is one such factor. Ad-36 induces obesity in animals, increases differentiation of 3T3-L1 rodent preadipocytes and is associated with human obesity. Here we show that replication, commitment to adipogenic lineage, differentiation and lipid accumulation of human adipose derived stem cells (hASC) isolated from lipoaspirates is induced by Ad-36. Ad-36 significantly increased replication of pre-confluent hASC, 32 and 48 h post-infection as shown by BrdU incorporation. Ad-36 induced commitment of hASC to adipogenic lineage, even in the presence of osteogenic inducers. In the absence of any inducers, Ad-36 significantly increases the expression of proadipogenic genes CCAAT/enhancer binding protein β (C-EBP-β), peroxisome proliferator activator receptor γ2 (PPARγ), aP2, and induces lipid accumulation as determined by Oil O assay. Ad-36 induced lipid accumulation in hASC is proportional to the viral load and does not require adipogenic inducers. Ad-2, a non-adipogenic human adenovirus is unable to induce adipogenic response in hASC, indicating that the adipogenic effect is not common to all human adenoviruses. Furthermore, Ad-36 increases mRNA expression of Lipoprotein lipase Glycerol kinase, genes responsible for lipid uptake and intracellular lipid recycling, respectively. As expected from increased Glycerol kinase expression, glycerol release in the media was reduced in the Ad-36 infected hASC. In summary, we showed that commitment, proliferation, differentiation and lipid accumulation of hASC ican be modulated by Ad-36 even in absence of the adipocyte inducers. These results indicate a novel regulatory control of ASC differentiation.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC DIFFERENTIATION ALONG CARDIOVASCULAR LINEAGE

VII-1 Cardiomyocyte differentiation using adipose-derived stem cells

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Background: Cardiomyocyte differentiation using adult stem cells is considered important and promising for the treatment of cardiac diseases. Thus, clinical trials using cardiomyocytes differentiated from bone marrow-derived mesenchymal stem cells (BSCs) have already started. In this study, we tried to differentiate adipose-derived stem cells (ASCs) into cardiomyocytes *in vitro* using two types of differentiation media.

Materials and methods: ASCs were harvested from inguinal fat pads of 5-week-old mice (C57BL/6). After two passages of the subculture, the cells were harvested and differentiated into cardiomyocytes using two types of media: 1) DMEM with 5-azacytidine, and 2) methylcellulose medium.

Results: Twelve days after the initial incubation in these media, about 5-10% of cells in both media started self-beating. The cells changed from a fibroblast like appearance to a round or elongated shape.

Conclusion: Although we are presentlythese cells using immunostaining and electron microscope, these self-beating cells were considered to be myocytes that differentiated from ASCs. In this study, 5-azacydine, referred to as carcinogenesis, was considered unnecessary for use in differentiation. However, the quantitative differences between cardiomyocytes incubated in the two types of media need to be studied, and the efficiency of differentiation investigated.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC DIFFERENTIATION ALONG CARDIOVASCULAR LINEAGE

VII-2 Cardiomyogenic differentiation potential of human adipose-derived Stem Cells (ASCs)

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Introduction: Myocardial infarction is a leading cause of mortality and morbidity in the United States. Myocardial infarction is characterized by irreversible loss of cardiomyocytes, which can lead to deterioration of cardiac function, and eventually heart failure. Since adult cardiomyocytes have limited ability to regenerate, cell transplantation targeted at replacing scar tissue or diseased cardiomyocytes with functional cardiomyocytes has emerged as a promising therapeutic strategy for improving cardiac function post-infarction. Adult stem cells present an attractive source of cells for cardiovascular cell therapies. Bone marrow-derived mesenchymal stem cells have been shown to differentiate into cells displaying features of cardiomyocyte-like cells after exposure to a variety of biochemical stimuli (the most widely studied chemical stimulus is 5-azacytidine or 5-aza). However, studies conducted by other investigators have failed to reproduce these findings. Adipose-derived stem cells (ASCs) have also been shown to be capable of cardiomyogenic differentiation via 5-aza stimulation, although there are no reports describing the effects of 5-aza on human adult ASCs. The purpose of this study was to examine the effects of chemical stimuli such as 5-aza on human ASC differentiation along the cardiomyogenic lineage.

Methods: Adipose-derived stem cells used in this study were extracted from human subcutaneous adipose tissue harvested during elective abdominoplasty from female donors between the ages of 20-35, and cultured as previously described. Low passage ASCs were seeded in 6-well polystyrene plates pre-coated with type I collagen at 50,000 cells /well, 100,000 cells/well, and 150,000 cells/well. ASCs were exposed to 5ìM or 10ìM of 5-aza transiently for 24 hours or repeatedly (one or three treatments per week) in regular plating media with 10% serum or without serum for up to 8 wks in culture. Controls consisted of ASCs cultured under the same conditions without exposure to 5-aza. Parallel experiments were conducted on non-collagen coated polystyrene well plates. Untreated and 5-aza treated ASCs were examined daily using light microscopy to monitor changes in cell morphology. Protein expression of sarcomeric α-actinin, cardiac troponin I, cardiac troponin T, connexin 43, and α-smooth muscle actin at different time points was assessed via immunocytochemistry and Western blot analysis.

Results: Single 24hr transient exposure of human ASCs to 5-aza did not result in spontaneously beating cell clusters nor the formation of myotubes after 8 weeks in culture. Expression of cardiac specific proteins such as sarcomeric α-actinin, cardiac troponin I, and cardiac troponin T in 5-aza treated cells was undetected. Repeated exposure to 5-aza inhibited cell proliferation, and reduced α-smooth muscle actin protein expression significantly. The absence of actin stress fibers was evident in ASCs that were subjected to repeated 5-aza exposure. Immunocytochemistry and Western blot analysis detected the expression of connexin 43 in control and 5-aza treated ASCs after 4 weeks and 8 weeks in culture, indicative of the formation of cellular gap junctions. Studies to examine the gene expression of early cardiac specific genes such as Nkx2.5 and GATA4 are currently underway.

Conclusion: DNA demethylation agent 5-azacytidine has been widely described in literature as an effective chemical stimulus used to promote cardiomyocyte differentiation in various cell types ranging from embryonic stem cells, P19 cells, bone marrow-derived mesenchymal stem cells, to adipose-derived stem cells. Of interest and importance is the observation that human female ASCs may not be capable of differentiating into cardiomyocyte-like cells when exposed to 5-azacytidine *in vitro*. This is perhaps due to its non-specific demethylating activity. Future studies include examining the effects of other chemical stimuli and direct cell-cell contact with mature or neonatal rat/chick cardiomyocytes on ASC differentiation along the cardiomyogenic lineage.

SCIENTIFIC SESSION

BASIC SCIENCE: ASC DIFFERENTIATION ALONG CARDIOVASCULAR LINEAGE

VII-3 Novel patterned conductive surfaces for electrical stimulation of adipose-derived cells for cardiovascular differentiation

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Introduction: Adipose tissue represents an abundant and easily available source of multipotent stem cells[1]. Notably, adipose-derived stem cells (ASCs) have significant potential for differentiation into cardiovascular lineages[2,3,4]. We previously showed that electrical field stimulation designed to mimic the signals that orchestrate the contractions of the heart substantially improved molecular, structural and contractile properties of engineered cardiac constructs[5]. We therefore expect that electrical stimulation of this kind will also enhance the cardiac differentiation of ASCs. In previous studies, electrical stimulation of ASCs on a clear, conductive Indium Tin Oxide (ITO) surfaces improved cell adhesion, caused cell alignment perpendicular to the electrical field and induced expression of both atrial natriuretic peptide and troponin I[6]. In the present study, the ITO surface was laser patterned to create a micro-scale interdigitated electrode array with significantly increased field strength over that achievable on non-patterned surface. The cells were seeded directly onto the ITO surface in a random monolayer or aligned perpendicular to electric field lines using aligned matrix of type I collagen, and the role of electrical stimulation on cardiac differentiation of ASCs was investigated.

Materials and Methods: C2C12 myoblasts and ASCs were used. Abdominal adipose tissue harvested from patients undergoing lipectomy was obtained under an approved protocol and digested with 0.1% type I collagenase for 5 hours at 37°C. Stromal vascular cells were fractionated by centrifugation, expanded for two passages, seeded onto patterned ITO surface of the bioreactor, and cultured in high glucose DMEM supplemented with 10% FBS. Identically prepared cultures of C2C12 myoblasts were used as controls.

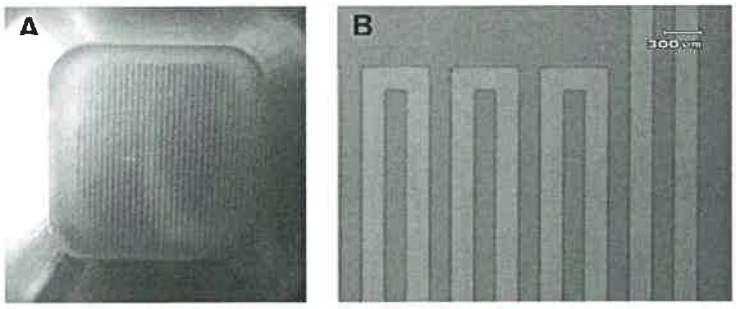

Figure1 Microscale interdigitated electrode array bioreactor. Top-down view of one of the wells of the patterned ITO surface bioreactor (A). High magnification image (B).

The bioreactors consisted of two glass slides (50mm x 75mm x 1.1mm) and a PDMS insert that contained two 15mm square x 10mm high wells. The lower glass slide was coated with ITO (resistance: 4-8Ohm/sq) on one surface (Delta Technologies, Stillwater, MN). An interdigitated electrode array was created by laser ablation of the ITO leaving behind 200ìm wide conductive traces. The PDMS insert was irreversibly bonded to this slide using plasma treatment, while the second glass slide served as bioreactor cover (**Figure 1**). Some of the conductive ITO surfaces were coated with type I collagen and aligned perpendicular to the electrical field[7]. Two days after seeding electrical stimulation (5mV square waves, 1ms duration, 1Hz frequency, electrical field gradient 0.25V/cm) was applied to the cells for four days.

Results: Electrode array stability was well maintained under the conditions of the experiments. Electrode properties were not changed after applying 1V pulses (1ms) for 3 weeks in PBS in an interdigitated array with 100 µm spacing and 200µm traces. The applied current was up to 15mA and the corresponding field strength was up to 100V/cm. C2C12 cells attached to patterned ITO surfaces within few hours after seeding. In contrast, only 10% of seeded ASCs were able to attach after two days of culture. Coating with type I collagen improved the attachment of ASCs by 30%. When aligned matrix of type I collagen was used, both the C2C12 cells and ASCs aligned along the coated type I collagen fibers, which were made perpendicular to the electrical field.

After electrical stimulation for three days on uncoated ITO surface, C2C12 cells and ASCs, which were initially randomly oriented, aligned perpendicular to the electrical field (**Figure 2**).

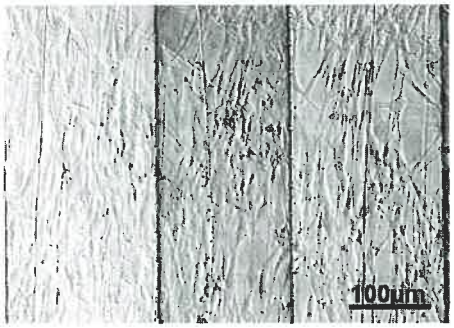

Figure 2 On uncoated ITO surface, C2C12 myoblasts aligned perpendicular to the direction of the electrical field gradient after four days of electrical stimulation.

Conclusions: These findings suggest that the patterned conductive ITO surfaces could be successfully used for cell electrical stimulation. Furthermore, by using type I collagen coating, the attachment of ASCs onto ITO surfaces improved and it was possible to achieve cell alignment with respect to the electrical field. Our ongoing studies involve characterization of adipose-derived cells' phenotype at different differentiation stages, and interrogation of the effect of the initial orientation of ASCs of their alignment in the electrical field. Overall, data collected thus far support the notion that electrical field stimulation can be used to modulate the behavior of ASCs.

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SCIENTIFIC SESSION

TRANSLATIONAL: NEUROLOGICAL

VIII-1 Migration and differentiation of mesenchymal stem cells from bone marrow and adipose tissue in mouse CNS

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There is increasing evidence that mesenchymal stem cells (MSCs) isolated from the bone marrow or adipose tissue are multipotent cells that can engraft and differentiate in the CNS. MSCs may offer advantages if developed for cell therapy because these cells are relatively easy to isolate from small volume of lipoaspirate or of bone marrow aspirate and can be readily expanded to provide a sufficient number of cells for transplantation. In this study, a series of CNS transplantation experiments were performed to characterize the engraftment, migration, homing, and differentiation patterns of human MSCs. Cell lines generated from human bone marrow derived MSCs (BMSCs) and adipose tissue derived MSC (ASCs) were injected into lateral ventricle of brain of NIHIII immune deficient mice by stereotaxic injection. The fate of MSCs was monitored by immunohistochemistry and PCR analyses 15 and 30 days post-injection. MSCs migrated along ventricular area both rostrally and caudally from the injection site. Cells were distributed throughout the entire rostro-caudal extent of ventricular zone. Many MSCs also were detected lining the ependyma throughout the ventricle. A significant number of donor cells were distributed along the length of the spinal cord. A higher frequency of MSCs was detected in animals injected with ASCs than BMSCs at 15 days following injection. Donor cells were dispersed into both lobes of the brain, numerous cells were detected in the cortex and different areas of cerebrum such as semilunar lobules, paraflocculus, postsuperior, fissure areas, and external and internal granular layers. Detailed analysis revealed that MSCs were predominantly seen in perivascular space in the brain. In animals injected with ASCs, the total number of detectable cells was significantly higher in 30 days post-engraftment

than 15 days, though the distribution pattern was unchanged. A significantly higher number of engrafted BMSCs were detected in the spinal cord of 30 days group. BMSCs were widely distributed throughout the brain at both time points. The distribution pattern of BMSCs at 15 days post engraftment was similar to ASCs. At 30 days, the majority of cells were found in the ventral areas of brain, with many detected in the immediate periphery of basilary artery. The results form these studies suggest that the MSCS effectively migrate throughout the CNS via the cerebrospinal fluid, as the pattern of detection of cells parallels the flow of cerebrospinal fluid in the brain. Numerous MSCs were found to be reactive to some specific neuronal antibodies such as NSE and MAP-2 at 30 days following engraftment indicating the differentiation of the grafted cells. The results of these studies demonstrated that transplantation of both types of MSCs into the ventricle resulted in the migration of MSCs throughout the brain within striatal, cortical, and cerebellar regions as well as and spinal cord.

SCIENTIFIC SESSION

TRANSLATIONAL: NEUROLOGICAL

VIII-2 Adipose stromal cells-secreted media protected hypoxia-ischemia-induced neonatal brain damage

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Recent studies suggest that adipose tissue contains pluripotent stem cells that are similar to bone marrow-derived stem cells. The beneficial effects of adipose-derived stem cells (ASCs) when delivered to diseased tissues appear to be related in part to their ability to secrete growth factors. Therefore, we tested the ability of factors secreted by ASCs to protect neurons in ischemic brains of rats. ASCs isolated from rat subcutaneous adipose tissue were grown to confluence in neural basal medium and the conditioned medium was removed and concentrated. Unilateral (left) carotid artery ligation was performed on neonatal (day 7) rats, followed by exposure to hypoxia (7%) for 2 hours. The concentrated conditioned medium was injected iv immediately or at 24hrs after ischemia. 7 days later, the areas of tissue in the hippocampus ipsilateral to the lesioned hemisphere were compared to the matching brain region in the contralateral to unlesioned hemisphere of the same rat. The percentage area loss was then determined for each animal. Treatments of ASC conditioned medium significantly protected against hippocampal volume loss induced by ischemic-hypoxic injury (61±11, n=3 vs. 98±6, n=5 (immediate) and 90±7, n=5 (24 h after), one-way ANOVA, *$p<0.05$). We conclude from these data that the delivery of a milieu of factors secreted by ASCs may be a viable therapeutic option for treatment of ischemic injury to the brain.

SCIENTIFIC SESSION

TRANSLATIONAL: NEUROLOGICAL

VIII-3 Neuroprotective effect of adipose tissue-derived stromal cells

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Background and Objective: Adipose stromal cell is a kind of the mesenchymal stem cells and has multi-potentiality of differentiation into various mesodermal tissues and neural lineage cells. Secretion of trophic factors such as vascular endothelial growth factor (VEGF), anti-apoptosis of endothelial cells and salvage of rat hind limb ischemia have been proved. Injection of neural differentiated adipose stromal cells into ventricle of ischemic rat brain improved neurologic deficits. But the neuroprotective effect of these cells was not verified yet, and transplantation of undifferentiated adipose stromal cells into animal stroke models was not reported. So the objective of this study is to prove the neuroprotective effects of these cells and the mechanisms. We also intended to trace the early migration of these cells when injected intravenously to the brain-ischemia induced rats.

Methods: Apoptotic cell death of rat pheochromcytoma cell line (PC12 cell line) induced with OGD (oxygen-glucose deprivation) or staurosporine was evaluated with addition of normoxic or hypoxic conditionded media of adipose stromal cells. MTS assay was used to measure apoptotic cell death. Using inhibitors of intracellular signal cascade kinase and VEGF receptor, the exact mechanism of the anti-apoptotic effect was verified. Fluorescent labeling of these cells and intravenous injection to rat brain focal ischemia were performed, then histologic evaluation of brain section with fluorescent microscope was done. We also measured the concentration of VEGF in conditioned medium with ELISA.

Results: Hypoxic conditioned media showed anti-apoptotic effect in OGD and staurosporine induced apoptosis via VEGF signaling. Intravenously injected cells migrated to peri-infarction areas in a few days. Human adipose stromal cells conditioned media contained VEGF in a large amount.

Conclusion: This study suggests that adipose stromal cells have *in vitro* neuroprotective effect and intravenous injection of these cells may be a promising cell transplantation therapy of stroke.

SCIENTIFIC SESSION

TRANSLATIONAL: NEUROLOGICAL

VIII-4 Comparative analyses of mesenchymal stem cells derived from bone marrow and adipose tissue

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The biological characteristics of mesenchymal stem cells (MSCs) isolated from two distinct tissues, bone marrow and adipose tissue were evaluated in these studies. MSCs derived from human and non-human primate (rhesus monkey) tissue sources were compared. The data indicated that MSCs isolated from rhesus bone marrow (rBMSCs) and human adipose tissue (hASCs) had more similar biologic properties than MSCs of rhesus adipose tissue (rASCs) and human bone marrow MSCs (hBMSCs). Analyses of *in vitro* growth kinetics revealed shorter doubling time for rBMSCs and hASCs. rBMSCs and hASCs underwent significantly more population doublings than the other MSCs. MSCs from all sources showed a marked decrease in telomerase activity over extended culture; however they maintained their mean telomere length. All of the MSCs expressed embryonic stem cell markers, Oct-4, Rex-1 and Sox-2 for at least 10 passages. Early populations of MSCs types showed similar multilineage differentiation capability. However, rBMSCs demonstrated an extended differentiation potential for more than 100 population doublings whereas rASCs and hBMSCs lost differentiation ability in about 45 population doublings. MSCs derived from bone marrow and adipose tissues use identical mechanisms to regulate self-renewal. Further analyses of the MSCs populations incorporated the use of Affymetrix oligonucleotide microarray data and proteomic analyses both stem cell types at several passages in each species. In light of the genetic sequence similarity between man and other primates (>90%) some investigators have suggested that the most important evolutionary differences relate to how genetic information is utilized. In

recent years the kinds of experiments needed to test this idea have become possible. The global comparison of gene expression between human and rhesus monkey mesenchymal stem cells in culture was performed by the analysis of the alterations in levels of RNA expression and protein. Microarray analysis has provided evidence that multiple genes involved in the nuclear localization of proteins and spliceosome assembly are differentially regulated between the cell populations and species. High level analysis of a partial dataset followed by an analysis of functional annotations for genes resulted in the identification of specific, biologically relevant processes that are differentially represented in each species and stem cell type in culture. Preliminary evidence from the proteomic analysis indicates a high degree of similarity between the BMSCs and ASCs; however, the expression of distinct proteins and marked changes in the levels of some proteins were detected. Efforts are now underway to identify these novel proteins. Overall *in vitro* characterization of MSCs from these two species and tissue sources revealed a high level of common biologic properties. These results should provide information that will help to design more direct studies of the roles these processes, pathways, and proteins play in each species, cell type, or passage.

SCIENTIFIC SESSION

TRANSLATIONAL: NEUROLOGICAL

IX-1 Vascularization during tissue injury repair is enhanced by topical treatment with adipose stromal cells

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Background: Cell-based therapies hold tremendous promise for treating cardiovascular diseases through promoting repair of ischemic tissues. Adipose-derived stromal cells (ASC) are pluripotent cells that secrete an array of angiogenic growth factors. We evaluated the potential of locally applied ASCs to enhance neovascularization and tissue healing, by assessing the fate of transgenic ASCs expressing green fluorescent protein (GFP), in a porcine skin-wound model.

Methods and Results: Yorkshire pigs underwent full-thickness skin wounding (132 total wounds, 3 pigs). ASCs were isolated from adipose tissues of transgenic pigs expressing GFP or normal pigs and applied to wounds after suspension in a fibrin matrix prepared from autologous blood using a GPS® device (Biomet Biologics, Inc). In total there were 6 treatment groups: Platelet-rich plasma (PRP), Platelet-poor plasma (PPP), PRP+autologous ASCs, PPP+ASCs, PPP+allogeneic GFP-ASCs, and Saline (6 wounds/group). Biopsies obtained on days 3, 7, 14, and 21 were evaluated histologically. Wound healing was normal in all groups, including allogeneic GFP-ASC treatment, without inflammatory cell infiltration. Detection of α-smooth muscle actin-positive arterioles indicated that microvessel densities were >1.4 fold higher ($p<0.05$) in the regenerated dermis of wounds treated with matrices containing ASC compared to other groups. This effect correlated strongly ($p<0.05$) with VEGF levels in cultures of PRP and PPP imbedded ASC (1,819+70 and 974+12pg/ml) compared to PRP (136+7pg/ml) and PPP or saline (both undetectable). Allogeneic GFP-ASCs were abundant at 3 wk following application, indicating immunotolerance by the host. Furthermore, GFP-ASCs predominantly engrafted at peri-vascular locations within the repaired tissues, suggesting a direct role in neoangiogenesis.

Conclusion: This data demonstrates a functional contribution of ASCs to repair injured tissues by supporting neovascularization, both through enhancing local angiogenic stimuli and stabilizing newly formed vessels, and also provides mechanistic understanding for our previous findings that ASCs promote recovery from acute ischemic injury both in rat myocardial infarction and mouse peripheral vascular disease models.

SCIENTIFIC SESSION

TRANSLATIONAL: NEUROLOGICAL

IX-2 Enhanced diabetic wound healing using human adipose stem cells: In search of predictable and reproducible efficacy

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Introduction: It is well known that diabetes is associated with abnormal wound healing and diabetic wounds represent a significant health care burden throughout the world. Lower extremity ulcers are a common reason for the hospitalization of diabetic patients, and these wounds often result in lower extremity amputation. The dysregulated wound healing associated with diabetes includes dysfunction of native stem cells and wound fibroblasts. We hypothesized that Adipose Stem Cells (ASCs) would be able to favorably impact the diabetic wound environment. To this end we investigated the effects of ASCs applied topically to 1cm full thickness excisional wounds in diabetic (db/db) mice.

Methods: Human ASCs were isolated and cultured using published methods. Diabetic (db/db) mice were monitored for the onset of diabetes via blood glucose levels, and then received dorsal 1cm full thickness excisional wounds. Non-diabetic (db/-) littermate mice were used as normal wound healing controls. Animals were randomized to receive either ASCs in delivery vehicle , or vehicle only (control) in a blinded fashion(N= 7/group). Wound healing rates were measured for each animal using photography and wound area tracings until complete wound healing.

Results: Preliminary results from a pilot study using ASCs from a single donor revealed a therapeutic response in which cell treated animals healed nearly one week sooner than controls. In a follow-up study using ASCs from three new and distinct donors, wounds reached 75% closure by 8.69 days with one patient line compared with 11.56 days for control diabetic mice ($p=0.024$). This treatment came close to restoring wound healing to those seen in non-diabetic controls (7.17 days; $p=0.755$). Repeated measures ANOVA confirmed the therapeutic effect of this particular cell line over time ($p=0.03$) but failed to find a significant effect associated with the two other cell lines tested.

Conclusions: This study raised the exciting possibility that adipose stem cells may one day improve healing in diabetic patients living with chronic and debilitating wounds. Interestingly, some ASC preparations displayed significantly enhanced wound healing rates, whereas others did not. The observed differences in ASC efficacy among the different cell lines/donors tested suggests that further research is required to understand the reason(s) for such biological variability. Additional studies are needed to identify and develop *in vitro* assays predictive of a cell lines potential therapeutic benefit.

SCIENTIFIC SESSION

TRANSLATIONAL: NEUROLOGICAL

IX-3 Accerelated healing of full-thickness wound of db/db mouse by murine adipose-derived stromal cells (ASCs)

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In recent years ASCs have been reported to contribute to neovascularization under ischemic environment (Planat-Benard 2004, Miranville 2004, Moon 2006) and, through their angiogenic property, they are also expected to contribute to cutaneous wound healing, which is assumed to depend greatly upon local blood flow. To test the applicability of cell therapy with ASC (derived from BKS. Cg^{-} m^{+}/m^{+}) to prolonged cutaneous wound healing, we administered murine ASC or, as negative control, dermal fibroblast (DF) to full-thickness skin defect freshly made simultaneously with the cell on the back of db/db mouse (BKS.Cg^{-}Lepr(db)/Lepr(db)=congenic with BKS.Cg-m^{+}/m^{+}), in which healing by secondary intention is greatly prolonged for as long as more than 2 weeks. When cultured cells were resuspended with trypsin and injected into the skin surrounding with the skin defect, there were no statistically significance in the area of defect at any time point among the groups of mice administered with ASC, DF, and vehicle (PBS) only. However, when cells were detached mechanically from the culture dish bottom with their extracellular matrix undestroyed and surgically administered subcutaneously in the vicinity of the skin defect, the wound areas are significantly smaller at postoperative day 10 to 14 in the group administered with ASC than in those administered with DF and not administered with any cells. Histololical findings of the wound tissues, which could explain the acceleration of wound healing exclusively by ASC by enhanced neovascularization, will also be described. These results indicate that ASC could be a new option for cell therapy of intractable skin ulcer, and that method of administration or, more specifically, the processing protocol of cells to be administered, could be crucial to their effect.

SCIENTIFIC SESSION

TRANSLATIONAL: NEUROLOGICAL

IX-4 Angiogenic effect of ASCs in rat ischemic hindlimb model and *in vivo* tracking of the cells.

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According to the recent discovery of adipose tissue derived adult stem cells (ASCs), many researchers are working on ASCs for the possibility of clinical application. It has been already known that bone marrow stem cells, umbilical cord blood stem cells and vascular endothelial cells are effective in vascular regeneration within ischemic tissue. The purpose of this study is to confirm the angiogenetic effect of ASCs in ischemic animal model and *in vivo* tracking of ASCs after the administration of cells.

With a method of ligating superficial and deep femoral artery, 24 ischemic hindlimbs were made in female Sprague-Dawley rats. At the same operative event, small amount of autologous inguinal fat pad was taken from each rat to harvest autologous ASCs. Adipose tissue dissociation was performed with a routine method using collagenase type I and ASCs were harvested. Culture proliferation of ASCs was done for 2 weeks. Cultured cells were tagged with two different materials, Feridex® (iron particle) and Cy7 fluorescence for *in vivo* tracking and identification of cells after administration. As a control group, normal saline was injected for 8 rats and $1x10^{7}$ASCs were injected into muscles of ischemic hindlimb for 8 other rats. For the other 8 rats, $1x10^{7}$ASCs were injected intravenously through the tail vein.

After 3 weeks, ischemic hindlimb specimens were harvested and various techniques of analysis including infrared thermography, immunohistochemistry, angiography, fluorescein skin test with Wood lamp showed visual evidence of improved blood supply. Histologic study was also revealed increased vascularization within ischemic tissue. Definitive evidence of incorporation of stem cells into the new vessels was confirmed with immunohistochemistry for VEGF and Prussian blue (+) reaction of Feridex® labeled cells. Autopsy of the animals on serial time schedule are undergoing and hopefully we could get meaning information on migration and targeting of stem cells.

Autologous ASCs are actively engaged in the neovascularization process of ischemic rat hindlimb model. It is obvious that ASCs are an effective therapeutic agent for the revascularization of ischemic tissue. Meaningful information on the pathway of cellular migration and targeting of stem cells is going to be presented.

Poster Abstracts

Poster Abstracts

Human adipose-derived stem cells (ASCs) as an *in vitro* model of peripheral circadian oscillators

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Recent studies have shown that circadian clock genes are expressed in adipose tissue and display an oscillatory expression profile. Our initial transcriptomic analysis of murine adipose tissue depots indicates that >20% of all expressed genes exhibit a circadian rhythmicity within these tissues. To date, most studies of circadian biology have primarily relied on *in vivo* rodent models.

In this study, we demonstrate that human ASCs derived from subcutaneous lipoaspirates can be used as an *in vitro* model to examine circadian mechanisms. Following a 2-hour exposure to dexamethasone (1M) or fetal bovine serum (50%), undifferentiated human ASCs (n=4 donors) show a robust and coordinated expression of circadian oscillator genes (*Bmal1, Per1-3,* and *Cry1-2*), and their downstream target genes (*Rev-erbA, Rev-erbB*) over a 48-hour period. Dexamethasone and serum induction of adipocyte-differentiated human ASCs elicited a similar circadian gene expression; however, the nadir of individual genes was greater in adipoctye-differentiated cells relative to undifferentiated ASCs, and the temporal dynamics were accelerated. In both undifferentiated and adipocyte differentiated ASCs, the periodicity of the *Bmal1* and *Per/Cry* genes were phase displaced 8-12 hrs relative to one another, consistent with their *in vivo* profiles in adipose tissue. Furthermore, the additiomn of GSK-3beta inhibitors, known to regulate circadian clock activity, resulted in a phase shift of circadian gene expression. These findings demonstrate our ability to study circadian regulation in a humanized adipocyte model and use this model to evaluate the effects of drugs targeting selected metabolic pathways on peripheral circadian mechanisms *in vitro.*

Scarless skin repair in immunodeficient mice

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Scarring, the end result of the wound healing process in adult mammals, is a problem of significant clinical importance. We observed that Athymic Nude-*nu* mice, similar to mammalian fetuses, are able to restore the structure and integrity of injured skin through a process resembling regeneration, where scar formation is absent. Among the post-injured skin tissues collected from Athymic Nude-*nu*, wild-type controls (C57BL/6J), immunodeficient SCID and Rag (lack of B and T cells), Athymic (thymectomized neonates and adult C57BL/6J) and mice treated with an immunosuppressant (cyclosporin A), only Athymic Nude-*nu* mice revealed (1) lack of scar (H&E and Masson's trichrome staining), (2) low levels of collagen (hydroxyproline assay), (3) high levels of hyaluronic acid, (4) a statistically significant increase in elastic modulus for injured samples over unwounded (biomechanical testing) and (5) low levels of pro-scarring cytokines (PDGF-B, TGFβ,1). Additionally, immunohistochemical and Western Blot analyses of post-injured tissues as well as flow cytometry analysis of blood samples showed the presence of CD8 positive cells in all studied animals except Nude-*nu* mice. In summary, scarless skin healing in Athymic Nude-*nu* mice provides a new model to study the influence of the immune system on tissue regeneration.

ASC mediates suppression of Th1 responses *in vivo* through the action of Treg

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Similarly to what has been described for bone marrow-derived mesenchymal stem cells (BMSC), adipose-derived stem cells (ASC) have been reported to suppress T-cell proliferation responses *in vitro* by an unknown non MHC-restricted mechanism. Previous work by other authors has also demonstrated a therapeutic effect of BMSC of allogeneic origin in both animal and clinical studies in some immune disorders, such as graft versus host disease.

We decided to study the possible anti-inflammatory effect of human ASCs in a xenogeneic context in two different murine models of inflammatory disease: TNBS-induced inflammatory bowel disease,, a murine model for human Crohn's disease and ulcerative colitis, and collagen-induced arthritis (CIA), a murine model for human rheumathoid arthritis. In our experiments, human ASC were administered itraperitoneally. into a part of the animals, which have developed symptoms of the corresponding disease. In the case of CIA animals, some of them were administered with ASCs by direct injection into the joints. At the end of the experiments, disease was scored, and different immune parameters were analyzed, including cytokine and antibody expression, as well as T cell proliferation.

Our studies indicate that human ASCa are able to suppress acute local inflammatory Th1 responses in different tissues by means of a mechanism mediated by Treg lymphocytes.

17-β estradiol enhancing osteogenic differentiations of human adipose-derived stromal cells

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Adipose-derived stromal cells (ASCs) possess multiple differentiation potentials and may serve as a cell source, for regenerative medicine and tissue engineering, if effectively modulated. Due to estrogen's function in tissue and organ development through regulating cell proliferation and differentiation, we hypothesized that an estrogen supplement may effectively enhance the multiple differentiation potentials of human ASCs. 17-β estradiol (E2) was investigated for modulating *in vitro* osteogenic and adipogenic differentiation in human ASCs isolated from a healthy female donor. After exposing ASCs' to osteogenic and adipogenic differentiation medium supplemented with different concentrations of E2, osteogenic markers (alkaline phosphatase activity, extracellular matrix, calcium deposition, and osteocalcin expression) and adipogenic parameters (lipid accumulation and differentiated cell population) significantly improved. The enhancement by estrogen is dose-dependent and linked to differing alpha and beta estrogen receptors (ER). We preliminarily demonstrated that estrogen as a modulator of differentiation, can potentially improve the function of ASCs in stem cell-based tissue engineering and regeneration.

Characterization of ASC migration toward a physiological concentration of PDGF-BB

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Introduction: Several recent studies have hinted at the ability of human adipose-derived stem cells (ASCs) to behave in a similar fashion to perivascular cells (pericytes or smooth muscle cells). While transcription of RNA for PDGFbeta receptor and production of RNA coding for PDGF-BB suggest a responsiveness to perivascular guidance cues, the functional chemotactic effect of physiological concentrations of PDGF-BB has not been examined for this cell type. If ASCs were to play a perivascular role *in vivo*, they would first need to respond to perivascular guidance cues such as PDGF-BB. In this model, migration of ASCs through a Boyden chamber system in response to differential presentation of PDGF-BB is measured in order to elucidate the potential perivascular functionality of the cell type.

Methods: DiI-labeled ASCs were cultured in DMEM/F12 with 3% FBS for 48 hours in order to induce quiescence before being seeded onto a Transwell insert membrane. 24 hours before the migration experiment, half of the cells were placed in a hypoxic environment using a Modular Incubation Chamber purged of oxygen with 5%$C0_2$, 95% N_2 gas mixture. A suspension of ASCs at passage 6 in DMEM/F12 with 10% FBS was used to deliver cells to the upper surface of the membrane at a concentration of 50kcells/cm^2. The lower chamber of the Transwell migration system was filled with DMEM/F12 with 10% FBS containing 1ng/mL human PDGF-BB, 1ng/mL human PDGF-BB plus 2.5 µg/mL of PDGFbeta receptor antibody, or PBS vehicle. Cells were allowed to migrate for 90 minutes and 3 hours before being fixed with 4% paraformaldehyde. Nuclei were labeled with Hoechst 33258 nuclear stain. Images were then analyzed with a confocal microscope using a 20x objective lens and quantified by counting cells in on both upper and lower membrane surfaces and expressing the quantity migrated by percentage of total cells in each field of view (n=5).

Results: A significant increase in ASC migration after three hours was seen in cells exposed to PDGF-BB as compared to those exposed to PBS vehicle, while no such increase was seen this those treated with PDGF-BB and PDGFbeta receptor antibody compared to PBS vehicle. Additionally, significantly more ASCs cultured in hypoxia migrated to the lower compartment of the Transwell system after 90 minutes compared to those presented with PDGF-BB with PDGFbeta receptor antibody.

Conclusions: These data suggest that ASCs can migrate toward a chemotactic PDGF-BB signal even at low physiological concentrations of growth factor. Furthermore, ASCs appear to suffer no adverse effects from 24 hours of culture in hypoxic conditions.

Identification of preadipocytes in human adipose tissue: techniques and applications for staining adipocyte precursors

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Background: Adipose tissue engineering holds promise for the future of plastic and reconstructive surgery. Specifically, preadipocytes may be the most resilient cell type in fat transplantation. Preadipoctye Factor-1 (Pref-1/dlk-1) is a reliable marker for the immunohistochemical identification of preadipocytes. As such, we used Pref-1 staining to identify and quantify the numbers of preadipocytes seen at different time points after the transplantation of human fat tissue into an established murine model.

Methods: Fifteen athymic nu-nu mice were divided into three groups of five. Each mouse was injected with 0.1cm^3 of human fat, mixed with sustained-delivery polyglycolic acid (PGA) beads and one of the following three growth factors: bovine serum albumin (BSA), basic fibroblast growth factor (bFGF), or neuropeptide Y (NPY). Mice from each group were sacrificed at two and four months. Tissues were processed histologically, and immunostaining was done for Pref-1.

Results: Within the BSA (control) group, there was no significant change in the number of cells staining positively for pref-1 at the two-month versus four-month time points. However, in both the bFGF and NPY groups, there was a significant decrease in the number of stained preadipocytes between two months and four months, and a corresponding increase in adipose cell formation over this time period as well.

Conclusions: The present study demonstrates the usefulness of Pref-1/dlk-1 in the immunohistochemical identification of adipocyte precursors at different time points after fat transplantation. This technique will have vast applications in future efforts to better understand fat cell biology.

CD34 expression of adipose-derived stem cells (ASCs)

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Objectives: Stromal vascular fractions isolated from adipose tissue contain CD34$^+$ cells, which seem to be adipose derived stem cells (ASCs), but many of them lose CD34 expression with culture time. The aim of this study is to examine how CD34 expression of ASCs changes and how different CD34$^+$ ASCs and CD34$^-$ ASCs are each other.

Methods: To examine the cell composition of freshly-isolated SVF, multicolor FACS assay was performed. Changes in CD34 expression of ASCs over cell culture were examined. Using cultured ASCs (passage 2), CD34$^+$ ASCs and CD34$^-$ ASCs were sorted. The two populations were examined for doubling time, vascular network formation and differentiation capacities for adipogenic, osteogenic and chondrogenic lineages.

Results: Freshly isolated SVFs contained several cell populations in view of surface antigen expression profiles. A main population of ASCs was identified as CD31$^-$CD34$^+$CD45$^-$CD90$^+$CD105$^-$CD146$^-$ cells, but it expressed CD105 after plating. CD34 expression of ASCs gradually declined but 10-20% of ASCs preserved its expression even after cell culture for 20 weeks. About a half of CD34$^+$ ASCs lost CD34 expression after 1-2 weeks culture. CD34$^+$ ASCs proliferated more rapidly than CD34$^-$ ASCs. After 3 weeks of differentiation cultures, CD34$^-$ ASCs contained significantly more lipid and calcium contents compared to CD34$^+$ ASCs. Both cell populations similarly formed vascular networks in 3-D matrigel culture.

Conclusions: Almost all ASCs were initially CD34$^+$, but some of them turned to CD34$^-$ with culture time. CD34$^+$ ASCs may asymmetrically divide into CD34$^+$ and CD34$^-$ ASCs. It was suggested that ASCs acquire more potentials of mesechymal differentiations after losing CD34 expression.

Role of toll-like receptors on human adipose-derived stromal cells

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Background: Adult mesenchymal stem cells (MSCs) are promising tools for such applications as tissue engineering and cellular therapy. It is not clear how stem cells exposed to unfavorable conditions (eg, hypoxia or inflammation) respond to signals of danger after *in vivo* transplantation. Toll-like receptors (TLRs) play a major role in the immune

system, participating in the initial recognition of microbial pathogens and pathogen-associated components. This study was designated to determine the role of TLRs in human MSC.

Methods and Results: Reverse-transcriptase polymerase chain reaction and flow cytometry analysis demonstrated that MSCs derived from human adipose tissue and bone marrow express TLR 1, 2, 3, 4, 5, 6, and 9. We investigated induction of the differentiation and proliferation of human adipose tissue stromal cells (hADSCs) by TLR agonists, including flagellin (FGN), peptidoglycans (PGN), lipopolysaccharide (LPS), the synthetic double-stranded RNA analog poly I:C, and synthetic CpG oligodeoxydinucleotide (CpG-ODN). None of these agonists, except ODN, affected the proliferation of hADSCs. LPS and PGN increased osteogenic differentiation, but CpG-ODN decreased it. Poly I:C itself did not affect adipogenic or osteogenic differentiation, but exerted a synergistic effect on LPS- or PGN-induced osteogenic differentiation. RT-PCR analysis demonstrated that LPS and PGN induce osteogenic markers in hADSCs. TLR agonists affected the expression of chemokines and cytokines differentially. Furthermore, hADSCs affected the expression of specific TLRs *in vitro* under hypoxic conditions.

Conclusion: These data provide evidence of a nonimmune role for TLR signaling on MSCs, which may provide clues to the behavior of transplanted MSCs *in vivo*.

Identifying modifier genes influencing the properties of adipose stromal cells

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Adipose stromal cells (ASC) isolated from fat are a readily available source of autologous stem cells. ASCs exhibit pluripotency having been shown to develop into myogenic, adipogenic, neurogenic, osteogenic, chondrogenic endothelial and hepatic cell lineages *in vitro*. In addition, ASCs in culture include a significant percentage of highly proliferative cells enabling for rapid expansion of pluripotent cells *in vitro*. Several recent publications suggest their potential value as a source of autologous, adult progenitors for cell therapy applications.

Understanding the factors that influence the behavior and properties of ASCs will contribute to our understanding of the regulation of basic biological processes such as proliferation and cell differentiation. Additionally, such insights will facilitate the use of ASCs for biomedical applications. While most studies have focused on the role of external factors (growth factors, matrix composition, etc.) the focus of this program is to identify the genetic factors that influence ASC properties. The fact that the proliferative potential of bone marrow stromal cells isolated from wild-type mice of 5 different strains vary by as much as 10-fold, reinforces the contention that genetic factors modify the properties of stem/progenitor cells,

The proliferative potential of ASCs derived from C57Bl/6 and 129X1/svj were compared. ASCs isolated from subcutaneous fat from both lines were plated at very low density in 48-well plates. The frequency of proliferative ASCs varies by 3 fold between strains (1.5% and 4.4% for C57Bl/6 and 129X1/svj, respectively). In addition, the developmental potential and ability of ASCs to recruit endothelial cells between strains are being compared. Using a breeding scheme of selective backcrosses we will develop mice with ASCs with a 129X1/svj phenotype in a C57Bl/6 background (or vice versa). High density SNP screening of genomic DNA from these mice will permit identification of chromosomal regions containing elements important for the properties of ASCs.

Rapid immune cytokine suppression induced by adenovirus-36 infection

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Adipose tissue growth is associated with increased in inflammatory cytokines. Due to the association of proinflammatory cytokines with diabetes and other comorbidities, understanding the regulation of adipose tissue induced proinflammatory response is critical. Ad-36 is a group-D human adenovirus that increases adiposity in experimental animal models, shows association with human obesity and induces preadipocyte proliferation and differentiation *in vitro*. We sought to identify the role that Ad-36 infection plays in the *in vitro* and *in vivo* modulation of cytokine expression and obesity development. Human adipose-derived stem cells (hASC) isolated from subcutaneous adipose tissue biopsy of a healthy donor (BMI=25) were infected with either Ad-36 or Ad-2 (moi=3.8). Ad-2 is a non-adipogenic human adenovirus used as a negative control. On day 1, mRNA expression of macrophage migration inhibitory factor (MIF) was significantly higher in Ad-36 infection and significantly lower in Ad-2 infection suggesting that Ad-36 is suppressing the recruitment of macrophages, while Ad-2 is promoting macrophage recruitment (**Table 1**). By day 2 monocyte chemoattractant protein-1 (MCP-1) and IL-18 mRNA expression were both significantly lower in the Ad-36 infected group, suggesting a coordinated immune suppression is induced by Ad-36 infection in isolated cells of adipocyte lineage. To determine the anti-inflammatory response of Ad-36 in adipose tissue, we infected human adipose tissue explants obtained by lipoaspiration with Ad-36. Similar to the findings in hASC, the Ad-36 infected group showed greater MIF and lower MCP-1 mRNA expression compared to the uninfected control (**Table 1**). To determine the *in vivo* impact of Ad-36 induced modulation of inflammation, 5 week old male Wistar rats were inoculated intranasally either with media, UV inactivated Ad-36 or replication competent Ad-36. Four days later, serum IL-6 levels were reduced in both virus infected groups suggesting a receptor mediated immune suppression (**Table 1**). Whereas, IL-18 and MCP-1 levels were significantly reduced in replication competent Ad-36 infection alone, suggesting that active viral replication is required for these effects. Additional studies are underway to identify the tissue source for the effecter molecules *in vivo*. In summary, these data suggest that despite its pro-adipogenic effect, Ad-36 is able to rapidly induce coordinated immune suppression and may play a direct role in modulation of adiposity.

	MCP-1	MIF	IL-18	IL-6
hASC*: Ad-36 infected	42% (p<0.003)	154% (p<0.001)	79% (p<0.007)	N/A
hASC*: Ad-2 infected	136% (p<0.05)	51% (p<0.03)	94% (p=NS)	N/A
hAT explants* Ad-36 infected	70% (p<0.07)	167% (p<0.056)	113% (p=NS)	N/A
Rat serum Ad-36 infected**	52% (p<0.05)	N/A	51% (p<0.005)	22% (p=NS)
Rat serum UV Inactivated Ad-36**	92% (p=NS)	N/A	76% (p<0.054)	28% (p=NS)

Table 1: Percent mRNA* or protein** compared to the respective uninfected control groups. Data are expressed as Mean % change (p value).
N/A; Data not available; NS: Statistically not significant. Funded by NIH R-01 DK066164 to NVD

Autocrine and paracrine signaling in adipogenesis

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While increases in fat during adult life were historically thought to be a hypertrophy of existing mature adipocytes via increases in lipid load, a complementary mechanism has been more recently suggested, involving an ongoing process of preadipocyte differentiation within the stromal vascular fraction of adipose tissue. Previously, multiple

groups have demonstrated *in vitro* the ability to mimic these adipogenic events by differentiating a population of the non-adipocyte fraction of processed lipoaspirate into mature lipid-laden adipocytes. Now we are seeking to better understand the autocrine and paracrine events that potentially regulate this adipogenic process. Here we show a quantitative secretory profile of classic adipokines as well as novel anti-apoptotic and angiogenic factors that our lab has previously shown arise from these cells (namely: Leptin, Resistin, Adiponectin, IL-1β, IL-6, IL-8, TNF-α, MCP-1, PAI-1, HGF, and NGF) and an analysis of lipid load at regular intervals during a fourteen day differentiation period *in vitro*. Definition of the temporal sequence of adipokine and angiogenic factor secretion during adipogenic differentiation, and correlation of such soluble factor release with changes in lipid load will provide a more comprehensive understanding of unique signaling events that are concomitant with maturation of adipocytes.

The impact of aging on stem cell differentiation induced by nutrition-related hormones

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Introduction: Glucose-dependent insulinotropic peptide (GIP) is a hormone released from endocrine cells in the intestine in response to nutrient ingestion to potentiate insulin secretion. Leptin is a hormone released from adipocytes in response to nutrient ingestion to regulate appetite. GIP modulates leptin expression and secretion from the adipocyte. We have previously shown in both *in vitro* and *in vivo* studies that GIP and leptin are anabolic for bone. This anabolic effect is mediated in part through direct modulation of osteoblastic activity. Studies were performed to determine if these two hormones could also modulate stem cell differentiation to promote osteoblastogenesis. We also wished to determine if aging had any impact on stem cell differentiation.

Materials and Methods: Bone marrow was harvested from C57Bl6 mice at three, six twelve, eighteen and twenty four months of age. Mouse femora and tibiae were flushed with αMEM and dispersed to single-cell suspension. For induction of osteoblast differentiation, the mMSCs were plated in 96-well plates in triplicates at a density of 1500 cells/cm^2. When the cells reached confluence, they were treated with osteogenic induction media, in the presence or absence of GIP and leptin. The alkaline phosphatase activity (ALP staining) and mineralized bone nodule formation (von Kossa staining) were performed at day 10 and day 21, respectively, after the treatment was initiated. For adipogenic induction, the mMSCs were seeded in 24-well plates at a density of 30,000 cells/cm^2. Two days after the cells reached confluence, the cells were treated with adipogenic induction medium in the presence or absence of GIP and leptin for 3 days and then switched to adipogenic maintenance medium with media replaced every other day. The cells were stained with Oil Red O 10 to 14 days after the treatment. For real time PCR total cellular RNA was isolated from cells using TRIZOL Reagent. Total RNA was reverse transcribed using TaqMan Reverse Transcription Reagents. The transcription reaction was diluted 1:1 with water, and 1μl of the diluted cDNA was used as template for real-time PCR analysis using a SYBR Green RT-PCR Kit and a Chromo-4 real-time RT-PCR machine.

Results: Receptor expression for both GIP and leptin was highest in stem cells from three month-old mice and decreased progressively and dramatically with age (over hundred fold difference between the 3 vs 24 month old mice). GIP (at 1nM) significantly increased both alkaline phosphatase staining and nodule formation by von Kossa in stem cells with a peak effect on cells from mice of eighteen months of age. Leptin (at 100nM) did not increase either alkaline phosphatase or nodule formation. Both GIP and leptin increased adipocytic differentiation with a peak effect in cells from mice of 12 months of age. Paradoxically, if GIP and leptin were used in combination an inhibitory effect on adipocytic differentiation was now observed.

Conclusions: We demonstrate that mesenchymal stem cell receptor expression of two major nutritional hormones decreases with age. Further, we demonstrate that GIP's anabolic effect on bone may be mediated in part through the commitment of stem cell differentiation to the osteoblastic pathway. In view of the importance of nutritional signals on maintaining bone mass these data would suggest that age related declines in GIP and leptin receptor expression may play a pathophysiolgical role in age related bone loss.

Resveratrol-mediated regulation of PPARγ activity and turnover in adipocytes

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Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear hormone receptor predominantly expressed in adipocytes that plays a role in lipid metabolism, insulin sensitivity, obesity and type 2 diabetes. Sirt1, a deacetylase activated by calorie restriction, has been shown to regulate PPARγ transcriptional activity in adipocytes. Resveratrol, a polyphenol found in grapes and berries, activates Sirt1 and may regulate PPARγ activity. We sought to determine the effects of resveratrol on PPARγ activity using 3T3-L1 adipocytes and qRT-PCR to assay the expression of three PPARγ regulated genes. Resveratrol treatment decreased the expression of aP2 and LPL while the expression of PEPCK was unchanged from control levels. In contrast, the PPARγ agonist rosiglitazone activated aP2, PEPCK, and LPL expression.

PPARγ protein levels are regulated by the ubiquitin-proteasome system under basal and ligand-activated conditions. Resveratrol affected the turnover rate and steady-state levels of PPARγ. In each case, resveratrol-mediated decreases in PPARγ protein levels were abrogated by proteasome inhibition. A link between resveratrol-mediated inactivation of PPARγ and increased turnover of PPARγ protein is significant given genetic studies of heterozygous PPARγ deficiency showing decreases in PPARγ levels improve insulin sensitivity. Therefore, we initiated studies examining the effect of resveratrol on insulin signaling in adipocytes. Western blot analysis indicated resveratrol treatment is associated with increased insulin-dependent Akt phosphorylation, suggesting a role for resveratrol in mediating insulin sensitivity. Ongoing studies are focused on examining the effects of resveratrol on glucose uptake in adipocytes. In addition, we are beginning to develop the ability to examine resveratrol-mediated regulation of PPARγ in human adipocytes using human ASC-derived adipocytes.

Our results indicate resveratrol mediates PPARγ activity and proteasome-dependent degradation in adipocytes. Resveratrol-mediated changes in PPARγ, along with increased insulin-dependent Akt phosphorylation, suggest that resveratrol is a potential bioactive regulator of insulin sensitivity in adipocytes and may offer a botanically based approach in modulating insulin resistance.

Secretome of primary cultures of human adipose-derived stem cells (ASCs): Modulation of serpins by adipogenesis

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Studies of adipogenic protein induction have led to a new appreciation of adipose tissue's role as an endocrine organ. Adipocyte-derived "adipokines" such as adiponectin, leptin, and vaspin (visceral adipose tissue-derived serine protease inhibitor) exert hormone-like activities at the systemic level. In this study, we examined the secretome of

primary cultures of human subcutaneous adipose-derived stem cells (ASCs) as an *in vitro* model of adipogenesis. Conditioned media obtained from four individual female donors after culture in uninduced or adipogenic induced conditions were compared by 2-dimensional gel electrophoresis and tandem mass spectrometry. Over 80 individual protein features showing more than 2-fold relative differences were examined. Approximately 50% of the identified proteins have been previously described in the secretome of murine 3T3-L1 pre-adipocytes or in the interstitial fluid derived from human mammary gland adipose tissue. As reported by others, we found that the secretome included proteins such as actin and lactate dehydrogenase, that do not display a leader sequence or transmembrane domain and are classified as "cytoplasmic" in origin. Moreover, we detected a number of established adipokines, such as adiponectin and plasminogen activator inhibitor 1 (PAI-1). Of particular interest was the presence of multiple serine protease inhibitor proteins (serpins). In addition to PAI-1, these included pigmented epidermal derived factor (PEDF, confirmed by western immunoblot), placental thrombin inhibitor, pregnancy zone protein, and protease C1 inhibitor. These findings, together with the recent identification of vaspin, suggest that the serpin protein family warrants further proteomic investigation with respect to the etiology of obesity and type 2 diabetes.

PPARα and its role in lipid metabolism through circadian rhythm analysis

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Previous research has shown that the alpha isoform of peroxisome proliferators-activated receptors (PPARα) exhibits circadian rhythmicity and plays a significant role in lipid metabolism. PPARα is known to be a major transcriptional regulator in fatty acid oxidation (FAO) through several processes such as fatty acid transport and activation of acyl-CoA esters. Additionally, PPARα has been shown to display patterns of expression that are in coordination with the core circadian genes' profiles. Our prior research has demonstrated that peripheral tissues such as the liver, heart and fat depots (brown, inguinal, and epididymal) exhibit synchronized oscillatory patterns of the circadian genes (*Npas2, Bmal1, Per* and *Cry*). In an effort to better understand PPARα's role in FAO, we extended our circadian analysis to investigate the expression of both the circadian genes and lipogenic and cholesterogenic sterol regulatory element-binding protein (SREBP) controlled genes (*ACC, HMG-CoAR* and *SCD1*) in the peripheral tissues of PPARγ-null mice.

In this study, homozygous wild-type C57BL6/NxSV/129 mice served as controls against homozygous PPARα-null C57BL6/NxSV/129 mice. The animals were acclimatized to a regular chow diet and maintained on a strict 12h light/12h dark cycle. Both wild-type and PPARα-null mice were then further divided into two additional groups consisting of one ad libitum group, who received unrestricted access to food, and a restricted feeding group whose feeding time was limited to the 12-hour lights on period only. Animals from each of the four subdivisions were then sacrificed every 4 hours over a 24-hour period. RNA was extracted from the collected tissues and was followed by quantitative real-time PCR of selected genes of interest.

In our analysis of wild type versus PPARα-null mice without any dietary restrictions, we found little to no significant change in the expression profiles of the circadian genes in the liver, epididymal white adipose tissue (eWAT) and brown adipose tissue (BAT). These genes included *Npas2, Bmal1, DBP,* and *Per3*. However, upon the addition of a dietary stressor such as restricted feeding, there is a drastic change in the patterns of expression. When the wild type-ad lib mice are compared against the wild type-restricted feeding, there is an 8-12 hour phase shift in the profiles of the restricted feeding group. Additionally, when wild type mice are compared against PPARα-null mice under restricted feeding conditions, there is both a 4-8 hour delay and a dampening of gene expression in the knockout mice. This delayed shift is distinct and robust in the heart and BAT, where PPARα and fatty acid oxidation are most important.

Our research presents novel insights into the role of PPARα in lipid metabolism and its functionality during fatty acid oxidation, especially in times of dietary stress. It is apparent that restricted feeding is able to drastically change the entire circadian machinery, from the clock oscillatory genes to the downstream genes that actively participate in

fatty acid metabolism. Clearly, the timing of food consumption is an effective method of entrainment, resetting the circadian clock. Dietary restriction also reveals new perspective into the role of PPARα during fasting. Because there is little to no significant change in the gene expression profiles between wild type and PPARα-null mice in the ad lib state, PPARα seems to have a marginal role in maintaining basal metabolism. However, the phase shifts demonstrated during the restricted feeding state suggest that PPARα fulfills a much more important role during times of dietary stress. Thusly, there is an initial shift in gene expression under the restricted feeding state and an additional phase shift and dampening in expression due to the lack of PPARα. This further shift is not only present in the expression profiles of genes downstream from PPARα, but also the core circadian genes, particularly in the BAT. These phase shifts and changes in amplitude reveal that PPARα has a significant role in setting the circadian clock and regulating levels of circadian and fatty acid metabolism gene expression.

Fluorescent stereoscopic photography for quantifying the histologic characteristics of adipocytes from different regional anatomic sources

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Purpose: Autologous fat grafting has many advantages over other soft tissue augmentation strategies; however, it remains problematic due to its unpredictability. Various techniques have been employed to minimize damage to harvested adipocytes in an attempt to improve graft maintenance, but comparison at a cellular level is time consuming and laborious. We present a model that allows rapid quantification of adipocyte volume, membrane deformation, and membrane integrity. This model will assist clinicians in comparing techniques of fat harvesting and processing in a way that can be quantified and compared using statistical analysis.

Methods: Human fat samples were collected from six anatomic sites in the same patient using a 3mm Coleman liposuction cannula. The samples were formalin-fixed, embedded in paraffin, and processed into 5micron sections for hematoxylin and eosin staining. One representative slide was selected from each of the anatomic locations, and specimens were reviewed under fluorescent microscopy at 100x magnification based on the autofluorescent properties of fat cell membranes after they have been stained with hematoxylin. Ten different fields were analyzed from each slide, and images were acquired with Metamorph (Stemmer Imaging) computer software. Statistical analysis was then performed for the average cell volume, degree of adipocyte membrane deformation, and loss of membrane integrity. These characteristics were chosen based on the premise that an intact cellular membrane signifies a viable cell.

Results: Statistical analysis was used to compare the resilience of the harvested fat cells from different anatomic sites. We have hypothesized that the sample population containing the highest number of round, viable cells is the most appealing donor site for autologous fat grafting.

Conclusion: We have created a novel technique for assessing the viability of adipocytes harvested for autologous grafting. This will have value in scientifically determining the premium harvest site for autologous fat grafting, as well as for assessing the soundness of various harvest techniques.

Cell growth characteristics and differentiation frequency of adherent equine adipose tissue-derived mesenchymal stromal cells: Adipogenic and osteogenic capacity

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Objectives: Characterization of equine adipose tissue-derived mesenchymal stromal cell (ASC) growth characteristics and frequency as well as their adipogenic and osteogenic differentiation potential.

Study Design: *In vitro* experimental study.

Animals or Sample Population: Foals (n=3, range: 17 to 51 days) and young horses (n=5, range 9 months to 5 years).

Methods: Equine ASCs were harvested from gluteal adipose tissue and isolated to a stromal vascular fraction (SVF) and grown up to passage 10 to determine cell doubling (CD) characteristics. Limit dilution assays were performed on primary and passaged MSCs to determine the frequency of colony forming units with a fibroblastic phenotype (CFU-F), and alkaline phosphatase expressing cells (CFU-ALP), as well as the frequency of ASC differentiation into adipocytes (CFU-Ad) and osteoblasts (CFU-Ob).

Results: ASC isolates exhibited an average cell doubling time (DT) of 2.0 ± 0.4 days during the first ten cell doublings. Approximately 1 in 2.28 ± 0.35% of the total SVF nucleated cells were ASCs, based on the CFU-F assays, and 1 in 3.64 ± 1.29% expressed ALP. Primary ASCs differentiated in response to adipogenic and osteogenic inductive conditions and maintained their differentiation potential during subsequent passages (P2 and P4).

Conclusions: Equine adipose tissue ASC frequency, *in vitro* growth rate, and adipogenic and osteogenic differentiation potential are similar to those documented for ASCs of other mammalian species.

Clinical Relevance: The results have direct relevance to the use of adipose tissue as a potential source of adult stem cells for tissue engineering applications in equine veterinary medicine.

The use of the stromal vascular fraction of adipose tissue for cartilage and bone regeneration

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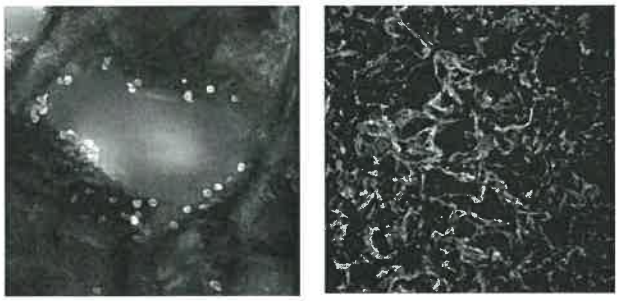
The stromal vascular fraction (SVF) of adipose tissue provides an abundant source for adipose tissue derived stem cells (ASCs), which may be used for bone and cartilage regeneration. Currently, ASCs are often being purified and expanded using lengthy and costly *in vitro* culturing procedures. From a clinical point of view it would be ideal if the heterogenous mixture of SVF cells could be directly seeded onto a carrier material, making these lengthy purification and expansion procedures superfluous. This requires that, among the various cell types within the SVF, at least the ASCs adhere to a carrier material, within a short time frame.

We investigate the feasibility of directly seeding SVF cells onto a 70:30 Poly(D,L-lactide-co-caprolactone) scaffold in the context of bone and cartilage tissue engineering, whereby expensive and lengthy *in vitro* expansion steps are avoided. Therefore, we phenotypically characterized the SVF cells and examined which cells of the SVF adhere to the scaffold and in what time frame. In addition, we investigated whether the attached cells can differentiate towards the osteogenic and chondrogenic lineage.

The SVF cells of 3 human donors were seeded onto the scaffolds. Adherence, proliferation and cell phenotype were monitored using DNA assays and fluorescently labeled antibodies against cell-specific membrane markers visualized with confocal microscopy. Osteogenic and chondrogenic differentiation was assessed using RT-PCR, (immuno) histochemistry and confocal microscopy.

SVF cells attached to the scaffold within 10min. While the SVF contains 34% of ASC-like cells, 72.3% of the adhered cells had an ASC-like phenotype. Only low frequencies of endothelial cells, leucocytes and other cell types were found, indicating that the stem cells preferentially bind to the scaffold. The attached ASC-like cells were capable of differentiating into the chondrogenic and osteogenic pathways.

Our study suggests that the SVF of adipose tissue can be used for bone and cartilage regeneration without expensive and lengthy *in vitro* expansion steps. This opens up the possibilities to perform a cost-effective, patient-friendly surgical procedure in which the SVF is harvested and directly returned to the defect site within a single surgical procedure.



Images: Confocal images showing the distribution and morphology of $CD34^+$ cells that adhered to the 70:30 Poly(D,L-lactide-co-caprolactone) scaffolds, 1 hour and 6 days after seeding of the SVF. Images are topview projections of a z-stack (50 images), which were taken as horizontal cross sections within the scaffold and subsequently stacked on top of each other. The green cells indicate positive staining for CD34. The red dots are the nuclei of all cells (either $CD34^+$ or $CD34^-$), which were stained with propidium iodide after fixation

Clinical therapy of flexor tendonitis with adipose-derived stem and regenerative cells in the horse

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It has been reported that performance horses have a greater than 10% prevalence of flexor tendonitis. The lower limb tendons in horses are under constant tension and cumulative damage from the stress of performance sports leads to clinical tendonitis and often a rupture of the fibers. The success rate of return to prior level of performance of horses treated with adipose-derived stem and regenerative cells (Vet-Stem) is greater than 73%. This abstract will present a retrospective analysis of the return to performance of sport horses with flexor tendonitis by injury severity, breed, sport, and age. An analysis of cell dose and concentration will be presented.

Osteogenic potential of frozen-thawed adipose derived adult stem cells

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We have investigated the ability of frozen-thawed human adipose tissue derived adult stem cells (ASCs) to form a colony using a colony-formation-unit (CFU) assay {Guilak et al., J. Cell Physiol. 2006,206,229-237}. For the initial series of experiments, the ASCs were placed in an isopropanol freezing container and put into the -80 freezer for 24 hours and then transferred to a liquid nitrogen. The freezing media consisted of 80% FBS, 10% DMSO and 10% DMEM/F-12 media. As shown in **Figure 1**, ~7.94 unfrozen ASCs are required to form a fibroblast colony(CFU-F) while ~7.23 frozen-thawed ASCs are required to form a similar colony. For the adipogenesis (CFU-AD) column, this number indicates that 1 out of every 15.16 induced ASCs will differentiate into adipocytes while 1 out of every 13.03 frozen-thawed ASCs will do the same. In contrast, analysis of colony forming activity related to osteogenesis, as detected by alkaline phosphatase (ALP) or alizarin red (OB) staining, indicated that there is a 2- to 3-fold reduction in osteogenic potential following cryopreservation. In another study, we have investigated the ability of ASCs stored in liquid nitrogen for 30 days and then thawed to form a colony. The ASCs were frozen at three different cooling rates (1, 20 and 40°C/min) in a controlled rate freezer {Thirumala, et al., Biotech. Prog. 2005,21,1511-1524} in two different media: i) in stromal media or DMEM/F 12 Ham's with 10% fetal bovine serum (Hyclone, Logan, UT), 100U penicillin, 100µg streptomycin and 0.25µg Fungizone and ii) DMEM/F-12 media with 10% (v/v) of DMSO. The data is summarized in **Table 1** and shows that ~2.9 ASCs are needed to form a CFU-Ob colony when frozen at 1°C/min in the presence of DMSO while in the absence of DMSO, the number of ASCs required to form the same colony is significantly higher at 49.6 ASCs. These initial studies suggest that the post-freeze osteogenic potential of human ASCs can be retained even when they are frozen in the absence of serum.

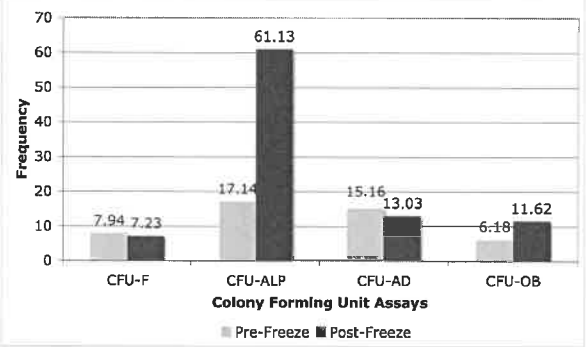

Figure 1. Colony-Formation-Unit (CFU) analysis of ASCs, pre- and post-freeze. CFU-Fibroblast (CFU-F), CFU-Alkaline Phosphatase (CFU-ALP), CFU-Adipocytes (CFU-AD) and CFU-Osteoblast (CFU-Ob) were performed using passage 2 ASCs from n = 3 donors; mean values are displayed.

Table 1: CFU-Osteoblast Frequency of ASCs frozen at 3 cooling rates.

Freezing Media Composition	Cooling Rate (°C/min)		
	1	20	40
Stromal Media	49.6 ± 12.3	42.5 ± 4.2	286.6 ± 177
DMEM/F-12 + 10% (v/v) DMSO	2.9 ± 0.42	14.1 ± 13.0	7.7 ± 2.0

*ASCs were stored in Liquid Nitrogen for 30 days. As shown in Fig. 1, approximately 6.18 unfrozen ASCs are required to form a CFU-Osteoblast colony.

Selective expansion of mesemchymal stem cells by culturing stromal vascular fraction obtained from human omentum in low serum medium

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The omentum is a unique and versatile organ that can be utilized for a variety of surgical procedures. The omentum is rich in lymphatics and is a source of blood vessels and fibroblasts. It is a thin organ with very little structural support but it can be utilized to cover other organs and to fill lost space. Based on our success in obtaining selective expansion of mesenchymal stem cells by culturing stromal vascular fraction (sSVF) obtained from human subcutaneous adipose tissue, we tested whether the same procedures can be applied to human omentum.

When the sediment fraction (oSVF) obtained after centrifugation of cell populations dispersed from human omentum by collagenase-digestion was directly analyzed by flow cytometry, CD34-strongly positive / CD31-weakly positive population was detected. A contrast from a parallel flow cytmetry of sSVF was that another population of CD34-weakly positive / CD31-strongly positive was absent in oSVF. As it was for sSVF, CD34-strongly positive population in oSVF was not positive to CD106 or CD13. The cells in oSVF from 48-year-old female, 64-year-old male and 83-year-old male patients proliferated faster in low serum medium than in high serum medium but this contrast was not evident as observed for sSVF. The cells proliferated from oSVF both in high and low serum medium showed the potential of adipogenic differentiation *in vitro* but it tended to higher after selective expansion in the low serum medium. The cells proliferated from oSVF exhibited low potential of osteogenic differentiation *in vitro* and the cells expanded in low serum medium showed higher amount of calcium accumulation. Staining of chondroitin sulfate by alcian blue suggested low potential of TFG-β dependent chondrogenic differentiation of the cells expanded in high-serum medium. The cells expanded in low serum medium exhibited alcian blue staining independent of the addition of TFG-β to chondrogenic induction medium.

Thus, we found that mesenchymal stem cells with adipogenic, osteogenic and chondrogenic differentiation potential can be selectively expanded in a low serum medium.

Exogenously applied recombinant BMP-2 induces adipogenesis rather than osteogenesis in human adipose-derived stem cells

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Introduction: Bone morphogenetic proteins (BMPs) are known to promote osteogenesis. Nevertheless, compared to rodent cells, human mesenchymal stem cells respond quite poorly to BMPs *in vitro* and only large doses of the protein are effective in human clinical trials. Some reports also describe an effect of the protein on adipogenesis ranging from stimulation to inhibition of the differentiation process. As latter work was mostly performed with murine pluripotential mesenchymal precursor cell lines we wanted to investigate the influence of BMP-2 on the differentiation behavior of human adipose-derived stem cells *in vitro*.

Materials and Methods: Stem cells were either isolated from liposuction material or excised adipose tissue by enzyme digestion. Recombinant BMP-2 was administered at 0.5 and 1µg/mL in either control or osteogenic medium.

Osteogenic differentiation was confirmed by von Kossa and Alizarin Red staining as well as quantitative assessment of calcium and intracellular alkaline phosphatase activity after 1, 2, 3, 4 and 5 weeks. Lipid droplets indicative for adipogenic differentiation were demonstrated by Oil Red O staining. The relative expression of the bone markers alkaline phosphatase, osteopontin, bone sialoprotein II and osteocalcin as well as the adipogenic markers aP2 and PPARγ was weekly determined by RT-PCR during 5 weeks.

Results: On its own, even high doses of recombinant BMP-2 did neither stimulate osteogenesis nor adipogenesis. However, combined with osteogenic medium (containing dexamethasone and β-glycerophosphate) it exerted a strong adipogenic effect on human adipose-derived stem cells whereas no enhancement of osteogenic differentiation could be observed.

Conclusion: In future bone tissue engineering approaches using BMPs and human adipose-derived stem cells, possible adipogenic effects should be taken into consideration. Maybe other growth factors are needed for the differentiation of human adipose-derived stem cells into the osteogenic lineage.

Preliminary characterization of canine and equine cell preparations obtained by enzymatic digestion of adipose tissue

Theodore Sand from VetStem in California

Cells isolated from adipose tissue through the action of enzymatic digestion are heterogeneous in nature, consisting of many of the cell types found in the blood stream as well as cells residing in the extracellular matrix. Studies were undertaken to characterize the composition of cells obtained following digestion of equine and canine adipose tissue with either collagenase alone or in combination with hyaluronidase. The dual enzyme protocol resulted in an average increase of 70% for nucleated cell yield per gram for equine tissue (n=19) and an 86% increase for canine tissue (n=8) compared to collagenase alone. Cytological assessment and CFU-F of the isolated cell preparations were performed. The profile of cell types identified in preparations obtained from digestion of both canine and equine adipose tissue samples includes mononuclear cells, neutrophils/lymphocytes, eosinophils (low number but seen with most samples) and monocytes (not seen in canine samples, and very rarely seen in equine samples). There was a noticeable increase in the presence of "tissue" cells in preparations obtained with the dual enzyme protocol. Generally, the yield of adherent cells (on a per gram basis) determined in a CFU-F assay was higher with the dual enzyme treatment for both equine and canine samples compared to collagenase alone. Cells obtained from equine tissue were cultured with induction media and were found to differentiate into osteogenic and chondrogenic lineages. Furthermore, the cells were found to be positive for CD44, CD90, CD133, and ABCG2 (with anti-human monoclonal antibodies). These results support the use of a digestion protocol that includes both collagenase and hyaluronidase in producing cell preparations for therapeutic applications, since the cell yield was higher on average, the yield of adherent cells was higher for both canine and equine adipose samples and cells cultured from the preparations display markers indicative of MSCs and can be differentiated into osteoblasts and chondrocytes .

Ultrasonic treatment of human abdominal adipose tissue improves tissue viability *in vitro*

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Introduction: Autologous adipose tissue injection is an effective, minimally invasive and widely used method of soft tissue augmentation. Controlled retention of the implanted tissue is critical, especially when fat is placed into biomechanically sensitive areas such as the vocal fold. Therefore, methods that can improve the short and long term survival of grafted cells, such as adipocytes, preadipocytes and adipose derived stem cells (ADSCs), should improve fat graft stability and survival. Ultrasound treatment of cells and tissues has been shown to increase mass transport, which could benefit tissue grafts during the immediate post-implant period when blood supply to the implanted tissue is sub-optimal. In this study we show that indirect ultrasound stimulation of adipose tissue *in vitro* improves the ADSCs viability and survival.

Materials and Methods: Human adipose tissue from abdominoplasty surgeries was gently scraped with a scalpel to remove most of the fibrous component. The tissue was cultured for 7 days with or without indirect ultrasound treatment (Lysonix™ 2000, 22kHz). Metabolic rate, viability, histology and expression of molecular markers (Annexin V, aP2, CD105) by the adipose tissue were assessed.

Results: We first assessed whether the ultrasound treatment changes temperature and found that temperature was not increased in the culture media significantly (23±2°C). Viability of the fat cells was indirectly assessed by measuring glycerol-3-phosphate dehydrogenase (GPDH) released into the culture media. The GPDH activity in ultrasound treated samples was inversely related to ultrasound intensity. Ultrasound treatment significantly increased glucose consumption, suggesting that the treated cells were more metabolically active. Total RNA content of the ultrasound stimulated samples was comparable to the control. Real time PCR analysis demonstrated that in ultrasound stimulated samples, expression of the annexin V was not significantly increased. However, the CD105 expression was upregulated and the expression of a P2 was significantly down-regulated in ultrasound treated samples.

Conclusions: Our data suggest that ultrasound treatment might improve ADSCs viability and survival.

Analyzation of the cloning capacity of porcine adipose-derived adult stem cells

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Human adipose-derived adult stem cells (ADAS) are self-renewing, can be differentiated into multiple tissue lineages, and exhibit a high expression of both CD34 and CD44 stem cell markers. At this time, these stem cell characteristics have not been identified in porcine ADAS. The development of an animal stem cell model may enhance important biomedical applications, such as tissue engineering and cord blood transplants, which can be utilized in the human species. The objective of this study was to test the hypothesis that stromal cells isolated from porcine subcutaneous fat tissue contain a population of stem cell-like cells that have the capacity to self-renew. Ten grams of subcutaneous fat were collected from three mature female pigs, finely minced and washed with PBS containing 2% penicillin streptomycin (P/S) and 2.5 µg/ml Fungizone. The tissue was homogenized with 1% collagenase in PBS with 0.1% bovine serum albumin in a shaker incubator at 37°C for 2 h. The cell suspension was filtered and stained with Hoechst 33342 to determine the number of nucleated cells in the suspension. Cells were plated at 50 cells/ml in 10ml of DMEM supplemented with 10% fetal bovine serum, 1% P/S and 2.5 µg/ml of Fungizone in a 100mm dish and incubated at 39°C in 5% CO_2. Fresh medium was supplied every 3-4 d. Adherent cells formed colonies after 10 d in culture and were selected for ring cloning (passage 0) based on the following criteria: colonies contained at least 100 cells and colonies were located a minimum of one microscopic field from all other colonies. Cells were passaged by trypsinization and cultured in a 4-well dish (passage 1) and at 90% confluence passaged into a 6-well dish (passage 2), a T25 flask (passage 3), and a T75 flask (passage 4). Clonal analysis was performed on three cell lines from three mature female pigs and passage 4 was reached in ~23 d. From each line 12 clones were successfully isolated and expanded giving a total of 36 clones. Currently, multiple differentiated phenotypes of these clones are being assessed by changing culture conditions to medium demonstrated to induce differentiation. Stains specific for intracellular lipids, glycosaminoglycans and calcium deposition will be used to detect adipocyte, osteocyte and chondrocyte

differentiation, respectively. Many treatments have already resulted from the use of tissues generated through advancements made in the porcine animal model. Additional future biomedical applications include the use of these less differentiated cells as the donor cell in nuclear transfer. These cells could be more efficiently reprogrammed following fusion with an oocyte, thereby increasing nuclear transfer success rates and averting resultant anomalies. The true nature and potential of these cells must be determined to further develop this animal model and generate cutting edge techniques to be applied to the medical field.

Therapeutic protein secretion from genetically modified adipose tissue

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Adipose stromal cells (ASCs) isolated from fat are a readily available source of autologous stem/progenitor cells. ASCs exhibit pluripotency having been shown to develop into myogenic, adipogenic, neurogenic, osteogenic, chondrogenic endothelial and hepatic cell lineages *in vitro*. In addition, ASCs in culture include a significant percentage of highly proliferative cells enabling for rapid expansion of pluripotent cells *in vitro*. Several recent publications suggest their potential value as a source of autologous, adult progenitors for cell therapy applications regenerating damaged tissue.

Rather than using ASCs to repair damaged tissue, the goal of this study is to engraft genetically modified ASCs to secrete therapeutic proteins into the blood. In particular, can ASCs transduced with an expression cassette for adipocyte expressed coagulation Factor IX, form FIX secreting fat tissue and rescue hemophilia B individuals.

A human FIX cDNA was cloned downstream of the hCMV early promoter in a mammalian expression vector. The murine preadipocyte cell line 3T3-F442A and murine ASCs have been transfected with the expression plasmid to test for adipocyte expression of human FIX. In parallel, Rosa26 ASCs (and preadipocytes derived from the ASCs) are being transplanted into syngeneic recipients using several different matrix carriers with and without proangiogenic factors. The generation of adipose tissue from genetically modified donor ASCs secreting plasma FIX could lead to a cell therapy for hemophilia B. Given the ready availability of these cells, genetically modified, autologous ASCs may have broad therapeutic application for specific blood protein deficiencies by generating artificial adipose secretory tissue.

Differences in replication and differentiation of preadipocytes among visceral adipose tissue sites in baboons

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We measured differentiation and proliferation of preadipocytes isolated from omental, mesenteric, and retroperitoneal adipose tissue from 6 juvenile male baboons. Preadipocytes from second passage were plated in duplicates in 24 and 96-well plates at a density of 1 and 3 x 10^4 cells/cm^2 in DMEM/F12 medium enriched with 10% fetal bovine serum. Confluent cultures were induced to differentiate by switching to serum free DMEM/F12 medium supplemented with 10 mg/ml transferin, 33 µM biotin, 17 µM calcium pantothenate, 0.5 µM insulin, 0.1 µM dexamethasone, 2 nM triiodothyronin, 0.5µM Roziglitazone, and 540 µM IBMX for 11 days (the last two ingredients for 2 days only). Cells were lyzed and the activity of the enzyme glycerol-3-phosphate dehydrogenase (GPDH) was measured as a marker of differentiation. To mark dividing preadipocytes, subconfluent cultures were labeled with the thymidine analogue

BrdU for 12 h that was detected by immunofluorescence. In addition, nuclear content of growing cultures were measured at days 2 and 5 of last replating using CyQuant proliferation kit and the fold increase of nuclear content was calculated. The results from ANOVA followed by pairwise comparisons using Tukey adjustments are given in the table, in means ± SEM (different letters in the superscript indicate significant difference, $p < 0.05$). Our data suggest that adipose site may affect adipocyte precursor proliferation and differentiation in agreement with similar observations in rats and humans.

	Omental	Mesenteric	Retroperitoneal
GPDH, U/ml/min/g protein	2.5 ± 1.3^{a}	3.6 ± 1.6^{a}	9.3 ± 1.2^{b}
Nuclear content, x increase	$2.1\ \ 0.3^{a}$	1.7 ± 0.2^{ab}	1.2 ± 0.03^{b}
$BrdU^{+}$ cells, % of total cells	23.0 ± 5.4	25.6 ± 5.4	15.3 ± 5.4

The efficacy of BMP-4 as an osteoinductive agent for ASC-based pediatric craniofacial reconstruction

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Background: Pediatric craniofacial surgeons face a shortage of autologous bone for grafting in the repair of osseous defects after trauma or congenital anomaly reconstruction. In the pediatric population, an under-developed diploic space renders local calvarial bone graft sources non-existent. Practical concerns ranging from donor site morbidity to low tissue yield limit distant bone graft sources. Recent work by our group has evaluated the efficacy of a bone morphogenetic protein-2 (BMP-2) /adipose-derived stem cell (ASC)-based treatment paradigm for craniofacial reconstruction. While BMP-2 has been investigated as an osteoinductive agent in this context, less attention has been paid to BMP-4. The objective of this project is to broaden the surgeon's options by investigating the efficacy of BMP-4-osteoinduced ASCs for reconstruction of the craniofacial skeleton.

Methods: ASCs harvested from New Zealand White rabbits were cultured for four weeks with either control basal media or BMP-4-based osteoinductive media. Both media types were tested on cells in two-dimensional (2-D) and three-dimensional (3-D) culture. The 2-D culture was conducted in standard tissue culture-treated dishes, while the 3-D culture was conducted on fibrillar collagen sponges. After four weeks, the ASCs in 2-D and 3-D culture were analyzed for progression towards an osteogenic phenotype by polymerase chain reaction (PCR) for markers of osteogenesis (e.g. Runx2 and alkaline phosphatase) as well as by histology (e.g. alizarin red and alkaline phosphatase staining). Finally, the 3D scaffolds were subjected to Faxitron imaging to identify radio-opacities consistent with matrix calcification.

Results: Preliminary data from alizarin red and alkaline phosphatase staining are consistent with calcium deposition and alkaline phosphatase activity, respectively, in 2-D culture for the BMP-4 treated cells. These studies were negative in controls. Faxitron, histology, and PCR analysis is currently underway for the 3-D cultures.

Conclusions: Craniofacial surgeons' options for reconstruction of bony defects in the pediatric population are limited. While some progress has been made in evaluating the efficacy of BMP-2 / ASC-based therapies for this purpose, little attention has been focused on BMP-4 as an ASC-osteogenesis-enhancing agent. By meeting its objective of evaluating the efficacy of BMP-4 as an ASC-osteoinductive agent in 2-D and 3-D culture, this study may elucidate another option for the resolution of a difficult problem in pediatric craniofacial surgery.

Angiogenic potential of adipose-derived stem cells in murine flap model

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Introduction: Total or partial flap loss due to ischemia is occasionally encountered in reconstructive surgery. Previous literatures have shown that cell population isolated from adipose tissue has an angiogenic potential both *in vitro* and *in vivo*. In this study, we sought to determine if this cell population, Adipose-derived stem cells (ASCs), could selectively induce neovascularization and extend the survival area of skin flaps.

Materials and Methods:<Random pattern flap model> ASCs were isolated from ICR mice and expanded ex vivo for 3 passages. After the elevations of cranially based random pattern skin flaps, DiI-labeled ASCs were then injected subcutaneously into the flap. Millimetric measurements were taken at postoperative day 7. Specimens were harvested from the same position for histological analyses. Flaps with no ASC injection group were served as a control group. <Prefabricated flap model> ASCs were isolated from Wistar rats and expanded ex vivo for 3 passages. The superficial femoral artery and vein and the fascia over were used as a vascular crane. The full thickness skin graft was used for prefabrication. ASCs were injected to the fascia and to the vascular crane. The viability of the flaps was evaluated macroscopically, microangiographically and histologically.

Results:<Random pattern flap model> ASCs led to a statistically significant increase in flap viability in the experimental group by postoperative day 7. Histological examination also demonstrated statistically significant increase in capillary density in the experimental group in comparison with the control group. Moreover, some of the endothelial cells were stained positively for DiI which was labeled with ASCs.<Prefabricated flap model> Macroscopic evaluation revealed the viability of the transferred prefabricated flap in the first week in the experimental group, whereas the viability was encountered in the second week in the control group. Microangiographical and histological examinations demonstrated that abundant neovascularization was found in the experimental groups when compared to the control group.

Conclusions: These findings suggest that ASCs have a potential for enhancing the blood supply of murine skin flaps. The mechanism of this enhancement might be both the direct differentiation of ASCs into endothelial cells and the indirect effect of angiogenic growth factor released from ASCs.

Comparison of immunomodulation by untreated and cytokine treated human stem cells from adipose tissue andamniotic membrane

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Human adult stem cells (SC) isolated from sources like adipose tissue and amnion can be expanded in an undifferentiated state or differentiated along multiple lineages and are regarded to be promising candidates for regenerative medicine and tissue engineering. We have evaluated the *in vitro* immunomodulatory potential of human adipose-derived stem cells in comparison to human amniotic mesenchymal and human amniotic epithelial cells under identical experimental conditions. Peripheral blood mononuclear cell (PBMC) stimulated in mixed lymphocyte reactions (MLR) or in phytohemagglutinin activation (PHA) assays were cocultured with SC at different cell ratios and PBMC proliferation was evaluated. Additionally, the applied SC populations were pre-exposed to cytokines like interferon-γ and tumor necrosis factor-α.

All investigated SC inhibited activated PBMC proliferation in a cell dose-dependent manner in MLR (66-93% inhibition at equal amounts of SC and PBMC) and PHA assays (67-96% inhibition at equal amounts of SC and PBMC). The lowest effective SC to PBMC ratio was 1 to 8. The immunoinhibitory properties were independent of passage number (passage 2-6) but were significantly reduced by prior cryopreservation. Furthermore immunosuppression was not limited by the presence of proinflammatory cytokines. From these *in vitro* data we conclude that all three stem cell types may be considered for future allogeneic transplantation in cell therapy and regenerative medicine.

Safety of administration of adipose-derived stem and regenerative cells in the horse and the dog

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"First, do no harm." This statement is one of the founding principles of medicine. As with any medical technology, safety has the single largest impact on commercial, regulatory, and medical viability. Veterinarians in the United States have treated over 1,500 commercial orthopedic cases in western performance horses, race horses and show jumping horses and nearly 100 canine cases. The incidence of reported adverse events (AER) is less than 0.43% in the horse and 0.0% in the canine. No AER has resulted in death of a patient and most have clinical resolution with only transient injection site reactions. No adverse systemic effects have been reported with either local or intravenous administration of cells. This presentation will cover the key elements of the Vet-Stem adipose-derived stem and regenerative cell therapy safety profile, including (a) Processing Quality Assurance (QA) and Quality Control (QC) measures, (b) minimally-manipulated, autologous cell-based medicine, (c) controlled study data, and (d) field safety profile.

Clinical liposuction for collection of adipose-derived stem and regenerative cells in the horse and the dog

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Vet-Stem is a commercial adipose-derived regenerative cell therapy company. We have treated over 1,500 commercial orthopedic cases in western performance horses, race horses and show jumping horses and nearly 100 canine cases. Liposuction, as opposed to surgical lipectomy, provides a more cosmetically acceptable collection method. The techniques, efficacy of collection, and safety issues will be detailed along with the physiological and anatomical differences between species. Data on liposuction techniques including equipment, cell yields, and cell characterizations will be presented. Implications for the business model of delivery of adipose-derived regenerative cell therapy using tumescent liposuction will be discussed.

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