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Biotechnology in the AO Foundation

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The AO Biotechnology Advisory Board (BAB) was founded by the AO Academic Council (AcC) in 2003 to support AO in their Clinical Priority Programs in areas concerning biotechnology. AO's clinical research funded by the AO Research Board is increasingly emphasizing and applying biotechnology. Across the three clinical priority fields, new implant materials, and cell engineering for bone and cartilage healing and repair are frequently proposed. As most of this research comes from clinicians, most projects are driven towards clinical translation.

Biotechnology broadly represents diverse fields, including cell biology, biochemistry, materials science, drug delivery and pharmacokinetics, bioengineering and much more. As biotechnology continually and rapidly evolves, with important breakthroughs continually reported, no single expert or even small panel of experts can hope to accurately track all progress. Hence, tapping into the international external expert network is essential to provide an up-to-date and informed consensus that the BAB seeks in advising and evaluating AO research programs. Leaders within the AO Foundation recognize that various specialities are not sufficiently represented, requiring the foundation to find experts in these fields who additionally have connections to clinical specialities like trauma, CMF and spine. Exploiting synergies between research and clinical networks, high quality research programs are created to help to further improve the reputation of AO as clinical and research-driven foundation.

BAB has four main foci which include: (1) advising the AO Foundation on important biotechnology developments relevant to the clinical priority areas, (2) peer review and monitoring of all biotechnology research programmes and proposals submitted for AO support, (3) creation and funding of new research programmes through research calls that support AO Clinical Priorities, and (4) creation and maintenance of a network of external experts involved with biotechnological aspects of orthopedic medicine that provide an input on AO research programmes and directions, and link to existing AO networks.

BAB-funded projects seek to deliver an additional value by combining basic research knowledge and innovative approaches with the expertise from clinicians for possible use of theses approaches in clinical applications. BAB therefore promotes research and development synergies where they are most appropriate within the AO. Through such partnerships, clinical practice and patient care. can both benefit from new technologies. BAB will continually survey clinical priorities to attempt to produce new research calls that fit and partner into these clinical goals. Hence, both fundamental and applied research can be integrated across the AO, and biotechnology can be selected to fit as these priorities evolve. This is a challenging goal for a focused but open research foundation like the AO.

Within the last 3 years interfaces have been created by: a) the Academic Council with its suggestions for clinical priority programs in which BAB may recommend biotechnology as one research focus; b) the AO Research Board, in which projects already in the creation phase can be considered for potential to integrate biotechnology approaches, and c) BAB assistance with the AORF in reviewing biotechnology-related research projects.

The AO Foundation: ready for the future?!

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AO Research was founded on the collaboration between the clinician and the scientist to solve clinical problems. Müller understood the potential of internal fixation, Allgöwer understood the need to determine its biological basis and hence, AO made internal fixation a success based on excellent surgery and science. Over the years, basic research and development in fracture surgery has evolved. At the present, there remains an interest in mechanics of fracture stabilization but the future rests in biology and understanding conditions that affect bone strength. In the recent past, AO research has not maintained its focus on the collaboration between the clinician and the scientists. The Research Institute has been able to develop a strong basic science program in musculoskeletal research but the prime focus of solving clinical problems has been lost. To restore this important surgeon/scientist collaboration for the future, the Foundation has readied itself by prioritizing its research so as to focus its resources through its clinical priorities programs. The Biotechnology Advisory Board and a fair and equal peer review process for research within the Foundation have been established. The development of specialty academic councils will stimulate the clinician/scientist involvement through the clinical priority programs. With these changes, the AO Foundation's research program is now ready to tackle the future.

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Clinical Applications of Mesenchymal Stem Cells

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Human mesenchymal stem cells (MSC) have been isolated from various tissues including bone marrow, cord blood, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and deciduous teeth. These cells have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage and muscle. A great deal has been learned in recent years about the isolation and characterization of MSC. Several methods are currently available for isolation of the mesenchymal stem cells based on their characteristics. Because of the ease of their isolation, their multilineage potential and their extensive differentiation potential, mesenchymal stem cells are among the first stem cell types to be introduced in the clinic. MSC have generated a great deal of interest because of their potential use not only in regenerative medicine and tissue engineering, but also in the treatment of autoimmune diseases or even as an adjuvants in hematopoetic stem cell transplantation. Various studies – pre-clinical and clinical - illustrate their therapeutic value. Furthermore, MSC seem to be hypoimmunogenic and modulate lymphocyte function. Therefore, allogenic mesenchymal stem cells transplantation seems possible. It is envisaged that mesenchymal stem cells can be used in systemic transplantation for generalized diseases, local implantation for local tissue defects, as a vehicle for genes in gene therapy protocols or to generate transplantable tissues and organs in tissue engineering protocols. The results of these initial trials are very encouraging and several clinical trials are under way to study the efficacy and long-term safety of therapeutics based on mesenchymal stem cells.

Biotechnology in Trauma

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The musculoskeletal system has the potential to regenerate defect situations within an appropriate time. In some cases, however, this regeneration fails resulting in a delayed healing or non-union that requires further treatment. Delayed bone defect healing in Europe alone leads to socio-economic costs of up to €14.7 billion per year [1].
This fact demonstrates the need for alternative treatment options in defect regeneration of the musculoskeletal system.
Besides delayed healing implant related infections are a feared complication in orthopedic and trauma surgery with tremendous consequences for the patient. To reduce this risk, administration of perioperative antibiotic prophylaxis is a routine procedure in orthopedic surgery.
To intensify the research in this field in order to improve and simplify therapeutic procedures and develop new strategies for treatment and prevention, the WHO declared the "Bone and Joint decade 2000-2010".

Using different approaches the osteosynthetic implant can be more "biological".
Changing the structure and composition of the implant surface can result in a better osteoconductivity. Using biological factors, such as growth factors, the osteoinduction can be improved. And implant related infection might be prevented by the use of anti-infective surfaces.
The disturbance of the bone and the vascularization, however, makes a delivery of systemically applied substances to the defect side problematic. Therefore a controlled local delivery system is important for the optimal application of the therapeutic factors in trauma surgery.

To meet this requirement a new bioactive coating method for implants, which is based on a biodegradable poly(D,L-lactide) (PDLLA, coating thickness: 10 µm) was developed [2]. This coating allows the incorporation of e.g. growth factors and antibiotics. The implant therefore serves for stabilization and as a local drug delivery device. The effect of different growth factors such as IGF-I, TGF-ß1 and BMP-2 locally released from coated devices (intramedullary nails, plates and cages for spinal fusion) on bone healing was investigated in different animal models [3-6]. The radiological, biomechanical and histological examinations revealed a stimulated healing in the growth factor treated animals.
More detailed studies showed an enhanced cell proliferation and maturation in the growth factor treated fractures.
The results demonstrate that the local growth factor application enhances bone regeneration in the early phase without alteration of the physiological healing process.

Using a new developed non-viral gene therapy for local transfection of cells with BMP-2 coding plasmids from coated implants, a high transfection rate and stimulating effect on fracture healing could be demonstrated in first experimental studies. The effect of plamid transfection was as high as the effect of the protein application.

To optimize prophylaxis for implant associated infections the above mentioned local delivery system was used for the application of antibiotics [7].
In a rodent animal experiment the efficacy of local prophylaxis was investigated. The medullary cavities of rat tibiae were contaminated with *Staph. aureus* and titanium Kirschner wires were implanted. For local antibiotic therapy the implants were coated with PDLLA + gentamicin. All animals treated without local application of the antibiotic developed an osteomyelitis and all cultures of implants were tested positive on *Staph. aureus* 42 days after surgery. The local application of gentamicin delivered from the PDLLA coating reduced significantly the signs of osteomyelitis in all animals and three of ten implants remained sterile in the microbiological analysis.

This technique was transferred already to clinic. Patients with open tibia fractures were treated with PDLLA and Gentamicin coated implants. So far, onset of infection could be prevented using this technique. Further osteosynthetic implants will be coated in the close future.

In conclusion, the bioactive coating of mechanical well established implants could firstly stabilize the fracture and secondly serve as a local drug delivery system. The use of gentamicin coated tibial nails is approved in Europe and Canada and the first patients have been treated.

The biotechnological approach, however, can not substitute a well planed and performed surgery with the use of the optimal stabilization system.

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Strategies to Reconstitute the Proximal Femur after Failed Total Hip Replacement

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Background and Introduction:

The frequency of surgical reconstruction of a failed total hip replacement (THR) is increasing and is currently estimated to represent close to 10% of all THRs performed. This revision procedure remains a surgical challenge, as there often exists substantial associated bone loss. Impaction allografting (IA) is a revision technique where bone graft particles are used in an attempt to reconstitute a deficient proximal femur or acetabulum. Clinical results with IA have been variable, with some authors reporting massive subsidence of the femoral stem. This may be due to the fact that the graft does not appear to remodel completely into new live bone. Over the past several years, our group has developed a research program to characterize the IA procedure from anatomical, mechanical, and biological perspectives. Furthermore, we have begun research that aims to optimize the current surgical procedure and develop more innovative solutions through the use of novel biomaterials. This presentation will outline our previous work on this topic and our current status.

In-Situ Crosslinkable Osteoinductive Poly(lactide) Scaffold for Bone Regeneration

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Background in scaffolds for bone regeneration: Current clinical methods of treating skeletal defects involve bone transplantation or the use of other materials to restore continuity[1]. Autologous bone graft has been the gold standard of bone replacement because it provides such essential elements as osteogenic cells, osteoinductive factors, and an osteoconductive matrix for healing. However, the limited supply of autograft bone, donor site morbidity, and the long recovery time for segmental defects restrict its use in bone repair. Allograft bone, although available in abundant supply, has drawbacks that include reduced rates of graft incorporation compared to autograft bone. Furthermore, the long recovery time for segmental defects or partial recovery in the case of non-unions has prompted researchers to look for alternative bone grafts to accelerate the rate of fracture healing.
rhBMP-2 delivered in a biodegradable carrier has been demonstrated to accelerate the repair of bone defects[2]. Currently the most appropriate carrier for rhBMP-2 delivery is not determined. Recent studies demonstrate that implants made from collagen/gelatin or PLGA sponge soaked in rhBMP-2 have faster rate of fusion compared to autologous iliac bone graft[3]. The drawback of collagen/gelatin sponge is soft tissue compression which prevents bone induction at standard rhBMP-2 doses. On the other hand, proteins can not be immobilized in PLGA sponge because the sponge is fabricated by casting from organic solvents. Since proteins such as rhBMP-2 have significant solubility in organic solvents, a large fraction of the protein is lost or deactivated. We propose a novel carrier based on stabilization of rhBMP-2 in biodegradable poly(lactide-ethylene oxide-fumarate) (PLEOF) hydrogel microspheres and embedding of the microspheres in the *in-situ* crosslinkable poly(lactide fumarate) (PLA) scaffolds.

Rational to use *in-situ* crosslinkable poly(lactiede) scaffold: It is well established in preclinical or clinical studies that rhBMP-2 delivered in a biodegradable carrier accelerates the repair of bone defects. Implants made from PLGA sponge soaked in rhBMP-2 solution have faster rate of healing compared to autologous iliac bone graft. However, rhBMP-2 can not be immobilized directly in PLGA sponge because organic solvents which deactivate the protein are used in the fabrication of the sponge. Biodegradable *in-situ* crosslinkable hydrogels, due to their high water content, are ideal for immobilization of proteins[4]. A logical question is: Can injectable *in-situ* crosslinkable PLAF embedded with rhBMP-2 loaded hydrogel microspheres be used as an osteoinductive scaffold for repairing bone defects?

The hypotheses of this study include 1) that rhBMP-2 encapsulated in degradable PLEOF hydrogel microspheres retains its activity, 2) rhBMP-2 is released from the microspheres into the pore volume of the PLAF scaffold in therapeutic concentrations, and 3) the rhBMP-2 released from the scaffold promotes differentiation of BMS cells to osteoblasts and formation of mineralized matrix *in-vitro* and *in- vivo*.
Recently, in our laboratory, we have developed a novel method to encapsulate rhBMP-2 in hydorgel microspheres by emulsion crosslinking of a gel phase in mineral oil. The hydrogel microspheres are then lyophilized to a free-flowing dry powder. Since proteins do not have appreciable solubility in mineral oil, very high encapsulation efficiency can be obtained with this method. Subsequently, the dry hydrogel microspheres are embedded in a poly(lactide fumarate) (PLAF) based matrix, developed in our laboratory, to form biodegradable *in-situ* crosslinkable osteoinductive scaffolds for bone regeneration. The PLAF macromer is synthesized from poly(lactide) which is approved by FDA for certain clinical applications and fumaric acid (a substance that occurs naturally in the Kreb's cycle). We hypothesize that rhBMP-2 encapsulated in hydrogel microspheres retains its activity and is released in therapeutic concentrations to promote differentiation of bone marrow stromal (BMS) cells to osteoblast lineage and to accelerate the formation of mineralized tissue. rhBMP-2 is encapsulated in degradable poly(lactide-ethylene oxide-fumarate) hydrogel microspheres, embedded in PLAF scaffold, and its release kinetics is measured by enzyme-linked immunosorbent assay (ELISA). Porous PLAF scaffolds with well-defined pore geometry are seeded with bone marrow cells isolated from rats. The constructs are cultured *in-vitro* or implanted in segmental femur defects of rats to assess the extent of mineralization and bone formation. The *in-vitro* effect is evaluated using cell count, alkaline phosphatase activity, and mineralization. The *in-vivo* effect is evaluated radiographically and histologically.

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Compression enhances cell distribution and scaffold stability of osteochondral matrix constructs

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INTRODUCTION
Homogenous cell distribution and sufficient initial scaffold stability remain key issues for successful tissue engineered osteochondral constructs. The purpose of this study was to investigate the application of initial compression forces of cell culture followed by different stress patterns.

METHODS
Bone marrow stromal cells were harvested from the iliac crest during routine trauma surgery. The cells were expanded in a 2-dimensional culture and then seeded into the biologic hybrid scaffold with a concentration of 1x10E6 cells per ml. Pressure and vacuum forces were applied in a specially developed glass kit. The constructs were exposed to two different protocols of compression combined as oteochondral matrices of CaReS (rat collagen I, Ars Arthro, Esslingen, Germany) and Tutobone (bovine acelluar spongiosa, Tutogen Medical GmbH, Neunkirchen a. Br., Germany). Controls were resected osteochondral fragments from patients with articular fractures and uncompressed constructs. These effects were evaluated using microscopy to identify matrix penetration and vitalitity. Biomechanical tests were conducted, too using a modified biomechanical testing machine. The ‘constrained compression’, maximum load to failure, modulus, and strain energy density were determined.

RESULTS
Histology: Penetration and cell distribution was demonstrated homogenous and vital, respectively. Mechanical tests showed a significant enhancement of primary matrix stability. The following stress patterns did not enhance significantly stability over seven days.

DISCUSSION
The application of mechanical stimulation in the tissue engineering process leads to a progress in the structural and biomechanical properties of these tissues and offers new possibilities in the management of bone injuries and degenerative diseases. The influence on mesenchymal stem cell differentiation and in vivo cell viability have to be investigated in the future.

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Tissue Engineered Bone Replacements Systems

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Background and Introduction: Over one million operations for the repair of orthopedic irregularities such as trauma, fracture nonunions, and congenital defects are performed annually in the United States. Of these procedures, roughly 275,000 involve the use of bone grafts to assist the healing process [1]. Traditionally, bone grafts have been the material of choice for managing the repair of bone defects. Nearly $300 million is spent annually in the United States on bone grafts and bone substitutes [2]. Grafts and other materials currently used for the treatment of orthopedic defects have select advantages, but autografts and allografts are deficient in several aspects. Autografts suffer from donor site shortage and surgery at the donor site often produces complications such as donor site damage, pain, infection, and hematoma [3-5]. Sterilization of allografts can lead to a lessening of the allograft's osteoinductivity or to a reduction in strength [6]. Avascular autografts and allografts remodel slowly due to lengthy vascularization times; as a result, healing of the defects is highly unpredictable [7]. Neither autografts nor allografts can be easily shaped to fit a bone defect, particularly under operating room conditions, and incomplete resorption of the graft may occur [8]. As a result of the drawbacks associated with the use of traditional grafts, efforts to tissue engineer a more desirable bone substitute are underway.

Rational: A composite of demineralized bone matrix (DBM) and polylactide may be useful as a tissue-engineered bone substitute. Polylactide and DBM are both biocompatible and osteoconductive. Polylactide beads are appealing because characteristics such as their degradation profile, mechanical properties, biological interaction, and their capacity to deliver growth factors or drugs can be customized to fit particular applications. DBM fragments are desirable because they contain osteoinductive BMPs that are delivered naturally. The osteoinductivity and mechanical properties of DBM can be tailored to suit specific applications by carefully monitoring the level of demineralization. A composite containing polylactide beads and DBM fragments may be advantageous in that polymer beads having different functions such as cell delivery, drug delivery, and growth factor delivery could be used simultaneously with DBM fragments, thereby providing different mechanical properties and levels of osteoinductivity for the treatment of specific orthopedic defects or conditions. Thus, it is feasible to investigate the potential for a composite system consisting of DBM fragments and polylactide beads to support the attachment and proliferation of mesenchymal stem cells and to provide a structure for the cells' differentiation into the osteoblast lineage.

Methods:
Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to monitor the expression of select osteoblastic genes by D1 and MC3T3 cells cultured on specific composite systems. The sytems consisted of 100% DBM, 70% DBM:30% PL, 50% DBM:50% PL, and 100% PL. On days 4, 13, 24, and 36, the designated RT-PCR samples were rinsed twice gently with PBS. Ribonucleic acid (RNA) was then isolated from the samples at each timepoint using an RNeasy® Mini Kit (QIAGEN) and following the manufacturer's protocol. The purity and concentration of the isolated RNA was determined using an RNA 6000 Nano Assay Kit (Agilent Technologies) and following the manufacturer's protocol. Primer pairs were chosen to test for the expression of four mouse target genes in the RNA samples: osteoblast-specific genes including bone sialoprotein (BSP), osteocalcin (OCN), and runt-related transcription factor 2 (Runx2); and an endogenous control gene of β–actin (β-ACT).

Results: The level of BSP expression in D1 cells increased significantly ($p < 0.05$) between day 13 and day 24 for the 70/30 and 50/50 mixtures; BSP levels increased significantly ($p < 0.05$) between day 13 and day 36 for 100DBM and between day 4 and day 24 for 100PL. On days 4 and 13, BSP expression by D1 cells on 100PL was significantly higher ($p < 0.05$) than expression on 100DBM. By day 24, BSP expression by D1 cells on 100PL was significantly higher ($p < 0.05$) than expression on the other three mixtures.

Levels of Runx2 expression in D1 cells increased significantly ($p < 0.05$) between day 4 and day 24 for all mixtures. With the exception of the 50/50 mixture on day 13, Runx2 expression by

D1 cells on 100PL was significantly greater ($p < 0.05$) than expression on the other three mixtures on days 4, 13, and 24.

The level of OCN expression by D1 cells increased significantly ($p < 0.05$) (and exponentially) between day 13 and day 36 for the 70/30 and 50/50 mixtures; the OCN level increased significantly ($p < 0.05$) between day 13 and day 24 for 100PL. OCN expression by D1 cells on 100PL was significantly higher ($p < 0.05$) than expression on the other three mixtures on days 4, 13, and 24. By day 36, OCN expression in D1 cells on 100PL was significantly greater ($p < 0.05$) than expression on 100DBM only.

The level of BSP expression by MC3T3 cells decreased significantly ($p < 0.05$) between day 4 and day 13 for all DBM-containing mixtures. For the 100PL mixture, the BSP expression increased significantly ($p < 0.05$) between days 4 and 13. On day 4, BSP expression by MC3T3 cells on 100DBM was significantly higher ($p < 0.05$) than expression on the PL-containing mixtures. By days 24 and 36, the expression of BSP in MC3T3 cells on 100PL was significantly greater ($p < 0.05$) than expression on the DBM-containing mixtures.

Levels of Runx2 expression by MC3T3 cells increased significantly ($p < 0.05$) between day 4 and day 36 for the 100DBM and 70/30 mixtures. The Runx2 expression by MC3T3 cells on 100PL was significantly higher ($p < 0.05$) than expression on the DBM-containing mixtures on days 4, 13, and 24.

The level of OCN expression by MC3T3 cells increased significantly ($p < 0.05$) between days 24 and 36 for 100DBM and between days 4 and 36 for the 70/30 mixture. For the 50/50 and 100PL mixtures, OCN expression by MC3T3 cells increased significantly ($p < 0.05$) between days 13 and 24, but OCN expression decreased significantly ($p < 0.05$) between days 24 and 36; a similar pattern was observed for Runx2 expression. On days 13 and 24, OCN expression by MC3T3 cells on 100PL was significantly greater ($p < 0.05$) than expression on the DBM-containing mixtures. The large increase observed in OCN expression with time for D1 and MC3T3 cells signifies that extracellular collagen was becoming mineralized.

Discussion and Conclusion: A battery of qualitative and quantitative analytical methods indicates that D1 mouse marrow stromal cells attach, proliferate, and differentiate on four specific mixtures of DBM fragments and PL beads. Greater amounts of cell attachment occurred on DBM fragments than on PL beads due to factors that may include: inconsistent hydrolyzing of the PL beads with EtOH, more favorable conditions for serum coverage of DBM, mechanical agitation of the medium, and contact of bead surfaces with other substrates. Larger cell populations led to increased amounts of lactic acid production and more acidic medium in the DBM-containing mixtures. The cellular lactic acid buildup exceeded the medium's buffering capacity, as evidenced by the distinct color change in the medium. Despite the inhibiting effects of acidic medium on osteoblast differentiation, D1 and MC3T3 differentiation occurred on all mixtures; however, ALP activity and gene expression levels were higher on 100PL. With medium having a more neutral pH level, the 100PL mixture may have provided the best culture environment for D1 differentiation to occur. Similar studies with lower cell densities and the addition of HEPES buffer to the culture medium should be performed in an effort to keep the extracellular pH at a physiological level. Finally, studies should be conducted without osteogenic medium supplements to determine if D1 differentiation can occur solely as a result of the osteoinductive factors in DBM.

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Strategies for Segmental Bone Repair

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Background: The management of large bone defects caused by trauma, degenerative disease, or tumor resection is one of the most challenging problems faced by orthopaedic surgeons (Rommens *et al.*, 1989). Autogenous bone grafting is currently the clinical standard for osseous reconstruction. However, the use of autograft bone has several disadvantages including limited available tissue for transplantation, lack of structural integrity to withstand functional loads, and increased patient morbidity at the site of harvest. Because of these limitations, structural allografts are increasingly being used clinically. Allografts provide structural integrity in the short-term but are associated with a high rate of complications and late fractures. The 25 – 35% failure rate of allografts due to nonunions and fractures 1-2 years after implantation is directly associated with their recognized lack of ability to revascularize and remodel (Berrey *et al.*, 1990; Koefoed *et al.*, 2005). These problems have led to the search for improved methods for stimulating bone repair. We recently collaborated on a study led by Drs. Regis O'Keefe and Xinping Zhang that showed the reduced healing potential of allografts is due to the absence of cellular activity and that revitalization of allografts via incorporation of an engineered periosteum containing BMP-2 expressing mesenchymal progenitor cells significantly improves allograft incorporation (Zhang *et al.*, 2005).

Tissue engineering strategies may therefore be used to improve graft repair or alternatively to develop bone graft substitutes. The basic elements required for successful bone repair include an extracellular matrix scaffold, cells, a vascular supply, and osteoinductive factors (Bruder and Fox, 1999). If these elements are not available from tissues surrounding the injury site, they may need to be provided in some combination within an implantable tissue-engineered construct. The transformation from bone grafting to bone tissue engineering began with the introduction of osteoconductive bone graft substitutes or scaffolds and is now evolving to include local delivery of osteogenic cells and bioactive factors. Osteoconductive scaffolds facilitate invasion of capillaries, attachment of osteoprogenitor cells and subsequent appositional mineralized matrix formation in large bone defects. In addition to the scaffold material, microarchitectural parameters, such as porosity, pore size, interconnectivity, surface morphology, and anisotropy, strongly influence the mechanical properties of, and biological responses, to porous scaffolds (Lin *et al.*, 2003). Scaffolds may be used to locally deliver osteoinductive or angiogenic proteins or genes. A major remaining barrier to the use of bioactive factors for tissue regeneration has been the identification of effective and safe doses and delivery methods. However, sophisticated temporal and spatial release strategies may ultimately overcome these limitations (Rose *et al.*, 2004). Cell-based strategies for engineering bone regeneration involve the implantation of differentiated osteoblasts or osteoprogenitor cell populations, derived from a growing number of tissue sources. Cellular augmentation may be especially important for difficult clinical cases involving older patients, smokers, patients receiving chemotherapy or radiation, and patients with severely damaged wound beds or metabolic diseases in which the endogenous cellular supply may be diminished (Bruder and Fox, 1999).

Strategies to Augment Bone Repair Scaffolds: We have established a challenging 8 mm rat segmental defect model to test different strategies for inducing functional bone repair. The model facilitates use of micro-CT imaging to quantify 3D ingrowth of bone and vascularity and mechanical testing to evaluate functional integration. Using these models and methods, three distinct strategies are being investigated involving the delivery of proteins, cells, or genes. For protein delivery, our goal is to identify combinations of growth factors and/or sustained delivery strategies that induce functional bone repair at lower and therefore safer and less costly doses. Studies involving protein release from RGD alginate and nanoparticles will be presented. For cell delivery, we seek to

quantitatively evaluate the ability of different cell sources to promote bone repair, with and without in vitro predifferentiation. In addition to marrow-derived cells, we are evaluating the osteogenic potential of amniotic fluid derived cells both in vitro and in vivo. Finally, our gene delivery strategy is to use an rAAV coating approach developed by Dr. Edward Schwarz at the University of Rochester to augment the bioactivity of bone repair scaffolds. This approach has recently been shown to improve structural allograft healing (Koefoed *et al.*, 2005).

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Biphasic constructs for cartilage repair

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Damage to articular cartilage, either by trauma or disease, can affect joint function as adult articular cartilage has a limited capacity for repair. Currently resurfacing the articulating surfaces of synovial joints with synthetic prostheses still represents the optimal treatment for end-stage disease.[1] Although primary joint replacements have shown reasonable success rates, these have their limitations as failure rates of up to 20% after 10 years have been reported depending on the type of implant.[2] It has been speculated that all joint replacements made of synthetic materials will need replacement if the patient lives long enough. Furthermore, this treatment is not appropriate for focal defects. Recent efforts have focused on developing new treatments that result in biological repair and preclude the need to use non-degradable alloplastic implants. One of these approaches entails using tissue engineering methods to regenerate articular cartilage.[3] However, the bearing surface of synovial joints is comprised of a layer of articular cartilage which is integrated with the underlying subchondral bone by mechanical interlocking. Recreation of this cartilage (soft tissue)-bone (hard tissue) interface is a major problem for bioengineering tissues for repair or replacement of joint surfaces.

Although a variety of methods have been proposed to repair osteochondral defects, the approach we have developed to bioengineer these articulating surfaces is to generate biphasic constructs composed of cartilage tissue overlying and integrated with a substrate that serves as the bone interfacing component ("osteochondral-type" biphasic constructs).[4] We have developed a porous biodegradable ceramic substrate, composed of calcium polyphosphate (CPP), which has mechanical properties approximating cancellous bone and is suitable to use as a bone substitute material.[5] To generate the biphasic construct articular chondrocytes are placed on the intended articulation surface of the CPP substrate and grown in culture for up to 8 weeks. As the cartilage forms *in vitro* the developing tissue fills the pores that open to the surface and in this way integrates with the top portion of the substrate. Biphasic constructs (4 mm diameter by 6mm long) were implanted into focal defects created within sheep knees (trochlear groove). The implants successfully integrated with host tissues (bone and cartilage) with maintenance of the implant cartilage after 9 months *in vivo*, confirming the validity of our approach. The repair cartilage which had been subjected to the forces of normal activity matured significantly between 3 and 9 months as evidenced by the increase in mechanical properties, tissue thickness and collagen content. Bone grew into the pores of the CPP and there was no adverse reaction. In some regions the cartilage had fused with bone suggesting the possibility of recreating a natural subchondral interface.

The use of biphasic constructs to repair focal articular surface defects holds great promise. More importantly, after appropriate modification, they will also be suitable to use as a biological surface replacement which should prevent many of the problems associated with nondegradable prostheses.

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Gene therapy for the regeneration of traumatic articular cartilage defects

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Traumatic focal defects of articular cartilage, those who penetrate the subchondral bone (osteochondral defects), heal with a repair tissue that degenerates in the course of the time. No clinically available treatment leads to complete and durable cartilage regeneration. Although the concept of gene therapy for cartilage damage appears elegant, current research indicates that an adaptation of gene transfer techniques to the problem of a circumscribed cartilage defect is required in order to successfully implement this approach. In particular, the localized delivery into the defect of therapeutic gene constructs is desirable. Current strategies aim at inducing chondrogenic pathways in the repair tissue that fills such defects. Among the most studied candidates, polypeptide growth factors have shown promise to enhance the structural quality of the repair tissue. Our group investigates the regulation of chondrogenesis in cartilage defects. We evaluated different therapeutic candidates in two- and three-dimensional systems of chondrogenesis *in vitro*. Using an osteochondral defect model in the rabbit knee we showed that nonviral overexpression of a human insulin-like growth factor I (IGF-I) and fibroblast growth factor 2 (FGF-2) cDNA by transplanted articular chondrocytes encapsulated in alginate spheres improved articular cartilage repair and accelerated the formation of the subchondral bone compared to control implants. In addition, the direct application of recombinant adeno-associated virus (rAAV) vectors to sites of cartilage damage allows for efficient and sustained transgene expression. We could further demonstrate that rAAV-mediated overexpression of FGF-2 is sufficient to significantly improve the overall repair, filling, architecture, and cell morphology of osteochondral defects in rabbit knee joints. These data demonstrate that implantation of transfected articular chondrocytes encapsulated in alginate spheres into deep articular cartilage defects augments cartilage defect repair *in vivo* via stimulation of chondrogenesis. The data also provide a basis for rAAV application to sites of articular cartilage damage to deliver therapeutic agents that promote cartilage repair. These results suggest that therapeutic growth factor gene delivery using either encapsulated and transplanted genetically modified chondrocytes or direct rAAV gene vectors may be applicable to sites of focal articular cartilage damage. A better understanding of the basic scientific aspects of cartilage defect repair, together with the identification of additional molecular targets and the development of improved gene-delivery techniques, may allow a clinical translation of gene therapy for cartilage defects.

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PHOTO-CROSSLINKING COLLAGEN GEL FOR TISSUE ENGINEERED CARTILAGE

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INTRODUCTION:

Tissue engineering is a novel approach for regenerating articular cartilage using three-dimensional biomaterials as cell scaffolds. Collagen is one of the ideal natural materials that can be used as a scaffold because of its biodegradability. However, collagen gels can shrink when seeded with cells and may not allow the construct to fit the shape of a cartilaginous defect. Therefore, integrity between the implanted construct and adjacent tissues may not be obtained.

Kochevar et al. reported that type I collagen can be crosslinked using photoreactive dyes, such as Rose Bengal or Riboflavin, and exposure to visible light. *In situ* gel crosslinking could induce molecular interactions with the native cartilage surrounding the lesions to stabilize the gel during cartilage formation. Based on these data, the governing hypotheses of this work are: 1) Photochemical crosslinking can be used to generate a hydrogel that permits chondrocyte encapsulation; 2) Chondrocytes in the hydrogels have the capacity to form neocartilage with improved biochemical properties for cartilage repair in the knee; 3) The neocartilage will integrate with existing native tissues forming.

METHODS:

The following experiments were performed using swine (aged 3-6 months) articular chondrocytes as cell source and male athymic mice (aged 6-10 weeks) for implantation. All animal experiments were approved by the IACUC.

Chondrocyte Isolation: Swine cartilage tissues were harvested from the shoulder and knee joints. They were cut into small pieces, washed, and digested for 16 to18 hours at 37°C in 0.05% collagenase solution.

Experiment I

In order to determine optimal dose of the photo-initiator, Riboflavin, and visible light, *in vitro* cellular viability was performed. Macroscopic pictures were taken to examine whether shrinkage of the collagen gels was suppressed.

Photoencapsulation of Chondrocytes and Culture: Single passaged chondrocytes were resuspended in various test concentrations of Riboflavin solution (0.1–1 mM). Subsequently, the cell suspension was mixed with an equal volume of 0.5% type I collagen solution. The suspension with a final cell concentration of 40×10^6 cells/ml was poured onto 6 well culture plates and photocrosslinked using various irradiation test doses of visible light. Table 1 shows the combination of different factors that was tested to generate photocrosslinked collagen hydrogels. Control samples were not subjected to irradiation. The constructs were cultured up to 10 days for *in vitro* study using HAM-F12 supplemented with 0.05% ascorbic acid, 10 mM L-glutamine, 0.01 M MEM non-essential amino acids, 1% penicillin-streptomycin, and 10% fetal bovine serum. The *in vitro* constructs were incubated at 37°C in a humid environment with 5% CO_2. Medium was replaced every 2-3 days.

Encapsulated Chondrocyte Viability (N = 5): DNA content analysis was performed as relative cell number using Pico Green fluorescent procedure at day 0, 1, 3, and 7 of the culture. Samples were digested in a papain solution for 15 hours at 60°C to extract DNA. The dye was added to the extracted DNA and fluorescence emission was measured.

Table 1. Combination of factors for photocrosslinked collagen hydrogels

Concentration of Collagen	Photoinitiator	Irradiation Time
Type I collagen (0.5 %)	Riboflavin (0.1 – 1 mM)	Light: λ= 440-500 nm (40 – 300 sec)

Experiment II

Implantation into mice (N=8): The samples were implanted on the dorsum of mice and harvested at 6 weeks to determine whether this novel method would allow the construct to make hyaline cartilage in the *in vivo* environment.

Histological and immunohistochemical evaluation: The samples were fixed in 10% formalin for 24 hours, embedded in paraffin and sectioned. Tissue sections were stained with hematoxylin and eosin to observe chondrocyte morphology and distribution, and Safranin-O was used to study the distribution of GAGs. For immunohistochemistry, sections were stained with primary antibody against swine type II collagen.

Experiment III

To investigate integrity of newly formed tissue to native hyaline cartilage, vital cartilage chips were mixed with the gel and examined histologically and immunohistochemically.

Encapsulation of chondrocytes with native cartilage chips (N=8): Vital native cartilage was minced into 2mm in average diameter. These minced chips were mixed into the gel before the irradiation.

RESULTS:

Experiment I

DNA assay revealed that higher relative cellular number was acquired in the combinations of Riboflavin/irradiation-time at 0.25mM/40 sec. Under this condition, the relative cellular number was 195.9 ± 24.6% at day 7 of culture (Fig 1). Macroscopic pictures demonstrated that this crosslinking technology could prevent shrinkage of the collagen gel.

Experiment II

Figure 2 demonstrates that newly formed tissues included glycosaminoglycan (GAG) and type II collagen, which indicated hyaline cartilage.

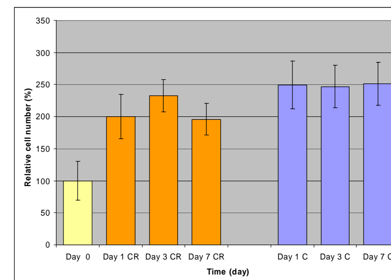

Fig 1: Relative cell number (%) = given cell No×100/initial cell No. CR indicates experimental group. C indicates control group.

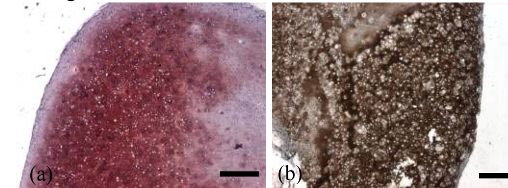

Figure 2: (a) Safranin-O staining; (b) Immunostaining for type II collagen. Bar scale is 200μm.

Experiment III

Figure 3 demonstrates a newly formed tissue was integrated with adjacent native cartilages.

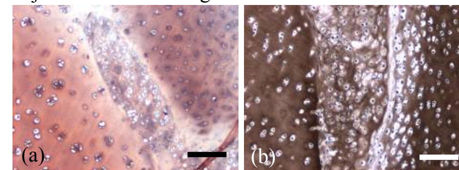

Fig 3: (a) Safranin O staining. (b) Immunostaining for type II collagen. Neocartilage was observed between native tissues. Bar scale is 100μm.

DISCUSSION

This would allow cell-collagen constructs to regenerate hyaline cartilage and to integrate with native cartilage in mice. Additionally, this novel photocrosslinking technology for collagen gel may prevent the gels from shrinking. Although the regeneration occurred in subcutaneous space of mice, which is not a natural environment for articular cartilage, this study encourages further study in large animal joint models.

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Numerical Simulation of Functional Tissue Engineering for Articular Cartilage

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INTRODUCTION: Mechanical pre-conditioning of implantable chondrocyte-seeded constructs for cartilage repair is of increasing clinical and scientific interest. However, the determination of the 'correct' parameter setting is still object of contrary discussion. Therefore, in this study, a coupled experimental-numerical procedure has been performed to correlate the spatial distribution of biological activity with the development of selected mechanical field variables.

MATERIALS & METHODS: To determine the mechanical loading conditions for a single cell, and deducing from the cell response the prediction of local cell stimulation, a multiscale finite element approach (FEA) has been chosen. Starting with a macro-model of the scaffold-cell construct, the local load history of a selected element of the FEA macro-mesh provided the boundary conditions for a micro-model with a single cell and its neighborhood. Using the biphasic, poroelastic features of a commercially available FEA-code, the viscoelastic effects of the porous polyurethane (PU) scaffold were implemented using a newly developed non-linear bimodular hyperelastic function with a special structural tensor modeling the anisotropic behavior of the solid phase[1]. Further, an incorporated stochastic "tissue growth function" helped to determine the time course as well as the spatial distribution of matrix development.

Experimentally, the cylindrical PU scaffolds were seeded with bovine chondrocytes[2]. The scaffolds were hold in culture for three days under various loading and boundary conditions to study the effects on cell response. Loading was performed with a recently developed bioreactor[3]. To localize regions with biological activity, the scaffold was cut into different spatial segments. A biochemical analysis of mRNA expressions of important cartilage genes[4] was then performed for each segment separately.

RESULTS: Boundary conditions and load frequencies affected the results globally and especially locally. The spatial distribution of gene expression of collagen I, II, aggrecan, COMP, HAS, and PRG4 as well as their time dependent development showed partially different, in some cases even contrary trends. Comparing the spatial distribution of cell messages with the numerically detected development of several mechanical field variables, information about possible mechanical stimuli can be provided. It was shown that the fluid flow and the distribution of the pore pressure as well as its gradient essentially depend on the permeability of the construct. The dependency on load velocity in the range of frequencies under consideration is less distinct.

CONCLUSIONS: In summary, the observed variations in mRNA expressions indicate a gene specific metabolic cell response to different mechanical stimuli. A coupled experimental-numerical procedure would allow a time dependent analysis of process parameters during construct conditioning in tissue engineering in future studies.

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A comparison between autologous chondrocyte implantation and cartilage progenitor therapy in the healing of chondral defects.

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Arguably, the 'gold-standard' of the biological repair of chondral defects is antilogous chondrocyte implantation (ACI). However, a major limitation of the technique is that the size of the defect to be treated is limited by the amount of chondrocytes that can be generated that maintain chondrogenic potential. In humans, chondrocytes expanded in monolayer culture lose their chondrogenic potential after around 7 population doublings. We have isolated a progenitor cell population from articular cartilage that maintains chondrogenicity through over 40 population doublings thus generating ample cells for potential repair application and for treating much larger defects.

We have initiated a large animal study to compare ACI with cartilage progenitors in a chondral defect model in goats. We have conducted and completed a pilot study and the main study that will last 20 months is underway. I shall present the data from the pilot study together with the design of the main study. I will also briefly describe our work in humans that shows that similar cells exist in adult human cartilage.

Silk fibroin as an adaptable 3-D scaffold for defect repair in subchondral bone

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Background: Fractures and bone defects in the subchondral bone have a common problem regarding bone healing: the balance of bone homeostasis is disturbed and even in the presence of autogenous bone grafts or synthetic bone replacements, newly formed bone may be resorbed, cyst formation may develop and on the long run collapse of the overlying articular surface and/or degeneration of the articular cartilage may occur[1, 2]. There is evidence that in subchondral bone the stiffness geometry and structure of the applied synthetic material as well as the local inflammatory response of the tissue to the surgical trauma play a major role in the failure of bone healing and subsequent degeneration of the articular cartilage. The aim of the first part of this study was to demonstrate that through adaptations of geometrical, mechanical and structural properties of a 3-D silk scaffold to the local requirements of the subchondral bone in the proximal tibia in sheep *i)* bone healing could be achieved, and *ii)* degeneration of overlying cartilage could be avoided. In the second part of the study biocompatibility issues of the silk scaffold were addressed by comparing different preparation methods for the 3-D silk matrix in a drill hole model in sheep.

Materials&Methods: For both parts of the study adult, Swiss Alpine sheep between 2 to 3 years of age were used. Surgeries were performed with the sheep under general anesthesia and appropriate postoperative analgesia. After recovery, the sheep were kept in stalls in small groups until they were allowed to roam free on pasture at 4 weeks after surgery.

Part I: Three groups were made, where as bone replacement one group received the 3-D test implants (UPW) sterilized by ethylene oxide. Controls were hydroxyapatite (HA) granules and autogenous bone grafts. Implants were followed for 2 weeks (silk, autografts) and 2 months (silk, HA, autografts). A total of 30 sheep were operated.

A rectangular defect of 15mm width, 10mm heights and 18mm depth was made at the medial aspect of the proximal tibia, just cranial of the collateral ligament and 4mm distally from the proximal rim of the tibia shaft. The overlying articular cartilage was never touched and neither was the joint capsule opened.

Part II: Four different silk preparations were tested, which differed in the source (2 silk distributors) and in the silk scaffolding process, namely silk dissolved in an organic solvent using NaCl salt crystals as porogen vs. a water based scaffolding process using paraffin spheres as a porogen (UPW) (n=4 groups à 6 samples). Leaching of the porogen out of the scaffolds was either by aqueous buffer (hexafluoroisopropanol; HFIP-scaffolds) or hexane (UWP-scaffolds)[3, 4]. For sterilization all scaffolds in part II of the study were steam autoclaved. A previously established drill hole model in sheep was used to test biocompatibility. Briefly, drill holes of 8mm diameter and 13mm depth were created bilaterally in the proximal and distal metaphyses and epiphyses of the humerus and femur. The holes were filled with the 4 different silk scaffolds and followed for 2 months, when sheep were sacrificed.

For both, part I and II, bone samples containing the silk scaffolds were harvested, radiographed (Faxitron), and processed for histology of non-decalcified bone samples embedded in plastic resin. Ground (30-40µm) and thin (5µm) sections were prepared and either (surface-) stained with toluidine blue or von Kossa/McNeal. (Semi-) quantitative and qualitative evaluation for both studies was focused on cellular reactions and new bone formation. For part 1 degradation of hyaline cartilage was assessed biochemically and histologically, with the latter in relation to the measured distance from the subchondral defect to the overlying cartilage surface. Statistical evaluation of the measured variables was performed using factorial analysis of variance followed by a posthoc test according to Scheffe.

Results: After 2 months silk scaffolds were infiltrated with cells even in the most central parts in both studies. Disparate biocompatibilities were

obtained for part I and part II of the study. Whereas the (foreign body) reaction in response to UPW scaffolds was strong in part I, a substantially better biocompatibility was demonstrated in part II Overall, the UPW scaffolds were equal or slightly better as compared to the HFIP scaffolds regarding infiltration with giant foreign body or mononuclear cells, but there were slight differences between combinations of scaffolding protocols and silk sources regarding new isles of bone formation at 2 months after implantation. The source of the silks did not have an effect on the experimental outcome.

Degeneration of the hyaline cartilage overlying the rectangular defect occurred in 100% of the sheep in part I, although the cartilage itself was never touched. Proteoglycan content was lowest in the group with the hydroxyapatite bone substitute and equal in the autograft or silk scaffold group. The severity grade of cartilage degeneration was related to the distance measured between the proximal rim of the tibia and the defect.

Discussion& Conclusion: It could be demonstrated this study, that silk scaffolds can be used as bone substitutes. At this point there is no clear indication about the cause(s) for the difference in biocompatibility between the two parts of the study. Potential causes could be differences in sterilization protocols (ethylene gas vs. autoclave) resulting in ethoxylated silk and/or the final residual solvent concentrations in the scaffold after manufacturing and sterilization, rather than the biocompatibility of silk fibroin per se derived from different sources.

Furthermore, it was shown that degeneration of hyaline cartilage overlying a rectangular defect was mostly related to the distance between the defect and the calcified cartilage zone as well as the type of the implant used as bone replacement. The use of stiff hydroxyapatite granules resulted in more proteoglycan depletion compared to the more elastic silk scaffold or autogenous grafts. Local inflammation seemed to play a minor role in relation to cartilage degradation.

It can be concluded that *i)* subchondral defects in close proximity to the calcified cartilage zone result in degeneration of the overlying hyaline cartilage already after 2 weeks, *ii)* silk scaffolds may have some advantage from a mechanical (stiffness) point of view when used as bone replacement in subchondral bone and, *iii)* if scaffolding, leaching and sterilization protocols are validated local (foreign body) reaction may be well controlled using silk fibroin scaffolds. The possibility of the new scaffolding protocol (UPW) to accommodate growth factors[5] that enhance bone formation may even further ameliorate the situation of the overlying hyaline cartilage and slow down its degradation as a response to injury to subchondral bone.

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Challenges in bone tissue engineering

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The application of tissue engineering principles is commonly described by using a combination of cells, matrix and growth factors in order to regenerate or to replace tissue. Bone tissue engineering currently uses either growth factors on a variety of carriers or osteogenic cells seeded onto an even larger number of different materials. The former approach has been very successful in preclinical animal studies using bone morphogenic proteins for the healing of critical size skeletal defects in various locations.

Osteogenic growth factors

Clinical applications in the head and neck area, however, have shown ambiguous results or required extremely high dosages when compared to the natural content of BMPs in bone. Besides other reasons, the unfavourable release characteristics of carriers such as collagen or inorganic materials have been considered responsible for these results. Loading with BMP has been commonly performed by simply soaking the growth factor solution into the carrier. However, this type of loading is associated with rapid delivery of growth factors after implantation within the first 48 hours. As many polypeptide growth factors provide a heparin binding domain, modification of collagen carriers by covalent binding of heparin to the respective sites in the collagen molecule can substantially retard the delivery of loaded growth factors.

Alternatively, degradable polymers that give way to bone regeneration during resorption can be used as slow release systems for bone morphogenic proteins. Incorporation of growth factors into the polymer has been accomplished using organic solvents to liquefy the solid polymer and by gas foaming. As the former approach may be associated with residual amounts of solvents the later appears to be preferable. Gas foaming of amorphous poly-DL-lactic acid with incorporation of BMPs has shown controlled release from the porous implants that induced alkaline phosphatase *in vitro*. In vivo, bone formation was induced in heterotopic sites in the gluteus muscle of rats and bone regeneration was found in critical size defects in rat mandibles. This approach could help to provide a more controlled manner of growth factor delivery and thereby reduce the amount of growth factor that is required to induce bone regeneration.

Osteogenic cells

An alternative way to enhance bone regeneration is the use of osteogenic cells seeded onto biomaterials and implanted into skeletal defects. In adult human individuals these cells are most frequently derived from iliac crest bone marrow aspirates. Cytofacs analysis and magnetic bead sorting has shown that only a minor portion of aspirated cells are compatible with the surface markers of undifferentiated mesenchymal cells such as STRO-1. Extensive ex vivo expansion is, thus, required to obtain adequate numbers of cells for the repair of clinically relevant defects.

Despite a decade of experimental evaluation a number of unresolved questions remain. i) The expansion protocol has to prevent premature aging and differentiation during expansion. ii) Cell viability and proliferation is difficult to control during cultivation in three-dimensional scaffolds. iii) There is no agreement so far whether differentiated osteogenic cells or rather undifferentiated mesenchymal cells are preferable for implantation and subsequent bone formation *in vivo*. iv) It is largely unknown which parameters control the behaviour of cultivated human bone marrow stroma cells (hMSCs) after implantation in the *in vivo* environment.

Experimental evaluation of expansion protocols have shown that media using FCS or human serum tended to result in premature differentiation and growth arrest. Media using PDGF-BB and other growth factors have shown reliable expansion through a high number of passages. Short term vs. long term culturing as well as dynamic vs. static culturing of hMSCs both in organic and inorganic carriers porous carriers have not shown to result in significant differences in bone formation in critical size defects in athymic rats. Poor cell survival and inferior bone specific cellular activity after transplantation have to be considered as reasons which may result from premature differentiation and subsequent growth cessation and/or poor revascularization *in vivo*.

Future research has to focus on the combination of slow release scaffolds with growth factors and osteogenic cells that can improve vascularization and provide stabilization and/or stimulation of the seeded cells after implantation thereby enhancing the specific function of all three components of the engineered construct.

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Craniofacial Regeneration in Wounds Compromised by Radiation Therapy

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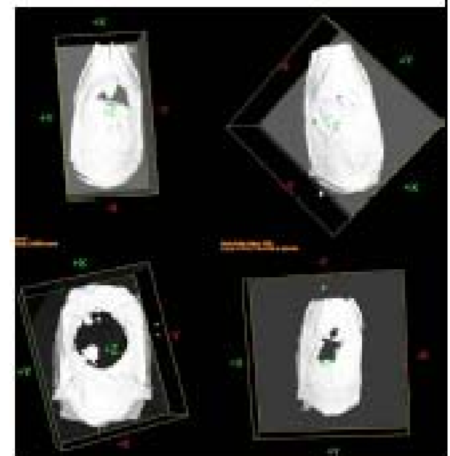
Introduction: The etiologies of craniofacial defects that require bone grafting are related to ablative tumor surgery, ischemic conditions (osteoradionecrosis), infectious causes (osteomyelitis and periodontitis), traumatic injury, or developmental/congenital causes. These defects are more likely to be in wounds complicated by microbiologic contamination, infection, and radiation therapy. One practical obstacle for successful regeneration in the craniofacial region is the exposure to radiation that is characteristic of patients with head and neck malignancies. Radiation therapy complicates reconstructive surgery by increasing apoptosis and decreasing the vascular supply to the surgical field.

The consequence is poor healing, scaring, fibrosis and a surgical site that is extremely difficult to reconstruct. Although protein therapy, gene therapy, and stem cell therapy may heal critical sized craniofacial defects, none of these approaches successfully heals clinically relevant, critical-sized bone defects previously treated with a therapeutic-equivalent dose of radiation. A potential strategy for overcoming the negative effects of radiation includes the use of anabolic dosing of parathyroid hormone (PTH), which effectively augments bone formation during stem cell directed osteogenesis. Based on this observation, we hypothesized that combining anabolic PTH treatment with gene therapy approaches would successfully overcome the negative effects of radiation for bone tissue engineering.

Methods: Three weeks before surgery, rats received a therapeutic equivalent 12 Gy radiation dose to the calvaria. Syngeneic dermal fibroblasts were transduced *ex vivo* using an adenoviral vector coding bone morphogenetic protein-7 (BMP- 7). Nine mm diameter bony defects were created and implanted with a BMP-transduced cell-gelatin scaffold, covered by either LD-SAM flap or no flap. For anabolic PTH groups, animals were given daily subcutaneous injections of either recombinant human PTH [1-34] (60 mg/kg) or vehicle (0.9% sodium chloride). Regenerated defects were harvested at 6 weeks and analyzed by micro-CT, histology, and levels of hypoxia.

Results: Pre-operative radiation therapy significantly limited tissue regeneration even when treated by BMP-7-transduced cells, (12.64 ± 2.52% in control; 18.87± 2.85% with PTH injection). However, the combination of flap transfer, significantly increased percent bone volume/total defect volume (37.09.16 ± 8.00% of flap transfer versus 61.48± 20.03% of flap transfer combined PTH, $p<0.05$). Bone mineral density was also significantly increased by the addition of PTH treatment in both the cell graft and combination of flap groups. Histology revealed the regeneration pattern of multiple bone nodule formation. Flap and PTH also improved regional hypoxia status significantly.



Conclusion: The combination of a LD-SAM flap, transduced cell graft and anabolic dosing of PTH markedly increases bone formation by restoring a well vasularized environment with a responsive mesenchymal stem cell population, and can overcome the negative effects of pre-operative radiation therapy in rat cranial defects.

Fig. 1 Micro ct scans of bone regeneration in craniofacial defects. Upper left: no radiation, BMP transduced cells. Upper right: no radiation, BMP transduced cells, PTH. Lower left: XRT, BMP transduced cells. Lower right: XRT, BMP transduced cells, PTH.

Hard tissue regeneration in the maxillary sinus using methods of tissue engineering

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Introduction

Hard tissue defects of the alveolar process after trauma, tumor or the atrophy after the loss of teeth are a common problem. For the dental rehabilitation of the patient dental implants is often the method of choice. Sinus floor elevation is a standard procedure prior to implant insertion in the atrophic maxilla when vertical bone height is limited. Augmentation by autologous cancellous bone from the iliac crest is considered as today's gold standard. Harvesting of pelvic cancellous bone has its own morbidity. Most biomaterials used in sinus grafting are considered to be bioinert fillers with only osteoconductive properties. Using tissue engineering methods we combine the the advantages of autologous canellous bone with limited donor site morbidity.

Material and methods

In animal experiments and human studies we have investigated different methods of tissue engineering. In 9 adult sheep we performed a bilateral sinus floor elevation using the growth and differentiation factor rhBMP-2. In the augmented sinus dental implants were inserted. In another similar sheep study we augmented the sinus using BioOss in combination with stem cells from bone marrow aspirate. Autologous cancellous bone was used as a control standard in both animal experiments. After the successful use of stem cells in the animal study we treated human patient in a pilot study with autologous stem cells in combination with a biomaterial. The augmented sinus was examined radiologically and histologically.

Results

In the maxillary sinus augmented with BMP the volume, the bone to implant contact and the bone density was investigated. In the experiments using stem cells and BioOss the cell number, the volume and the new bone formation was measured using radiography and histomorphometry.

BMP showed a similar bone volume but a higher bone to implant contact and a higher bone density than the gold standard. The combination of stem cells with the biomaterial resulted in a high new bone formation rate with no complications in the maxillary sinus. The density of the biomaterial and the new bone was higher than the canclous bone control. In the patient study biopsies harvested during implant insertion showed new bone formation and no signs of infection or inflammation. Stabil implant insertion was possible in all patients.

Conclusion

Methods of tissue engineering might a promissing alternative to augmentation methods in sinus floor elevation.

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Tissue Engineering for Mandibular Bone Lengthening in Smokers

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It is a clinical challenge to treat heavy smokers due to their compromised healing ability, particularly on the need for reconstructive surgery in craniofacial deformities or tumor treatment. Nicotine is one of the major components in tobacco products responsible for tobacco addiction. It is known to cause the retardation of skeletal development and bone tissue healing. Distraction osteogenesis is a newly developed technique to treat severe craniofacial deformities. It provides a tool to explore the biochemical mechanism of bone induction and formation.

This study attempts to establish a nicotine-induced compromised bone healing model of rabbit mandibular distraction osteogenesis.

Twenty New Zealand white rabbits were randomly assigned to 4 groups: low dose nicotine of (0.75g), high dose nicotine (1.5g), placebo control, and sham control. 60-day time release nicotine pellets and placebos were embedded subcutaneously. One week after nicotine embedding, osteotomy and active distraction was performed. Plasma nicotine levels were measured before nicotine embedding, on the day of osteotomy and before sacrifice. After seven weeks exposure of nicotine, the rabbits were sacrificed and the mandibular samples were subjected to plain x-ray, micro-CT and histological study.

The plasma nicotine levels in the low dose nicotine group were less than 9.0ng/ml, and in the high dose nicotine group were in the range from 20.9-47.4ng. When compared to control groups, the bone healing process was significantly compromised only in the high nicotine dose group.

The rabbits administrated with time-released nicotine pellets can simulate human smokers. The plasma nicotine level attained in the high dose nicotine group was equivalent to the nicotine concentration (10 to 49ng/ml) commonly observed in human heavy smokers, whereas the low dose nicotine group was comparable to the light smokers. When compare to the control groups, nicotine exposure in high dose for seven weeks has a significant impact on the bone healing during the rabbit mandibular distraction osteogenesis.

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Small chemicals to enhance bone repair

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INTRODUCTION: Bone morphogenetic proteins (BMPs) are the key cytokines in bone formation and repair. Since the cloning of the first members of the BMP superfamily in 1988 recombinant BMPs failed to substitute autologous bone as gold standard in clinical treatments for bone repair, mainly because the first clinical trials showed, that milligram doses of rhBMP-2 are required for effect [1].Therefore, BMP-presentation and release kinetic mainly determined by the delivery system have to be optimized for an efficient clinical application of rhBMP. Another strategy to decrease the BMP dose in clinical applications is to combine the cytokine with enhancers of BMP activity. Here we show that NMP (N-methylpyrrolidone) is an enhancer of BMP activity and can be used to generate biomaterials of the 3rd generation, where biocompatibility, biodegradabi-lity, and bioactivity are combined.

METHODS: MC3T3-E1 pre-osteoblastic cells were tested for different cell maturation responses: ALP (Alkaline phosphatase activity) and Alizarin Red mineralization assay. At the molecular level, cell extracts were analyzed by Western Blotting for Smad 1,5,8 and p38 phosphorylation, as well as by quantitative real time PCR (qRT-PCR) for specific osteoblastic markers (Osteocalcin-OCN, Bone Sialoprotein-BSP). Histological and histo-morphometric analysis of bone repair *in vivo*: non critical size 6 mm defects were created in rabbit calvarias and subsequently treated with three different membranes, namely PLGA, and NMP-PLGA, or left untreated (control).

RESULTS: NMP increased ALP activity of MC3T3-E1 cells concentration dependent. and mineralization of MC3T3-E1 cells. NMP action depended on extracellular bone morphogenetic protein (BMP), because in the presence of the BMP antagonist Noggin ALP activity in the presence of NMP was reduced below control levels (67+/-10%). In combination with rhBMP-2 NMP showed a synergistic effect on ALP activity, mineralization p38 and Smad 1,5,7 phosphory-lation. This synergistic effect depends on active PKD. The synergistic effect on p38 phosphory-lation is also PKC dependent. The *in vivo* results in the guided bone regeneration model showed that in the presence of NMP healing of the defect was 79.17±5.61% compared to 49.31±8.75 % without NMP.

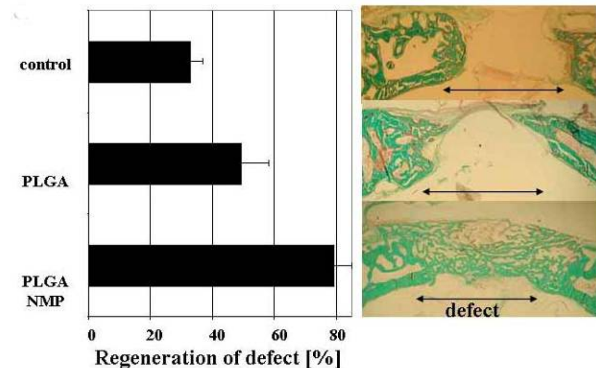

Figure 1: Enhancing of bone repair by guided bone regeneration and NMP: Defects of 6 mm in diameter created in the calvarial bone were treated with PLGA membrane, NMP-PLGA membrane or left untreated (control). The left panel shows the percentages of the area where bone regeneration in the defect has occurred in relation to the original defect area.

DISCUSSION & CONCLUSIONS: The results show that NMP improves the biological activity of BMP *in vitro* and *in vivo* by enhancing the kinase activity of the BMP-BMP-receptor complex. Since p38 and Smad 1,5,7 phosphorylation is increased, both the preformed receptor complexes and the BMP induced receptor complexes exhibit an NMP dependent enhanced in kinase activity. In contrast to normal BMP activity which is only PKD depen-dent, the synergistic effect for p38 is also PKC dependent. Thus, NMP not only enhances the BMP activity but also induces other, PKC dependent pathways, and creates in the presence of BMP an orchestrated signalling pattern favouring bone repair and regeneration. In the end, our finding could translate into novel treatment strategies for bone regeneration under the influence of autologous BMP for non-critical size defects and recombinant human BMP for critical size defects.

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AO Spine Research Network: Global Collaboration to Address Disc Degeneration and Regeneration

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Background and Rational:

Degeneration of the disc has been implicated as a principal factor in the onset of debilitating back pain and consequent limitations on daily activity. Surgical treatments to either fuse the affected segment, or to preserve motion through the use of mechanical implants, are not the ultimate solution to the problem. Attention has shifted to the biological restoration of the degenerated disc. The Spine Research Network (SRN) was established by AOSpine to draw together the multidisciplinary expertise of several leading research institutions to pursue a common scientific goal. The projects of the SRN are closely aligned with the AO clinical priority of "Degeneration and Regeneration of the Intervertebral Disc". The SRN's overall objective is to investigate the pathogenic mechanisms of disc degeneration and to develop long-term biological or non-biological treatments which preserve spinal motion and eliminate pain.

Program:

The current projects of the SRN emphasize the importance of the underlying biological mechanisms. The group is working on bioreactor design and tissue preparation methods for functional ex vivo studies of whole discs, for example, to improve the understanding of nutritional demands and limitations in the degenerating disc. In vitro studies and computer simulations are employed to characterize the intradiscal transport mechanics of relevant nutrients, cytokines and therapeutic agents.

Projects are underway to better understand the processes of disc inflammation and pain development. The ingrowth of blood vessels and nerve fibers is being studied as a potential source of disc-related pain. The effect of age-related accumulation of specific proteins which play a role in the inflammatory cascade is being investigated.

To progress towards the production of engineered disc tissue for the biological restoration of disc function, the use of stem cells is being evaluated. Genes which are most suitable as markers of disc cell phenotype have been identified and will be evaluated to demonstrate the specificity of the cultured tissue. To provide an appropriate biological and mechanical environment for tissue engineering, a variety of scaffold materials are being studied.

The SRN draws together expertise crossing the boundaries of multiple disciplines to address the treatment of disc disorders by employing a strategy which focuses on the underlying biological processes which drive the degenerative processes, and which are required to support a regenerative treatment.

ACKNOWLEDGEMENTS: This research work is supported by the AO Foundation (Davos, Switzerland) and by grants from AOSpine (Dubendorf, Switzerland).

NOVEL POLYMERS FOR STUDIES IN STEM CELL DIFFERENTIATION AND NUCLEUS PULPOSUS SUPLEMENTATION

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Background: Degenerative disc disease has been implicated as a major component of spine pathology. Currently, the two major clinical procedures for treating disc degeneration are disc excision and spinal fusion. Although these procedures offer relatively good short-term clinical results in relief of pain, in many instances, these treatment modalities have been disappointing because of altered spinal mechanics leading to subsequent degeneration at adjacent disc levels.

Biological repair of the degenerate disc would be the ideal treatment and recent advances in tissue engineering offer the unique opportunity to engineer a replacement nucleus pulposus (NP) using polymer-cell constructs and growth factors.

Rational for using chitosan and mesenchymal stem cells: Chitosan has been chosen because it is injectable (very soluble at room temperature but gelling at 37°C), biocompatible and can retain more than 80% of the proteoglycan and collagen produced by entrapped cells (1,2). This is of particular importance because any scaffold designed for disc use must be able to retain proteoglycan if a functional tissue is to be achieved.

Furthermore, the scaffold will allow implantation without major surgical disruption of the annulus fibrosus (AF). We use human adult mesenchymal stem cells (MSCs) because the use of stem cells is essential for clinical application if an autologous source of cells is to be used.

Rational for using nitrogen rich plasma polymers: MSCs are pluripotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament disc and fat. However, recent evidence indicates that a major drawback of current cartilage and intervertebral disc tissue engineering is that human MSCs isolated from some arthritic patients (a clinically relevant source of stem cells) express type X collagen (a marker of chondrocyte hypertrophy associated with endochondral ossification) (3). Some studies have attempted to use growth factors to inhibit type X collagen expression, but none has addressed the possible effect of the chemical composition of the substratum on stem cell hypertrophy.

Here, we examine the growth and differentiation potential of human MSCs cultured on extremely N-rich plasma polymer layers, which we call "PPE:N" (N-doped plasma-polymerised ethylene, containing up to 36% [N]). We show that PPE:N almost completely suppresses the expression of type X collagen. In contrast, neither aggrecan nor type 1 collagen expression was significantly affected. These results indicate that PPE:N coatings may be suitable surfaces for inducing MSCs to a disc-like phenotype for tissue engineering of intervertebral discs, in which hypertrophy is suppressed.

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Enhanced Spinal Fusion by Tissue Engineering of Mesenchymal Stem Cell/ Tricalcium Phosphate Ceramics Composite – An Experimental Study with Non-Decorticated Posterior Spinal Fusion

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In conventional posterior spinal fusion surgery, limited source of autograft and excessive bleeding during harvesting of autograft and intra-operative decortication could lead to significant morbidity. We investigated the alternative procedure to enhance osteogenesis in posterior spinal fusion by mesenchymal stem cells (MSCs) in an animal model. Rabbit bone marrow derived MSCs were differentiated into osteogenic cells by osteogenic supplement (OS group) *in vitro*. The autogenous MSC derived osteogenic cells were then loaded on beta tricalcium phosphate ceramics (β-TCP) and implanted onto non-decorticated transverse processes of lumbar vertebrae L5 and L6 in rabbits. The cell-freeβ-TCP served as control (Control group). The implantation of autogenous iliac crest with decortication was used as positive control (Autograft group). The spinal segment samples were harvested at 7-week post-operation for detail evaluations. The microCT analysis showed that all samples in the autograft group were fused and incomplete fusion was found in the OS and control group. In comparing the OS and control group, the gap distance between transverse processes in OS group (2.33±1.51mm) was significantly less than the control group (7.26±0.27mm). Moreover, the volume of fusion transverse processes in OS group were increased by 54.3% ($p<0.05$). The tissue volume of the newly formed bone was also significantly higher in the OS group (2.3 fold at L5 and 3.6 fold at L6). Comparing with the autograft group, the volume of fusion of the transverse process in OS group was 16.1% greater than autograft group significantly. Under fluorescent microscopy, more active bone formation and remodeling was observed in the OS group. The polarized light microscopy showed the bony tissue at the mineralization front in OS group was less mature than the control group. In conclusion, the TCP composite seeded with differentiated MSC were found to be effective bio-complex in enhancing non-decorticated posterior spinal fusion.

Stress and homeostasis in the bone and the intervertebral disc

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The skeleton is continuously subjected to several stresses. However, although intense stresses can be detrimental, it is well-documented that mild stresses represent an important regulatory factor during development and in postnatal life. Nevertheless, although data available today provide some insights into how cells sense these stimuli and convert them into biochemical responses, the precise molecular mechanisms that underlie these phenomena are still obscure. Here, we present some evidence on the role of exogenous stresses on the maintenance of homeostasis.

By using an in vitro model for the application of static mechanical stretching in osteoblast-like cells, we have found that this stress, very rapidly stimulates the activation of major signaling pathways (e.g. members of the MAPK family) and transcription factors (e.g. the c-Jun and c-Fos proteins). These events lead to the stimulation of DNA synthesis in these cells. Interesting, in contrast to other cell types, this stimulation is unrelated to autocrine growth factor action[1, 2]. In addition, this mechanical load is able to directly up-regulate the expression and DNA binding of the master regulator of osteoblast differentiation Cbfa1, and this effect seems to be regulated by the stretch-triggered induction of distinct MAPK cascades[3].

The intervertebral disc is also subjected to various types of stress, such mechanical, nutritional, hypoxic and osmotic. We have studied some of these stresses, hypothesizing that they can, in some extent, regulate the homeostasis of this tissue. More specifically, we have investigated the effect of hyperosmotic stress on intervertebral disc cells and have shown that it affects their proliferation via the regulation of signaling pathways, such as the MAPK pathways, and cell cycle regulators, e.g. the p53 oncosuppressor protein[4]. Finally, as it has been reported that growth factor secretion is a consequence of disc degeneration, we have studied the effect of isolated growth factors on disc cell proliferation and the activation of pivotal signaling pathways, as well as the role of the extracellular matrix components in these responses[5].

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Intervertebral disc mechanobiology and the kinetics of gene expression

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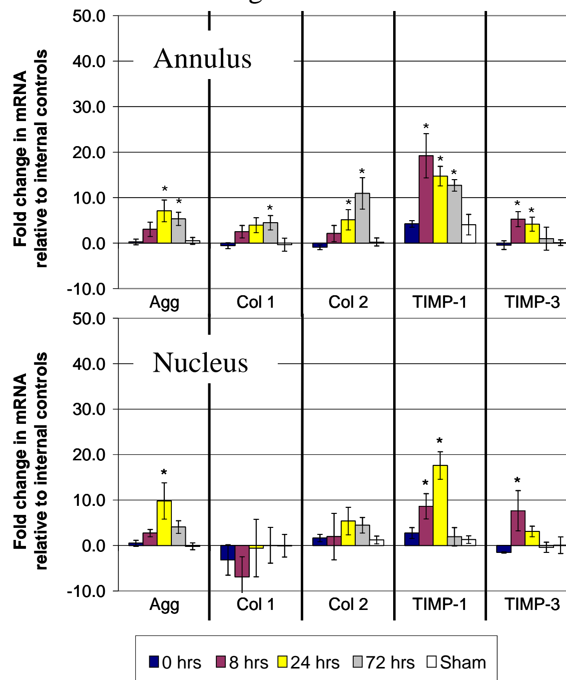
Introduction: Disc degeneration may be accelerated through mechanical loading. In vivo studies on rodent models defined a clear relationship between mRNA expression and compression loading magnitude, frequency, and duration. It is unknown when changes in gene expression peak and for how long they remain altered following loading. Defining the kinetics of gene expression in response to mechanical loading is a research priority, represents a large gap in the spine literature, and has clinical implications related to how frequently strenuous activity may be repeated. The purpose of this study was to investigate kinetics of gene expression by recording mRNA levels 0, 8, 24 and 72 hours after a single loading event.

Methods: This study was approved by the University of Vermont IACUC. Forty-nine skeletally mature Wistar rats (>12 weeks) were instrumented with an Ilizarov-type device spanning caudal disc 8-9. 72 hours after surgery rats were anesthetized and the c8-9 discs were subjected to 1.5 hours of loading at 1MPa and 1Hz. Shams (n=9) were subjected to 1.5 hours of anesthesia without loading. Animals in the loading groups were euthanized either immediately (0 hrs) or awakened and allowed to recover for 8, 24, or 72 hours (n=10 per group). The instrumented disc along with the proximal and distal control levels from each rat were harvested, and annulus (AF) and nucleus (NP) tissue separated. Control and loaded disc levels from all animals were dissected and annulus and nucleus tissue were separately analyzed by real-time RT-PCR for levels of rat-specific collagen 1A1, collagen 2A1, aggrecan, stromelysin (MMP3), collagenase-3 (MMP13), gelatinase (MMP2) aggrecanase-4 (ADAMTS-4), TIMP-1, and TIMP-3 mRNA. Each gene was normalized to 18S rRNA levels. ANOVA with post-hoc testing was performed.

Results: In the annulus, peak gene expression occurred after 8hrs in TIMP-1 and TIMP-3; after 24 hrs in agg, ADAMTS-4, and MMP-3; and after 72 hrs in Col-I, Col-II, and MMP-13. No change was observed in MMP-2 (Figure, catabolic results not shown). In the nucleus, maximum changes in gene expression occurred after 8hrs in Col-1 and TIMP-3; and after 24 hrs in aggrecan, TIMP-1, ADAMTS-4, MMP-13, and MMP-3. No changes were observed in collagen-II or MMP-2.



Discussion: In the nucleus genes were generally up-regulated within 1.5 hours after initiation of compression, were significantly modified after 24 hours and were returning to sham levels by 72 hours. Annulus displayed similar patterns but message persisted longer than in the nucleus with several genes significantly up-regulated 72 hours following load cessation. The increased levels in the annulus at later time points could reflect a prolonged time of elevated expression or a slower rate of message degradation. TIMP-1 and TIMP-3 genes displayed the most rapid response to loading with peak levels occurring within 8 hours, indicating mechano-sensitivity of these regulatory genes. This in vivo study on the mRNA kinetics response to loading defined recovery times required for gene expression to return to baseline levels and may more precisely characterize healthy and risky loading conditions. This information is also useful for optimizing tissue harvest time and for determining the frequency of repeated loading episodes in chronic mechanobiology studies.

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Clinical needs and applications of Biotechnology in Cranio-Maxillofacial Surgery

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Background and Introduction:

Cranio-Maxillofacial (CMF) surgeons are the specialist surgeons who treat the anatomical region of the head and neck. This surgical discipline is truly multi-disciplinary encompassing maxillofacial surgeons per se, oral and maxillofacial surgeons, plastic surgeons, neurosurgeons, ENT surgeons and ophthalmic surgeons.

Managing the hard and soft tissues of the face and cranium places CMF surgeons in a similar regional position to orthopaedic and general trauma surgeons in terms of their treatment of the rest of the skeleton. We have similar problems and similar needs but also ‘vive la difference’.

Clinical Needs:

1. Reconstruction of bony and soft-tissue defects of the cranio-maxillofacial skeleton especially:
   - Tumour resection defects
   - Trauma, especially gun shot injuries
   - Deformity – mainly congenital

2. Enhancement of bone growth in children with congenitally hypoplastic tissues especially of the facial skeleton and overlying soft-tissues

3. Improvement in the quality and time of consolidation after distraction osteogenesis by the use of growth factors and other tissue engineering techniques

Applications:

1. Developments in tissue engineering of bone and soft tissue are highly relevant to CMF especially for reconstruction of defects of the jaws and cranium, for example continuity defects of the mandible and cranioplasty defects of the cranium

2. The use of free tissue transfer through microvascular anastomosis remains the state of the art in large defect reconstruction in the head and neck especially after resection of malignant tumours, for example for squamous cell carcinoma of the oral cavity

3. New techniques and others in development of prefabricated flaps supported by tissue engineering could well play a significant role in the future especially in reconstruction of large defects

4. Distraction osteogenesis (DO) which is used mainly in the head and neck for congenital defects in children to allow the process to be speeded up and more stable.

5. DO as transport osteogenesis also has a role to play in reconstruction of continuity defects and similarly would be greatly enhanced if TE techniques could be used to enhance and speed up the process

From basic research to clinical application

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Background and Introduction:
To make a success of a translation research, several requirements must be joined together. Initially, a geographical situation and a local political conjunction which place at the disposal the high essential technological schools, infrastructures specialized and for the medical field a teaching hospital or equivalent. There must be a political good-will and a governorship to stimulate the blossoming of such project. It is not enough to want to make translation research, it is necessary to have the organizational means of them. The caricatured situation of the high school of technology which develops ideas on its side and which sees appearing a possible application in the field of health is not any more topicality. Indeed, social and economic requirements require a quasi-tripartite management of the projects. There are the world of technological sciences, the world of medical sciences and the concept of public health which introduces dimensions not only economic but such ethical and social.

The Facts
The story of the university orthopedic and traumatology department is remarkable. At the beginning there is more than 20 years, it is the medical world, which on the pressure and the passion of its Head of current department pushed a form of collaboration with the engineers of EPFL. The first projects of medical or anatomical nature parasitized the finite element analysis and research laboratories in the objective to support the comprehension of articular biomechanics. This very technical work led to many fundamental results of order. Little by little the pressure and enthusiasm, this time common, made it possible to widen the field of research and to associate a biological dimension gradually there. A paradox is observed, medicine sought technological answers and the mixture of the ideas results little by little in creating a group whose studies lead to a techno-biological concept.

By way of example the analysis of articular biomechanics by the finite elements naturally resulted in being interested in the articular implants, then with the interface of the these implants with bone tissues. The following stage was the modeling of the reaction of the bone to these implants. Thereafter, will to include/understand the biological reaction (gene expression) of the bone to thorough the researchers (physicist) to direct itself towards again scientific horizons of the biological world. So gradually a well-defined structure - the institute of research in orthopedics could take shape on the level institutional. Its perennially is ensured by under-groups which join together around the same project engineers and doctors. They work in concert forming and learning from each one.

Discussion and Conclusion:
Finally, the translation in the clinical world requires legal competences and of management of quality. These tools must be placed at the disposal by the institutional framework, if one wishes to make bear fruit and benefit the populations from our projections. And in this dimension, EPFL and Hôpital Orthopédique-CHUV bring to the level of the assistance to the technology transfer, intellectual property and the ethical commission the possibility of giving a realistic dimension to our projects.

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Fracture healing and its potential as a bioreactor for skeletal tissue engineering

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Background and Introduction:

Significant advances have been made in prosthetic replacement of components of the skeletal system using synthetic materials. Although the success of prosthetic replacement is good, the increasing demands both in terms of implant life with increasing longevity and level of activity still leave room for improvement. Long term aseptic loosening of prostheses, primarily attributed to wear debris related osteolysis results in a continuing need for revision surgery. The advances in biological tissue engineering, using cells and scaffolds in vitro, although promising, have not yet reached the levels of use seen with synthetic prostheses. Increased longevity is associated with an increase in degenerative disease of the skeletal system, particularly degenerative joint disease.

The skeleton can adapt to the ever changing mechanical demands throughout life, optimizing functional morphology through dynamic adaptation. However, accumulative fatigue damage or gross monotonic overload lead to gross fracture. It is well established that bone as both a tissue and structure has great capacity for repair and unlike many tissues can restore both microscopic and gross architecture under optimal fixation conditions. The mechanical environment at the fracture site is key in determination of both the pattern and progression of repair. The repair process is acutely sensitive to mechanical conditions particularly in the early stages. Conventionally, the repair process comprises three phases, inflammatory, reparative and remodeling. Indirect bone repair comprises a unique cascade of connective tissue differentiation from the fracture haematoma through the entire spectrum of connectives tissues with the optimal outcome of regenerated lamella bone. Indirect bone repair also encompasses both intramembranous and endochondral ossification. There is also a suggestion from some studies of transdifferentiation during the repair process.

Lower order animals have the capacity to regenerate whole limbs after amputation, in mammals this regenerative capacity is lost, however, the process of fracture repair demonstrates that regeneration of connective tissues can occur within the adult skeleton. Changing mechanical conditions at a fracture site may lead to delayed or non-osseous union, with fibrous or cartilaginous tissues remaining in the fracture gap. Modelling studies indicate that hydrostatic stresses lead to chondrogenesis and deviatorial strains to fibrous tissues. Specific stresses applied to bone in *in vivo* bone chamber experiments have also been shown to induce cartilage. Thus the repair process in bone can be manipulated by mechanical influences to modulate the tissues present as a function of time.

Rational:

It may be hypothesized that specific hydrostatic stresses in the order of 0.5- 3MPa applied to a bone healing process will induce cartilaginous tissues. Furthermore, this process could be used in an *in vivo* bio-reactor to resurface joints with biologically engineered articular cartilage.

Methods:

Three groups of six sheep received unilateral hip hemi-arthroplasties were sacrificed 24 weeks post-operatively to harvest the acetabula. At operation, acetabular cartilage was removed completely and the subchondral bone was reamed to bleed. Three femoral head sizes, 25-, 28-, and 32-mm, were used to induce different contact stress levels. In a fourth group three polyethylene pegs were inserted into the bone to support the femoral head and protect any regenerate tissue from load. Vertical ground reaction force (GRF) data were measured and normalised by body weight for both limbs pre-operatively and every 4 weeks post-operatively. Five Specimens each from the 25- and the 28-mm group and eight un-operated controls were processed and stained with Safranin O and Sirius Red. Cartilage proteoglycans in the regenerated tissues from four specimens in 25-mm group were detected by immunoblotting using specific monoclonal antibodies. Type II and type I

collagens and Proteoglycans (PGs), including aggrecan, biglycan and decorin, were detected by immunoblotting with specific antibodies.

Results:

Peak vertical ground reaction force (pvGRF) of the operated limb had recovered to an average of 85 % comparing to the contralateral un-operated ones at the end of the study, with no significant difference between groups.

Regenerated tissue was observed overlying the bone on all specimens and was picrosirius positive for collagen.

In the load protected group, all 4 operated specimens were Safranin-O negative. In the loaded groups, 4 out of all 6 specimens in each group were Safranin-O positive. In all three loaded groups GAG content in the regenerated tissues was significantly lower than the contralateral control cartilage ($p < 0.01$).

Regenerated tissues from loaded groups showed two clear -chain bands, 1 and 2, at the expected sizes. Western blotting results confirmed a mixture of type II and type I collagens in all regenerated specimens. The presence of cartilage aggrecan, biglycan, and decorin in the loaded groups was also confirmed by immunoblotting.

Discussion and Conclusion:

This preliminary study indicates that the cascade of connective tissue differentiation seen in indirect bone repair can be exploited in *in vivo* tissue engineering to resurface synovial joints with *de novo* cartilaginous tissue. Translation of this concept to clinical use could not only provide a biological re-surfacing but may also allow a hemiarthoplasty approach with greater prosthetic longevity in the absence of wear debris and potential issues associated wit metal ion release.

Although in early stages of development refinements can be introduced in terms of the compliance of the prosthetic femoral head, also the progression to resurfacing of both articular surfaces.

We conclude that a cartilaginous-like tissue with a mixture of hyaline and fibro cartilage can be regenerated on a bone surface using controlled contact stresses applied from selected prosthetic femoral heads on the acetabular articulation in the ovine hemiarthroplasty model.

The data show the potential for regeneration of hyaline cartilage by *in vivo* tissue engineering using a mechano-biological approach in an integrative physiological environment.

Initial hybridisation of synthetic and autologous biological tissue replacement may allow a more rapid introduction and use of biological constructs in orthopaedic practice.

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The role of periosteal cells and mesenchymal stem cells in the physiological healing process of long bone defects. Potential regeneration acceleration by autologous cell transplantation.

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Introduction: Reconstruction of the periosteum has received little attention in most fundamental research works on the healing of bone defects, partially due to the lack of phenotypic markers for periosteal cells. The exact course of periosteal reconstruction and its influence on fracture healing still remain unclear. Mesenchymal stem cells (MSCs) offer new options for research into the healing of bone defects and their transplantation may help to overcome some of the problems during the treatment of critical healing conditions. We hypothesise that: a) the integrity of the periosteum is essential for bone healing processes, beyond its function as a source of progenitor cells, and b) autologous MSCs have the potential to enhance bone healing in an impaired situation that is known to lead to an atrophic non-union.

Methods: 120 male Sprague Dawley rats (410-460g) (3 groups: sham, non-union, MSC) received an osteotomy of the left femur, which was stabilized with an external fixator. In the sham group, no further intervention was performed. In the non-union and MSC groups, the bone marrow was removed up to the inner K-wires and the periosteum was cauterized 2 mm proximally and distally of the osteotomy. 3 weeks prior to the osteotomy, bone marrow was harvested from the right tibia of each animal and MSCs were cultivated in vitro. Two days post-osteotomy ~2 M MPCs were injected percutaneously into the osteotomy gap of the MSC group. The non-union animals received an injection of culture medium. Animals were sacrificed 14 or 56 days post-op and evaluated by radiology and biomechanical testing, as well as by histological, histomorphometrical and immunohistochemical analysis.

Results: The mean torsional stiffness was significantly larger ($p<0.001$) in the sham group than the non-union group (136.2±34.5% vs. 2.3±1.2%). No maximum torsional failure moment was measurable in the non-union group. The MSC group showed higher torsional stiffness, but no significant difference ($p=0.141$) compared to the non-union group (5.3±5.6% vs. 2.3±1.2%).

At 56 days, the X-rays of the sham group showed a completely bridged osteotomy and evidence of cortical remodelling. The non-union group showed no callus at the osteotomy site and a partially widened osteotomy gap with rounded, hypodense cortical ends. The MSC group showed partial callus with some degree of bridging and X-rays similar to the non-union group. Interestingly, most animals of the MSC group had mineralization of the intramedullary cavity at the osteotomy site (Fig. 1), as supported by the histology and histomorphometric data. Whilst the sham group showed a bony bridging of the osteotomy at 56 days, the non-union group displayed bone resorption at the cortical ends and no sign of bridging. The MSC group showed signs of bridging as well as of resorption (Fig. 2).

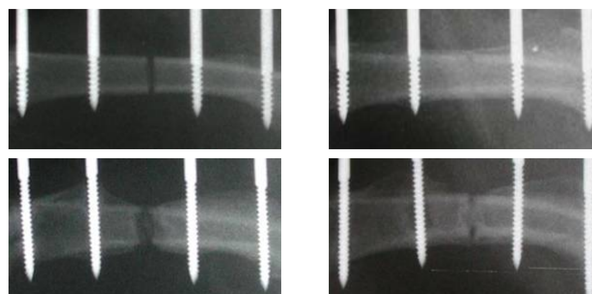

Fig. 1) X-rays of osteotomised femur at 56 days. Top: directly post OP (left) and sham (right); Bottom: atrophic non-union (left) and MSC treated (right).

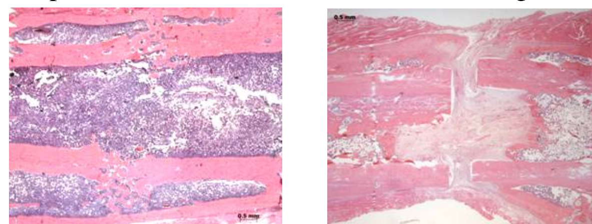

Fig. 2) Hematoxilin Eosin staining at 56 days. Top: sham (left) and atrophic non-union (right); Bottom: MSC treated.

Discussion & Conclusions: The cauterisation of the periosteum and removal of the bone marrow, in combination with a high stiffness of the external fixator, may create an atrophic non-union under well defined biomechanical conditions and with minimised interaction between the healing zone and the implant. The diversity of the results strongly indicates that the regenerative potential of MSCs has not been exploited consistently enough. Nevertheless the results are promising and demand further investigations, especially during the initial phase after transplantation.

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Processing of Metals by Human Bone and Immune Cells

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The purpose of this study was to explore functional and morphological intracellular detection of immunogenic metal ions by osteoclasts (bone resorbing cells) and human dendritic cells (most potent antigen presenting immune cells). We exposed the human cells in vitro to a variety of metal surfaces, metal particles and metal ions. Newport Green™ was used to fluorescently label intracellular protein-metal complexes. Confocal imaging and flow cytometry analysis showed specific staining for cells containing aluminium, chromium, nickel, titanium and zirconium ions. The intensity of staining varied between ion types, whereby Ti^{3+} resulted in the brightest fluorescence signal. Metal particle and metal ion distribution within the cells could be confirmed using transmission electron microscopy. In addition, human immune cells were tested for reactivity against metal ions in vitro, indicating that patients treated with metal implants develop immune reactivity against metals of their implant. In conclusion we can say that human osteoclasts corrode pure metal surfaces and that dendritic cells process intracellular metal particles and metal ion protein complexes. Metals processed by immune cells induce immune reactions in vivo.

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Treatment of non-union fractures with fetal cell therapy

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Background in non-union fractures : This project has been developed in order to solve the clinical problem of *atrophic non-unions* (osseous fractures). This situation is observed in 5 to 7% of all tibial and femoral fractures and represents an important and difficult population to treat. Progressively, surgeons have come to realize that fracture healing is not only a mechanical problem but also a biological or a cellular challenge.

There are two major expected outcomes in this research program, one at the level of understanding wound healing and one at the clinical level. For wound healing, by specifically looking at differences in human fetal and adult protein production, we would like to identify proteins produced by fetal cells but not by adult cells in the context of wound healing. These results would be fundamental in our comprehension of the phenomenon involved in wound healing in general and in specific for bone and associated osteogenetic factors. This approach could open new avenues of research with the clear view of enhancing wound healing as it has been demonstrated recently for the skin. At the clinical level, the actual treatment of non-unions is challenging the orthopedic community. Developing new solutions for this clinical problem is of primary importance and the approach of using fetal cells, if suitable, may revolutionize the medical practice. Obvious enhancements will be obtained for the patients suffering from non-unions who often have their quality of life profoundly affected due to the associated handicap and especially due to the associated pain. Delivering fetal cells percutaneously would then be an easy, non-traumatic procedure with substantial improvement in morbidity to unhealed fractures.

Rational to use fetal cells : Fetal associated tissues such as placenta, amniotic liquid or umbilical cord are described to be potential sources of cells for tissue engineering[1]. In contrast to embryonic tissue derived up to the end of the 8th week, fetal tissue begins at the 9th week and is considered as an organ donation. Human fetal liver cells have already been used for transplantation to treat severe immunodeficiencies, haematological disorders and inborn errors of metabolism when there was no perfectly matched donor for marrow transplantation[2]. Neuronal affections such as Huntington's[3] or Parkinson's Disease[4] have been treated by transplantation of fresh fetal neuroblasts. Unfortunately, these cells are difficult to expand in culture and have to be transplanted freshly therefore needing large quantities of fresh tissue[5]. Recently, human fetal skin cells derived from one cell bank (1-4 cm^2 tissue results in over 10.5 million fetal skin constructs) were used in clinical trials and new advances in tissue therapy are possible with cellular constructs obtained from *ex vivo* cultures[6]. Engineered regeneration of human skeletal adult tissues could be also developed using human fetal bone cells. To evaluate their potential integration in a bone engineering strategy, a biological characterization of these cells is necessary.

Age dependent biology of osteoblasts is generally accepted. The particular biology of human primary fetal bone cells was partially demonstrated with cells isolated from calvaria[7]. They were found to secrete primarily matrix constituent proteins in culture, whereas adult cells produce additional proteins involved in matrix turnover. Age dependent differences regarding osteoblastic synthesis of osteoanabolic peptides and their impact on the regeneration of osseous defects were observed with a rat calvaria model[8].

Recently, we performed a study to specifically evaluate the characteristics of human primary fetal bone cells for a better comprehension of their biology *in vitro* and to evaluate their potential use for tissue engineering in comparison to adult bone cells and mesenchymal stem cells[9]. Compared to primary adult bone cells, it was shown that fetal bone cells could be of great interest for bone research, due to their rapid growth rate and their ability to differentiate into mature osteoblasts. Importantly, human primary fetal bone cells represent an interesting and promising potential for therapeutic use in the bone tissue engineering field as these cells can be easily stocked "frozen for use" when necessary.

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Bone Regeneration with Platelet-rich Plasma, Calcium Phosphate Ceramics and Stem Cells

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INTRODUCTION: Ceramics such as β-tricalcium phosphates (β–TCP, specific surface area < 0.5 m^2/g) or Calcium-deficient hydroxyapatite (CDHA, specific surface area 48 m^2/g) can be combined with expanded mesenchymal stem cells (MSC) and growth factors to accelerate bone healing. In 1998, Marx and coworkers reported that adding platelet-rich plasma (PRP) to an autogenous cancellous bone graft resulted in a faster maturation rate and higher bone formation rate in alveolar defects. The aim of this study was to evaluate whether a combination of expanded MSC with PRP in resorbable calcium phosphate ceramics can promote osteogenesis and enhance ectopic bone formation in a SCID mouse model. We evaluated whether the effects of PRP depend on the type of carrier and the stage of osteogenic differentiation of the applied MSC.

METHODS: CDHA and β–TCP ceramic blocks were loaded with human MSC. Half of the specimens were treated with five-fold concentrated PRP. Furthermore, we compared undifferentiated MSC with MSC that were cultured under osteogenic conditions for 2 weeks *in vitro* on the scaffolds. Bone formation and osteogenic differentiation were evaluated by histology, alkaline phosphatase (ALP) enzyme activity, and osteocalcin (OC) content 4 and 8 weeks after ectopic implantation in SCID mice.

RESULTS: Ectopic bone formation was enhanced in MSC/CDHA (7/32) compared to MSC/β-TCP (2/30) composites; however, there was only a trend to more bone formation on CDHA after addition of PRP.

The addition of PRP to the composites increased the specific ALP activity significantly (P=0.006) on CDHA, but on β-TCP a similar trend did not reach significance.

The specific ALP activity was significantly higher in MSC-loaded samples compared to empty scaffolds ($p<0.001$) in CDHA and β-TCP ceramics. Mean ALP activity values were significantly higher in the undifferentiated MSC/β-TCP group compared with biocomposites subjected to osteogenic induction.

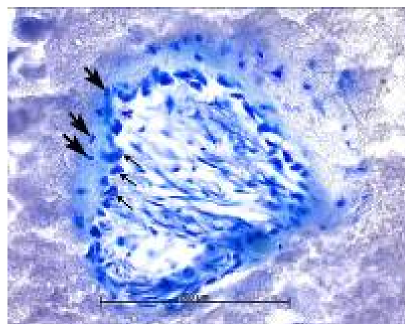

Fig. 1. Toluidine blue staining of a CDHA sample with PRP and undifferentiated MSC after 8 weeks shows bone formation in the outer parts of a peripheral ceramic pore with osteoblasts (small arrows) and osteocytes (large arrows) (200x magnification).

Although higher mean values of OC were obtained in cell-loaded CDHA with PRP versus without PRP, this difference did not reach significance. In contrast to β-TCP biocomposites, MSC/CDHA samples revealed a significantly higher OC content than the empty ceramic (P=0.031) but no significant difference was seen between the undifferentiated and induced MSC/CDHA samples.

DISCUSSION & CONCLUSIONS: PRP in combination with MSC loaded on CDHA had a positive effect on osteogenic differentiation regarding ALP activity, but due to a large donor-dependent MSC variability a trend towards better ectopic bone formation did not reach significance. MSC/β-TCP groups failed to profit from the addition of PRP. In conclusion, the effect of PRP depended on the ceramic and the differentiation status of the MSC, however it did not clearly promote osteogenesis of human bone-marrow-derived MSC.

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Estrogen-Withdrawal Increases the Mechanosensitivity of Bones to Loading

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Estrogen, the primary non-mechanical regulator of female bones, has been suggested to enhance the mechanosensitivity of bones to loading[1]. In this virtually life-long experiment, we evaluated whether the estrogen-status of the rats modulates the skeletal responsiveness to loading.

A total of 105 growing [5-17-week-old, normal estrous cycle (E+)], adult [47-59-week-old, normal estrous cycle (E+)] and senescent [90-102-week-old, menopausal (E-)] female Sprague-Dawley rats were used. At entry, rats were randomly assigned into 6 groups; 3 control groups (n=15/group) and 3 exercise groups (n=20/group). In the exercise groups, rats were subjected to an identical, 12-week period of progressive treadmill training. At each time point, a comprehensive structural analysis of the femoral neck was performed (peripheral quantitative computed tomography and mechanical testing). The menopausal status of the senescent rats was confirmed by microscopic examination of the ovaries and uterus (the absence of functional follicles in the ovaries and uterine atrophy denoting the menopausal status) in age-matched control rats prior to the execution of the exercise period.

In comparison to their age-matched controls, twelve weeks of treadmill training resulted in significant increases in femoral neck total bone mineral content (tBMC) in growing (E+) and senescent (E-) rats. In addition, a significant exercise-induced increase in the total cross-sectional area of the femoral neck (tCSA) was observed in senescent (E-) rats. These changes resulted in significantly increased breaking load of the femoral neck (Fmax) in growing (E+) and senescent (E-) rats. In contrast, no exercise-induced benefits were observed in femoral neck parameters in the adult (E+) rats.

In conclusion, these findings are in perfect agreement with both experimental and clinical studies[2], as we observed an estrogen-induced packing of mechanically excess mineral into fertile (adult) female skeleton, and consequent damping of the mechanosensitivity of these bones. At menopause, this extra “bone stock” was shed, and consequently, the mechanosensitivity of bones to loading was regained.

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Mechanoregulation of cell phenotype for musculoskeletal tissue engineering

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INTRODUCTION: For tissue engineering of musculoskeletal tissues, e.g. cartilage and bone, it is often difficult to harvest enough mature differentiated cells, without substantial morbidity. Hence, the recruitment of progenitor cells to form the desired tissue type is crucial. In addition to biochemical factors and gene therapy, biophysical signals have also proven to alter cell differentiation and proliferation. As such signals may be induced through load bearing in vivo, or in bioreactors in vitro, mechanical stimulation may be applied over long durations and adjusted during tissue regeneration.

It is well-documented that both chondrocytes and osteoblasts respond to various mechanical signals, e.g. fluid flow, strain and hydraulic pressure. Moreover, in vivo experiments have demonstrated that mechanical conditions play a crucial role in bone regeneration, where progenitor cells differentiate into fibroblasts, chondrocytes and osteoblasts. Theoretical models have been developed to mathematically describe the regulatory role of biophysical stimuli in tissue differentiation (e.g. Prendergast 1997), and when incorporated into simplified computational adaptive models have been shown to often corroborate with temporal and spatial tissue distributions during bone healing (Lacroix 2002, Isaksson 2006). However, because bone healing is a complex biological phenomenon and adaptive computational models are sensitive to many unknown cellular and biological processes, which are simplified, the validity of mechanoregulation theories are still debated.

This study investigates the validity of one established mechanoregulation theory in a simple tissue engineering system for the purpose of defining mechanical signals which may aid in the future stimulation of chondrogenic and osteogenic cells from progenitor cells.

METHODS:

Fibrin scaffold FEA: Cylindrical fibrin hydrogel specimens were prepared and allowed to equilibrate in normal saline at RT. Unconfined stress relaxation tests were then conducted. The specimens were sequentially compressed between polished stainless steel and glass surfaces at 2, 4, 6, 8, 10, 15 and 20% strain, held till equilibrium for each step. Reaction force was recorded throughout, and the equilibrium radial expansion of the disc at the glass surface was imaged. Equilibrium modulus, E, and Poisson's ratio, ν, were directly calculated.

A poroviscoelastic axisymmetric finite element model of the fibrin specimens was created consisting of user defined elements with an Upper Convected Maxwell viscoelastic constitutive formulation of the solid matrix (Baaijens 2005). This formulation describes the material behavior of the fibrin with 5 parameters: E, ν, relaxation time, λ, viscoelastic shear modulus, G_v, and permeability, κ. The values of the latter three material parameters were found by fitting the stress relaxation reaction load measurements at each strain level to the finite element model simulations using a multidimensional unconstrained nonlinear minimization algorithm.

In vitro progenitor cell stimulation: Bone marrow mesenchymal stem cells (BMSCs) were isolated from the tibiae and femora of Wistar rats. The adherent cells were expanded up to the third passage in monolayer culture with αMEM and 15% FCS. 10^7 cells/ml were cast into fibrin cylinders.

The fibrin/cell constructs were placed individually into chambers that held the fibrin cylinder between two polished stainless steel platens. The chamber design allowed continuous circulation of culture media (αMEM, 1% ITS, 1% aprotinin, 10 mM βGDP, 60 mg/l ascorbic acid) around the outer cylindrical surface. After 3 days without loading, the chambers were placed in a multi-unit pneumatic actuator and dynamically loaded under displacement control. Control construct were collected at this stage (day 0 controls). Constructs were cyclically-loaded for 2h daily, for 7 and 14 days, in uniaxial unconfined compression (see below for strain magnitudes). The constructs then remained in the chambers with circulating media for 7 more days without load.

Mechanoregulation corroboration: Prior to in vitro experiments, the fitted parameter values as well as boundary conditions for the in vitro experiments were input into the FE model of the fibrin constructs. For a variety of realizable loading

conditions, the distribution of stimulated cell phenotype in the fibrin according to the mechanoregulation theory based on octahedral shear strain and interstitial relative fluid velocity (Prendergast 1997) was predicted. The MSC seeded fibrin constructs were then loaded under selected loading conditions similar to that used in the FE model. Finally, the mechanoregulation theory predicted cell phenotype was compared to the gene expression profile (RT-PCR: loaded, n=8/ε; unloaded, n=12) and histology assessment (IHC: CD105, n=2) in the core of the fibrin construct from the in vitro experiments.

RESULTS:

Average Poisson's ratio was 0.15 (range 0.11-0.19). Equilibrium modulus did not differ between 2-10% ε (0.02 MPa), but was lower for 15-20% ε (0.01 MPa). A good fit between measured and simulated force data was found for all strain levels (ave. R^2=0.94, range 0.87-0-97). The optimization procedure was found to yield a unique solution independent of initial values. λ and G_v were found to vary linearly with ε whereas κ was similar for the 2-10% ε range (1.24×10^{-12} m^4/Ns)).

Various loading waveforms were explored with the FEM of the fibrin cylinders in the bioreactor. As the shear strain and fluid velocity were similar for the range of ν and κ found above, constant values were used. A trapezoidal waveform (100 μm/s ramp-up, 5 s hold, 100 μm/s ramp-down, 5 s hold) was finally selected. With this waveform, steady state was reached in 10-15 cycles for the range of strain magnitudes explored. As expected, shear strain was constant throughout the carrier whereas fluid velocity differed and had the highest gradients at the free outer surface. This led to a radial distribution of mechanical stimuli, but at some strain magnitudes all or the majority of the construct was predicted to have a homogenous cell phenotype. The strain magnitudes of 2, 5 and 10% were predicted to stimulate osteogenic, chondrogenic and fibrogenic cells respectively at the central core of the fibrin carrier.

The isolated and expanded (2 passages) MSCs were checked for their multi-potentiality. MSCs when grown in osteogenic media (Sakai 2003) for 21 days in monolayer developed mineralized nodules as confirmed by ^{45}C assay and von Kossa staining. When grown in chondrogenic media (Sakai 2003) embedded in fibrin for 14 days, aggrecan and collagen-II mRNA were up-regulated.

The MSCs when loaded into the fibrin carrier were found to be well distributed throughout the carrier (qualitatively checked on H&E stained images) and did not change in cell viability over the 17 day culturing period (calcein AM stain compared quantitatively at day 3 and day 17).

After 7 days of loading: only mRNA levels of OPN was significantly up-regulated 4x with 2% strain compared to 0% strain. There was no difference in coll-I mRNA levels between different strain magnitudes. Aggrecan mRNA levels tended to increase with increasing strain levels. Coll-II mRNA levels were similarly slightly up-regulated for 2, 5 and 10% strain. SOX9 was only slightly up-regulated for 10% strain.

CD105 was highly expressed in cells before loading (day 0) and after 7 days at 0% strain. At strain levels 2 and 5% after 7 days of loading, CD105 was also partially expressed at a much lower level. CD105 expression for 10% strain was very low but still detectable.

All results after 14 days of loading are pending.

DISCUSSION:

Surprisingly a simple biphasic or poroelastic description of fibrin at a concentration in the range used for tissue engineering constructs was not valid. Especially the peak reaction loads and pressures were quite inaccurate. In contrast, a poroviscoelastic description was more accurate in describing the entire transient and equilibrium response. Curve fitting to calculate parameter values was very robust.

MSCs maintained their phenotypic cell marker for up to 17 days of culture at 0% strain. Although gene expression changes were modest after 7 days of stimulation, cells exposed to 2% strain were more osteogenic and those at 5 and 10% were more chondrogenic. No fibrogenic cell phenotype tendency was observed after 7 days of stimulation.

However, 7 days of stimulation may not be sufficient as some cells continued to express an MSC phenotypic cell marker. This will be resolved with further duration of stimulation.

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HLA-independent transplantation of human mesenchymal stem cells from bone marrow and adipose tissue for regeneration of bone

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Mesenchymal stem cells (MSC) are an attractive cell population for the regeneration of bone tissue. Some of the potential sources from which these cells can be isolated are bone marrow aspirates and adipose tissue. MSC from bone marrow are negative for immunologically relevant surface markers such as MHC-II and inhibit the proliferation of allogenic T cells *in vitro*. With due consideration for these observations, MSC from bone marrow must be described as immunologically privileged or even immunomodulating cells and could potentially be available for an allogeneic cell therapy.

Before and after osteogenic induction the influence of MSC on the proliferative behaviour of resting and activated allogeneic lymphocytes was studied as a measure of the elicited immune response (mixed lymphocyte culture). At the same points the expression of immunologically relevant surface markers (e.g. MHC-I, MHC-II, CD40, CD40L) was measured and correlations between the different sets of results were sought. In subsequent *in vivo* experiments, MSC from bone marrow and fat tissue seeded on mineralized collagen sponges have been transplanted in a xenogenic mouse model (heterotopic subcutaneous transplantation). Engraftment of MSC has been investigated using in situ hybridisation of human specific Alu-repeats. The pattern of surface antigen expression of mesenchymal stem cells isolated from bone marrow is largely the same as that of stem cells isolated from adipose tissue. Analogous to bone marrow derived cells, undifferentiated cells isolated from adipose tissue lack expression of MHC-II, this being a characteristic that is not lost in the course of the osteogenic differentiation process. With reference to their influence on allogenic lymphocytes, independently of their origin, MSC have analogous immunological features. In co-culture with allogeneic lymphocytes, both cell types fail to lead to any significant stimulation, and they both retain these characteristics during the differentiation process. In co-culture with activated lymphocyte cultures MSC from bone marrow and adipose tissue inhibit proliferation before and after differentiation. 4 and 8 weeks after xenogenic transplantation, engraftment of both cell types could be demonstrated using in situ hybridisation. On a histological level, no relevant immune response against transplanted xenogenic cells could be detected.

Our results confirm that MSC are immune modulating cells. These properties are retained even with osteogenic induction *in vitro* and seem to be characteristic for both MSC from bone marrow and cells isolated from adipose tissue. With regard to the engraftment of MSC after xenogenic transplantation, our results suggest that HLA-unmatched transplantation of human mesenchymal cells from bone marrow and from adipose tissue would be possible, for example in the context of tissue engineering. Nevertheless, for this purpose the ability for adaequat tissue specific matrix synthesis compared to autologous MSC needs to be further investigated.

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BIOREACTOR-BASED ENGINEERING OF OSTEOINDUCTIVE GRAFTS

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INTRODUCTION

A major challenge to be faced in order to introduce cell-based therapies for bone repair into wide-spread surgical practice is to translate a research-scale production model into a manufacturing design that is reproducible, clinically effective, and economically viable. One possible means by which to achieve this goal is via a bioreactor system capable of controlling, automating, and streamlining all of the individual phases of the bone-tissue engineering process.

Bone marrow stromal cells (BMSC) can easily be expanded in monolayers from a marrow aspirate, and when loaded into porous ceramic scaffolds, are capable of generating osteoinductive constructs [1], which have been demonstrated to support bridging of large segmental bone defects in humans [2]. Moreover, when BMSC are cultured under controlled regimes of perfusion, after monolayer expansion, the cells have been shown to have an increased capacity to differentiate and deposit mineralized matrix [3].

We have previously described a bioreactor for efficient and uniform cell seeding of 3D scaffolds under direct oscillating perfusion, and demonstrated its applicability to seed expanded BMSC on ceramic scaffolds [4]. Working towards the ultimate goal of automating and streamlining the process of generating osteoinductive grafts, in this work we combined both the seeding and culturing of BMSC into porous ceramic scaffolds within a single bioreactor, and eliminated the phase of monolayer expansion. Specifically, we used this bioreactor to test whether under perfusion i) BMSC could be seeded directly into a porous ceramic scaffold, starting from nucleated cells freshly isolated from a bone marrow aspirate; ii) BMSC would proliferate within the ceramic; and iii) the resulting constructs would be osteoinductive upon in vivo grafting.

METHODS

Bone marrow aspirates were obtained from four adult donors (36 to 54 years old) during routine orthopaedic surgical procedures involving exposure of the iliac crest, in accordance with the local ethical committee and after informed consent. Nucleated cells were separated by Ficoll density gradient centrifugation and resuspended in medium supplemented with 10% FBS, 10^{-8}M Dexamethesone, 5ng/ml FGF-2, and 10^{-4}M ascorbic acid. Porous hydroxyapatite ceramic scaffolds (Finceramica, Faenza, Italy) were 8mm diameter by 4mm thick disks. An average of 18 million nucleated cells per scaffold were added to the bioreactor, and the cell suspension perfused through the pores of the ceramics at a superficial velocity of 1mm/s. After 5 days, medium was replaced, perfusion flow was reduced to 0.25mm/s, and constructs were cultured for up to an additional 2 weeks in the bioreactor. Following in vitro culture, one half of each ceramic was used to extract cells, count them and assess their phenotype by cytofluorimetry and clonogenicity by colony forming efficiency tests. The other half of each ceramic was implanted subcutaneously in a nude mouse for 8 weeks and subsequently histologically assessed by hematoxylin and eosin stain.

RESULTS

Colony forming efficiency assays indicated that the initial concentration of BMSC in the nucleated cell fraction of the marrow aspirates averaged 0.026%. The numbers of BMSC seeded within the ceramics thus averaged 4800 and increased over the culture period, up to an average of 9.0E+05 BMSC per ceramic after 19 days of perfusion. Based on the determined number of clonogenic BMSC initially loaded, this corresponded to an average expansion of 8.2 doublings. Cytofluorimetry analyses indicated the presence in the constructs of an additional component of hematopoietic cells (average of 4.2E+05 per ceramic), which could have played a role in conditioning the expansion of BMSC. BMSC extracted from the ceramic constructs were highly clonogenic (29% to 38%), in contrast to those typically expanded in monolayers (11%). When BMSC-ceramic constructs from each donor were

cultured under perfusion for 19 days, abundant bone tissue was observed throughout the ceramics, both in the peripheral and central regions.

DISCUSSION

In this study, we have demonstrated for the first time that BMSC can be seeded by perfusion onto porous ceramic scaffolds directly from the nucleated cell fraction of a bone marrow aspirate, without a prior monolayer expansion phase. The seeded BMSC proliferated within the ceramic during the in vitro culture period and retained a higher clonogenicity than monolayer expanded BMSC, possibly suggesting that they remained in a more ‘progenitor-like’ state. The reproducible, abundant and uniform bone formation by engineered constructs establishes that osteoinductive tissues for possible clinical use could be generated using autologous cells in a streamlined manufacture process based on perfusion systems [5]. Including monitoring and controlling features in the perfusion bioreactor will represent the next step to potentially provide an economically viable approach to the automated production of osteoinductive grafts [6].

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PTH AND ALENDRONATE IMPROVE OSTEOTOMY HEALING IN AN OSTEOPOROTIC RAT MODEL

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Introduction

Osteoporosis is a major health problem in older populations, and is directly related to the increased rate of fractures in the elderly. Additionally, both aging and osteoporosis are know to be frequently associated with decreased quality of fracture repair. A number of drugs have been developed for the prevention and treatment of osteoporosis. The most commonly prescribed class of drugs for this purpose are the bisphosphonates, which act by inhibiting osteoclast activity and subsequent bone resorption. Numerous clinical studies have shown that treatment with bisphosphonates results in increased bone mass and decreased fracture risk when compared with untreated control populations. Previous animal studies with bisphosphonates have shown increased callus size and bone mineral content when compared to controls; however callus remodeling and fracture line appearance are inhibited, and *in vivo* and post-mortem mechanical studies have not shown an increase in torsional stiffness related to callus size. While bisphosphonates appear to be relatively safe with respect to metaphyseal fracture healing, there is minimal clinical data on the effect of bisphosphonates treatment on diaphyseal fracture healing in the osteoporotic population.

Forteo® (recombinant human PTH) is the first commercially available drug with a demonstrated anabolic effect on bone in animals and humans. PTH enhances bone mass at all four levels: cancellous, endocortical, intracortical and periosteal. Intermittent dosing of PTH has been shown to increase callus volume and mechanical fracture strength in animal studies, and there is evidence to suggest that fracture healing may be accelerated in animals treated with PTH. Additionally, PTH can stimulate bone formation independent of resorption, and can restore cancellous and cortical bone volume in parathyroidectomized rats. It was hypothesized that pretreatment with bisphosphonates would (i) compromise fracture healing, (ii)that post-fracture treatment with PTH would produce superior results in fracture healing when compared to Alendronate-treated animals or controls, and (iii) that PTH would reverse pretreatment inhibition of bone metabolism secondary to bisphosphonates therapy.

Material & Methods

One hundred and twenty-six Sprague-Dawley rats were used for this study. The rats were ovariectomized and randomly divided into two groups: Group 1 (n=63) received 10 μg/kg/week of Alendronate by subcutaneous injection, and Group 2 (n=63) served as controls (no pretreatment). Surgery was performed on all rats at eight weeks post-ovariectomy. At the time of surgery, the group that received pre-treatment with Alendronate (Group 1) was randomly divided into three groups: Group 1a (n=21) continued weekly injections with Alendronate, Group 1b (n=21) received daily injections with rhPTH (Forteo®) (10 μg/kg/day), and Group 1c (n=21) received no treatment; the rats that did not receive Alendronate pretreatment were randomly divided into the same three post-surgical treatment groups (groups 2a-c). Surgical technique involved an open femoral osteotomy with a Stryker saw at the mid-diaphyseal level. After anesthesia and preparation, a 3 cm skin incision was made over the lateral femur. The femur was exposed by separating the vastus lateralis and rectus femoris muscles, and an osteotomy was made using a Stryker micro saw through the mid-diaphysis of the femur. A smooth Kirschner wire (1.1 mm) was then inserted in a retrograde manner through the medullary canal at the osteotomy site, exiting through the greater trochanter. The osteotomy was then reduced by inserting the K-wire through the distal femoral diaphysis. All animals were euthanized six weeks after surgery. Samples were analized by two-factor analysis of variance (ANOVA) with interaction. Mechanical testing was performed in all samples in four point bending using an ELF 3200 high precision mechanical testing apparatus (Endura TEC, Minnetonka MN). Bending stiffness, failure moment and failure deflection was determined. Material properties were estimated based on bending results and cross-sectional

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geometry. Osteotomy healing was evaluated by manual palpation, biomechanical testing, μCT analysis and qualitative histology. Statistical analysis was done using χ^2 and two-factor ANOVA tests.

Results

At 6 weeks the osteotomized femurs were tested. The union rates for animals pre-treated with Alendronate were as follows: Group 1a – 82%, andGroup 1b – 78%, (p=0.021) compare to Group 1c – 44%. For the rats that did not receive pretreatment (Group 2), the union rates were as follows: Group 2a and 2b – 56%, Group 2c – 41%. The μCT analysis showed increased volume of callus formation in rats treated with Alendronate and PTH over untreated controls ($p<0.05$).

Estrogen deficient rats from the control group showed a 59% nonunion rate at 6 weeks. Those rats that received pharmacologic intervention before osteotomy presented with a non union rate of 54% statistically insignificant compare to the control group.

Intervention with either Alendronate or PTH was statistically significant for all osteotomized samples after mechanical testing.

Mechanical results from the osteotomized callus evidenced homogeneous results non-dependable on the pharmacological intervention. Also, at six weeks the osteotomized callus samples demonstrated 30 % of bending stiffness compared to the non osteotomized side.

More important, Estrogen deficient rodents that received Alendronate or PTH did decrease the rate of nonunion compared to those estrogen deficient rats that both received Alendronate before osteotomy that was stopped after intervention or the control group. (table. 1)

Conclusions

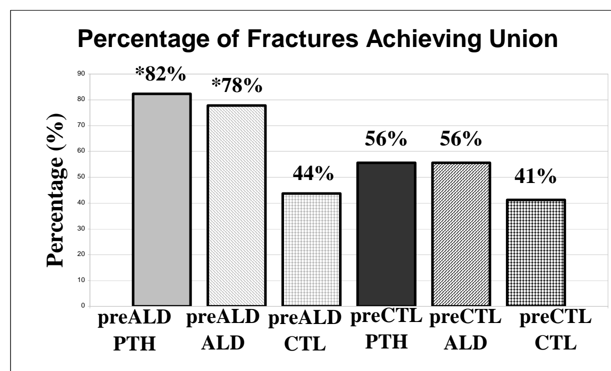
In the setting of Estrogen deficient osteoporotic rat model, at 6 weeks, pretreatment with Alendronate and post treatment either with Alendronate or PTH prevented from the development of nonunion compared to the control group.

Pretreatment with Alendronate did not inhibit osteotomy healing, but prevents the development of nonunion when continued after surgical intervention in the estrogen deficient rat model. Comparable union rates results were evidenced by the use of PTH or Alendronate after osteotomy in both Alendronate pretreated group animals even though the pharmacological mechanism of action is known to be different.

In the osteoporotic animal model both PTH and Alendronate are enhancers of osteotomy healing.

*This study was supported by a grant of the Orthopaedic Trauma Association and the AO Research Fund.

Table 1.



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AN ENGINEERED VASCULARIZED GRAFT FOR LARGE BONE DEFECT

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Background and Introduction: Autologous bone grafting is the current golden standard for the repair of large bone defects, despite its drawbacks like limited availability of grafting material and donor site morbidity. Possible alternatives like allografts or xenografts have also serious limitations, like the risk of infections, possible immune reactions and ethical issues. Due to these problems, researchers in the area of bone repair have explored alternative solutions. Calcium and phosphate based materials as well as polymer scaffolds have shown some interesting osteoconductive properties. Nevertheless, the lack of osteoinductive potential prevents the healing of large bone defects treated only with such alloplastic materials. Many studies have shown that the lack of osteoinductive potential of such scaffolds can be partly overcome by seeding mesenchymal stem cells (MSC) onto the scaffold prior to implantation. However, a major problem still remains, namely the insufficient vascularization of the central part of these large grafts (>4cm).

Rational: Therefore, the aim of this study was to in vitro evaluate the interaction of MSC and endothelial cells (EC) combined within a 3D scaffold on MSC differentiation into osteoblasts and whether the EC were showing any early indications of vessels formation. Furthermore, in an earlier study, we have shown that activated platelet-rich plasma (PRP) had the potential to strongly promote osteoblastic differentiation of MSC (1). This osteoblastogenesis-promoting property of PRP combined with its ability to form a gel upon activation suggests that PRP could act as a carrier for cells, and could at the same time deliver autologous biological stimuli necessary to improve angiogenesis and bone formation within a construct seeded with the appropriate cells. We have therefore studied the potential of a complex 3D-construct composed of polyurethane (PU) (2) scaffold seeded with MSC and EC (3) embedded in a PRP gel in a controlled *in vitro* environment.

Methods: Aspirates of bone marrow and blood were obtained from patients undergoing hip surgery after informed consent (KEK Bern 126/03). MSC were isolated using a Ficoll gradient and were expanded in IMDM, 10% FCS and 5ng/mL FGF-2. Primary human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics (cat# C-003-5C) and were expanded in M200 (Cascade M200-500) supplemented with LSGS (S-003-10). PRP was produced by two consecutive centrifugation steps and was activated by 5U/mL bovine thrombin in $CaCl_2$.

Four types of 3D-constructs were evaluated (Fig. 1) Constructs were analyzed for gene expression of typical osteoblastic and endothelial markers as well as genes involved in angiogenesis using real-time RT-PCR. Histological analyses were performed on cryosections using toluidene blue and van kossa staining, immunohistochemistry (laminin, vWf) and immunofluorescence (CD31, Osf2).

Results from two independent experiments performed in triplicates (n=6) are shown as mean±SEM. Statistical analyses were performed using the Mann-Whitney U-test, $P<0.05$ was considered to be statistically significant.

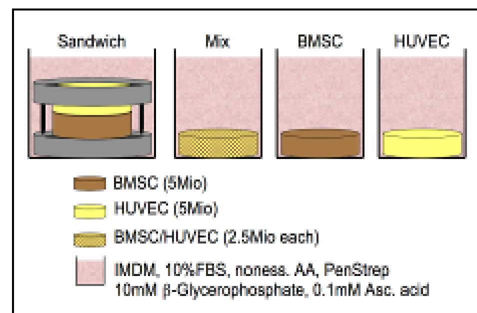

Figure 1. Experimental set-up.

Results: MSC and HUVEC embedded in PRP and seeded on PU scaffold were able to survive in shared culture medium for more than 35 days in IMDM, 10% FCS, without further supplementation.

Mix-constructs showed a significant up-regulation of osteoblastic markers compared to MSC alone or Sandwich-constructs after 35 days (Fig. 2). The same was true concerning endothelial and angiogenic markers (von Willebrand factor (vWf), VE-cadherin, EGFL7, VEGFR1,-2, and -3, PDGFRB, Tie1, Tie2, MMP-2 and MMP-9) compared to HUVEC alone or Sandwich-constructs after 35 days.

Histological analyses after 21 and 35 days revealed early mineral deposition only in Mix-constructs (by Van Kossa staining). Furthermore, cells in Mix-constructs showed formation of inter- and intracellular lumen (Fig. 3) as well as formation of tube-like structures, as assessed by immunostaining for vWf and laminin (Fig. 4), as well as immunofluorescence staining for CD31 (data not shown).

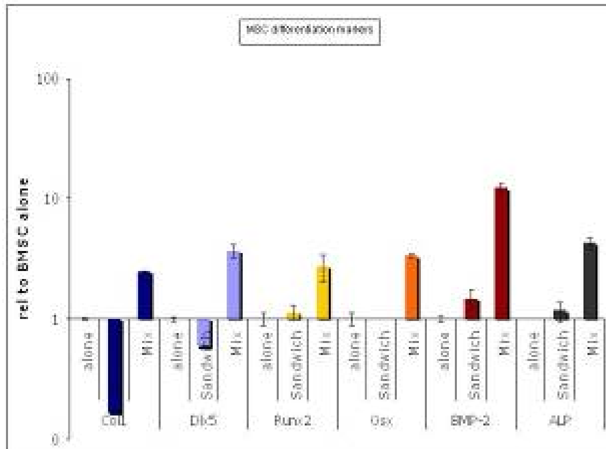

Figure 2: Mix-constructs are showing an up-regulation of osteoblastic differentiation markers.

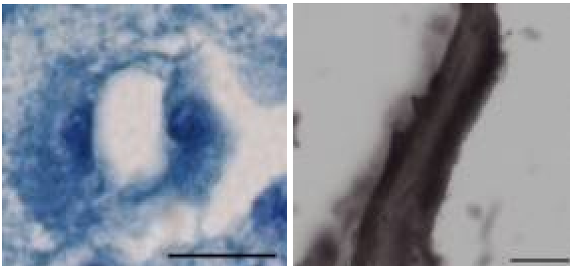

Figure 3: Intercellular lumen. Figure 4: Tube-like structure

Discussion and Conclusion: The aim of this study was to define an optimal construct *in vitro* to serve as a potential alloplastic bone graft *in vivo* that could overcome the problem of insufficient vascularization in large bone defects. Our constructs consisting of a polyurethane scaffold seeded with MSC and/or HUVEC in PRP showed an up-regulation of genes involved in osteoblastic and endothelial differentiation, as well as angiogenesis. Furthermore, constructs consisting of a mixture of MSC and HUVEC in PRP (Mix-construct) showed a much higher expression level of the above genes than the other evaluated construct types. This suggests that direct contact of MSC and HUVEC enhances osteoblastogenesis and vessels formation, at least at an early stage. In this respect, histological analyses revealed intra- and intercellular lumen formation as well as tube-like structures only in Mix-constructs. All these observations were done up to culture day 35, proving the high stability of these tube-like structures. These *in vitro* results suggest that our 3D-construct consisting of a mix of MSC and HUVEC in PRP seeded on a PU scaffold might have the potential to significantly improve vascularization and therefore bone formation within the construct when implanted into a large bone defect *in vivo*.

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The Swiss MD-PhD association (SMPA): a way to bridge the gap between the bench and the patient's bed

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The complexity of basic medical science is rapidly growing. The high level of technologies applied in basic science demands specific and extensive training for researchers. This questions the "late-bloomer" pathway of clinicians that switch to basic research after their clinical training and begins to set researchers and clinicians apart. The weakening of the bridge between bench and bedside has led to the creation of MD-PhD programs, in Europe as well as in the US. MD-PhD programs should improve the efficiency of medical research by creating a seed for close collaboration between basic research and clinical sciences.

The Swiss MD-PhD association (SMPA) (http://www.smpa.org) is an organization of physician-scientists (MD-PhDs), which promotes scientific and personal cross-talks between clinicians and basic scientists. The SMPA is a young association created in June 2003 and currently comprises around 70 physicians with an M.D.-Ph.D. education, M.D.-Ph.D. students and M.D. with a strong activity in research. The design of an optimal MD-PhD education and career planning for young physician-scientists is one of the main focus of interest of the society. In that respect, a close collaboration with the Swiss Academy of Medical Sciences (SAMS), the Swiss Medical Association (FMH) and other institutions has been established. Once a year, the SMPA organizes a general assembly embodied in a scientific meeting. The latter offers the opportunity to invite well known speakers, to engage scientific and political round table discussions and to bring clinicians and basic scientists in contact to promote translational and clinical research.

Biotechnology Funding in the AO Foundation

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Background and Introduction:

The AO Research Fund has existed since 1983 and its principal function for some years has been to offer research grants in the general areas of interest to the AO Foundation and primarily seed money for young research workers. Applications were open to anyone and funding was up to a maximum of CHF 60,000 for 1-3 years with little monitoring.

The mission of the AORF now is to support basic scientific, pre-clinical and clinical research in all areas of trauma, surgery of the musculo-skeletal system and related problems.

It still provides predominantly seed money to individual researchers and research groups, finances pilot studies and supports investigation of new and unconventional ideas or hypotheses. These are called Start Up grants and are for a maximum of CHF 60,000 for up to 2 years.

But now it also funds projects focused on specific clinical problem areas (the AO Clinical Priority Programmes) as defined by the Academic Council. These are called Focus grants and are for a maximum of CHF 100,000 for up to 3 years.

The principal clinical areas encompass general trauma, spine, cranio-maxillofacial and veterinary surgery.

What about Biotechnology?

In recent years, Biotechnology has figured highly among applications and grants awarded.

How are grants obtained?

There are two deadlines per year:
February 15
August 15

All details are on the AO website at
www.aofoundation.org/aorf

Application forms are available for downloading and are submitted by email

Forms must be submitted in full and must comply exactly to the guidelines available. In particular the finances must be well documented. Normally we do not accept applications for salaries for investigators. Forms which fail to comply are returned without review.

All applications are reviewed by two reviewers who are usually members of the AORF Commission but may be external reviewers. Focus grants in the field of Biotechnology are also reviewed by two members of BAB and the four reviewers will try to agree a recommendation.

All decisions are made by the AORF Commission which meets twice a year.

Social and Ethical Issues in Regenerative medicine: towards a governance of 'bench to beside'

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Discussion:

Regenerative medicine (RM) is still in its infancy and need to be defined. We will consider it as the field of 'replacement, repair and regeneration' of tissues or deficient organs. Advanced therapies such as gene therapy, somatic cell therapy and human tissue engineering are part of RM. Like any new technologies, the development of RM is balanced between opportunities and uncertainties, hope and hype, risk and benefits.

The field of RM has enormous potential and existing advances are being made. Nevertheless, numerous concerns arise concerning:

- Appropriate regulatory processes and new regulation for 'combination products' (living cell and scaffold for example), etc.
- Risk assessment and safety standards for the donation, testing, procurement, processing, storage, distribution and preservation of tissue and cells, (pure material, toxicology of the biomaterial, contamination, diffusion in the body), etc.
- Complexity of the issues and multidisciplinary science (how to go beyond the cultural divide between clinicians and scientists), communication skills (e.g. converging technologies), etc.
- Links between academic research and industries, competitiveness, intellectual property etc..
- Ethical issues e.g. consent, ownership, animal material, traceability of donors, surveillance of recipients, visions about ageless bodies, boundaries between therapy and enhancement etc.

This list is not an exhaustive one.

As has been learned with high profile controversies over BSE, GM crops, public attitudes play a crucial role in the realization of new technological advances. Our societies depend on science and technologies, the public awareness of the dangers and possibilities continues to increases. There is a clear mistrust in institution concerning the governance of technologies. Nevertheless, risk assessment should not constitute the only issues of the development of RM. Scientific community has the challenge today to listen and value the public knowledge. In biomedical research, concerned public such as Voluntary Health Organizations (like patient groups) can contritube by their personal experience of the disease to the development of the research and the clinical introduction. Innovation processes concerning RM must be opened at an early stage. RM development offers the challenge to integrate social and ethical reflections into the process of technology design in order to contribute to further developments in this promising field.

The upstream nature of RM gives an opportunity to generate a constructive debate about the future of these advanced therapies. The most effective models of care need to be developed. Upstream engagement, transparency, accountability, openness and confidence building will contribute to a governance from 'bench to beside'.

ACKNOWLEDGEMENTS: I would like to thank Dr. M. Hofmann and Dr. L. Otten for the constructive exchanges that we had.

Nonviral DNA Vectors in Bone Regeneration

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Background and Introduction: There exists an ongoing need for technology to enhance gene transfection in vivo in an orthopedic setting, both in terms of vector design and in terms of DNA design. From a perspective of translation to clinical impact, nonviral approaches to gene delivery in orthopedics present a far simpler pathway than viral approaches. Candidate genes for induction of bone formation are genes that encode the bone morphogenetic proteins, of course, as well as transcription factors involved in mesenchymal stem cell differentiation into osteoblasts, including RUNX2.

Rational: As an approach to obtain sustained levels of therapeutic protein, such as of the BMPs, gene delivery presents a reasonable approach to sustained release approaches, since one transfection leads to prolonged expression. As an approach to obtain sustained levels of intracellular proteins such as transcription factors, transfection is also a logical choice, being far more straightforward than extracellular delivery of the transcription factor, e.g. with a membrane translocalization tag. In the environment of an orthopedic defect, created by surgery or by trauma, an early competition for the delivered gene between macrophages and fibroblasts will exist. Presumably, transfected fibroblasts will have a much more prolonged impact in production of expressed extracellular proteins such as BMPs than will macrophages, given their longer lifespan. Thus, there exits a need to develop targeting approaches for fibroblasts in the environment of an orthopedic defect.

Methods: Multifunctional peptides are being explored as nonviral vectors for plasmid DNA.[1] Bifunctional peptides comprising a DNA-binding domain (lysine hexamers or nonamers), are flanked by cysteine resides to allow stabilization of formed nanoparticles by disulfide bonding in the extracellular environment. This DNA-binding domain is fused to a fibrin-binding domain, to retain DNA within a fibrin ingrowth matrix used as a surgical implant to localize administration of the DNA within a surgical defect.

Results: Preliminary results on the delivery of an angiogenesis-inducing gene, namely a mutant of the hypoxia-sensing transcription factor HIF1α have been collected. The gene, condensed with the bifunctional peptide, has been delivered in dermal wounds of mice, and histomorphological indications of expression of genes downstream to this transcription factor, most notably vascular endothelial growth factor, are measured.[2] If effective transfection in this assay were taking place, then induction of angiogenesis within the tissue defect should be present. Such angiogenesis was indeed observed at prolonged durations.[2]

In order to adapt this system for gene delivery in bone, a number of changes are currently being made. Most notably, additional bifunctional peptides are being synthesized to bind to integrin receptors expressed on wound fibroblasts but not on macrophages. These peptides will be fused to DNA-binding peptides in an attempt to target the peptide-DNA nanoparticles to fibroblasts for selective uptake and transfection. Moreover, substantial engineering to the gene itself is underway. Notably, we are expressing the BMP-2 protein with a FLAG tag attached, to enable localized, quantitative determination of BMP-2 expression. This BMP fusion protein was demonstrated to be biologically active. Secondly, we are localizing the BMP-2 gene within a genetic environment that should enhance nuclear localization based on DNA sequence, rather than in earlier work based on nuclear localization sequences incorporated within the peptide-DNA nanoparticle.

Discussion and Conclusion: This project aims to adapt a soft tissue transfection system for use in forming new bone in bony defects, focusing on prolonged BMP-2 expression from transfected fibroblasts in the bone defect wound environment. Engineering is being conducted on the nonviral vector and on the gene itself, to optimize its uptake, nuclear localization and expression.

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PDGF Gene Therapy to Promote Oral Implant Osseointegration

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Background and Introduction: Tooth loss is a common consequence of oral disease or injury to the craniofacial complex. Over 240 million people in the industrialized world are missing one or more teeth. Of these, only 2% receive tooth replacement dental implants. Despite the costs associated with oral implants, tooth replacement sales are growing at a year-over-year rate of 15-18%, and are estimated to exceed $1.1 billion USD globally, thus representing a significant health care burden. Tooth loss can lead to the destruction of nearly half of the original tooth-supporting (or alveolar) bone[1]. Traditional techniques for enhancing bone formation for oral implant placement include bone autografts, allografts or guided bone regeneration[2]. The use of osteogenic growth factors such as platelet derived growth factor (PDGF) to regenerate tooth-supporting and peri-implant alveolar bone in preclinical animal models[3,4,5,6,7] and in early human trials[8,9] offers significant potential for periodontal regenerative medicine. However, outcomes of these therapies are limited in terms of regeneration and predictability, in part due to drug instability at the site of delivery. Therefore, the utilization of gene therapy to control the release and bioavailability of osteogenic GFs offers potential for tissue engineering of osseous defects[10].

Recently, our group has demonstrated the potential of using gene delivery to regenerate alveolar bone and cementum around teeth and alveolar bone associated with dental implant fixtures [11,12](Jin, et al. 2004; Dunn, et al. 2005). These studies have demonstrated strong potential for the use of gene therapy for bone regeneration.

Delivery of PDGF by gene transfer has been shown to stimulate gingival fibroblast, PDL and tooth-lining cell (cementoblast) mitogenesis and proliferation above that of continuous PDGF administration in vitro[13,14]. PDGF has also demonstrated positive effects in regenerating bone around teeth and dental implants. The primary goal of this application is to validate novel PDGF gene delivery regenerative medicine strategies and apply them to animal models with a long-term goal of human application.

Rationale: For gene therapy to become a clinical reality for human application in the treatment of disease or injury, safety must be a primary concern. The use of viral vectors for growth factor delivery to bone defects requires evaluation of specific vector biodistribution properties (i.e., dissemination of vectors from the osseous site to other extraorthopic tissues and organs). Various safety assessments have been performed using growth factor transgenes[15] and for bone-sparing agents preclinically[16] demonstrating lack of significant local and systemic toxicity. The continued diligence in carefully evaluating both short-term and long-term safety of gene therapy vectors will be important if gene therapy is to become a viable treatment alternative for bone repair applications. Thus, the major goal of this application is to determine the potential of PDGF gene delivery to repair alveolar bone defects and to comprehensively assess safety in an attempt to grow bone where success has not been met using traditional growth factor application.

Methods: The maxillary first molars of male Sprague-Dawley rats were extracted bilaterally and the extraction sockets and soft tissues were allowed to heal for 30 days. Osteotomy defects were subsequently created and 1 x 2 mm titanium oral implants were then press fit into position (Figure 1). The remaining surface of the implants and the osseous defects received the following treatments: adenovirus encoding PDGF-B (AdPGFB 8 E11 particles/ml) in 2.6% collagen gel (n = 8) or collagen alone (n = 8). In an alveolar defect model utilizing AdPDGFB virus, viral copies within the blood and organs were detected using real time PCR in all of four groups: high dose Ad-PDGFB (8 E11 particles/ml), low dose Ad-PDGFB (8 E10 particles/ml), collagen alone, and untreated control. In addition, biodistribution of the virus was evaluated using in vivo bioluminescence using Ad-

luc as a reporter to evaluate dissemination of the vectors beyond the craniofacial complex.

Results: By four weeks post-surgery, both the AdPDGFB and collagen only groups demonstrated more mineralized tissue when compared to the two week time points ($p < 0.05$). Viral copies detected in the blood were not significantly different between treated and untreated rats at all time points. Viral copies within the organs were also not significantly different between treated and untreated rats for all time points except for a slightly elevated level found in the high-dose group at 14 days post surgery in the liver and spleen. Bioluminescence results demonstrated the localization of the vector to the defect site, with minimal dissemination to the organs over the course of 70 days. Finally, from the set of preliminary micro-CT images, representative images were converted to a 3D finite element model for simulated biomechanical testing.

Discussion and Conclusion: These preliminary and ongoing data suggest the feasibility of administering and targeting PDGF gene therapy vectors to oral implant defects and the ability of this model to be assessed in terms of efficacy and safety. Finally, these data confirm the potential of CT imaging of titanium implants for quantification of alveolar bone and simulated biomechanical testing.

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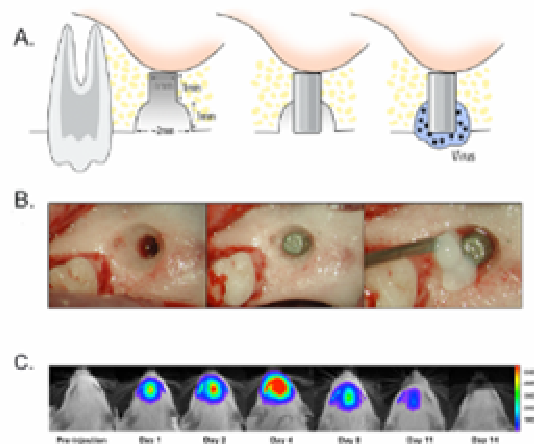

Figure 1: A. Oral Implant Osteotomy Defect Model for PDGF Gene Delivery. "Well-type" osteotomy defects B. High magnification photos from the surgical operation including defect creation (left panel), dental implant placement (middle) and gene delivery (right). C. Optical imaging of reporter gene, Ad-luciferase targeting to oral implant defects. Color enhancement demonstrates localization of luciferase protein production following injection of luciferin substrate into animals from baseline to 14 days post-gene delivery to oral implant defect sites.

Gene terapy in regeneration of the intervertebral disc

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Background to regeneration of the intervertebral disc:

Our research is directed towards exploring the importance of the extracellular environment on the function of cells from the central nucleus pulposus (NP) of the intervertebral disc (IVD) and utilizing that information to facilitate strategies for regenerating the degenerate IVD.

The NP is a key component of the IVD. The matrix produced by NP cells is highly hydrophilic, generating sufficient swelling pressure to force the vertebrae apart even under the loads induced by gravity and muscle action that operate on the erect human spine. The NP is also the site of the earliest and ultimately most severe matrix changes in the IVD in degeneration.

Degeneration of the IVD (DIVD) leads to loss of disc matrix, approximation of the vertebrae *("loss of disc height"),* and mechanical back pain; indeed it is held to be the most common cause of significant chronic back pain and sciatica and as such is one of the most important causes of morbidity and loss of work in the Western world.

Restoring IVD height is a major goal in the management of DIVD and chronic back pain.

Tissue engineering +/- IVD regeneration (IVDR) are obvious approaches to restoring disc height. Before adopting either it is important to understand what roles might be required of the components of putative regenerates and how the local conditions within the degenerate disc might influence their behaviour.

Our work:

Our approach has been to study the basic biology of IVD degeneration and to formulate a regeneration strategy with our front-line clinical colleagues.

Briefly, we have established:

- An understanding of the pathobiology of IVD degeneration and particularly matrix loss. In particular we have shown that the IL-1 family is key to initiating production of the matrix degrading enzyme cascades that lead to matrix loss and reduction in disc height. And that the best source of cells to populate a regenerate is mesenchymal stem cells (MSC).
- Clinical colleagues would ideally want a regenerative medicine based therapy that could be simply delivered and would result in restoration of the normal anatomy of the motion segment around the degenerate IVD.

Our vision is therefore to produce an injectable proto-regenerate, with: the basic building blocks (cells, biomatrix) from which to regenerate the IVD; and built in regulatory processes to both promote regeneration (programmed extracellular signaling messages) and to inhibit the underlying disease processes within the target IVD.

Part of this strategy is to test the use of gene therapy approaches to regulating cell function.

This presentation:

In this presentation we will describe two gene therapy experiments made possible through funding from the AO Foundation.

The first is to inhibit IL-1 mediated events in the IVD by using a gene delivery system for the IL-1 inhibitor IL-1Ra.

The second is to promote cell survival, differentiation towards a chondrogenic phenotype and matrix synthesis by MSC using PLLA scaffolds s a paradigm.

Briefly in the first we have been able to totally inhibit MMP and ADAMTs protein expression by native human nucleus pulposus cells in IVD explants by injecting into the explant MSC transfected with IL-1Ra gene in an adenoviral vector.

In the second we have rescued MSC from cell death, and caused them to differentiate into chondrogenic cells synthesizing an NP matrix by transfecting the MSC with the gene for the master chondrogenic regulator gene Sox-9, again in an adenoviral vector and matrix synthesis of stem cells

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Bone Formation Enhancement by BMP-2-Functionalized Implant Coatings - A Study in Miniature Pigs

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The objective of this investigation was to compare the osteoinductive efficacies of four different BMP-2-carrier modes at a maxillary implantation site in miniature pigs: (i) uncoated implants bearing adsorbed BMP-2; (ii) implants bearing a calcium-phosphate coating upon which BMP-2 was adsorbed; (iii) implants bearing a calcium-phosphate coating into which BMP-2 was incorporated; (iv) coated implants bearing incorporated and adsorbed BMP-2. The screw-like implants (n = 6 per group) were inserted within the partially edentulous maxillae of 18 adult miniature pigs. They were retrieved 1, 2 and 3 weeks later for the histomorphometric analysis of new bone formation (a) along the implant (coating) surface, and (b) within the core of the implant chambers. The analysis revealed a complex biological picture. During the early (1-week) post-implantation phase, BMP-2 stimulated the recruitment and activity not only of osteoblasts but also of osteoclasts. Hence, particularly along the implant (coating) surface, new bone was both deposited and degraded, and to proportionally different degrees in each group. Within the core of the implant chambers, newly-formed bone was degraded to a lesser degree. By the third week, the implant chambers were generally so well filled with bone as to preclude an adequate discrimination between the osteoinductive efficacies of the three coated-implant groups. Significantly less bone was associated with the uncoated implants bearing adsorbed BMP-2. Our findings reveal: (1) the importance of evaluating osteoinductivity both temporally and spatially; and (2) the need for a more “challenging” experimental implant model.

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Characterizing cell adhesion and migration on surfaces biofunctionalized with nanopatterned collagen matrices

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We have recently developed a method for creating ultrathin collagen type I matrices on non-biological surfaces in which individual collagen fibrils are almost perfectly ordered on the nanoscale. Initial experiments showed that fibroblasts seeded on our nanopatterned collagen matrices polarize strongly and migrate in the direction of the aligned fibrils. The ability to regulate the adhesive and migratory behaviour of cells, combined with the excellent biocompatibility of collagen, make nanopatterned collagen matrices a promising tool for the surface bio-functionalization of so-called “smart” biomaterials.

Using live-cell/time-lapse atomic force microscopy (AFM), we have studied the interaction between fibroblast cells with the matrix on a nanoscale level and show that cell alignment involves collagen matrix deformation. Because of the high tensile strength of collagen fibrils, cells are able to build up traction and move directionally when they pull longitudinally on the fibrils. In contrast, due to their high-pliability, collagen fibrils offer only low mechanical resistance when cells pull on them laterally, preventing the creation of traction and cell movement. Cell polarization therefore reflects anisotropic mechanical properties of the collagen matrix.

Cell alignment and matrix contraction was strictly Mg^{2+}-dependend, pointing towards a role for collagen-binding integrin receptors in mediating cellular adhesion to these matrices. In agreement, CHO wild-type cells, which lack endogenous collagen-binding integrin receptors, adhered poorly to the collagen matrix, while CHO cells stably expressing the integrin α_2 subunit (CHO-A2) adhered and spread rapidly. This indicated that $\alpha_2\beta_1$ integrin was able to mediate a firm attachment of these cells to collagen. In order to quantitate $\alpha_2\beta_1$ integrin-mediated adhesion, we measured adhesion forces of single cells to the collagen matrix by AFM force spectroscopy. Forces required for cell detachment were significantly higher for CHO-A2 cells compared to wild-type cells. In conclusion, we have characterized the adhesion of different cell types to highly-ordered collagen type I matrices and show that cell polarize on these matrices by anisotropically deforming them in a $\alpha_2\beta_1$ integrin-dependent manner.

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Quantification of Porosity, Connectivity and Material Density of Calcium Phosphate Ceramic Implants Using Micro Computed Tomography

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Introduction: Calcium phosphate ceramics have been widely investigated in orthopaedic tissue engineering and surgery as bone extensor. Attention has been given to manufacturing of a porous ceramic that mimics the trabecular bone structure for better osteoconduction. Although different methods have been applied to manufacture the porous structure, it was unable to quantify the pores and their interconnection within the ceramics. With the advance of biomedical imaging technologies, the study attempted to quantify the pore structure of different ceramics using a high-resolution micro-computed tomography (microCT).

Materials and Methods: Three kinds of ceramic blocks with product names, BSC, ChronOS, and THA, respectively, were synthesized by three methods from three different manufactures and evaluated in the study. The specification claimed the porosity of the ceramic ranged from 40% to 80%. Six blocks of each ceramic were evaluated by conventional water immersion method and µCT. The pore size and connectivity of the pores were evaluated with standardized protocols. By the water immersion method, the porosity of three ceramic was ranged from 60% to 78%. The three-dimensional analysis of the pores by µCT showed that the porosity of the ceramics was 26.2% for BSA, 59.9% for ChronOS, and 67.7% for THA. The pore connectivity was 2.7 for BSC, 39.7 for ChronOS, and 7.1 for THA. The ChronOS had more functional pores (200-400µm in diameter) than that of the BSC (52.8%) and THA (43.2%) ($p<0.05$).

Discussion and conclusions: It was shown that the distribution of the pore size of three different ceramics has different characteristics. We speculated that different combination of structure parameters may have different in vivo property in osteogensis while the chemical property of the ceramics cannot be neglected in the in vivo performance. Providing objective information on the functional pores, the microCT evaluation serves as a good standard for specification of the ceramic-related implants in the future characterization of scaffold biomaterials for orthopaedic and related medical applications.

Significance of the study: The results and methods developed above have been adopted into our ongoing studies, including the one on 'Cocktail Therapy Developed for Repair of Steroid-induced Osteonecrosis', which evaluates the potential of a combination of biological (autologous bone marrow mononuclear cells), biomaterial (ChronOS), and biophysical (low- intensity pulsed ultrasound) intervention for improvement of prognosis of core-decompression in steroid-associated osteonecrosis.

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Optimization of Extracellular Matrix Production and Gene Expression by Human Annulus Cells in Three-Dimensional Constructs

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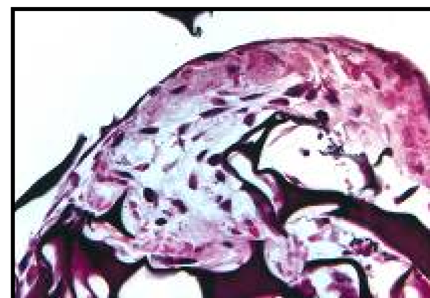
Introduction: Tissue engineering offers the potential to correct a number of conditions fundamental to disc aging and degeneration: low cell numbers in the aging disc, altered cell-cell and cell-extracellular matrix (ECM) interactions, and production of inappropriate ECM. Tissue engineering for the disc is a relatively new field, and knowledge of how disc cell microenvironments influence disc cell proliferation, ECM gene expression and ECM production is only now being obtained. This information is needed to help formulate criteria for successful cell-carrier interactions.

Objectives: To optimize gene expression, cell proliferation, and proteoglycan production by human annulus cells in three-dimensional carrier constructs.

Methods: Studies were approved by our human subjects Institutional Review Board. Annulus cells were obtained from surgical procedures performed on individuates with herniated discs or from discs procured from the Cooperative Human Tissue Network. Agarose, alginate, fibrin gel, collagen gel, and collagen sponges were used as cell carriers for annulus cells cultured from discs of 28 individuals (mean age 45.3 years, 5 Thompson grade II discs, 12 grade III discs, 12 grade IV discs). Cells were cultured in a standardized experimental protocol with 2-5 replicates for 10-14 days using previously published protocols from our lab. In situ hybridization assessed ECM gene expression of types I and II collagen, aggrecan and chondroitin-6-sulfotransferase. Assays were carried out to determine cell proliferation and proteoglycan production (S-GAG) (using the 1,9-dimethylmethylene blue (DMB) technique), and paraffin-embedded specimens were evaluated for cell and ECM features.

Results: In situ hybridization showed optimum expression of types I and II collagen, aggrecan and chondroitin-6-sulfotransferase by cells cultured in the collagen sponge microenvironment. Morphologic assessment showed production of abundant ECM between and around cells. Although collagen gels could often support good growth, these constructs did not result in either abundant ECM production or good EMC gene expression. Growth and ECM production in alginate and fibrin microenvironments were inferior.



Although agarose culture showed high S-GAG levels compared to the collagen sponge (2.94 $\pm$ 2.2 µg/ml (mean $\pm$ S.D. vs 0.94 $\pm$ 0.77), this was off-set by the significantly lower proliferation rate associated with culture of cells in agarose (agarose: 6,127 $\pm$ 2,150 cpm ^{3}[H]thymidine/µg DNA) compared to rates in the collagen sponge (12,729 $\pm$ 6,729, $p = 0.032$). As shown in the figure to the right, abundant ECM is produced by cells cultured within a collagen sponge.

Discussion and Conclusions: Human disc cells offer special challenges for in vitro studies because of the slow-growing nature of these cells. This work shows that collagen and S-GAG production can be modulated by choice of the carrier type into which human annulus cells are seeded. Cells seeded into the collagen sponge microenvironment attached well, proliferated, showed abundant gene expression for types I and II collagen, aggrecan and chondroitin-6-sulfotransferase, and produced abundant ECM evident upon histologic examination. These findings may have future application in cell-based tissue engineering strategies for biologic therapies for disc degeneration.

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Targeted Delivery of Natural TGF-β antagonist Suppresses Scar Formation during Wound Healing

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We have used a phage library (CX_7C) to identify peptides that bind to angiogenic vasculature in regenerating wound tissue. We identified a nona- and hexapeptides that selectively target phage to skin and tendon wounds. Peptide 1 shows preference for the early stages of the wound healing. Its sequence is homologous to a sequence in the heparin-binding site of the bone morphogenetic protein 4 (BMP4). Peptide 2 (CRK) is homologous to a part of thrombospondin type 1 repeat (TSR1) and binds preferentially to wounds at the later stages of wound healing. We constructed fusion proteins in which the peptides serve as a homing element and decorin as a therapeutic protein. Decorin is a natural antagonist to transforming growth factor-β (TGF-β), which is thought to be responsible for scar formation. The wound-targeted decorin fusion proteins were vastly more effective than non-targeted decorin in suppressing scar formation in skin wounds upon systemic administration. These results identify new therapy option for surgical wounds as well as for various kinds of internal trauma that goes beyond approaches based on the direct, topical application of therapeutic molecules at the wound site.

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"Early" endothelial progenitor cells (EPC) on ß-Tricalcium Phosphate scaffold under osteogenic conditions are useful for vascularisation in Bone tissue engineering

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Background and Introduction:

Early vascularisation of bone defects is a prerequisite for ingrowth of osteogenic reparative cells to regenerate bone in vivo. The size of the bone defect may limit the ingrowth of bone forming cells, since lack of vessels does not ensure a sufficient nutritional support of the bone graft. For bone tissue engineering endothelial progenitor cells (EPC) may provide a powerful cellular therapeutic strategy for vascularisation of a bone matrix (1). This investigation tests in vitro the ability of two different types of human EPC seeded on suitable scaffold like ß-tricalcium phosphate (TCP). At least two types of EPC can be cultured from peripheral blood mononuclear cells: "Early" EPC which were derived from myeloide or dendritic cell precursors, they still express leukocyte markers such as CD45 and differentiate within 5 days; secondly, "late" EPC which were derived from bone marrow stem cells and express stem cell markers such as CD133 (2,3). Cell adhesion, survival and differentiation on TCP scaffold were evaluated in order to develop an efficient cell seeding protocol to improve the vascularization and therefore accelerate the healing process of large bone defects in vivo.

Rational:

Which type of EPC can be cultivated on a ß-TCP scaffold (cell adhesion, survival, differentiation)? Which kind of precoating (collagen, fibronectin) is more effective on TCP? Do EPC remain their endothelial cell specific gene expression also under osteogenic culture conditions?

Methods:

For the in vitro study the cells were first characterized for their expression of typical EPC markers, followed by expansion in culture and loading onto a TCP scaffold. "Early" EPC were isolated from a buffy coat by density gradient centrifugation. Cells were cultivated on a fibronectin coated 24-well culture dish in endothelial basal medium (EBM) supplemented with endothelial growth medium (EGM) for 3 days. "Late" EPC were isolated from a punction of bone marrow using CD133 microbeads. The CD 133+ cells were cultured in presence of vascular endothelial growth factor (VEGF) and SCF approximately for three weeks until a sufficient number of cells was reached (4,5).

To examine the seeding efficacy and the necessity of a fibronectin or collagen G pre-coating of the scaffold, β -TCP scaffolds were precoated with fibronectin or collagen G in a well of a 24-well culture dish. EPC were loaded onto β-TCP scaffold (6,7) *in vitro* and seeded in a density of 2,5 x 10^5 cells per well and subsequentely the seeding efficacy was determined. EPC were evaluated for cell adhesion and cell viability. RT-PCR quantification of important genes involved in metabolism, endothelial and osteogenic differentiation was used to monitor the survival and differentiation process of cells seeded on ß-TCP. After 24 h, 48 h, 72 h TCP-granula were taken and adherent cells were lysed, RNA of the adherent cells was isolated, reverse transcribed and subjected to RT-PCR using specific primers for GAPDH (control), vWF(von Willebrandt factor), VEGF, Osteonectin, CBFA-1, Osteocalcin and Collagen-1.

To evaluate the influence of osteogenic differentiation, cells were cultivated for 4, 8, 12 days with osteogenic medium (Ascorbic acid, beta-glycerolphosphat, dexamethason in standard concentration), afterwards measurements of gene expression using RT-PCR was performed.

Results:

Early EPC were cultivated successfully for over 72 h on ß-TCP (seeding efficacy 93%+/-5%) if they were differentiated over a period of 3 days after isolation from buffy coat. These "early" EPC expressed over the whole period on β -TCP vWF and in part VEGF. Even under osteogenic culture conditions they still show vWF gene expression. A weak gene expression of CBFA-1 and osteonectin was also detectable. In comparison, late EPC demonstrated a weaker adhesion on ß-TCP (seeding efficacy 65%+/-5%). A significant GAPDH gene expression was detectable only at 24h after seeding only on collagen precoated

TCP-Granula. Moreover, under osteogenic culture conditions late EPC lost vWF gene expression and started to express the genes for collagen-1, osteonectin and osteocalcin.

Discussion and Conclusion:

Our data suggest that “early” EPC could successfully cultivated on ß-TCP-scaffold, they did not lose their endothelial characteristics and remained, at least in part, in a functional state since VEGF gene expression was still detectable in a number of samples even if these cells were cultured under osteogenic conditions. “Late” EPC showed only a weak adherence to coated ß-TCP and adopt osteogenic marker if they were cultivated under osteogenic conditions *in vitro*. In conclusion, “early” EPC, even they are not stem cells per definition, are an interesting candidate for improvement of vascularisation in bone tissue engineering.

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Enhanced Bone Formation by Low Intensity Pulsed Ultrasound in Posterior Spinal Fusion with Hydroxyapatite/ Tricalcium Phosphate Implant

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Low intensity pulsed ultrasound (LIPUS) was reported to increase the fusion rate of spinal fusion with autograft. The aim of the study was to investigate the effect of LIPUS on posterior spinal fusion implanted with synthetic hydroxyapatite/tricalcium phosphate (HA/TCP) bioceramics.

The HA/TCP bioceramics was implanted onto decorticated L5 and L6 transverse processes in adult rabbit. The LIPUS was applied for seven weeks post-operation while animal without treatment acted as sham group. After harvesting samples, undecalcified histology was performed. The inter-process distance and bone volume/tissue volume ratio of fusion mass was evaluated in micro-radiography. Hematoxylin & eosin as well as safranin O staining were performed for histological analysis.

LIPUS treatment group was observed to have smaller fusion gap and more fusion mass in micro-radiograph. It was further verified by significant decrease of inter-process distance in LIPUS treatment group (29%, p=0.006). Furthermore, the bone area ratio of fusion processes in LIPUS group was 50% greater than sham group (p= 0.007).

The histology of LIPUS group showed high cellularity in the large area of whole fusion bed and especially in newly formed bone while sham group showed less area near newly formed bone area of transverse processes. Moreover, the proteoglycan rich cartilage stained by safranin O was observed near the newly formed bone area in LIPUS treatment group while very less or even no cartilage tissue in samples of the sham group.

LIPUS exerted micro-mechanical stress on fusion bed in spinal fusion model. Mesenchymal stem cells, osteoblasts and chondrocytes were reported to be stimulated by LIPUS *in vitro*. They all involve in decorticated spinal fusion. The enhancement of both intramembranous and endochondral bone formation by LIPUS treatment was clearly demonstrated in this study. Thus LIPUS treatment is feasible to promote bone regeneration in posterior spinal fusion with biomaterials.

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Mesenchymal Stem Cells Pre-exposed to Basic Fibroblast Growth Factor Did Not Enhance Additional Bone Formation in Posterior Spinal Fusion

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Basic fibroblast growth factor (bFGF) has been shown to maintain the osteogenicity of bone marrow derived mesenchymal stem cell (MSCs) *in vitro*. This study is to investigate whether bFGF with osteogenic supplements could further enhance bone formation of posterior spinal fusion in an undecorticated model.

Rabbit bone marrow was aspirated from proximal femur. Bone marrow derived mesenchymal stem cells were selected by adherence on plastic culture-ware. The MSCs were treated by dexamethasone with (bFGF group, n=6) or without bFGF (OS group, n=6). Treated cells of two groups were loaded on beta-tricalcium phosphate ceramics and cultured for one day. The cell-ceramics composite was implanted onto L5 and L6 transverse processes of the same animal in posterior spinal fusion without decortication. The ceramics acted as control (n=6). Three fluorochromes were injected sequentially as tetracycline at week 2, xylenol orange at week 4 and calcein at week 6. The spinal segments were harvested at week 7. The bone mineral content (BMC) and volume of transverse processes was measured by peripheral quantitative computed tomography. The specimens were underwent undecalcified histology. The mineralization process was examined by fluorescent microscopy.

The BMC of transverse processes in OS group was found to be 16% greater than bFGF and control group significantly. The volume of transverse process in OS and bFGF group was significantly greater than control group by 54% and 46% respectively. The volume of transverse processes in OS group was 6% greater than bFGF group though not statistically significant. In histology, newly formed bone grew from two processes towards each other resulting in a relatively short gap distance in OS and bFGF group while less regenerated bone was observed in the control group. At the mineralization front, calcein which was injected into animal at week 6, was predominately labeled in bFGF group. In OS group, both xylenol orange (at week 4) and calcein labeled were found.

The MSCs were induced by osteogenic supplement to give active bone formation and promote bone remodeling during process of spinal fusion as shown by fluorescent microscopy. The bFGF with osteogenic supplements treated MSCs enhanced bone regeneration mainly on week 6. The bone formation of bFGF group might be slower than OS group. The bFGF with osteogenic supplements treated MSCs might be quite primitive in osteogenic lineage. The morphology of them was spindle shaped, like untreated mesenchymal stem cell in cell culture. They might take time to differentiate into mature osteogenic cells after implantation. Moreover, they might be able to differentiate into other cell types depending on the micro-environment *in vivo*.

In conclusion, mesenchymal stem cells pre-exposed to bFGF were not found to have additional enhancement effect on bone formation in the posterior spinal fusion model.

Effect of nicotine on mandibular distraction osteogenesis: a radiological and immunohistochemical study

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Background: Cigarette smoking is a social problem. It is a clinical challenge to treat heavy smokers receiving reconstructive surgery due to their compromised tissue healing ability. Nicotine has been implicated as the primary ingredient in cigarette smoke responsible for tobacco's physiologic effects. Although the influence of nicotine on bone is still controversial, our pilot study has demonstrated a dosage dependent response of nicotine on inhibiting bone healing on a rabbit model of mandibular distraction osteogenesis.

Objective: To evaluate the effect of nicotine on bone healing process, and on the expression pattern of bone growth factors during mandibular distraction osteogenesis.

Methods: Twenty New Zealand white rabbits were averagely assigned to the nicotine treatment group and control group. 1.5g 60-day time release nicotine pellets or placebos were embedded subcutaneously one week before osteotomy. After three days latency period, active distraction was performed at 0.9mm/day for eleven days. Five rabbits in each group were sacrificed after two and four weeks of consolidation respectively, the mandibular samples were subjected to plain x-ray, micro-CT, histological and immunohistochemical study.

Results: The bone healing process was significantly compromised in the nicotine treatment group. The presence of nicotine affected the growth factors expression associated with bone healing.

Conclusion: nicotine exposure had a significant impact on bone healing in a rabbit model of mandibular distraction osteogenesis.

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A study of the activity of patients treated with Ilizarov fixator using the physical activity monitor (PAM)

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Abstract: Fractures are common orthopaedic problems, and the Ilizarov technique of distraction osteogenesis is becoming a more common way of treating complicated fractures. Mechanical load plays a vital part in the healing of musculoskeletal system. The Ilizarov frame allows the fracture segments to have micro movement within the facture gap and to bear weight on the fractured limb, while the fracture segments are still held relatively stable. The technique and data reported here are part of a larger study of patients being treated with the Ilizarov technique. This study is investigating whether the loading applied to the bone varies between individuals and how this might modulate the healing process. The range of methods used in this study include questionnaires (assessing activity, pain and psychological parameters), radiographs, IRS, and blood sampling. In addition, we have developed an array of displacement transducers, together with an alogarithm to analyse the data, which we intend to use to assess actual loading in the fracture area. The data obtained from the Human Activity Profile (HAP) questionnaire and PAMs will therefore be fitted into this mosaic of techniques, to provide a detailed picture of the inter-relationship of these various aspects of fracture healing in vivo, when the Ilizarov fixator is applied.

Monitoring the patient's daily activity will provide vital critical information for research and for clinical diagnosis. Obviously it is very difficult to monitor the fracture patients' routine mobility for a continuous 24 hour period. In this study, we used the Physical Activity Monitor (PAM) to study the patients' continuous ambulatory activity over 24 hours.

The Physical Activity Monitor (PAM) is a TriTrac acceleration monitor which can be used to record data for 24 hours. It weighs 55 grams, with dimension of 64x62x13 mm, which allows us to track a patient's daily activities without affecting his normal activity and life pattern. PAM has been used to study Obstructive Pulmonary Disease[1], Rheumatoid Arthritis[2], knee problems[3-6] and also chronic disease, back surgery and tumor surgery of the leg. It is presumed that the fracture site will bear some loading during the action period, e.g. walking, stair climbing etc.

In order to prevent extraneous movement during use, the PAM is firmly secured to back with a backpack belt. The PAM was taken home by the patient and it is suggested that he switches it on when he feels that he is undertaking normal activities. The very large amount of recorded data obtained was analyzed using Matlab to identify the acting and non-acting duration. This entailed writing a novel program to analyze the mass of data.

The HAP is a self- reporting questionnaire. It consists of a list of 94 activities ranked in ascending order of level of energy required to perform each activity. The questionnaire is calculated using the Maximum Activity Score [7] and the Average Activity Score (AAS). MAS gives the maximum activity the patient can still do, while AAS gives a better assessment of the range of activities performed and of the presence of impairment. These scores will be compared with the PAM result to study the patient's activity times in each study period and throughout the fracture healing process.

We have previously validated this approach by giving the HAP questionnaire to 12 Ilizarov patients over the distraction period. The data obtained showed that, as healing progressed, the HAP score for an individual increased. We report here on the relationship between the HAP score and actual activity, measured using the PAM.

A limb lengthening patient, with 41mm distraction length, was studied for the whole lengthening period of 113 days. The average acting time, as measured by the PAM, was 4277 seconds over a 24 hours period. The AAS was 47, and the correlation coefficient r between the two results was 0.611. This result suggests that the activity as measured by PAM recording and the HAP score have a strong positive correlation, with high-levels of PAM results associated with high-levels of HAP scores. It also found that the patient's time of activity had just slightly increased during the limb lengthening. This could be attributed to less new bone formation during the lengthening phase. We have recently

In conclusion, these preliminary data demonstrate that it is feasible in the clinical situation to use both the patient-reported activity (the HAP) and real-time activity measurement techniques (the PAM) for assessing the actual activity of patients treated by the Ilizarov technique.

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CHARACTERIZATION OF THE OSTEOTOMY CALLUS USING FT-IRI IN A RABBIT ULNAR MODEL

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Introduction

Natural course of fracture healing includes: (i) interfragmentary stabilization by periostal and endosteal callus formation, (ii) fibro-cartilage differentiation and (iii) substitution of avascular and necrotic areas by haversian remodeling. This type of healing is often referred as secondary healing. Radiologic characteristics are more or less abundant callus formation, temporary widening of the fracture gap by osteoclastic resorption and a slow disappearance of the radiolucent line due to fibro-cartilage mineralization and bone formation.

On the other hand, direct or primary bone healing secondary to anatomic reduction and stable fixation. Lack of external callus formation and gradual disappearance of the narrow of the fracture line is the main criteria.

The process of mineralization is different in the two different scenarios due to the inherent rigidity provided by plate fixation when compared to the natural biologic process of bone healing.

FT-IRI has the capacity to measure the quality of lamellar bone, woven bone and calcified cartilage; evaluating different parameters that include: amount of mineral, amount of organic matrix, Mineral/matrix ratio, orientation of appatite and collagen, quality of bone defined as crystallinity/maturity ratio, amount of type B carbonate and maturity of collagen.

In order to compare the two different process of bone mineralization FT-IRI was applied to the following animal model. It is hypothesized that the degree of rigidity during fracture repair will alter the mineral properties of the fracture callus.

Methods

A rabbit ulnar model similar to that previously described by Bostrom et al. was used. Forty-two Male New Zealand white rabbits (6 moths old - 4.0-5.0 kg) were used in an IACUC approved study by the Institutional Animal Care and Use Committee.

After general anesthesia was provided, each rabbit's right and left forelimb was shaved and prepared with povidine and alcohol solution, and then sterilely draped. A longitudinal incision was made over the mid-portion of the ulna and the mid-ulnar diaphysis with extra-periosteal exposure.

82 limbs were available for the study; each forelimb was randomly designated to one of the following groups (21 in each group):

1. 1mm osteotomy 3 cm proximal to the ulnocarpal joint fixated with compression plate applied using AO technique.
2. 1mm osteotomy 3 cm proximal to the ulnocarpal joint fixed without compression
3. 1mm osteotomy with no fixation.
4. 3mm osteotomy with no fixation

The osteotomy was made using a 1 mm high speed dental burr. After surgery the soft tissues were closed in layers. Animals were euthanized Euthanasia was performed with an overdose of intravenous Phenobarbital at the following timelines, 2, weeks, 4, weeks and 8 wks postweeks after surgery.

Fourier Transform Infrared Microspectometry (FT-IRI) was used to determine: (i) the relative amount of mineral present (ratio of integrated v1,v3 phosphate to Amide I contours, (ii) carbonate/phosphate ratio (integrated areas carbonate(v2) and phosphate peaks), and (iii) crystallinity (peak height ratio of subbands at 1030cm $^{-1}$/ 1020cm^{-1} peak). Bone specimens for FTIR were fixed in 90% ethanol, embedded in PMMA and cut into longitudinal 2μm sections using a Jung Model K Microtome (Hedelberg, Germany) as described elsewhere. Sections were placed onto BaF2 spectral windows for analysis. *I*maging spectra were collected on a Perkin-Elmer Spotlight Spectrometer at resolution of 8cm-1 in transmission mode.

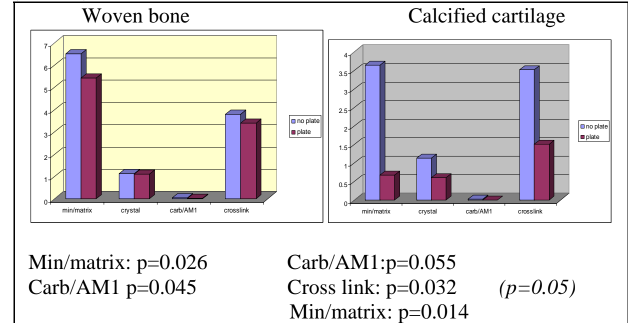
Statistics

ANOVA with p< 0.05 taken as significant was use between all groups. Student's t-Test for significance between the plated and non plated groups was used. Difference was consistent significant at (p<0.05)

Results

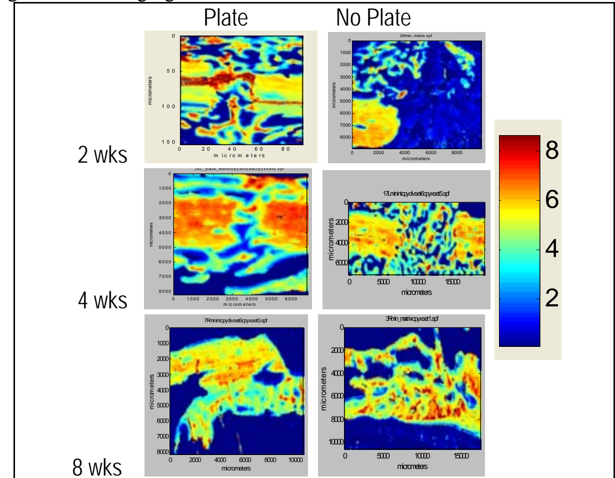
2 weeks: Mineral to matrix ratio and the carbon-Amine ratio was higher in the woven bone; also the carbon amine ratio was also higher in the non plated group compared to the plated group. When comparing woven bone between groups, the non-plated group demonstrated a more primitive less mature woven bone.

(Table 1). 2 weeks results



4 weeks: There was no statistical difference between woven bone and cortical bone but still a statistical difference was found in the amount of calcified cartilage.

Fig.1 FTI-IR imaging at 2, 4 and 8 weeks



8 weeks: a statistical difference mineral quality was not found between groups.

Conclusions

The previous data suggests that plate fixation accelerates the process of mineral development since the FT-IRI analysis demonstrated that during the early process of osteotomy healing (2 weeks) the amount of immature bone matrix and calcified cartilage was higher in the non plated osteotomy when compared to plate fixation; at four weeks, only the amount of calcified cartilage was statistically significant. At 8 weeks the mineral quality was equally comparable in both groups.

Plate fixation accelerates mineralization apposition and crystal maturation in the early stages of healing over no fixation but the amount of mineral is equally comparable in both groups after the bone has healed at 8 weeks. This study, demonstrates that fracture fixation not only affects connective tissue sequence but also the quality of the tissue.

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Synopsis of Soft Tissue Engineering Methods in Reconstructive Oral and Maxillofacial Surgery

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Background and Introduction: Operating on tumors in the oral cavity causes loss of tissue. Standard secondary surgical techniques on soft tissue are freeing of the tongue, lowering of the floor of the mouth and open vestibuloplasty. In routine interventions split skin grafts have been used for many years to cover extensive oral mucosal defects. The disposition to hyperkeratosis and growth of hair are the major disadvantages of this keratinized epithelium. Split skin grafts do neither differentiate into mucosa nor fulfil the demands of mechanical stress. Mucosal grafts do not show the disadvantages described above. In larger reconstructive procedures the limited availability of mucosa is a major concern. In reconstructive surgery the radial flap is a widely used microvascular, fasciocutaneous graft, too. To avoid the above mentioned disadvantages the the radial flap with *in vitro* cultivated mucosa transplants has been prelaminated. The aim of this study was to test the feasibility of applying tissue engineered mucosa in the above mentioned surgical procedures.

Methods: Three to four weeks prior to the transplantation a 4 to 8 mm^3 biopsy of oral mucosa and a 40 ml venous blood sample were taken to cultivate autologous cell layers. These procedures were performed in local anaesthesia. The tissue was transferred into sterile isotonic salt solution and transported to the lab. After separating the serum from the blood by centrifugation the serum was added to the cell culture medium. The medium for culturing gingival keratinocytes consists of Dulbecco's modified Eagel's medium and nutrient mixture Ham's F-12 at a ratio 3:1 with 10% autologous patient serum and additives. Primary gingiva epithelial cultures were established according to the explant culture technique. The oral tissue was separated from connective tissues by microdissection, washed with 70% ethanol and rinsed three times with phosphate buffered saline (PBS). The biopsies were cut into small pieces (1 mm3), resuspended in 2 ml culture medium and seeded into a culture flask. After 14 to 21 days the cell layer of the primary culture was detached with trypsin for 20 min at a temperature of 37°C. The trypsinized keratinocytes were transferred onto membranes in a density of 2 x 10.000 cells per cm^2. After a period of 3 to 4 weeks 2 to 3 layers of cells with an extension up to 15 cm^2 on top of the foil resulted. Then these constructs were transplanted. Biopsies were taken and examined immunohistochemically.

Results: Tissue engineered oral mucosa was applied successfully in all four surgical methods. Six months after transplantation a regular epithelial layering with a histological delimitation of the stratum, epithelial crest and a strong basal membrane appeared. According to the reception site the tissue engineered oral mucosa differentiated in several ways.

Vestibuloplasty and Freeing of the Tongue: .When the wound dressing was removed after seven days the surface still tended to bleed easily when being touched. After ten days it already seemed to be palely epithelialized. The wound generally stabilized within the next few days and healed after 20. There was a severe loss of vestibular dept within the first 6 months after the operation. Then the decrease ceased at this level. After freeing of the tongue there was one case of delayed healing, combined with severe scar formation. In the other cases healing proceeded without clinical complications.

Prelaminating the Radial Flap: After primary wound closure the donor site healed without complications like impairment of hand movement or finger force. At the recipient site no dehiscence between the borders of the flap and the resection was observed. All anastomosis functioned without failure. One case of delayed healing occurred due to an infection of the resection cavity. This was caused by a retention of saliva. Drainage of the right parotid duct was affected by the tumor resection. While the infection was drained it settled without endangering the flap. This incident resulted in a limited mouth opening. In the postoperative period the transplants showed a tendency to shrink. As table 3 shows the opening of the mouth and the movability of the tongue were good after 3 to 6 months. It was no longer possible to differentiate between the transplant and the local mucosa.

Discussion and Conclusion: The clinical results prove that cultured autologous tissue and tissue engineered grafts can be successfully applied in reconstructive oral and maxillofacial surgery. The application in clinical routine is often limited by the costs, which result from the quantity of preoperative work and laboratory preparations. These expenses could be decreased if the method was used for more indications, in other surgical disciplines and in a larger scale. To improve long term results it is necessary to achieve modifications in the field of cell cultivation and carrier materials as well as to obtain a better understanding of the physiological part of the healing process. To gain a more solid clinical evidence further controlled and randomized studies are needed, too.

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Sinus Augmentation with Periosteum derived Tissue Engineered Bone

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Background and Introduction:

Tumors in the oral cavity cause loss of bone and soft tissue. More often than the reconstruction of soft tissue the improvement of the hard tissue bearing of dentures and dental implants is required. For the reconstruction of bone defects the gold standard is still the autologous bone harvested from the iliac crest, the mandible or the maxilla. Tissue-engineering procedures for hard tissue augmentations of the maxilla offer significant advantages compared with conventional grafts, as there is minimal or no donor site morbidity, limited availability of bone and the necessity of an additional surgical procedure. Periosteum has been demonstrated to have cell populations, which include chondroprogenitor and osteoprogenitor cells, that can be isolated in tissue culture and form both cartilage and bone. The aim of this prospective study was to evaluate the feasibility of the clinical application of periosteum derived tissue engineered bone.

Methods:

Periosteal cells were isolated from a biopsy of periosteum, resuspended and cultured. The cell suspension was soaked into polymer fleeces and polymerized. The periosteal grafts were transplanted via sinus lift eight weeks after harvesting. The patients were divided into two groups. One underwent a one-stage procedure sinus lift with simultaneous implantation. In the other group the implants were inserted three month after augmentation. A control group was augmented just with autologous bone.

Results:

Periosteal harvesting from the mandibular angle via an intraoral approach under local anaesthesia was tolerated well by the patients, and throughout the complete procedure no complications occurred. The wounds resulting from replantation of the engineered tissue healed without complications. The implants were inserted into the grafted areas. When the implant was inserted primary stability was tested and proven clinically. Radiographs demonstrated a tight implant-bone interface. A total number of 118 (TE) and 183 (control group) ITI implants (Straumann AG, Waldenburg, Switzerland) were inserted into the grafted areas. All patients could be provided with fixed prosthetic rehabilitation like either crowns and bridges or dentures. The biopsies taken during the two-way protocol revealed mineralized trabecular bone with remnants of biomaterial. Osteocytes were apparent within the bone lacunae. In seven patients of the two-stage protocol group and in 17 patients if the one-stage procedure, an excellent clinical, radiological, and histological result could be proved 3 months after augmentation. In comparison to the initial situation, the clinical inspection showed a good formation of new bone without signs of resorption. This was confirmed by radiological imaging. In 10 patients (16 Sinus) of the two-stage protocol group the biopsies showed that the grafts had turned into a clinically appearing connective tissue-like consistency. In seven of these patients the situation was complicated by infection. The relation of augmentation procedures with BioSeed® - Oral Bone to infections as for example sinusitis and abscess is highly significant (p=0,000). All these cases required an additional augmentation procedure with autologous bone and bone substitutes. Only one patient of the control group which had been operated on just with autologous bone required secondary surgery. Also no infection was seen in this group. The loss of augmented material is significantly higher in the group in which the tissue engineered material was applied (p=0,000).

Discussion and Conclusion:

Our experiences from this pilot study with tissue engineered bone transplants reveal the necessity to limit the indications for tissue engineered bone. Its application is restricted to the sinus augmentation with simultaneous implant insertion at sites providing a sufficient bone bearing. These techniques are a new approach in hard tissue-impairment therapy and were applied in the region of the jaw and the face for the first time. To improve long term results modifications in the field of cell cultivation and carrier materials and a better understanding of the physiological part of the healing process are necessary.

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Bone Engineering: Allogenic and Alloplastic Bone Transplants vitalized by Osteoblast-like Cells

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Background and Introduction:

The search for suitable techniques and materials for the reconstruction of bone defects is a primary goal in many clinical disciplines. Implants made of synthetic polymers, ceramics or metals as well as allogenic materials like collagen or cartilage are used for bone grafting. Up to now no grafting material exists with the quality of the original tissue. These artificial materials show problems in anchoring and mechanical stability or induce immunological reactions. A new approach in therapy is the application of tissue engineered bone grafts. The possibility of cell culturing *in vitro* and the exclusive use of endogenous cells opens the way for a "self cell therapy" and thus avoids problems like limited resources. Additionally, the risk of donor site morbidity is decreased because only small biopsies have to be harvested. In this study, we focused on the search for a biomaterial which represents a suitable matrix for three-dimensional growth of human osteoblast-like cells *in vitro* and for the surgical management of intraoral applications.

Methods:

Human osteoblast-like cells were cultured on two different biomaterials: a human demineralised bone matrix (DBX® Mix, Musculoskeletal Transplant Foundation, NJ, USA, distributed by symthes) and a non-sintered, nanocrystalline, phase-pure hydroxylapatite (Ostim® Paste, Heraeus Kulzer, Hanau, Germany). Cortico-lamellar bone was obtained during dental surgery. For the staining of osteoblast-like cells an alkaline phosphatase assay kit (Sigma, Deisenhofen, Germany) was used. The evaluation of collagen type-I was done by light microscopy and the computer program Analysis 3.1 after immuno-staining with anti-collagen I antibody. Osteocalcin was analysed using a competitive EIA kit and an ELISA-Reader. For cell proliferation analysis, the nonradioactive assay EZ4U was used. The cell vitality was evaluated by fluorescence microscopy and a dichromogenic PI/FDA-staining. For the cell colonization analysis the samples were examined by scanning electron microscopy.

Results:

All cell culture supernatants of human osteoblast-like cells examined were osteocalcin positive with approximately 10 ng/ml osteocalcin and the alkaline staining of these cells typically resulted intensively positive (about 36.9%). Immuncytochemistry of the fixed cells showed the presence of collagen type-I in about 10.5% of the cells. Osteoblast-like cells seeded onto the human demineralised bone matrix (DBX®) showed a ten times higher rate of proliferation capacity than the cells cultivated on hydroxyapatite Ostim® (Fig.1). After 3 weeks of cultivation the vital cells migrated over the biomaterial and a beginning vitalization could be observed on DBX® (Fig.2). The surface of Ostim® was sparsely covered by human osteoblast-like cells after 3 weeks of cultivation indicating that there is no vitalization in vitro (Fig.3). Thin sections of the demineralised bone matrix (DBX®) showed a multilayered growth of human osteoblast-like cells already after 2 weeks of cultivation (Fig. 4). In comparison, Fig. 5 shows thin section of osteoblasts after a period of two weeks grown on Ostim®. Scanning electron microscopy after 3 weeks of cultivation on DBX® a dense network of multilayered polygonal shaped cells could be observed (Fig. 6). Fig. 7 shows an isolated and scattered growth of osteoblast-like cells upon Ostim®.

Discussion and Conclusion:

The topographic structure of the biomaterial surface could be a reason for different proliferation rates. Anselme (2000) described the decisive role of surface roughness, chemistry or surface energy regarding cell adhesion, cell migration or cell proliferation upon biomaterials. The mitogene effect of demineralised bone matrix can be attributed to the existence of various growth factors in the bone matrix, such as BMP´s (Urist 1965). Wozney *et al.* (1992) showed that BMP´s, belonging to the TGF-superfamily, are activated by the process of demineralization. Furthermore, Zhang *et al.* (1997) described that BMP´s are directly bound to the bone mineral and the demineralization process release them, indicating a proportional connection between the demineralization level, the accessible BMP´s and the osteoinductive effect. Further *in vivo* studies are necessary to examine if the present *in vitro* results correspond with the *in vivo* conditions. In future, it appears conceivable to produce made-to-measure and biological integrative biomaterials in combination with autologous cells. Pradel et al. (2006) clinically applied demineralized bone matrix (Osteovit, Braun, Melsungen, Gremany) cultured with osteoblasts in mandibular cysts. Nonetheless, further research with regard to the clinical application of such biomaterial/cell constructs are of essential importance for the further development of bone engineering.

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EFFECT OF 3D HYDROGEL SCAFFOLDS ON THE CHONDRODIFFERENTIATION OF SWINE MESENCHYMAL STEM CELLS IN VIVO

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INTRODUCTION: Bone marrow-derived adult mesenchymal stem cells (MSC) are a potential source of chondrogenic cells for reconstruction and repair of cartilage structures in the face, neck and extremities. MSC have been shown to differentiate into a chondrogenic lineage following a micropellet mass culture (3D) with specific chondrogenic media[1], but not in monolayer culture (2D). Previous studies in our laboratory demonstrated that swine Mesenchymal Stem Cells express type II collagen mRNA in micropellet mass culture without the addition of chondrodifferentiation factors (TGFβ). This suggests that chondrodifferentiation of MSC requires a 3 dimensional environment. Fibrin gel (FG)[2] and Polyethylene Glycol (PEG)[3] have been shown to be favorable 3D hydrogel scaffolds for tissue engineering cartilage from chondrocytes *in vivo*. The aim of this study is to evaluate the effect of FG and PEG 3D scaffolds in the chondrodifferentiation of swine MSC (sMSC) *in vivo.*

MATERIALS AND METHODS: All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital following the NIH Guide for the Care and Use of Laboratory Animals. Bone marrow was aspirated from the iliac crest of swine. sMSC were isolated by percoll gradient and *in vitro* culture. 2nd passage non-chondrodifferentiated sMSC were harvested from plates, encapsulated in both FG and no-degradable photopolymerizable PEG at $40X10^6$ cells/ml of hydrogel, and implanted into the subcutaneous tissue of nude mice for 6 weeks. After harvest, samples were analyzed for cartilage matrix formation by histology (H&E, Safranin O, Masson's Trichrome staining) and biochemistry (GAG, and hydroxyproline content).

RESULTS: Samples made with FG were soft and transparent in appearance, while samples made with PEG resembled cartilage in color and texture (Figure 1, gross appearance). sMSC encapsulated in PEG accumulated basophilic matrix, glycosaminoglycans, and collagen in the pericellular area (Figure 1, H&E, Safranin O, Trichrome respectively). sMSC encapsulated in FG did not produce cartilage matrix. Biochemical analysis demonstrated production of glycosaminoglycans (GAG) and collagen (hydroxyproline) in samples made with sMSC encapsulated in PEG (Figure 2).

CONCLUSION: This study demonstrated that sMSC cam be induced to a chondrogenic lineage in vivo when encapsulated in PEG, but not in FG, even without the use of chondrodifferentiation factors. This study demonstrates that the type of 3D scaffold has a strong influence in the chondrodifferentiation of swine MSC in vivo.

ACKNOLEDMENTS:
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AFFILIATED INSTITUTIONS:
** University of Colorado. Boulder, Colorado.3

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Figure 1

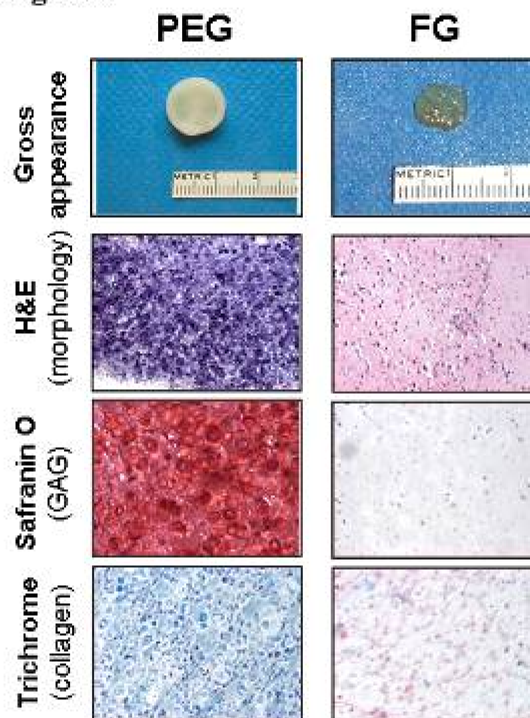
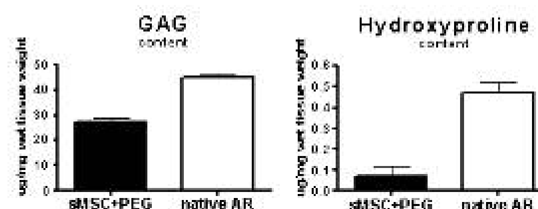


Figure 2



CELLULAR REPAIR OF MENISCAL TEARS IN THE AVASCULAR REGION

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INTRODUCTION: Spontaneous healing of traumatic meniscal tears occurs only in the vascularized outer third of the meniscus. Current repair techniques for tears in the avascular inner third of the meniscus, however, are prone to failure. Recent tissue engineering techniques using cell-based therapies have shown promise in attempts to heal meniscus tears, but complete healing has not been achieved thus far. To minimize donor-site morbidity, only very low numbers of autologous chondrocytes are often available for autologous chondrocyte transplantation. Therefore, efficient seeding of the cells onto scaffolds is essential for engineering new cartilage tissue. Previous research has shown that fluid flow in seeding conditions positively influences tissue engineered cartilage qualities. As an alternative to sutures or resorbable devices, we evaluated the healing capacity of a cell-seeded construct as an implant in artificially created tears in this avascular zone of the medial meniscus in swine as a large animal model. This two-part study addresses the issue of applying tissue-engineering techniques to repair meniscus injuries. Part I evaluates the efficiency of different seeding techniques using low numbers of cells. Part II investigates the potential for a cell-seeded scaffold to repair meniscal lesions in a large animal model—swine.

METHODS: All studies using animals, cells, or tissues were approved by the Institutional Animal Care and Use Committee (IACUC).
Part I: Scaffold Seeding. In the first part of the study, we evaluated two different chondrocyte sources at three cell concentrations on two types of scaffolds, Vicryl mesh® and a solid scaffold prepared from devitalized meniscus. Articular and auricular chondrocytes were harvested from three-month-old Yorkshire swine and seeded onto these scaffolds at cell concentrations of 1, 2, and 5 million/ml. The three seeding techniques were evaluated a) *Static*, in which the chondrocytes were allowed to attach to the scaffolds by gravity only, b) *modified centrifugal cell immobilization (CCI)*, in which the chondrocytes were seeded using centrifugal force and turning the scaffold, and c) D*ynamic Oscillating Seeding*, in which a continuous oscillating movement was applied to the cell-scaffold suspension in co-culture. The constructs were harvested after 7 days of culture and histologically examined for cell phenotype, cell distribution and cellular adhesion. Quantification of cellular attachment was evaluated biochemically by DNA count for each cell type on the mesh scaffold. Each parameter was statistically analyzed by the two-way ANOVA.
Part II: Meniscus Repair in Swine. In the second part of the study, we evaluated the healing capacity of a seeded implant inserted into a surgically made lesion in the avascular region of swine meniscus. Auricular and articular chondrocytes were harvested from three-month-old Yorkshire swine for seeding of the Vicryl mesh® scaffolds under the *Dynamic Oscillation* conditions for 8 days. Before implantation, the constructs were histologically analyzed for cellular attachment and homogenous cell distribution. Sixteen swine had a one-centimeter bucket-handle tear created at the medial border of the middle and inner avascular zone of their menisci. The controls consisted of 4 swine with no repair and 4 swine having suture repair only. The experimental groups were 4 swine receiving an implant with auricular cells, and 4 swine with articular cells. The implanted constructs were secured with 2 vertical mattress sutures to allow full weight bearing immediately after the operation. Menisci were harvested after 12 weeks and examined macroscopically, mechanically, and histologically for healing.

RESULTS: *Part I: Scaffold Seeding.* Auricular chondrocytes, in general, revealed better cellular attachment on both scaffolds when using any of the three seeding techniques. Examination of the S*tatic* seeding technique showed only few cells attached onto the different scaffolds over a period of 7 days. The greatest amount of cellular attachment was found on the solid scaffold, whereas nearly no cells were present on the Vicryl mesh scaffold. The same results were found using the *modified CCI* technique. The largest number of cells was found on the solid scaffolds using these two techniques, although the cells were attached only in defined small areas on the scaffolds. The most homogeneous cell distribution was found using the *Dynamic Oscillating Technique* using auricular chondrocytes. Although the articular chondrocytes covered all matrices completely, these cells were found to concentrate at points along the scaffolds. Cell-cell interdigitations were found throughout the Vicryl mesh scaffold, independent of the type of chondrocyte used. The devitalized meniscus chip was thoroughly covered with chondrocytes. Biochemical analysis of the chondrocytes attached to the PLGA mesh scaffold revealed that the *Dynamic Oscillating* technique resulted in up to 50% higher cellular attachment (**Figure 1**) than the S*tatic* technique and up to 150% higher than the *modified CCI* seeding**.**
Part II: Meniscus Repair in Swine. All 8 of the control samples failed to heal or show any new tissue formation inside the lesion. Closure of the meniscal lesions was observed grossly in all experimental samples after 12 weeks. Gross mechanical testing of the menisci samples with two Adson forceps demonstrated bonding of the meniscus tear. Histological analysis showed formation of new fibrocartilagenous tissue in all 8 experimental samples with integration into the native meniscus tissue. With the exception of one meniscus treated with auricular chondrocytes and one with articular chondrocytes, where partial healing was achieved, complete tear closure with newly formed fibrocartilagenous tissue was observed in all experimental samples.

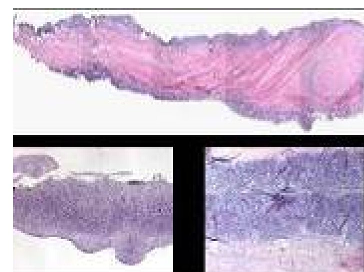

Figure 1. Auricular chondrocytes seeded dynamically onto meniscal cartilage (top) and Vicryl scaffold (lower left). Integration of Vicryl scaffold seeded dynamically with auricular chondrocytes between meniscal tissue (lower right).

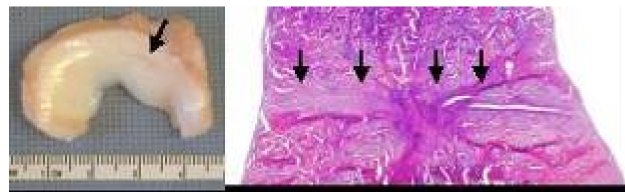

Figure 2. View of meniscal repair using auricular chondrocytes on Vicryl scaffold indicated by black arrow on left. Photomicrograph of healed meniscal lesion on right. Black arrows indicate region of the lesion.

DISCUSSION: This study demonstrates improved cell seeding of scaffolds under *Dynamic Oscillating* conditions. There was a clear trend towards a higher cellular content and a more evenly distributed cellular coverage over the entire surface of both types of scaffolds using *Dynamic Oscillating seeding* conditions. We conclude the *Dynamic Oscillating seeding* technique is an efficient technique for seeding low numbers of cells onto various scaffolds, which can be useful implants for meniscus tears.
Current repair techniques for tears in the inner avascular zone of the meniscus, such as sutures or resorbable devices, fail to effect healing. Lacking a suitable means for repair of the inner third of the meniscus, partial or total meniscectomy results in higher contact pressure of approximately fifty percent resulting in early onset of osteoarthritis after resection, even in young patients. Therefore, new meniscus repair techniques are needed. In previous studies, we demonstrated that cell-based implants in artificially created medial meniscus bucket-handle tears inserted into the subcutaneous pouch of nude mice developed a newly formed tissue integrating into the torn meniscus. The data from this present study extend those findings to a large animal model of meniscus repair. The results from this study provide evidence of the efficacy of this cell-based approach for treating meniscus tears and demonstrate the potential of tissue-engineered, cellular repair to provide successful healing of tears in the avascular zone in a large animal model.

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Creatine, pyruvate and glucose influence viability, metabolic activity and collagen content of normal and osteoporotic human osteoblast-like cells *in vitro*

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BACKGROUND. For the development of normal bone and during bone repair, cells require energy to survive, proliferate, differentiate and synthesize extracellular matrix, which then mineralizes. During these processes, ATP is generated by glycolysis, oxidative phosphorylation, as well as by the creatine kinase/phosphocreatine system that is present in these cells (*1, 2*); for review see (*3*). We have shown that external supplementation with the energy precursor, creatine, had clear stimulatory effects on the differentiation and mineral deposition by primary rat osteoblast-like cells (*4*). Bone cell bioenergetics may be a key element to understand not only bone formation as such, but also the pathological changes observed in bone diseases, e.g. osteoporosis. We therefore wanted to study the effects of creatine (Cr), pyruvate (pyr) and glucose (glc) supplementation on normal (nHOB) and osteoporotic human osteoblast-like cells (oHOB).

MATERIALS AND METHODS. HOB were isolated from bone chips of patients undergoing hip replacement surgery. HOB were inoculated as monolayer and micromass cultures. Micromass cell culture, representing an organoid system, promote the differentiation of rat osteoblast-like cells (*5, 6*).

The growth medium contained 5.55, 26.5, and 55.5 mM glc as well as 1 mM pyr and 5.55 mM glc. Osmotic effects of 55.5 mM glucose were compensated by the addition of 49.5 mM mannitol (man) to 5.55 mM glucose. Cr was added to the medium at a concentration of 10 mM. After 2 weeks, viability (neutral red uptake; NR), metabolic activity (MTT) and collagen content were measured..

RESULTS nHOB. Statistical analysis revealed that donors responded individually and differentially to monolayer and micromass culture in all parameters analyzed. Values for monolayer were higher than the ones for micromass cultures concerning viability and metabolic activity.

Viability. Addition of 1 mM pyr to glc 5.55 significantly promotes cell viability as compared to increasing glc concentrations. An increase in glc concentrations reduced the viability, but only glc 55.5 was significantly lower than glc 5.55. There was no osmotic effects of glc 55.5 as both glc 5.5 and glc 5.5; man were significantly are higher than glc 55.5. In addition, high osmotic pressure (glc 5.5;man) significantly increased number of viable cells over glc 5.5.

Metabolic activity. Under all conditions, 10 mM Cr significantly reduced metabolic activity as compared to Cr-free media. Addition of 1 mM pyr to glc 5.5 significantly promoted metabolic activity as compared to increasing glc concentrations and glc 5.5;man 49.5. There was an osmotic effect of glc 55.5 because glc 5.5 was significantly higher than both glc 55.5 and glc 5.5;man .

Collagen. Statistical analysis showed there were significant interaction effects of both Cr*medium and donor*medium. This means that the effects of Cr were dependent on the media used, and the donors respond individually to the media used. Addition of either 1 mM pyr or 49.5 mM mannitol to glc 5.5 seemed to reduce the values of celluar. collagen production as compared to the glc groups. Increasing concentrations of glc had hardly any effects. There were no osmotic effects of glc 55.5 as glc 5.5; man 49.5 is lower than both glc 5.5 and glc 55.5

PRELIMINARY RESULTS oHOB. Monolayer and micromasses were usually rather similar. NR and MTT were much lower in oHOB than in nHOB, but cellular collagen content was not negatively affected. Cr had beneficial effects on cellular collagen content, which seems to be more pronounced in the glc groups than in the other groups.

CONCLUSIONS. Pyruvate, glucose and osmotic pressure influence proliferation, metabolic activity and collagen content in HOB. Cell proliferation might be reduced in osteoporotic cells. Cell-cell contacts, as well as Cr supplementation, may have greater beneficial effects in oHOB compared to nHOB.

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The Swiss MD-PhD association (SMPA): a way to bridge the gap between the bench and the patient's bed

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The complexity of basic medical science is rapidly growing. The high level of technologies applied in basic science demands specific and extensive training for researchers. This questions the "late-bloomer" pathway of clinicians that switch to basic research after their clinical training and begins to set researchers and clinicians apart. The weakening of the bridge between bench and bedside has led to the creation of MD-PhD programs, in Europe as well as in the US. MD-PhD programs should improve the efficiency of medical research by creating a seed for close collaboration between basic research and clinical sciences.

The Swiss MD-PhD association (SMPA) (http://www.smpa.org) is an organization of physician-scientists (MD-PhDs), which promotes scientific and personal cross-talks between clinicians and basic scientists. The SMPA is a young association created in June 2003 and currently comprises around 70 physicians with an M.D.-Ph.D. education, M.D.-Ph.D. students and M.D. with a strong activity in research. The design of an optimal MD-PhD education and career planning for young physician-scientists is one of the main focus of interest of the society. In that respect, a close collaboration with the Swiss Academy of Medical Sciences (SAMS), the Swiss Medical Association (FMH) and other institutions has been established. Once a year, the SMPA organizes a general assembly embodied in a scientific meeting. The latter offers the opportunity to invite well known speakers, to engage scientific and political round table discussions and to bring clinicians and basic scientists in contact to promote translational and clinical research.

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Thymic epithelial cell-specific smad4 deficiency leads to lymphopenia and an increased suppressive capacity of $CD103^{+}CD25^{+}CD4^{+}$ T cells

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Background and Introduction:
The thymus constitutes the primary lymphoid organ for the development of T cells of the αβ TCR lineage. Regular thymic development depends on lympho-stromal crosstalk. Thymic epithelial cells (TECs) constitute the most abundant component of the stroma and express molecules critical for T cell development. In turn, thymocytes deliver signals that control TEC differentiation. However, the molecular mechanisms involved in TEC development allowing the formation of a fully functional thymus are at present poorly understood.
Defective thymic stroma leads to serious immunodeficiencies and autoimmune diseases in mice and humans (Nude phenotype, Bare lymphocyte syndrome and Autoimmune Polyendocrinopathy Ectodermal Dystrophy Syndrome).

Rational:
Smad4 takes a central position in the signaling cascade of members of the TGF-β family which are involved in many developmental processes. We hypothesized that smad4 is involved in the development of thymic epithelial cells. Since smad $4^{-/-}$ mice are embryonic lethal, we have generated mice that exclusively lack smad4 in TECs but not in other components of the thymus.

Methods:
To achieve tissue-restricted smad4 deficiency we took advantage of the cre/loxP system using the FoxN1 promoter that is exclusively expressed in thymic and skin epithelium to drive cre expression.

Results:
1) Mice devoid of smad4 mediated signaling in their TECs display a thymic cellularity which is decreased by up to 80% when compared to control littermates. Surprisingly, the overall architecture of the thymus remains intact revealing a proper distinction between cortex and medulla. Relative numbers of thymocyte subpopulations are normal.
2) Smad4 TEC deficient mice have a CD4 and CD8 T cell lymphopenia with an increased relative frequency of an activated/memory phenotype as well as an increased proportion of $CD103^{+}CD25^{+}CD4^{+}$ cells. These regulatory T cells are on a per cell basis more potent to suppress T cell proliferation of naive $CD4^{+}$ T cells than T Reg cells from control mice.
3) The increased regulatory capacity of $CD103^{+}CD25^{+}CD4^{+}$ cells is unrelated to FoxP3 expression.

Discussion and Conclusion:
Taken together, our results indicate that smad4 is essential beyond embryonic day 12.5 for regular function of TECs and demonstrate that genetic changes of the thymic stroma persistently impact on the behavior of peripheral T cells despite the absence of inherent defects in the T cells themselves.
Finally, we demonstrate a suppression potential by regulatory T cells which is unrelated to FoxP3 expression.

ACKNOWLEDGEMENTS:

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An optimized T cell epitope forecast approach for the identification of new human antigenic peptides derived from tumor-expressed splice variants

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The analysis of the human genome/transcriptome shows that ~ 60% of all human pre-mRNAs are alternatively spliced; nevertheless, only few tumor-specific splice variants have been already reported. It is unclear how widespread it is and to what extent aberrant alternative splicing contributes to tumorigenicity. Likewise, it is not clear the extent to which it might be a source of defective ribosomal products. We took a bioinformatic approach to identify splice variants of the Melan-A gene.

The next step consists of identifying putative antigenic peptides derived from these splice variants. Since it is known that the C-termini of antigenic peptides are directly produced by the proteasome, the peptides encoded by one splice variant of Melan-A are synthesized chemically and digested in vitro with purified proteasomes. The resulting fragments are identified by mass spectroscopy to detect cleavage sites. Using this information and based on the available anchor motifs for defined HLA class I molecules, putative antigenic peptides could be predicted. Their relative affinity for HLA molecules was confirmed experimentally with functional competitive binding assays and they were used to search patients' peripheral blood lymphocytes for the presence of specific cytolytic T lymphocytes (CTLs). CTL clones specific for a splice variant of Melan-A could be isolated; although they recognized peptide-pulsed cells, they failed to lyse melanoma cells in functional assays of antigen recognition.

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Long tendon constructs can be fabricated from rabbit adipose derived stem cells (rADSCs) and a collagen type I gel by cyclic stretching

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Background and Introduction:
Severely destroyed tendon tissue is often difficult to reconstruct. If the defect is too large in size direct suture of the tendon is not possible and it needs to be repaired by transplantation or transposition of other tendons. Since functional motor units have to be sacrificed to repair the defect there is the need for alternative sources of appropriate tissue.

Rational:
The field of Tissue engineering offers new methods for the reconstruction of damaged tissue. Progenitor cells of the adipose have proven to be an efficient cell source to create tissue-like complexes that regenerate tissue defects in vitro and in vivo. [1,2,3] These cells are multipotent, easy to obtain and to cultivate, and can be differentiated into the desired cell type. The authors investigated if useful tissue for tendon reconstruction could be fabricated from rabbit adipose derived stem cells (rASCs) in a collagen type I gel by cyclic stretching.

Methods:
Adipose was obtained from the inguinal fat pad of male New Zealand White Rabbits. To isolate rASCs the adipose tissue was minced, digested with collagenase, and cells were propagated in culture. Passages 3 or 4 were used for the experiments. Tendon constructs were fabricated by dispersing rASCs in a collagen type I gel (ArsArthro AG, Esslingen). Polymerization of the cell containing gel ($1x10^6$ cells/ml) occurred in special designed glass-cylinders with defined measurements. The constructs were transferred to a bioreactor and exposed to cyclic stretching for 5 weeks (Fig. 1). The stretching protocol included a stretching time of 8 hours followed by a 16 hours resting period. Stretching distance was 1 cm and stretching frequency 0.5 Hz. Non-stretched constructs served as control.

Results:
Tendon-like constructs with a length of 10 cm could be fabricated. They showed an increased opacity and a decreased diameter compared with the control. Histological analyses displayed longitudinal oriented, spindle-shaped cells, an organized dense matrix, and parallel collagen fibers. PCR analysis showed increased mRNA syntheses (collagen type I, III, fibronectin) compared with the control. Structural analysis showed a paratenon-like smooth surface and a parallel orientation of the collagen fibers of the stretched constructs (Fig.2 and 3).

Discussion and Conclusion:
Long tendon-like structures that display features comparable to regular tendon tissue could be fabricated using rASCs, a collagen type I gel, and a cyclic stretching bioreactor. The use of adipose derived progenitor cells seems to have a high potential for tendon tissue engineering. Further biomechanical studies and animal studies will investigate the potential clinical application of these long tendon constructs for tendon regeneration.

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ACKNOWLEDGEMENTS:
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Figures

Fig 1: Three tendon constructs after transfer into the bioreactor for cyclic stretching.

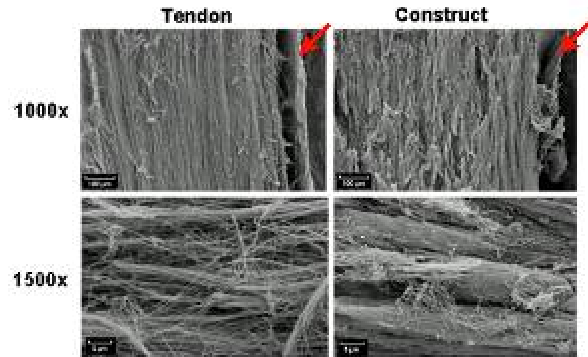

Fig. 3: Scanning Electron Microscopy of a natural tendon and the stretched construct. Note parallel alignment of collagen fibres and paratenon like structure marked with red arrows (first column: natural tendon, second column: stretched constructs, upper row: magnification 1000x, lower row: magnification 1500x).

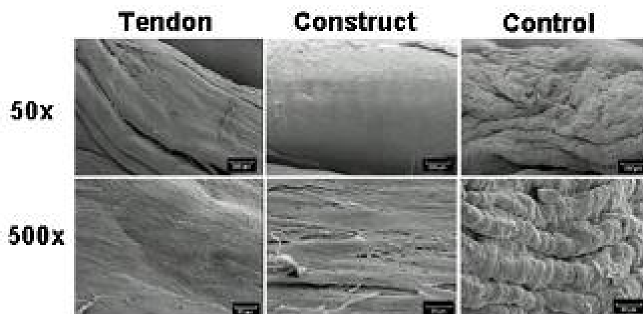

Fig 2: Scanning Electron Microscopy of the surface from a natural tendon, the stretched construct, and the control (first column: natural tendon, second column: stretched construct, third column: control, upper row: magnification 50x, lower row: magnification 500x)

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Controlling hard tissue integration at the bone-implant interface.

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Introduction: Complications and costs involved in internal fracture fixation implant removal may outweigh potential risks of device retention[1]. However, bacteriological, immunological, oncological and metabolic risks associated with long term implantation necessitate revision of this concept. In paediatric fracture fixation, removal is advocated to prevent plate migration and stress shielding in the fast growing skeleton. Approximately 10 % of all complications relate to difficulty in removing the implant due to the device becoming engulfed by bone (Fig. 1). Often this results in destruction of the device and further trauma to the patient. It is known that surface topography can be manipulated to enhance osseointegration at the bone-implant interface[2]. In this study, we investigated three forms of clinically available implant materials with varying micro-topographies with respect to osteoblast behaviour (proliferation, differentiation and gene expression)

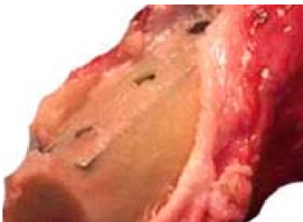


Fig. 1 Image of a fracture fixation plate engulfed by bone after 2 years implantation within a sheep.

Methods: Three clinically available materials were used (commercially pure titanium (cpTi); titanium6%Aluminium7%Niobium (TAN); titanium15%molybdenum (Ti15MO). Each had three distinct surface finishes (electropolished, paste polished, standard micro-rough). All surfaces were characterized using SEM, contact angle, XPS and non contact profilometry. Rat calvarial (RC) cells, originally isolated by sequential enzyme digestion from 6 day old Swiss Wister rats were cultured on 13mm sample discs in DMEM with 15% FCS, 50µg/ml of ascorbic acid, 1% penicillin-streptomycin and 5mM beta-glycerophosphate at 37°C 5% CO_2. Cell growth and morphology was assessed both qualitatively using SEM analysis, and quantitatively using tritiated thymidine incorporation (10µCi/ml) at 7, 14, and 21 day culture periods. Changes in relative gene expression for osteocalcin and collagen type I were investigated using quantitative real-time PCR normalised to the housekeeping gene 18S.

Results: Profilometry results showed that all micro-rough surfaces had comparable average roughness (Ra). All polished surfaces were comparable to the control (Ss) with the exception of NE, NP and TP which due to manufacturing problems were slightly rougher. Contact angle surface wettability showed all samples were hydrophilic as expected (Fig.2). Results for XPS showed that the surface chemistry was very similar after polishing as all samples underwent anodisation masking any possible changes (Fig. 3)

	TE	NE	ME	TP	NP	MP	TS	NS	MS	Ss
Mean Ra (µm)	0.142	0.409	0.293	0.410	0.243	0.207	0.86	0.75	0.82	0.112
CA °	70	69	74	68	83	75	68	72	78	49

Figure 2 Mean average roughness (µm) and contact angle (CA; degrees) of samples. Ss-stainless steel (control), T-cpTi, N-TAN, M-Ti15Mo, E-Electropolished,P-paste polished, S-standard micro-rough.

	Al 2p	C 1s	N 1s	Na KLL	Nb 3d	O 1s	P 2p	Ti 2p
TE		37.9	4.1	1.4		41.3	2.6	12.2
NE	1.4	28.2	1.1	0.3	0.2	49.2	2.3	17.1
ME		23.9	1.8	1.3		53.1	3.0	16.9
TP		30.4	0.8	0.4		49.2	1.7	17.5
NP	2.4	27.6	1.1	0.6	0.1	49.0	2.5	16.5
MP		35.7	0.6	45.3			2.5	15.4
TS		26.6	0.7	0.9		51.2	2.8	17.6
NS	2.1	26.0	0.9	0.5	0.2	50.9	2.0	17.2
MS		42.8	0.3	40.6			1.7	12.5

	C 1s	Cr 2p	Fe 2p	Mo 3d	N 1s	Na 1s	Ni 2p	O 1s	P 2p	Si 2p
Ss	28.0	7.3	2.3	0.4	1.9	2.6	0.4	49.5	2.1	5.5

Fig. 3 XPS results for samples as expressed as percentage of atomic concentration.

SEM analysis showed that despite the comparable Ra's for the standard surfaces, the micro-topographical features differed greatly (Fig.4).

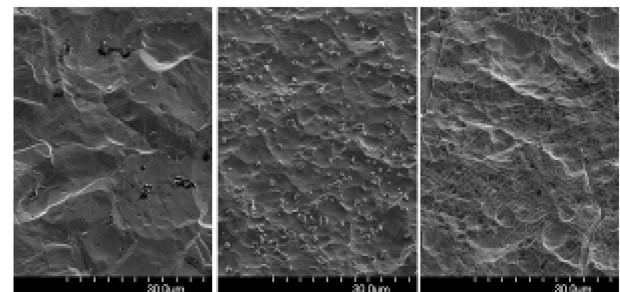
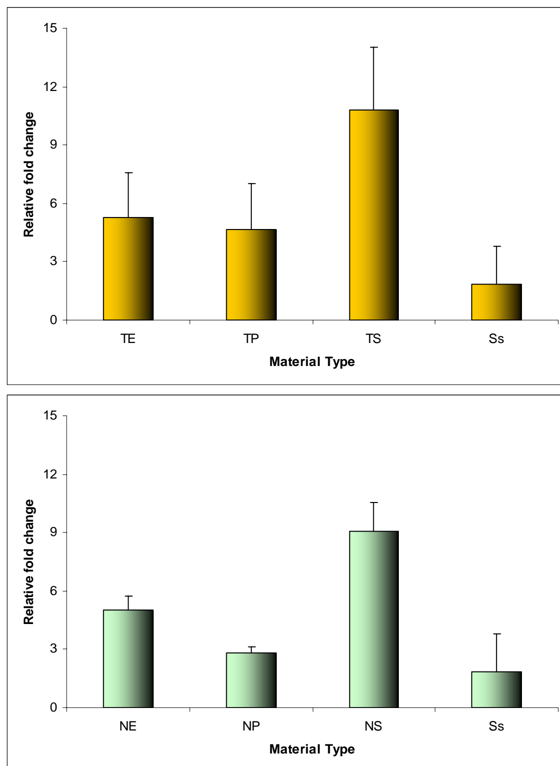


Figure 4 SEM images in secondary electron mode showing that despite similar average roughness', topographical features of the surfaces varied. A) TS B) NS C) MS

The control surface (Ss) had an almost flawless surface and was extremely smooth. Polishing enhanced the grain boundaries' contrast for both TE and MP. The slightly rougher Ra's for NE and NP may be due to the niobium rich inclusions 2 seen on the surface. Independent of material type, micro-rough standard surfaces showed a significant increase for both osteocalcin (Fig 5) and collagen I gene expression over both the polished variants. The highest increase for both genes was exhibited on the Ti15MO surfaces. A significant increase was also found between cpTi and TAN polished variants but not Ti15MO for osteocalcin gene expression. In contrast, for col I expression, a significant increase was observed between ME & MP. This increase for col I expression was not found however for cpTi or TAN. Incorporation of tritiated thymidine was highest on the polished surfaces and decreased in a time dependent manner.



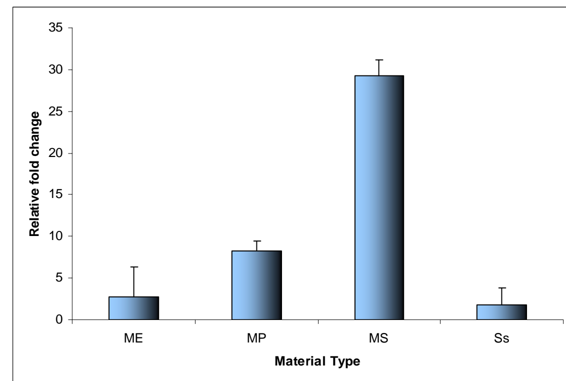


Figure 5 Representative real time qPCR results for osteocalcin gene expression at 21 days relative to 7 day expression (delta-delta Ct) (Gold – CpTi; Green – TAN; Blue - Ti15MO).

Discussion and Conclusion: Previous work in our lab has shown that micro-rough steel induces bone growth at the interface *in vivo*, becoming fully integrated at the implant surface. This has been supported by other studies for a variety of metal fixation devices[3,4.] In this study, we have shown that surfaces with a micro-roughness of approximately 0.7μm support a differentiated osteoblast-like phenotype pertaining to gene expression, in keeping with other recent studies[5,6]. The superior bone bonding property of titanium[7] over steel may contribute to the slightly higher expression observed for polished samples compared to the control. The data shows that surface polishing reduces osteoblast cell ability to differentiate and produce mature matrix compared to micro-rough surfaces. We propose that the polishing of clinically used metal implants instead serve to support a cell proliferative state as is suggested by the initial high rate of thymidine incorporation uptake observed for polished samples compared to micro-rough counterparts, and as documented elsewhere[6,10]. Regardless of time, thymidine uptake was markedly reduced on micro-rough surfaces compared to polished, thus supporting the notion that surface polishing advocates a proliferative cell state whereas micro-rough surfaces support a differentiated cell phenotype. It is possible that surface polishing may allow for either fibrous tissue formation or lower bony adhesion to occur on specific areas of implant devices to allow for easier retrieval.
This study highlights the promise of surface polishing in reducing extraosseous formation on internal fixation devices that require removal.

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Mechanically loaded *ex vivo* culture system for cancellous bone biopsies

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Introduction: In order to understand mechanical support and mineral homeostasis of bone, one must have both the cells and bone matrix combined in an isolated, 3D culture. A need exists for an *ex vivo* bone culture system, where a controlled biochemical and mechanical environment is created to be able to determine the influence of different parameters. The Zetos[1] model with mechanical loading has been validated (Fig.1) with ovine, bovine and human samples to keep cancellous bone tissue viable *ex vivo*. The samples maintain osteocytes, osteoblasts, osteoclasts and bone marrow cells in their natural 3D relationship to each other. Mechanical loading is a known anabolic stimulus for bone that is imperative to its natural development. The Zetos bioreactor may potentially be used for tissue engineering of bone to fill defects caused by tissue trauma or disease. The system could also be used to test possible tissue engineered constructs, reducing the amount of animal experimentation required. The goal of this study was to assess the response of three dimensional human explant cancellous bone to the addition of TGFβ_3 during long term culture with mechanical loading in the *ex vivo* loading bioreactor.

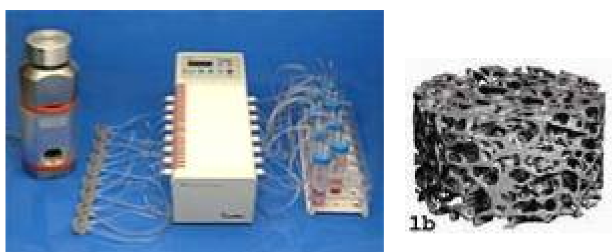

Figure 1. *Ex vivo* Zetos culture system. Microprocessor controlled pump allows perfusion of fresh media through the chambers. The bone cores (1b) are stimulated daily.

Methods: Human femoral heads (Ethic Commission Graubünden approval (18/02)) were processed into cylindrical cores (5mm height, 9.5mm diameter). The cores were inserted into the culture chambers, randomly assigned to groups and subsequently cultured for up to 14 days. Groups included, with or without TGFβ_3 (15ng/ml) and with or without loading (300 cycles at 1 Hz, giving 4000 microstrain) and heat treated dead cores as a control. As fresh tissue controls bone cores were fixed with 70% ethanol immediately after excision (T0). Post culture cell viability was assessed by cutting the cores into 250µm thick sections and the LDH assay was performed[2]. All remaining cores were fixed in 70% ethanol, dehydrated through an ethanol series and embedded into Technovit 9100 New[3] for subsequent histological and immunohistochemical evaluation.

Results: Histology of live cultured samples after 14 days in the Zetos system was comparable to fresh bone (T0). Non collagenous proteins such as bone sialoprotein and osteopontin were localised through immunohistochemical labelling of sections. The LDH assay displayed a uniform purple/blue staining (LDH positive) over the entire section on a macroscopic scale of the fresh, live tissue. Many dark stained osteocytes were seen in the bone cores cultured for 14 days in the loaded Zetos culture after administration of TGFβ_3. The dead sections however did not exhibit any dark, defined osteocytes and only the empty lacunae were visible (Fig. 2)

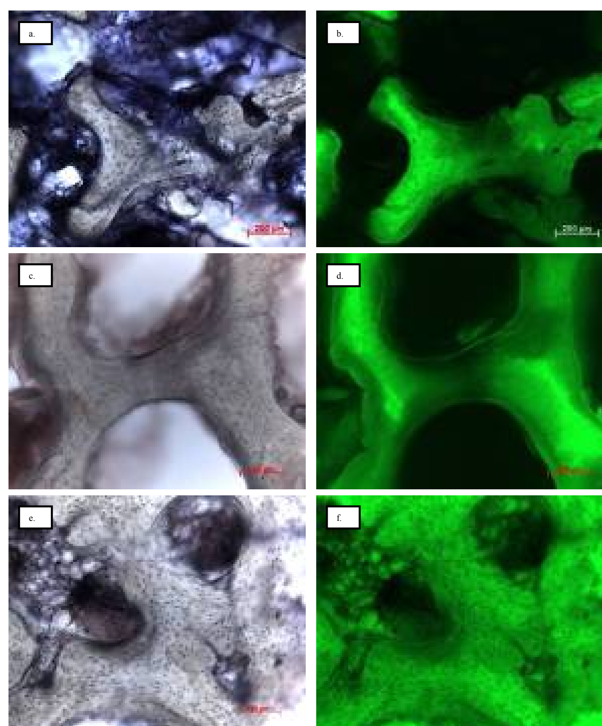

Figure 2. Human bone tissue (male, 54 yr). Mid-sections, stained for LDH-assay. a). and b). T0 section, darkly stained osteocytes and marrow can be seen. c). and d). A dead core taken after 14 days in loaded Zetos system. No darkly stained osteocytes or marrow can be observed. e) and f). Show an image of a core taken after

14 days in loaded Zetos culture with many darkly stained osteocytes and viable marrow can be seen.

The number of viable osteocytes observed in the fresh tissue (T0) was greater than after 7 and 14 days in Zetos culture. However, in all cases there appeared to be a positive effect of loading on the number of viable osteocytes present after 7 and 14 days in Zetos culture compared with the unloaded samples. In most cases the effect of loading plus TGFβ$_3$ on viable osteocytes was even greater. (Fig. 3)

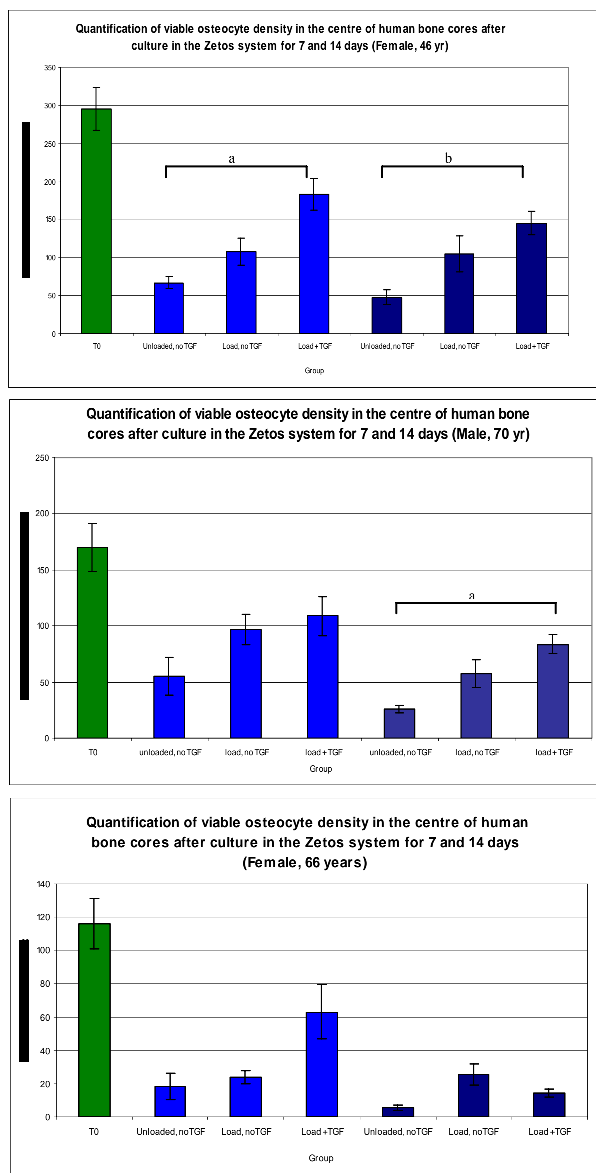

Figure 3. Graphical representation of the quantified osteocytes present in the central area of bone cores at T0 (fresh tissue) and after 7 and 14 days in Zetos culture under different experimental conditions. Light blue depicts 7 day time point, dark blue depicts 14 day time point.

Discussion and Conclusion: The Zetos bone bioreactor system permits the culture of viable 3D human trabecular bone cores up to 14 days. The outcome of this work shows that this *ex vivo* loading bioreactor is able to maintain a high percentage (over 50%) of viable osteocytes throughout the bone cores after 14 days in *ex vivo* culture. Further to this, the combination of daily loading and TGFβ$_3$ administration produced superior osteocyte viability at the core centres when compared to loading alone. The bioreactor has potential in pre-testing the integration of human bone with biomaterials, studying basic bone biology including osteoporotic bone.

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Viability in *ex vivo* cultured cancellous bone

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Background and Introduction: Biocompatibility studies are carried out either in 2D monolayer culture or in animal studies. Three dimensional bone organ cultures are required to reduce the number of animal studies performed, while simultaneously ensuring a more natural environment than that provided by monolayer culture of isolated cells. Due to the highly impervious nature of the calcified bone matrix and fatty marrow it is relatively difficult to ensure adequate supply of soluble factors to the central regions of the explant. The reduced rate of mass transfer to the central parts of the explant also produces an extra complication when using standard viability techniques, with the central regions often remaining completely unstained. A number of viability assays are toxic and the longer incubation time required for maximal penetration can cause complications by killing the external layers of cells. Radioactive labelling of viable cells is routinely performed by the addition of 25 µCi/ml 3H-glycine into the culture medium for 24 hours. This method of viability assessment however does have some major drawbacks. 3H is a weak β-emitter, resulting in long exposure times. Total assay time can be over five weeks, when including time for embedding the sample in resin and sectioning. Therefore we have developed improvements for various viability assays for their suitability in assessing the viability of 3D bone explants.

Methods: Cancellous bone cores 10 mm in diameter and 5 mm high were prepared from human femoral heads or ovine distal femurs. Assays were performed on either whole cores or 250 µm sections cut from fresh unfixed cores.

MTT- Whole bone cores were incubated with 10 ml of Thiazolyl Blue Tetrazolium Bromide solution (MTT-5 mg/ ml in DMEM + 10% FCS) at 37°C for 8 hours or at 4°C for 5 hours followed by 37°C for 3 hours. Alternatively 250µm sections were incubated with 500 µl MTT solution for 4 hours in a humidified 37°C environment.

Live/Dead- Whole bone cores were incubated with 10 ml staining solution (25 mM cell tracker green (live), 1 µg/ml ethidium homodimer 1 (dead) in DMEM) at 37°C for 6 hours. All incubations were carried out in the dark. After incubation, the cores were washed in PBS. The cores were then processed, embedded in methylmethacrylate (MMA) and 6 µm sections prepared.

Radiolabelling of Bone Cores - Whole cores were incubated with 2ml of DMEM containing 10% FCS and 25 µCi of ^{3}H-glycine for 24 hours at 37°C. The cores were dehydrated and embedded in MMA. Sections were cut and incubated with emulsion at 4°C for 3 weeks. The slides were developed, fixed and counterstained.

LDH- Bone explants were sectioned to 250µm. The sections were incubated in 500µl reaction medium (5% Polypep, 2 mM Gly- Gly, 0.75% NaCl, 60mM lactic acid, 1.75 mg/ml NAD, 0.3mg/ml Nitroblue Tetrazolium (NBT) pH8) for 4 hours at 37°C. Sections were then washed with warm (50°C) water, rinsed with PBS and fixed with 4% formaldehyde at 4°C.

Samples were imaged with a Zeiss photomicroscope (Axioplan Imaging) and processed with Axiocam and Axiovision software.

Results: MTT staining was temperature dependant. In cores incubated entirely at 37 °C, a ring of viability was seen around the outside of the core, while the central region remained unstained. This showed that the outside cells were actively using all the MTT. Pre-cooling cores and incubating at 4°C to reduce cell activity, prior to transferring to 37 °C lead to a uniform staining throughout the core. MTT staining of 250 µm sections demonstrated punctate staining within the osteocytes, often appearing as a ring (Fig 1).

The cells stained evenly throughout the sections, with central areas also staining as viable. Due to the thickness of the section, marrow integrity was well maintained and marrow cells produced a more diffuse type of staining (M). Fluorescent live dead staining was problematic both due to dye penetration problems and a low signal to noise ratio caused by the autofluorescence of the bone. It was also demonstrated that on whole cores neither dye penetrated more than 500 µm, leaving the central parts of the core unstained. Radio labelling with ^{3}H Glycine lead to stained osteocytes throughout the core. This method has the disadvantage of the inherent problems with radioactivity and the fact that total processing time is over 4 weeks.

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LDH staining of 250 µm sections led to excellent marrow structure preservation and uniform staining of marrow and osteocytes throughout the core (Fig. 2a). Exciting the bone with UV light caused autofluorescence which highlighted the stained live osteocytes as dark dots (Fig.2 b). As no embedding in resin is required, and the LDH assay only requires 4 hours incubation, viability can be assessed within 8 hours of harvesting the sample.

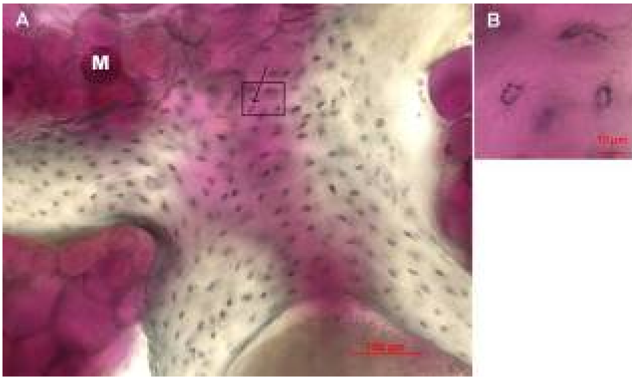

Fig. 1. Sections (250 µm thick) were cut from unfixed, undecalcified ovine cancellous bone cores and incubated with MTT for 4h. Punctate staining could be seen within the osteocytes, often appearing as a ring (arrowhead- magnified in B). The cells stained evenly throughout the sections, with central areas also staining as viable. Due to the thickness of the section, marrow integrity was well maintained and marrow cells produced a more diffuse type of staining (M).

Discussion and Conclusion: Taken together these results of non-radioactive viability methods indicate that viability assessment of whole cancellous bone cores leads to artefacts which are caused by poor diffusion. All these assays are routinely used in 2D cell culture systems, yet each required modifications to be suitable for use with cancellous bone. These artefacts can be overcome by preparing 250µm thick fresh sections prior to application of the assays. Fluorescent live/dead staining had additional complications caused by the autofluorescence of the bone generating a high signal to noise ratio, making assessment of osteocyte viability impossible. MTT staining was difficult to interpret due to the punctate nature of the stain.

We found that lactate dehydrogenase staining of 250 µm thick unfixed sections led to excellent viability determination of osteocytes within the mineralised matrix. It also maintained marrow organisation. Decreasing the viscosity of the LDH assay solution used in published methods led to a improved penetration into the calcified matrix. Quantification of thick sections is aided by using the autofluorescence of the bone to highlight the darkly stained osteocytes against the fluorescing bone.The optimisation of viability methods is an extremely valuable tool in biomaterial assessment in explant cultures of bone and should also be of use for tissue engineering studies. As the LDH assay has few handling steps, data is obtained rapidly.

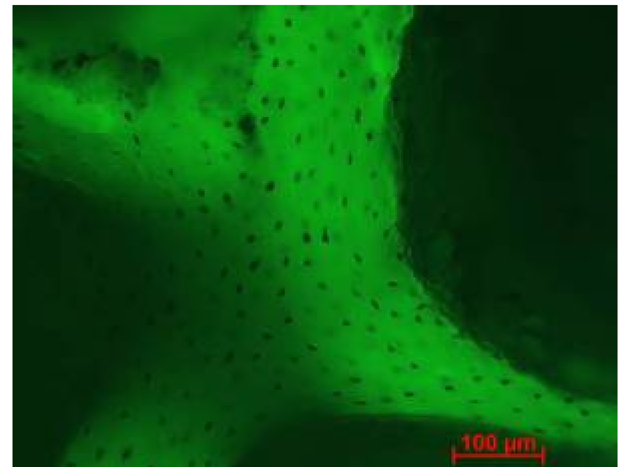

Fig.2. Sections of human cancellous bone (250 µm thick) were stained for LDH activity for 4h. Sections were mounted using a water based mountant and viewed immediately. The brightfield image shows the presence of numerous, darkly staining osteocytes (A). The corresponding image using the autofluorescence of the bone (515-565nm emission filter) enhances the contrast, leading to clearer definition of viable cells (B).

ACKNOWLEDGEMENTS: This work has been funded by 3R #7801 and ESA MAP AO99-122. Thanks to Dr. Thomas Perren (Davos Hospital), Dr.Heinz Bereiter (Chur Hospital) for supplying human tissue.

Bacterial adhesion to PLL-g-PEG modified surfaces

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INTRODUCTION: Infections associated with implant are estimated to cost £7-11 million per year[1]. With the rise in antibiotic resistant bacteria this is an important issue[2]. Once adhered many bacteria such as *Pseudomonas aeruginosa, Staphylococcus epidermidis*, and *Staphylococcus aureus* form biofilms on the implant surface. These can be difficult to clinically treat since the bacteria are protected from phagocytosis and antibiotics within the biofilm complex[3], hence the need to prevent initial bacterial adhesion.

One approach to prevent bacterial adhesion is to coat the surface with a protein resistant coating, such as PLL-g-PEG[4]. PLL-g-PEG is known to inhibit both eukaryotic cells and *S. aureus* adhesion[5-6]. The approach can be modified by functionalising the coating with an adhesion moiety (specific peptide sequence) e.g. RGD (Arg-Gly-Asp), functionalised PLL-g-PEG coating, which also minimises protein and *S. aureus* adhesion[5-6] but allows eukaryotic cells to adhere[5]. This study describes the visualisation and quantification of various common bacteria to PLL-g-PEG (PEG) and PLL-g-PEG/RGD (PEG/RGD) and PLL-g-PEG/RDG (PEG/RDG) control coatings on titanium surfaces, to see if they recognise the RGD adhesion moiety present in ECM proteins, that eukaryotic cells adhere to via their integrin-receptor mechanism.

METHODS: To visualise the adhesion of the various bacteria (Fig 1), the bacteria were cultured on uncoated titanium (Ti), PEG and PEG/RGD coated Ti surfaces for 2h, 4h and 18h at 37°C in brain heart infusion broth. For scanning electron microscopy (SEM) imaging, samples were fixed with 2.5% glutaraldehyde in 0.1M PIPES buffer for 5 min at pH7.4, post-fixed with 1% OsO_4 in PIPES at pH6.8 for 1h, dehydrated in an ethanol series, critical point dried, coated with 10nm Au/Pd and visualised with a Hitachi S4100 SEM. To quantify the amount of living bacteria adhering to the different surfaces, bacteria were cultured as before, then stained with fluorescent redox dye, 5-cyano,2-ditolyl tetrazolium chloride (CTC) for 1h, and imaged with a Zeiss Axioplan2 Epifluorescence microscope fitted with an Axiocam camera[6]. The density of adhering live bacteria observed in each image, were counted using KS400 software. The experiment was repeated three times and statistical analysis was performed using a one-way ANOVA with Tukey pair wise posthoc test.

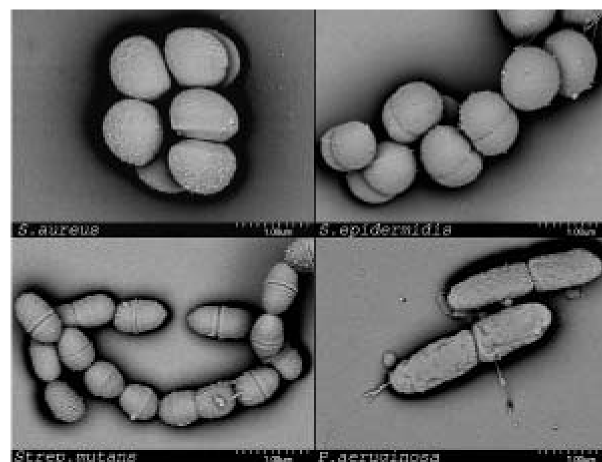

Fig. 1: SEM images of the four different bacteria strains used in the study. Note their different shapes and cell wall topography.

RESULTS: SEM images showed all 4 bacteria strains adhering to the uncoated Ti surfaces and over time the amount adhering increased due to the colonisation of the surface by the bacteria. With all four bacteria, less bacteria were observed on the PEG, PEG-RGD and PEG-RDG coated surfaces (Fig. 2). The observations made with the SEM were confirmed by quantification of adherence using the fluorescence microscope (Fig. 3).

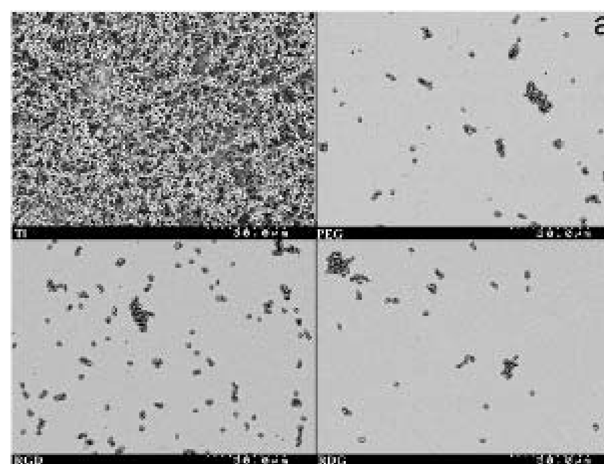

Fig. 2 SEM images of *S. aureus* on the different surfaces after 4h of culturing. There are less bacteria on the PEG, PEG-RGD and PEG-RDG coated surfaces than the uncoated Ti.

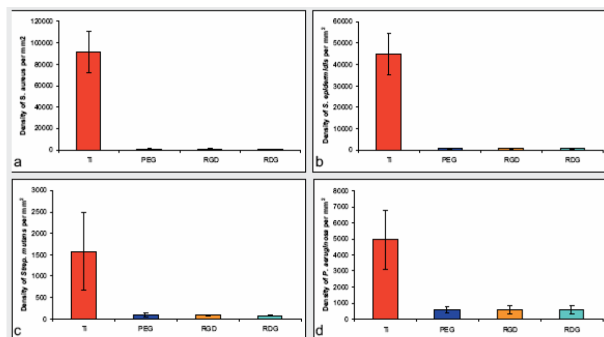

Fig.3: Graphs showing the effect of PLL-g-PEG coating on the density of a) S. aureus; b) S. epidermidis; c) Strep. mutans; and d) P. aeruginosa adhering to the different surfaces.

Statistical analysis showed that the difference between Ti and PEG coated surfaces as significant with all four bacteria strains ($p > 0.05$), but not between the PEG, PEG-RGD and PEG-RDG coated surfaces ($p > 0.05$).

DISCUSSION & CONCLUSIONS: PEG minimises non-specific protein adsorption, Eukaryotic cells do not attach to PEG or PEG/RDG (functionalised with a scrambled adhesion moiety), but do attach to PEG/RGD [5]. RDG (Arg-Asp-Gly) has no specific activity towards integrin receptors, therefore serves as a control for the presence/absence of specific interactions in bacteria-surface studies. The results from this study indicate that a PEG or PEG-RGD coating inhibits the adherence of *S. aureus, S. epidermidis, Strep. mutans* and *P. aeruginosa*. All four bacteria do not appear to recognise the eukaryotic cell adhesion moiety RGD.

Hence, PLL-g-PEG and PEGRGD coatings have potential as coatings on medical devices as they inhibit initial bacterial adhesion, and depending on the polymer used either encourage or discourage host cell adhesion.

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ACKNOWLEDGEMENTS: Thanks to Salvatore Chessari and Martin Schuler for help in synthesising & characterising the PLL-*g*-PEG.

Super-paramagnetic nanoparticles for enhancement of bone growth and differentiation

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Background and Introduction:

The development of super-paramagnetic nanoparticles opens new possibilities for directed growth factor delivery to enhance healing of bone defects. Proteins or plasmids (pDNA) can be coupled to super-paramagnetic nanoparticles (SPION), be directed to intra- or extracellular targets and then be released by magnetic force e.g. an intermittent magnetic field.

Rationale:

Since SPION have been developed recently there are only a few data about the influence of nanoparticles on cellular proliferation and differentiation. As a first step to use nanoparticles for biomedical purposes we examined the biocompatibility of plain coated SPION and its effect on osteogenic differentiation in vitro.

Methods:

Osteogenic precursor cells (MC3T3) and mesenchymal stem cells (C3H10T1/2) were cultured in presence or absence of amnio-polyvinylalcohol- (aPVA) and carboxy-polyvinylalcohol- (cPVA) coated SPION for 1h, 4h, 3d, 7d, 14d or 21d. The samples were stimulated in a static or intermittent magnetic field (1Hz, 1 or 4 h/d). The intracellular iron content, the cellular proliferation and the activity of bone specific alkaline phosphatase were measured and Cy-3 labelled SPION were detected by confocal laser scanning microscopy. The staining of the actin skeleton, focal adhesions (vinculin/paxillin) and calcified matrix was performed.

Results:

It was shown that the coating of SPION with aPVA enables and with cPVA inhibits the intracellular uptake of the SPION by changing of the particle load. In presence of aPVA-coated SPION (intermittent magnetic field, 4h) a weaker expression of the actin skeleton was observed. Beside this, no morphological changes were found. In presence of a static magnetic or intermittent (1Hz, 4h) field the particles seemed not to have any effect on cellular proliferation and differentiation. If frequency was increased to 2 Hz and applied for a longer daily time (8 h) aPVA-coated particles increased cell proliferation while alkaline phosphatase activity per µg DNA was decreased.

Discussion and Conclusion:

SPION have been shown to deliver proteins or pDNA to cellular targets. In absence of magnetic fields particles seem not to change the cellular properties of MC3T3 and C3H10T1/2 cells. In presence of an intermittent magnetic field SPION might change proliferation and differentiation properties depending on the surface coating and the used frequency. In future delivery- and release-mechanisms of SPION-coupled proteins or pDNA have to be examined to develop SPION-based applications for the clinical use.

Nanotechnology in Medicine
Nanoparticles/structures for drug and gene delivery and hyperthermia

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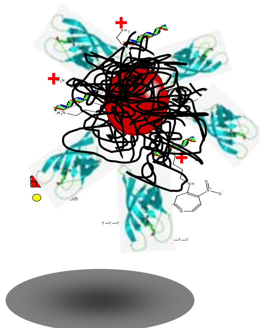
Background and Introduction:
Nanomedicine is the application of nanotechnology to health. It exploits the novel physical, chemical and biological properties of materials at the nanometer range as well as the use of nanosized particles for surface engineering.
The aim of nanomedicine may be broadly defined as the comprehensive monitoring, repair and improvement of all human biological systems, working from the molecular level using engineered devices and nanostructures to achieve medical benefit.

The Nanoparticle



The most interesting nanosized particles for medical application are superparamagnetic iron oxide with tailor-made coatings and derivatizations. Such particles have a size of 10 nm and are only magnetic in the presence of an external magnetic field. They are used as contrast agent for MRI, but in future also as non-viral vector for drug and gene delivery or as very local heat source for hyperthermia. Polymer particles are used for structuring surfaces at the nanoscale which allows the manufacturing of drug reservoir and controlled release by diffusion through nanochannels.

Targeted Drug Delivery and Controlled Release
The drug delivery systems enabled by nanotechnology aims to target selected cells or receptors in the body. The aim is that nanoparticles will carry therapeutic payloads or genetic content into diseased cells, minimizing side effects as the nanoparticles will only become active upon reaching their ultimate destination. They may even check for over dosage before becoming active, thus preventing drug released poisoning.
Two concepts are in development in the laboratory of LTP:

1. Drug or gene delivery with superparamagnetic iron oxide in joints using magnetic forces for guiding and targeting the drug. Our main applications are joint diseases because joints are ideal closed areas and the application of magnetic fields is relatively easy. We have shown that the targeting of the sinovia membrane with nanosized particles is possible. First results of in-vitro as well in-vivo gene delivery will be presented.

2. Nanostructuring of implant surfaces with nanoparticles: Tailor made pores in the size range from micro meter down to several nanometer in a hierarchical arrangement allow generating a novel drug delivery system on the surface of implants.

For structuring of the surface, colloidal coating processes were used applying soft-lithography techniques for the formation of large as well as small pores. The ceramic matrix guarantees high biocompatibility and adhesion of the structured layer.

Hyperthermia
Applying an AC magnetic field, SPION generate heat which allows a local heating of the tissue up to 44 to 45 °C. This is enough for necrosis of cancer cells. Combing this particle with bone cement, a very interesting system for stabilizing and treatment of cancerous bone was developed.

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NANOSTRUCTURED SMART DRUG DELIVERY COATINGS

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INTRODUCTION

Failure of a therapy based on systemically administered drugs has many origins, but one classical reason is the inability to achieve the required dose at the site to be treated. This effect is especially true for drug treatments following implantations.
In the orthopaedic field a large number of total hip replacements failures have been observed on patients younger than 60. This is mainly due to peri-implant bone loss occurring during the first two years after implantation. In order to treat these failures, implants with a coating containing a drug can be used. For example bisphosphonate is known to decrease the osteoclastic bone resorption activity and therefore reduce the risk of total hip repladement failure.

TECHNOLOGY

The use of thin and nano-structured ceramic coatings for orthopaedic medical implants is well established. These "nano" materials have shown advantages in terms of wear and corrosion resistance. However, as ceramics are dense materials, most of these coatings are only used as inert protective layers.

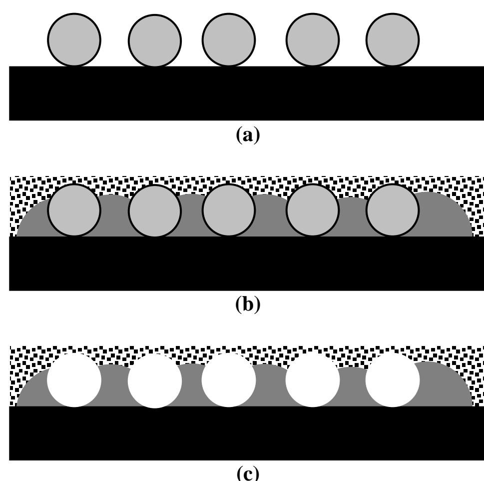

Figure 1: Processing steps. (a) Deposition of the polymeric template, (b) coating of the substrate with the ceramic, (c) thermal treatment to remove the template

We present here a new technology that combines the inertness and stability of ceramics with a high drug loading capacity.

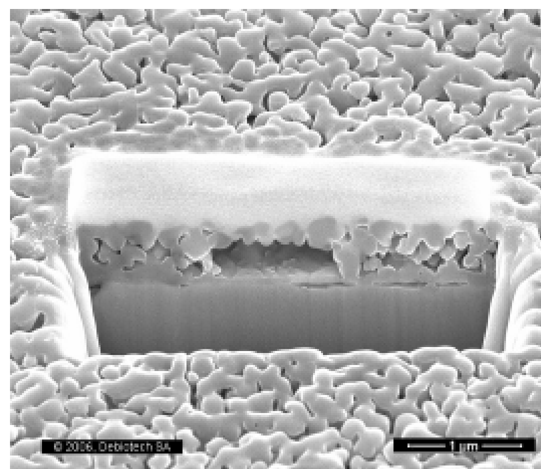

Figure 2: Structured coating with a micro-sized cavity for drug loading and a nano porous membrane for drug release control.

In order to store and release a drug over a few days to a few weeks, the coating combines two porosities: one of large size acting as a reservoir and where the drug is stored and another of size similar to the released molecules that acts as a diffusion membrane. Micrometer size cavities are created by depositing a template onto the implant. This template is made of mono disperse polystyrene particles that are deposited by dip coating onto the substrate (Figure 1(a)). It is then covered by the ceramic while the diffusion membrane is produced by adding a second layer of porous ceramic (Figure 1(b)). The nano porous membrane is created by a stacking of ceramics nano particles. Finally the template materials are removed by a thermal treatment and cavities are created (Figure 1(c)). The final coating has a thickness between a few hundred of nanometers and a few micrometers (Figure 2). First results on coating fabrication will be presented

ACKNOWLEDGMENTS

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FETAL SPINE CELLS FOR INTERVERTEBRAL DISC REGENERATION: PRELIMINARY CHARACTERIZATION

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INTRODUCTION

Degeneration of the intervertebral disc (IVDD) is though to be one main causes of low back pain. IVDD begins early in the nucleeous pulposous (NP) with decreased cellular content and a loss of proteoglycan and water. Our hypothesis is that matrix synthesis could be stimulated by porteoglycan producing cells. Fetal disc cells could be a promising cell source for regeneration of the degenerated disc. The aim of this study was to study the feasibility of using fetal disc cells for disc tissue engineering.

METHODS

Fetal spinal column tissues (1cm) were obtained from fetuses after voluntary interruption of pregnancy at 14-16 weeks of gestation (n=3). The spinal tissue was cleared of adherent tissue dissected and put into culture in tissue culture dishes. Alcian blue staining was performed on tissue section. Cell proliferation in monolayer was measured with the CellTiter colorimetric method and compared to adult NP cells (individual aged 30 to 40 years). mRNA expression was measured with real time PCR.

RESULTS

Histology of fetal spine showed vascularized cartilaginous vertebrae. Disc annulus consisted of concentric lamellae, containing proteoglycans (blue stain) and more or less collagen (pink stain) according to the age, with numerous isolated cells. The NP, representing 1/5 of the total disc surface, had a less organised matrix, which stained only for proteoglycans and was populated by cells in clusters. Isolated cells proliferated two times faster than adult NP cells. They expressed mRNA for proteins identified in NP cells, aggrecan, SOX9, Hypoxia-Inducible Factor-1and Glucose Transporter.

DISCUSSION

Fetal spine cells proliferate fast and express critical protein mRNA for survival in a disc environment. These cells seem to be a good source for disc tissue engineering

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In vivo evaluation of cpTi and TAN screws with modified surfaces to reduce bony integration.

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Introduction: Approximately 10% of paediatric patients undergoing surgery to remove implants experience complications[1] including increased operative time, increased blood loss, trauma to surrounding tissues, longer hospital stay and implant particulate debris contamination. *In vitro* studies show that surface topography of materials influences osteoblast proliferation, differentiation and phenotype. On smooth surfaces such as that of clinically used stainless steel, there is a loss of osteoblastic phenotype and an increase in proliferation and spreading of the cells. On microrough surfaces the osteoblastic phenotype is maintained and an increase in expression of genetic markers associated with bone formation has been found. We deduce that increased bone adhesion to implants is the major cause of the implant removal difficulties. We hypothesise that very smooth (polished) surfaces have less bone adhesion (strength and amount) than microrough implant surfaces. This study aims to assess the effect of surface topography of TAN (Titanium-6%Aluminium-7%Niobium) and commercially pure titanium (cpTi) screws on bone-implant adhesion in a new sheep cortical (tibia) and cancellous (rib) bone model.

Methods: Synthes cortical bone screws were used with a diameter of 3.5mm and a length of 10mm. The material types included stainless steel (SS) with an average surface roughness (SRa): 0.09µm (negative control), cpTi (TS), SRa: 0.82µm (as machined, positive control), TAN (NS) (as machined, positive control), SRa: 0.96µm, cpTi electropolished (TE), SRa: 0.09µm and TAN electropolished (NE), SRa: 0.30µm. Surface topography was assessed by non-contact white light profilometry and scanning electron microscopy (SEM) on a subset of screws. 18 female Swiss Alpine sheep were divided into 3 groups of 6 sheep each. One of each screw variant was implanted into the medial aspect of both tibial diaphyses (unicortical) and into the proximal 1/3 of ribs 8 and 9 (left side), each sheep having a total of 20 implants. The implants were left in place for 6 (group 1), 12 (group 2) and 18 weeks (group 3). The bones were then harvested and the peak removal torque was measured for screws from rib 9 and the right tibia from each sheep. The screws from rib 8 and the left tibia were left in situ and embedded in MMA before being stained with Giemsa and Eosin. The percentage of direct bone-implant contact was measured for each screw from the left tibia and the 8th rib.

Results: No surgical complications were detected in any of the groups. Measurements of the peak removal torque required to loosen the implants at 6 weeks, showed that polishing of screws made of both cpTi and TAN results in significantly lower torque for removal than standard microrough (as machined) screws when placed into cancellous bone (rib, $p<0.02$; Fig.1). There was also a significant decrease in peak removal torque of polished compared with standard cpTi screws when placed into the cortical bone of the tibia (Fig.2). At 12 weeks the difference between the standard microrough screws and their polished variants was even more apparent ($p<0.001$). A difference in the time course of response was found between the two bone types with regard to removal torque. In the rib, implants showed an increase in removal torque between 6 and 12 weeks followed by a decrease at 18 weeks. In the tibia, the implants demonstrated a continual decrease in removal torque over the 18 weeks. Histologically, the polished implants demonstrated a lower percentage bone contact than their standard micro-rough counterparts (Fig.4 & 5). In both bone types there was an increase in the mean percentage bone contact between 6 and 12 weeks (Fig.3).

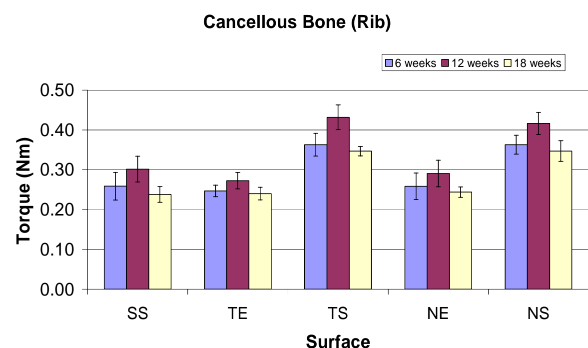

Fig1. Peak removal torque for implants placed into rib cancellous bone for 6, 12 and 18 weeks.

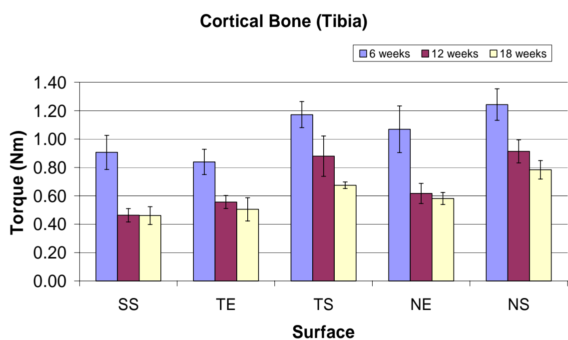

Fig2. Peak removal torque for implants placed into tibial cortical bone for 6, 12 and 18 weeks.

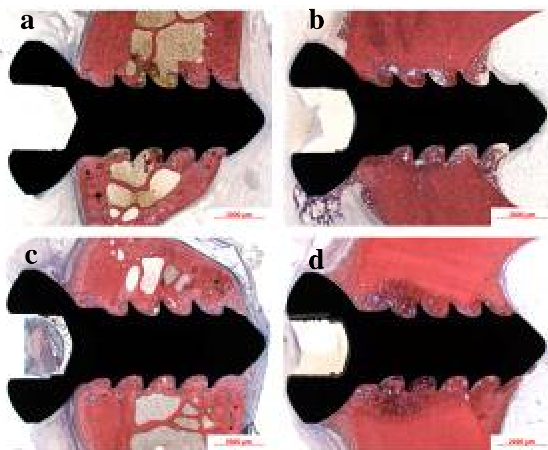

Fig3. Standard TAN screw a). Rib 6 weeks, b). tibia 6 weeks, c). Rib 12 weeks, d). Tibia 12 weeks (Giemsa-Eosin).

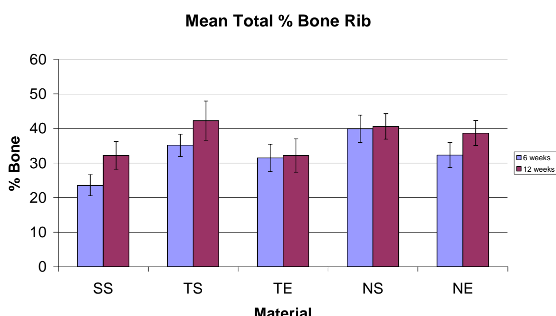

Fig4. Mean total % bone contact in rib implants at 6 and 12 weeks

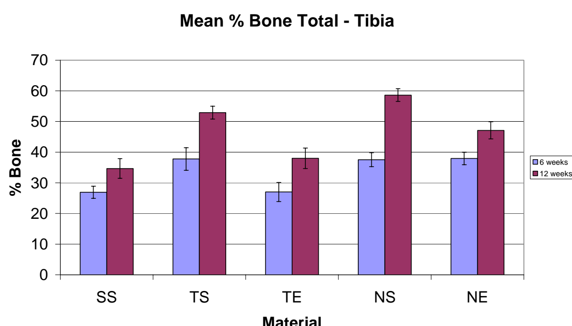

Fig5. Mean total % bone contact in tibial implants at 6 and 12 weeks

Discussion and Conclusion: This sheep model study successfully demonstrates the effect of implant polishing on both the strength and amount of bone-implant adhesion. The difference in the time course of response in removal torque between the rib and tibia may be due to a difference in the rate of bone turnover and remodelling between the ribs and tibia. Polig & Jee [2] have shown that in young adult beagles rib cortical bone has an annual turnover rate of approximately 18% while in the mid diaphysis of long bones it is less than 1%. In our study polishing of TAN resulted in a smaller decrease in removal torque compared to that of cpTi. This is probably due to the different effect of the polishing process on cpTi (pure α phase) to TAN (mix of α and ß phase) leaving numerous protruding hard inclusion particles of TAN within the surface after polishing, resulting in a greater surface roughness than in cpTi.

The histological results demonstrate that surface polishing can reduce the amount of direct bone-implant contact. The results would suggest that the removal torque should increase in the tibia between 6 and 12 weeks, however this is not demonstrated in the measurements of removal torque. The histological measurements do not give a quantifiable assessment of the bone quality at the bone-implant interface. A more accurate estimation of this is given by the removal torque measurements. Our results would suggest that while there is an increase in the percentage bone contact in the tibial implants, the quality of the bone at the implant interface and hence the strength of bone attachment is reduced over the first 18 weeks in the tibia.

We can demonstrate a good correlation between surface roughness and torque removal as well as percentage of direct bone contact independent of the implant material (for the materials tested). Therefore polishing implant surfaces has the potential to reduce the torque required for their removal. We believe that the results of this study may be used to modify surface design of implants destined for removal, especially in younger patients and thereby reduce implant removal complications.

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PERSPECTIVE OF CLINICAL APPLICATION OF HUMAN MSC-LIKE CELLS AFTER LASER DERMABRASION.

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Introduction: The skin is organ in which to high intensity there is a process of self-renewing of cells and restoration of its various damages. This process is provided by means of consecutive differentiation stem cells of skin. It's known, that besides epidermal stem cells located on basement membrane, in dermal layer of a skin there are undifferentiated cells which also participate in its regeneration. The aim of our studies was isolation and characterization of MSC-like cells from human skin, and an estimation of prospective of their application in regenerative medicine.

Methods: MSC-like cells were isolated from human dermis. The piece of skin has been received with the agreement of the patient during operation on abdominoplasty of the person with application of an endotracheal narcosis. Skin from abdomen was carefully dissected free of other tissues, mechanically fragmented and then digested with 0,1% Collagenase type IV (Gibco, USA) for 30 min at $+37^0$C. Isolated cells were resuspended in growth medium DMEM (Gibco, USA). The received cells were immunophenotyped by antibodies (Becton Dickinson) to surface antigens - CD10, CD13, CD29, CD31, CD34, CD44, CD45, CD49a, CD49b, CD49d, CD49f, CD71, CD73, CD90, CD105, CD106, CD117, CD166, HLA-ABC, -DP, -DQ, -DR. As the basic inductors for osteo-, adipo- and chondrogenic differentiations were used beta-glycerophosphate, indomethacin / isobutylmethylxanthin and TGF-beta-1 accordingly. For laser dermabrasion of patient's face was used erbium laser UltraFine Er-YAG (Coherent Inc., USA) with length of a wave 2,94µm, density of capacity 5J/cm^2, radius of a laser beam 2,5mm. With the agreement of patients have been generated 2 groups on 5 patients. For clinical trial MSC-like cells were washed from growth medium and free from infectious and other agents. In experimental group: on the postoperative laser surface were single applied MSC-like cells of skin in 3% hyaluronic acid (biomatrix) in isotonic solution and covered with an aseptic protective at 24h. Then were applied Ung. Solcoseryl (Poland) up to full epithelization. In control group regeneration of epithelium was spent by standard method - Ung. Solcoseryl.

Results: We isolated MSC-like cells from human dermis. Immunocytochemical analysis of the received cellular population has shown presence high expression of the following surface Ag's (%) - CD10 (84,1), CD13 (99,6), CD29 (99,8), CD44 (99,4), CD49a (96,1), CD49b (81,6), CD49d (67,7), CD73 (99,4), CD90 (99,3), CD105 (92,7), CD166 (97,6), HLA-ABC (99,9) and absence expression of Ag's (%) - CD31 (0,9), CD34 (1,1), CD45 (0,9), CD49f (1,9), CD106 (1,0), CD117 (0,7), HLA-DP (0,7), HLA-DQ (0,8), HLA-DR (0,8). At presence of inductors in growth medium the received cells were differentiated into cells of a bone, adipose and cartilage tissue. Clinical application of these cells has shown that using MSC-like cells in biomatrix allows to achieve complete epithelization practically on 5-7 day, in control group (without cells) - 9-11 days. During the postoperative period at observance by patients of a sun-protective regime complications don't noted. Hyperemic portions assumed normal coloration within 1-1,5 months, in control group – 2-2,5 months.

Conclusions: We derived MSC-like cells from adult human skin which are a perspective material for regeneration of skin in various fields of reconstructive medicine, particularly in combustiology and cosmetology.

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Effect of Metal Implant Surface Topography on Fibroblast Behaviour and Bacterial Adhesion

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INTRODUCTION: Internal fracture fixation implant surface finishes vary from electropolishing of stainless steel (SS) to micro-rough commercially pure' titanium (cpTi) and Ti-6%Al-7%Nb (TAN). Material comparisons are difficult as the surface finish of the materials vary considerably. 'Standard' SS has a smooth 'mirror like' finish while standard cpTi and TAN are microroughened for enhanced osseointegration. This *in vitro* investigation utilised the standard material finishes and electropolished variants of cpTi and TAN. These aided in distinguishing between both material, surface chemistry and topography effects upon fibroblast growth, cytoskeletal behaviour and adhesion. Roughened SS was not included as its reduced corrosion resistance rendered it biologically and commercially non-viable. Fibroblasts are the major cellular constituent of fibrous connective tissue adjacent to implants, therefore our *in vitro* model of soft tissue interaction used human fibroblast cell line (hTERT-BJ1). Modifying the actual metal implant surface to inhibit or reduce initial bacterial adhesion may have simultaneous benefits. Therefore visualisation and quantification of *S. aureus* bacterial adhesion to standard and electropolished finishes was also performed.

METHODS: The materials tested were standard and electropolished surfaces of titanium (ISO5832/2) and Ti-6Al-7Nb (ISO 5832/11) and electropolished stainless steel (SS) (ISO 5832/1). Surface characterisation was performed using Atomic Force Microscopy (AFM), Profilometry, Scanning Electron Microscopy (SEM) and X-ray Photoelectron Spectroscopy (XPS). Qualitative cell reactivity assessment utilised SEM for cell growth and morphology at 24h, 5 and 10d timepoints. Intracellular components, vinculin, tubulin, actin and DNA, were fluorescently labelled and imaged using the Fluorescence Microscope (FM) at 48h. DNA from cells cultured for 24h, 5 and 10d was extracted and quantified to confirm qualitative cell growth findings.

To visualise bacterial adherence, *S. aureus* were cultured on the different surfaces in a brain heart infusion broth at 37°C for 1 hour, then fixed for SEM imaging. To quantify the amount of adherent bacteria, they were stained with fluorescent 5-cyano,2-ditolyl tetrazolium chloride (CTC), and visualized with FM. The density of live bacteria on the surfaces in each image were counted using KS400 software and analysed statistically using a one-way ANOVA with Tukey test. This was repeated three times. Statistical significance was accepted at $p \leq 0.05$.

RESULTS: SS, TE and NE were all smooth samples with Ra's between 0.18-0.19μm (Table 1). While similar in Ra, AFM (Fig 1a) and SEM demonstrated that all materials had some variation in topographical morphology. SS had some surface scratches, TE had a topography of nanometric surface nodules and NE displayed an undulating topography. The rougher materials, TS and NS had higher but similar Ra's of 0.90 and 0.77μm. AFM and SEM demonstrated that the similarities end here with TS having a rugged irregular surface, NS demonstrating a 'microspiked' topography consisting of protruding particles. XPS showed that electropolishing the cpTi or TAN followed by anodisation did not add any contamination to the final surface chemistry (table 2).

Surface	code	Ra (μm)
Std micro-rough titanium	TS	0.90 ± 0.027
Electropolished TS	TE	0.19 ± 0.030
Std micro-rough Ti-6Al-7Nb	NS	0.77 ± 0.076
Electropolished NS	NE	0.18 ± 0.037
Std electropolished stainless steel	SS	0.19 ± 0.022

Table 1. Surface Roughness Parameter Ra for the different surfaces.

	Atomic Concentration [%]								
	Al 2p	C 1s	N 1s	Na KLL	Nb 3d	O 1s	P 2p	Ti 2p	
NS	1.7	25.5	0.9	0.8	0.2	51.2	2.1	17.6	
NE	2.0	27.8	1.1	0.4	0.1	49.3	0.6	18.6	
TS	n/a	26.95	0.6	1.2	n/a	50.7	3.0	17.5	
TE	n/a	32.1	1.3	0.3	n/a	47.0	2.6	16.4	
	Cr 2p	C 1s	Fe 2p	N 1s	Mo 3d	Na 1s	O 1s	P 2p	Ni 2p
SS	7.3	41.4	4.5	3.7	0.6	1.5	40.1	0.3	0.8

Table 2. XPS surface chemical analysis of the various surfaces (n/a – not applicable).

Qualitative cell growth on the samples, by SEM, demonstrates that cells reached confluency by the 10d timepoint on all samples except NS. This growth suppression was confirmed to a level of statistical significance (P>0.01) by DNA quantification. Cell morphologies were well spread on SS, TE and NE at both 24h and 5d timepoints. The morphology only changed due to lack of surface space, and cells adopted an elongated morphology. Cells on TS were not well spread at 24h with investigatory filopodia emanating from far within the cell body. By 5d, cells were better spread and 10d were elongated with confluent numbers. On NS, cells at all timepoints were rounded or elongated. At 24h and 5d filopodia could be seen attaching to the microspikes, however by 10d this exploration had ceased. On some cells, membrane integrity was reduced to a much coarser texture indicative of cell necrosis. Intracellular labelling demonstrated that on SS, TE and NE the focal adhesion (FA) sites were mature with associated actin cytoskeleton and a well conserved microtubule network. On TS, cells were less spread with smaller FA but a well-conserved actin and microtubule network. For NS (Fig 1b), cells were again unspread and FA's were small and could be observed avoiding the microspikes. The underlying microspikes could also be seen actively impairing for formation of the microtubule network.

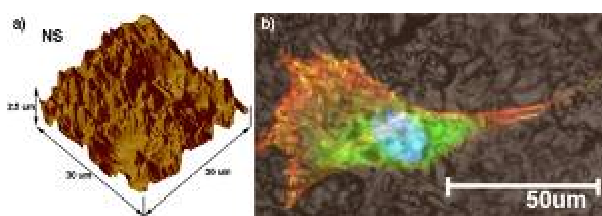

Fig1(a) AFM of NS. (b) Fluorescence microscopy of the cell cytoskeleton and adhesion sites on NS.

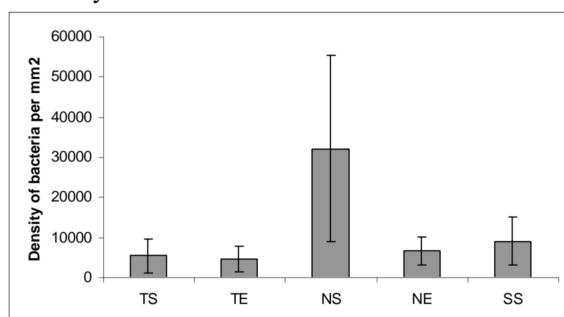

Fig 3. Graph showing the average density of living S. aureus adhering to the different surfaces (n=3cultures). The large variations are due to clumping of the bacteria on the surface.

Qualitative and quantitative results of *S. aureus* adhesion on the different surfaces correlated with each other, and showed significantly more live bacteria on NS than on the other surfaces, whilst there was no significant difference between the amount of bacteria on TS, TE, NE and SS surfaces (Fig 3). The results showed a significant decrease in the amount of bacteria adhering to the NE compared to standard NS surfaces.

DISCUSSION & CONCLUSIONS: Our findings indicate that surface chemistry is not paramount to cytocompatibility. NE's bio-performance (with regards to fibroblasts) was exceptional in comparison with its microrough counterpart, NS. A general roughened topography was also not the cause as both TE and its roughened counterpart, TS, performed well. As can be seen from the interaction of the filopodia, FA's and microtubules, the additional factor of NS is its unique microspiked topography, an inherent characteristic of the TAN microstructure. They are also present in a smoothed rounded edged form in NE, but are of no significance to subsequent cytocompatibility studies. Thus we propose that the topographical presence and dimensions of the particles present on NS inhibits the spreading of cells and development of mature FA's; two essential factors in the progression of the cell cycle and cell growth.

With regards to bacterial adhesion, this study found that electropolishing Ti-6Al-7Nb surfaces (NE) significantly decreased the amount of *S. aureus* adhesion compared to the standard Ti-6Al-7Nb (NS), which had a higher affinity to the bacteria than the other surfaces tested. This would indicate that the aforementioned microspikes of NS increase *S. aureus* adhesion and could lead to higher infection rates *in vivo*. Hence electropolishing Ti-6Al-7Nb surfaces could be advantageous in osteosynthesis areas as a measure to prevent soft-tissue irritation while also minimising bacterial adhesion, possibly even lowering the rate of infection. *In vivo* tests will have to be carried out to confirm this interesting development.

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Mechanically loaded *ex vivo* culture system for cancellous bone biopsies

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Introduction: In order to understand mechanical support and mineral homeostasis of bone, one must have both the cells and bone matrix combined in an isolated, 3D culture. A need exists for an *ex vivo* bone culture system, where a controlled biochemical and mechanical environment is created to be able to determine the influence of different parameters. The Zetos[1] model with mechanical loading has been validated (Fig.1) with ovine, bovine and human samples to keep cancellous bone tissue viable *ex vivo*. The samples maintain osteocytes, osteoblasts, osteoclasts and bone marrow cells in their natural 3D relationship to each other. Mechanical loading is a known anabolic stimulus for bone that is imperative to its natural development. The Zetos bioreactor may potentially be used for tissue engineering of bone to fill defects caused by tissue trauma or disease. The system could also be used to test possible tissue engineered constructs, reducing the amount of animal experimentation required. The goal of this study was to assess the response of three dimensional human explant cancellous bone to the addition of TGFβ_3 during long term culture with mechanical loading in the *ex vivo* loading bioreactor.

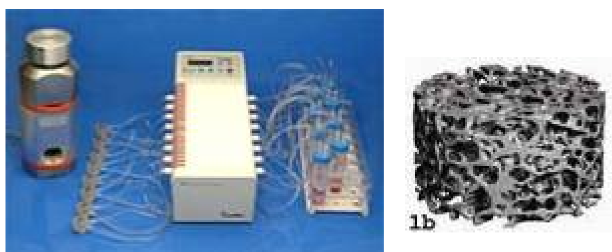

Figure 1. *Ex vivo* Zetos culture system. Microprocessor controlled pump allows perfusion of fresh media through the chambers. The bone cores (1b) are stimulated daily.

Methods: Human femoral heads (Ethic Commission Graubünden approval (18/02)) were processed into cylindrical cores (5mm height, 9.5mm diameter). The cores were inserted into the culture chambers, randomly assigned to groups and subsequently cultured for up to 14 days. Groups included, with or without TGFβ_3 (15ng/ml) and with or without loading (300 cycles at 1 Hz, giving 4000 microstrain) and heat treated dead cores as a control. As fresh tissue controls bone cores were fixed with 70% ethanol immediately after excision (T0). Post culture cell viability was assessed by cutting the cores into 250µm thick sections and the LDH assay was performed[2]. All remaining cores were fixed in 70% ethanol, dehydrated through an ethanol series and embedded into Technovit 9100 New[3] for subsequent histological and immunohistochemical evaluation.

Results: Histology of live cultured samples after 14 days in the Zetos system was comparable to fresh bone (T0). Non collagenous proteins such as bone sialoprotein and osteopontin were localised through immunohistochemical labelling of sections. The LDH assay displayed a uniform purple/blue staining (LDH positive) over the entire section on a macroscopic scale of the fresh, live tissue. Many dark stained osteocytes were seen in the bone cores cultured for 14 days in the loaded Zetos culture after administration of TGFβ_3. The dead sections however did not exhibit any dark, defined osteocytes and only the empty lacunae were visible (Fig. 2)

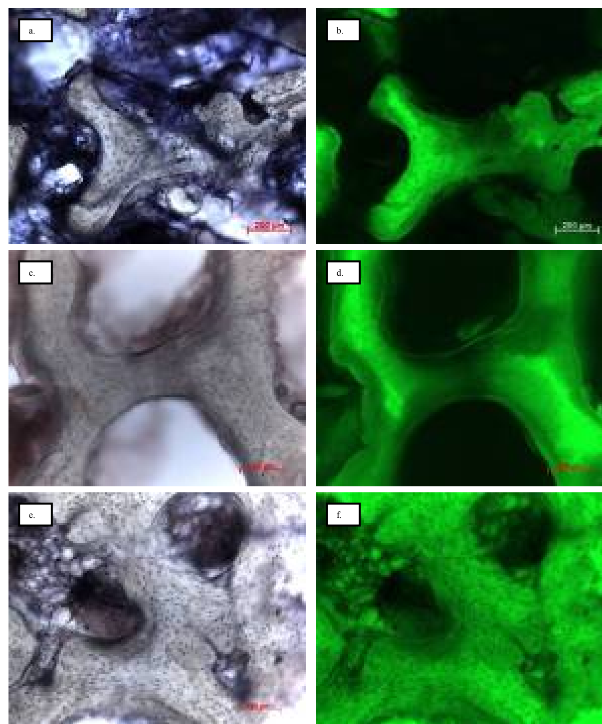

Figure 2. Human bone tissue (male, 54 yr). Mid-sections, stained for LDH-assay. a). and b). T0 section,

darkly stained osteocytes and marrow can be seen. c). and d). A dead core taken after 14 days in loaded Zetos system. No darkly stained osteocytes or marrow can be observed. e) and f). Show an image of a core taken after 14 days in loaded Zetos culture with many darkly stained osteocytes and viable marrow can be seen.

The number of viable osteocytes observed in the fresh tissue (T0) was greater than after 7 and 14 days in Zetos culture. However, in all cases there appeared to be a positive effect of loading on the number of viable osteocytes present after 7 and 14 days in Zetos culture compared with the unloaded samples. In most cases the effect of loading plus TGFβ_3 on viable osteocytes was even greater. (Fig. 3)

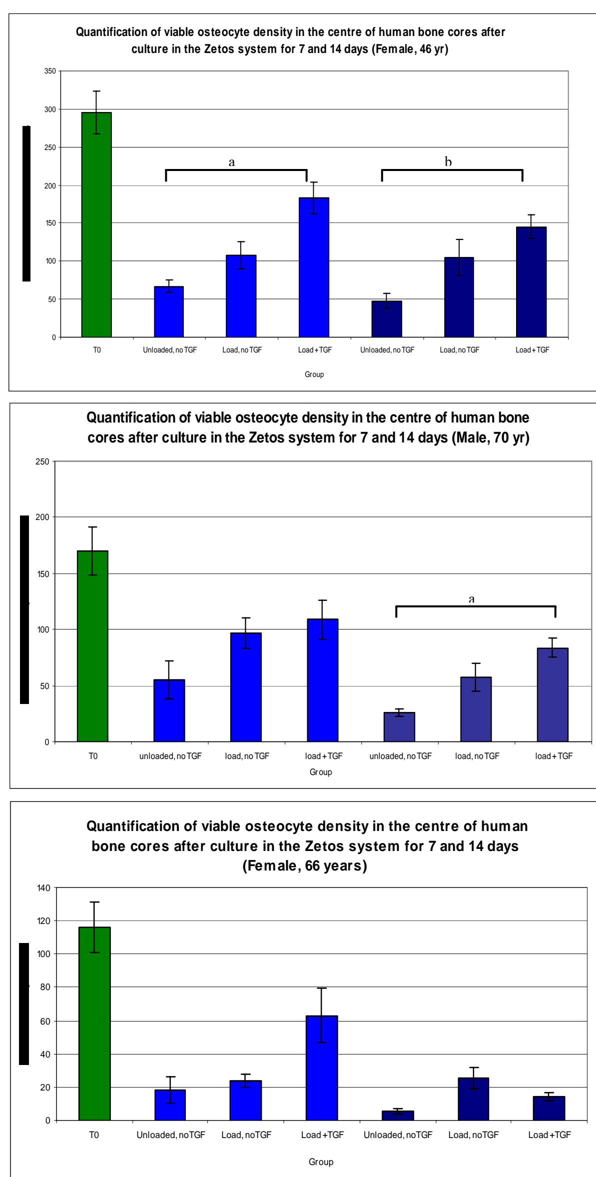

Figure 3. Graphical representation of the quantified osteocytes present in the central area of bone cores at T0 (fresh tissue) and after 7 and 14 days in Zetos culture under different experimental conditions. Light blue depicts 7 day time point, dark blue depicts 14 day time point.

Discussion and Conclusion: The Zetos bone bioreactor system permits the culture of viable 3D human trabecular bone cores up to 14 days. The outcome of this work shows that this *ex vivo* loading bioreactor is able to maintain a high percentage (over 50%) of viable osteocytes throughout the bone cores after 14 days in *ex vivo* culture. Further to this, the combination of daily loading and TGFβ_3 administration produced superior osteocyte viability at the core centres when compared to loading alone. The bioreactor has potential in pre-testing the integration of human bone with biomaterials, studying basic bone biology including osteoporotic bone.

References:

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