MESENCHYMAL STEM CELLS AND THEIR PROGENY: DEVELOPMENTAL PARADIGMS GOVERNING OSTEOBLAST DIFFERENTIATION & BONE FORMATION

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INTRODUCTION: Bone formation takes place in the organism not only during embryonic development, growth and remodeling, but throughout life in ongoing bone turnover, fracture repair and induction, e.g., by the implantation of decalcified bone matrix or purified or recombinant members of the bone morphogenetic protein family. This suggests there is a large reservoir of cells in the body capable of osteogenesis through life, but the nature of these cells over the lifetime of the animal, contributions from stem cell versus committed progenitor pools, whether there are different stages of committed progenitors recognizable, and identification of transitional steps from stem cell to committed osteoprogenitor to osteoblast remain important issues[1]. We will provide insights into the developmental paradigms governing osteoblast differentiation and bone formation from cellular and molecular analyses of developing bone colonies *in vitro*.

METHODS: Cells were isolated from 21 day Wistar rat calvariae, plated at different densities and cultured for up to 3-4 weeks in differentiation medium (αMEM, with antibiotics, 10% FBS, 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, and with or without 10 nM dexamethasone (dex). In some experiments, master colonies and their replicas were prepared on polyester cloth. Osteoblast development and bone formation or adipocyte development and fat formation were determined by morphology, histochemistry (Von Kossa, alkaline phosphatase (ALP) staining, Sudan IV or Oil Red O staining), immunocytochemistry with lineage-specific antibodies, and gene expression profiling by in situ hybridization, Northern blots, semi-quantitative Real Time PCR, RT-PCR, or global amplification poly(A) PCR. In some cases, myoblast/muscle and chondrocyte/cartilage development were assessed by semi-quantitative RT-PCR or RT-PCR. For isolation of a side population (SP), verapamil-sensitive exclusion of Hoechst 33342 was quantified and used as a sort parameter on a FACStarPlus (BD Biosciences). Populations were sorted on the basis of co-expression profiles of ALP and PTH1R.

RESULTS: Committed osteoprogenitors, i.e., progenitor cells apparently restricted to osteoblast development, can be identified by functional assays of their proliferation and differentiation capacity in vitro, i.e., the bone nodule assay or colony forming unit-osteoblast (CFU-O). In RC populations, they are present at low frequencies (~$1/10^2$ cells), a frequency that can be increased substantially by sorting the SP population or sorting on the basis of ALP and PTH1R expression, and have only a limited self-renewal capacity in culture. At least two distinct populations of osteoprogenitors are present: one appears capable of constitutive or default differentiation in vitro, whereas the other more primitive comprises inducible progenitors that are recruited by stimuli such as dex. Gene expression profiling revealed that multiple and complex transitions, not directly associated with proliferative lifetime of the progenitors, demarcate osteoblast development[2]. Notably, bioinformatics and statistical analysis have suggested that osteoblast development may not be governed by simple deterministic paradigms but that multiple developmental routes may lead to the functional osteoblast endpoint[3]. Co-expression profiles of osteoblast markers with markers of chondrocytes, myoblasts and adipocytes also suggest that multilineage precursors, possibly stem cells, may preface lineage programs prior to or even after commitment. The data also support a surprising level of osteoblast heterogeneity in vitro and in vivo that may underlie the growing number of examples of anatomical site-specific osteoblast responses to hormones, growth factors and other regulatory molecules.

DISCUSSION & CONCLUSIONS: Osteoblast development is a complex multi-step process in which osteoprogenitors arise from multilineage precursors whose fate may be governed by both stochastic and deterministic events and in which functional osteoblasts comprise a heterogeneous population that may be subject to different regulatory controls.

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CELL THERAPY AND BONE REPAIR

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INTRODUCTION: Bone damage, due to pathology or trauma, is a common occurrence, requires costly medical and/or surgical intervention and involves several human resources as well as a great deal of suffering in the patients. Present techniques are only partially successful, due to the length of treatment and shortage of donor bone tissue. In many cases grafts of new tissue are required to achieve functional recovery. Bone grafts or synthetic materials are needed to replace missing bone and enhance new bone formation. Grafts are either of autologous source (from the patient itself; autograft) or from other donors (allograft). In the first case, the autograft procedures require a second operation to harvest enough bone, increases risk factors, hinders the patient's recovery and substantially raises costs. The allografts are instead associated with the risk of blood-born diseases and are limited to the countries in which fresh frozen grafts are storaged and correctly donor-receiver matched; moreover, many grafts are produced from cadavers by heat and dehydration procedures which are expensive, time-consuming and strictly law-regulated.

At present, the use of autologous bone graft is considered the best option. Regenerative Medicine and Tissue Engineering are new research areas that investigate how to repair and regenerate organs and tissues using the natural signaling pathways and components of the organism (stem cells, growth factors, etc.) Cell biology gave several growth factors a role in the control of the proliferation and differentiation capacity of specific cell types. In addition, today's *in vitro* cell culture protocols allow the expansion of specifically selected cell populations. Cells harvested from the patient, including adult stem cells, are expanded in culture and associated with resorbable biomaterials both of synthetic and extractive origin. Therefore, damaged tissues that would normally not be repairable with the available traditional techniques could be in this way reconstituted. The employment of biomaterials as both vehicles and inductors of the *"ex vivo"* expanded cells, has and will always promote an increase in the research and development of a new generation of transplantable biomaterials. Of great relevance is the fact that these engineered tissues could be obtained and used with no quantity limitations.

METHODS: Cell cultures Stromal cells were obtained from iliac crest marrow aspirates from healthy donors. Donor age ranged between 3 and 50 years and donors were all white Caucasians. Informed consent was obtained from all donors and all procedures were approved by institutional ethical committee. Mononuclear cells (MNC) were counted, plated at 2-5x10^6 MNC/100mm dish in Coon's modified Ham's F-12 medium supplemented with 10% Fetal Calf Serum (FCS). Some plates were cultured in the presence of 1ng/ml human recombinant FGF-2. The medium was changed after 3 days and then twice a week. When dishes reached confluence, BMSC were detached with 0.05% trypsin/0.01% EDTA and replated. To evaluate CFU-f frequency (CFU-f assay), 100µl of the original marrow suspension were plated in 100 mm dishes. The medium was changed after 3 days and then twice a week. After 2 weeks of primary culture, cells were washed, fixed with 3.7% formaldehyde in PBS, and stained with 1% methylene blue in borate buffer. Colonies were counted.

Tissue engineering of bone: preclinical and clinical studies. Bone formation can be assessed in small animals by implanting BMSCs combined with mineralized tridimensional scaffolds subcutaneously in immunodeficient mice

To treat full-thickness gaps of tibial diaphysis in adult sheep, autologous cells isolated from bone marrow and expanded in vitro, were loaded onto highly porous ceramic cylinders (100% hydroxyapatite; 70- 80% porosity; pore size distribution: < 10 µm ~ 3% vol.; 10-150 µm ~ 11% vol.; > 150 µm ~ 86% vol) and implanted in critical-sized segmental defects in the tibia of the animals. External fixation was used to stabilize the grafts. For the human patient, the study protocol was approved by the ethical committee of the orthopaedic centers involved. Patients had non neoplastic pathologies, and were selected for this treatment after failure of an alternative surgical therapy, and/or to avoid important donor site morbidity, an expected long recovery time and likely future complications. The patients were informed of the nature of the treatment and gave their written consent.

RESULTS: Cultured Bone Marrow Stromal Cells (BMSC) can be regarded as a mesenchymal progenitor/precursor cell population derived from adult stem cells. They can differentiate into different lineages: osteoblasts, chondrocytes, adipocytes and myocytes. BMSC stimulation with particular growth factors (i.e. FGF2) induces a critical modification in the proliferation and differentiation ability of these cells, which remain in a osteo-chondrogenic stem/progenitor status. BMSC undergo limited mitotic divisions and do not express telomerase activity. We have also observed a sequential loss of lineage potential in tripotent cloned cell populations, suggesting a model of predetermined BMSC differentiation. Thus BMSC do not display full features of stem cells and should be regarded as early

mesenchymal progenitors. A search for specific markers expressed by BMSC at the early progenitor stage is in progress.

When implanted in immunodeficient mice, BMSCs combined with mineralized tridimensional scaffolds form a primary bone tissue highly vascularized. We have used this animal model to test efficacy of of cells expanded according to different protocols and different biomaterial scaffolds of different nature and with variable degree of reasorbability.

Autologous BMSC/bioceramic composites were used also to treat full-thickness gaps of tibial diaphysis in adult sheep. Gross morphology, x rays, histology, microradiography and SEM studies showed complete integration of ceramic with bone and good functional recovery. We have also reported the existence of complementary integration and disintegration mechanisms within HA ceramic (HAC) implants used to replace the critical-sized segmental defects

The healing process involves four main steps: 1) bone formation on the outer surface of the implant; 2) bone formation in the inner cylinder canal; 3) formation of fissures and cracks in the implant body; 4) bone formation in the bioceramics pores. By radiography and by tomography, bone formation was far more prominent over the external surface and within the inner canal of the implants. This might be due to a higher density of loaded cells and/or to a better survival of cells within the outermost portions of the HA bioceramics. Alternatively the implanted cells could stimulate, via a paracrine loop, resident osteoprogenitor cells, located within the skeletal tissues at the resection ends.

Given the results obtained in the animal models, similar composites were implanted at the lesion sites of six patients. External fixation was used. Some of the patients have been followed for more than 3-4 years. A full functional recovery of the treated limb occurred within 6 to 7 months from surgery. An initial integration at the bone/implant interface was already evident one month after surgery. Bone formation progressed steadily during the following months. A full functional recovery of the treated limb occurred within 6 to 7 months after surgery.

The pattern of the bone healing process in the patients was similar to the one described in the large animal model.

DISCUSSION & CONCLUSIONS: A tissue engineering approach has been followed in the orthopedics field. This approach via the composite biomaterial and cell technology, and the characterization of cell growth conditions, implantation protocols and surgical procedures is allowing significant progresses for bone repair.

Regardless of the low number of patients so far treated, we consider the results obtained very promising and we propose the use of culture-expanded osteoprogenitor cells in conjunction with HA bioceramics as a real and significant improvement in the repair of critical size long bone defects.

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Human jaw periosteal cells display reduced and delayed in vitro osteogenic differentiation as compared to bone marrow stromal cells

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INTRODUCTION: Human bone marrow stromal cells (BMSC) can differentiate toward the osteogenic lineage in vitro, generate bone tissue in vivo [(1)] and support the repair of large segmental defects in clinical cases [(2)]. Human jaw periosteal cells (JPC), more easily available than BMSC, have also been proposed for cell-based bone repair[(3)]. We compared the in vitro osteogenic differentiation of BMSC and JPC from the same individuals.

METHODS: Iliac crest marrow aspirates and jaw periosteum biopsies were taken from 6 healthy donors (21-69 years). BMSC and JPC were expanded using dexamethasone and FGF-2, previously shown to enhance proliferation and commitment of osteogenic cells[(4)]. Subsequently they were cultured in medium promoting osteogenic differentiation, containing dexamethasone, beta-glycerophosphate and ascorbic acid. After 1, 7, 14 and 21 days cultures were assessed for: DNA amount, alkaline phosphatase activity (AP), calcium deposition and mRNA expression of bone sialoprotein (BSP) and osteopontin (OP) using Real-Time RT-PCR[(5)]. Differences assessed by parametric (T-Tests) or non-parametric tests (Mann-Whitney, Wilcoxon) were considered statistically significant with $p < 0.05$.

RESULTS: Large variability was observed in both JPC and BMSC cultures from different donors. JPC proliferated faster, but had significant lower AP activity than BMSC *(figure 1a)*. Calcium deposition by JPC was lower than by BMSC up to day 14 and similar at day 21*(figure 1b)*. The expression of BSP *(figure 1c)* and OP was significantly lower by JPC than by BMSC up to day 7.

DISCUSSION & CONCLUSIONS: JPC proliferate faster but have a delayed and overall reduced capacity to differentiate toward the osteogenic lineage in vitro. Therefore, methods used to induce bone formation by BMSC might need to be adapted for JPC. Ongoing in vivo studies will help determine whether JPC are a valuable alternative to BMSC for cell-based bone repair.

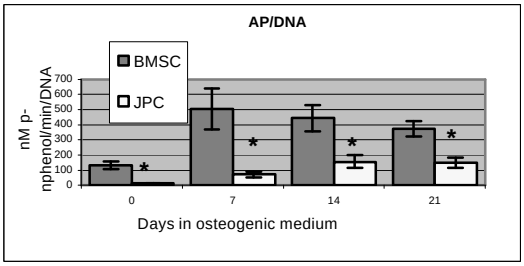

Figure 1a: alkaline phosphatase activity normalized to DNA amount

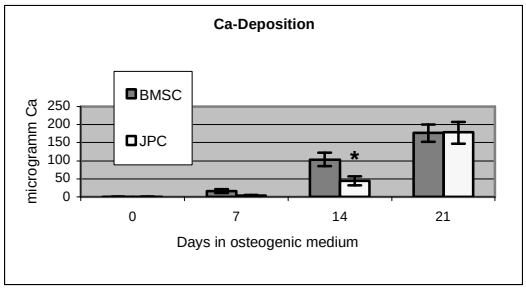

Figure 1b: Calcium deposition

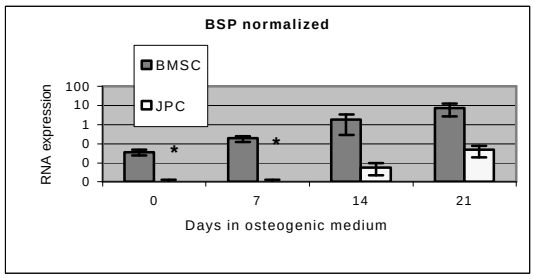

Figure 1c: BSP expression normalized to 18s and the expression level of osteoblasts[(5)]
(*) asterisks indicating statistical significance

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LONG-TERM EFFECTS OF LOCALIZED INSULIN-LIKE GROWTH FACTOR I DELIVERY ON BONE REPAIR AND REMODELING

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INTRODUCTION: Growth factor (GF) based approaches are an alternative avenue to autogenic and allogenic bone grafting for the enhancement of fracture repair [1]. Nevertheless, various safety issues are associated with the clinical use of skeletal GFs [2], and long-term performance characteristics of bone induced by the delivery of GFs have been hardly taken into consideration so far, even in preclinical studies. The aim of the present study was to investigate long-term physiologic consequences of localized insulin-like growth factor I (IGF-I) delivery on bone repair and remodeling. For this purpose biodegradable microspheres containing microencapsulated IGF-I were administered in 10 mm segmental tibial defects in sheep. Previously, we established the beneficial role of this treatment on bone repair. Here, we focus on macro- and microstructural changes in bone repair up to 18 months post operation by clinical, radiographic, gross pathologic, and histological techniques, as well as by novel microcomputed tomography (micro-CT).

METHODS: IGF-I was encapsulated in poly(lactide-co-glycolide) 50:50 (PLGA 50:50) microspheres (MS) using solvent evaporation technique [3]. Protein loading and encapsulation efficiency were determined by HPLC. Particle size was measured by laser light diffractometry, and surface morphology of the MS was analyzed by scanning electron microscopy. IGF-I in vitro release kinetics were monitored during 62 days and analyzed by ELISA. Bioactivity of encapsulated IGF-I was verified using a mitogenic bioassay in an osteosarcoma cell line (MG63). The in vivo study was performed in 6 adult sheep (Swiss alpine) according to Swiss federal guidelines and approved by the institutional animal welfare committee. Under anesthesia, unilateral, standardized 10 mm mid-diaphyseal tibial defects were established in either right or left hind legs. Each tibial defect received 100 mg PLGA MS loaded with 100 µg IGF-I. Afterwards, defects were stabilized with a dynamic compression plate.

Due to ethical considerations an untreated control defect was only authorized for an observation period of 8 weeks (negative control). Therefore, the corresponding intact tibiae served as a reference. Radiographs were taken at regular time intervals. Sheep were killed after 18 months. Inner organs were removed to examine pathological irregularities. Micro-CT images (µCT40, Scanco Medical, Bassersdorf, Switzerland) were made with a resolution of 30 µm. The bones were then cut, and slices prepared for histology (ground and thin sections stained with toluidine blue and von Kossa).

RESULTS: Radiographic evaluation revealed that remodeling of calluses and medullary cavities was well advanced after 18 months.

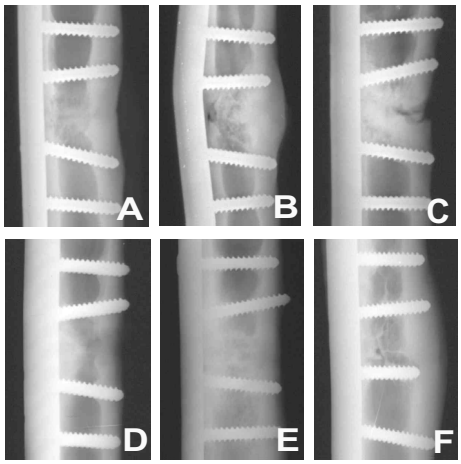

Fig. 1: Anteroposterior radiographs of six tibial defects (A-F) treated with 100 µg IGF-I in PLGA MS 18 months after surgery. All defects were clinically healed, and medullary cavities were fully recanalized in five sheep, but imperfect in sheep C.

Micro-CT images gave a detailed view of the 3D bone architecture within the defect zones (Fig. 2). New bone was nicely integrated into the osseous network of proximal and distal bone ends.

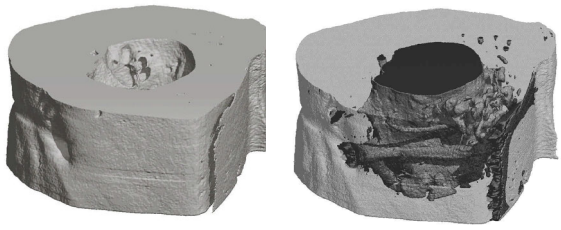

Fig. 2: Typical 3D micro-CT reconstructions of an IGF-I treated defect (sheep B) visualizing bone (A) and bone-marrow surfaces (B).

Bone morphometric data were also derived from micro-CT analysis (Table 1). Total tissue volume (TV) for the treated tibiae was significantly ($p < 0.01$) larger than for the contralateral tibiae. This was due to a higher bone volume (BV), whereas marrow volume (MV) was lower than normal. Bone surface was nearly three times higher than normal, allowing for ongoing remodeling.

*Table 1. Bone morphometric parameters (means ± SD, *p<0.01) of IGF-I treated defects and reference tibiae 18 months post operation.*

Parameters	IGF-I treated defects (n = 6)	Reference tibiae (n = 6)
TV [mm^3]	2833.9 ± 537.8*	2054.2 ± 172.1
BV [mm^3]	2463.9 ± 504.9*	1472.9 ± 153.2
MV [mm^3]	370.1 ± 109.8*	581.3 ± 39.9
BV/TV [%]	86.7 ± 4.1*	71.6 ± 2.0
BS/BV [mm^2/mm^3]	0.58 ± 0.22*	0.21 ± 0.02
Co.Th [mm]	2.6 ± 0.6*	4.1 ± 0.3

Histological sections showed that the osteotomy gaps were completely filled with new cortical bone, consisting of lamellar and woven bone. Considerable vascular ingrowth was visible in interspaces. Medullary cavities were still in the process of remodeling (Fig. 3b).

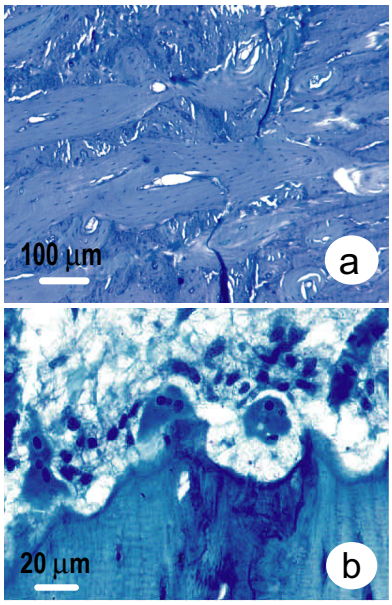

Fig. 3: Thin sections of a) newly formed cortex and b) marrow cavity stained with toluidine blue. a) Osteons have crossed the osteotomy, reestablishing structural continuity and providing the basis for mechanical restoration b) Ongoing bone remodeling is indicated by the presence of numerous osteoclasts actively resorbing bone.

DISCUSSION & CONCLUSIONS: Controlled local IGF-I delivery enhanced new cortical bone formation in sheep, and its application was safe in terms of local and systemic adverse events. Bone remodeling was towards normal 3D bone architecture. The observed delayed union was in conjugation with biomechanical instability. For the treatment of larger defects intrinsic structural support in combination with a suitably controlled IGF-I delivery regimen is suggested.

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BONE REGENERATION WITH RESORBABLE POLYLACTIDE MEMBRANE AND SPONGE IN AN UNSTABLE FRACTURE MODEL IN RABBIT RADIUS

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BACKGROUND:

Healing of segmental diaphyseal bone defects in animals can be enhanced by covering the defects with resorbable polylactide membranes. Based on the results of bone healing in defects 10 mm long in the rabbit radii, it was suggested that the membranes prevents muscle and soft tissue from invading the defect and maintains osteogenic cells and osteogenic substances within the space covered with membrane, thus promoting new bone formation.

OBJECTIVES:

1. To investigate and compare bone regeneration with resorbable polylactide membrane and polylactide sponge in a 20 mm bone defect in rabbit radii.

2. To determine and compare the biomechanical strength of the bone fixation construct with reinforcement by membrane and sponge of such bone defect which were rendered unstable by ulnar osteotomy

MATERIAL AND METHOD:

The material used was poly (L/DL-lactide) 80/20% in the form of membranes and sponges. The membrane was 0.1 mm thick and was porous in the defect side. The sponge has an average pore size of 300-500 μm with pore to volume ratio 95%. 20 mm long diaphyseal segmental defects were made in the left radii of adult New Zealand rabbits. Transverse ulnar osteotmies were made at midshaft to make the forearm unstable. The rabbits were divided into 5 groups. In Group 1, no fixation of the bone were performed and the limbs were immobilized in a plaster for 8 weeks. In Group 2, the bone defects were fixed with 1.5 AO miniplate, with 2 screws on each side of the defect. In Group 3, the bone defects were fixed similarly and polylactide membranes were used to cover up the bony defect. In Group 4, the bone defects were fixed similarly to Group 2 and the defects were bridged by sponge of 20 mm long, 3 mm in diameter, In Group 5, the bones were fixed similarly and the defects were bridged by a sponge of same dimensional and wrapped by polylactide membrane same as these in Group 2 from rabbit of same species. Histological study and biomechanical study were performed on the explanted forearm at 8 weeks.

RESULTS:

In Group 1, there was bone healing bridging the bone ends at 8 weeks. However, there was marked shortening of the limbs and all the limbs were deformed.

In Group 2, there was bone formation at the ends of both proximal bone stumps and distal bone stumps at 8 weeks. There was no bone bridging the defect.

In Group 3, 4, 5, there was bone formation dispersed across the defect. There was more abundant bone regeneration in Group 4 and 5. Evaluation at 1 year revealed good bone formation in the groups with sponges.

DISCUSSION AND CONCLUSIONS:

Polylactide membrane and sponge promote bone regeneration in 20 mm bone defect in the rabbit radii model.
It was postulated that membrane serves as a barrier to prevent fibrous tissue to grow into the bone defect and preserve the osteogenic factors and cells. It was highly likely that membrane surface served as an anchorage site for osteoblasts so they can proliferate and express their phenotype sponge provided more surface area for osteoblasts to proliferate and differentiate so more bone regeneration occurred. The size, 20 mm, well exceeded the critical size defect of the rabbit so resorbable polylactide sponge can be used to regenerate long segmental defect.

Key words: bone defects, bone regeneration, polylactide membrane, polylactide sponge

BLOOD VESSELS & MESENCHYMAL CELLS: CAN THEY MAKE BETTER BONE?

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INTRODUCTION: Mesenchymal cells are commonly used as a source of cells for new bone production. *In vivo* models clearly demonstrate that bone marrow-derived mesenchymal cells have an osteogenic potential, with new bone usually associated with a vascular supply. The blood vessels are generally thought to simply provide a source of nutrients for the donor cells rather than a direct role. *In vitro* models of osteogenesis provide valuable data on the regulating factors and genes involved in bone formation but most models rarely produce a piece of true bone and rely on markers of osteogenesis such as type I collagen, alkaline phosphatase or bone sialoprotein. Here, we present an *in vitro* organ culture model of osteogenesis where a piece of true bone forms from a human bone marrow plug. In the presence of endothelial cell-derived factors, a larger piece of bone develops more rapidly. Endothelial cell-derived factors stimulate a five-fold increase in adherent mesenchymal cell numbers within one week and these cells also differentiate into bone if pelleted and grown in organ culture. In addition, the endothelial cell-derived factors allow a plug of marrow to adhere to a slice of human bone and develop into a nodule of new human bone. These data suggest that blood vessels and endothelial cells play a direct role in osteogenesis and may aid in the production of larger, quicker and better bone.

METHODS: Human bone marrow was obtained with full consent from the opened sternum of patients undergoing cardiac surgery. A total of 133 organ cultures were established from 12 patients, age range 47 – 67 years. (10 males, 2 females). Bone marrow was dissected free from existing bone under a dissecting microscope and either grown as a plug in organ culture or mechanically dissociated into a single cell suspension. Marrow plugs or cells were established in αMEM, BGJb or conditioned media removed from confluent endothelial cells – ECCM (also αMEM). Cell cultures were harvested and counted at 7 days and if necessary pelleted for organ culture. Organ cultures were fixed at weekly intervals for up to six weeks and processed for histology and immunocytochemistry.

RESULTS: During the six-week culture period, αMEM allowed fibroblastic cell survival but no bone formation. When supplemented with β-glycerophosphate (+P), bone formation occurred with a frequency of 72% in 3-4 weeks, BGJb media produced bone with a frequency of 93 % in the same time period but ECCM (which is αMEM harvested from confluent endothelial cell cultures produced bone with a frequency of 95% within 7-10 days (Figure 1).

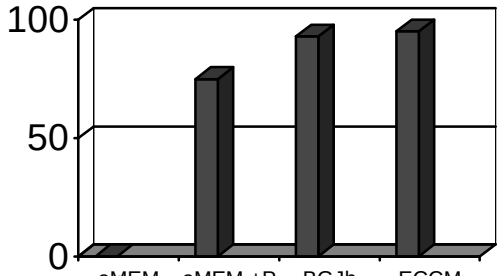

Fig 1. Frequency of bone formation of human bone marrow plugs in organ culture.

The bone resembled true bone with osteocytes embedded in a mineralized matrix and osteoblasts on the periphery.

DISCUSSION & CONCLUSIONS: In this report we show that endothelial cell-derived factors, released into tissue culture medium stimulate rapid bone differentiation from human bone marrow-derived mesenchymal cells. This bone develops in approximately one third of the time required in control cultures. Indeed, instead of being "spent" medium, this ECCM can stimulate a five-fold increase in mesenchymal cell number and these cells also can progress to true bone formation.

Normal and pathological bone formation requires a vascular supply, often associated with the removal of cartilage. We have recently demonstrated that ECCM will also specifically destroy hypertrophic chondrocytes. We suggest that during development, endothelial cells produce factors which can aid in the destruction and removal of hypertrophic chondrocytes, blood vessels transport osteogenic cells to the appropriate site and the endothelial cell products can stimulate cell proliferation and then differentiation of the osteogenic cells into new bone. The data presented here indicate that rather than just providing nutrients and a means for the appropriate cells to reach their destination, the endothelial cells play a direct and important role in bone formation. Thus, endothelial cells do help make better bone.

ACKNOWLEDGEMENTS: This work was funded by the Sir Jules Thorn Charitable Fund.

ENDOTHELIAL CELLS MODULATE BONE MARROW STROMAL CELL DIFFERENTIATION INTO OSTEOBLASTS: DEPENDENCY ON ENDOTHELIAL CELL MATURATION

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INTRODUCTION: Osteoblast precursor cells, which are present within the heterogeneous bone marrow stromal cell population, differentiate into mature osteoblasts in a tightly regulated process. It is known that endothelial cells communicate with osteoblast precursors and also with mature osteoblast cells. However, the interaction and the role of endothelial cells on the differentiation process leading to mature osteoblasts are not well understood. Vascular endothelial growth factor (VEGF) is a major factor in vasculogenesis and angiogenesis and is a known endothelial cell mitogen that might be important for endothelial cell maturation. We therefore investigated the effect of human umbilical vein endothelial cells (HUVEC cell line) on human bone marrow stromal cell (BMSC) differentiation towards the expression of the osteogenic phenotype.

METHODS: Two types of co-culture systems were used: indirect contact (providing a 2-way communication system) and EC-conditioned medium (providing a 1-way communication system from HUVEC to BMSC). The cultures were grown with and without the addition of Dexamethasone, a known inducer of osteogenesis *in vitro*. In addition, endothelial cells were stimulated with VEGF (a known EC mitogen and suggested to be an important factor for EC maturation) before being used in the co-culture systems. Using the quantitative real-time-RT-PCR technique, we measured at different culture times mRNA levels of representative genes expressed at various stages during osteoblastic differentiation such as osteopontin, bone sialoprotein II, osteonectin, osteocalcin, collagen I, MMP-13, BMP-2 and cbfa1. Cell proliferation (Hoechst 33258), matrix mineralization (Ca45 isotope incorporation), alkaline phosphatase activity (colorimetric assay) and VEGF levels (ELISA) in the medium were also quantified.

RESULTS: As expected, BMSC cultures stimulated with the steroid dexamethasone differentiate towards the expression of the osteoblastic phenotype, measured by an increased matrix mineralization, an elevated ALP activity and by the expression of specific osteoblastic markers, especially osteopontin, bone sialoprotein II and BMP-2. This differentiation process was delayed independently of the co-culture system used. Furthermore, when HUVEC were stimulated with VEGF before co-culture, the inhibition of osteoblastogenesis was even greater. BMSC proliferation was increased in the presence of HUVEC and even further increased in the presence of VEGF-stimulated HUVEC. Also this effect was independent of the co-culture type.

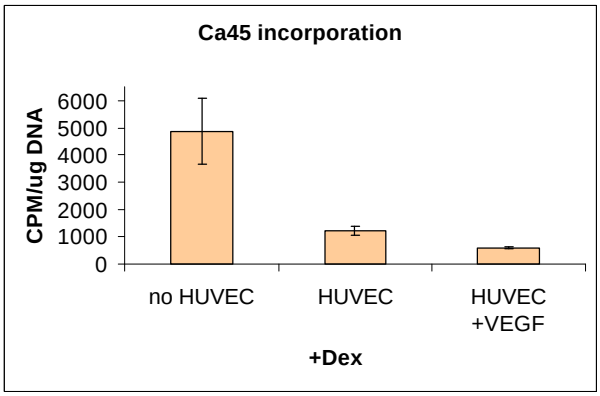

Figure 1: Matrix mineralization by BMSC at day 12

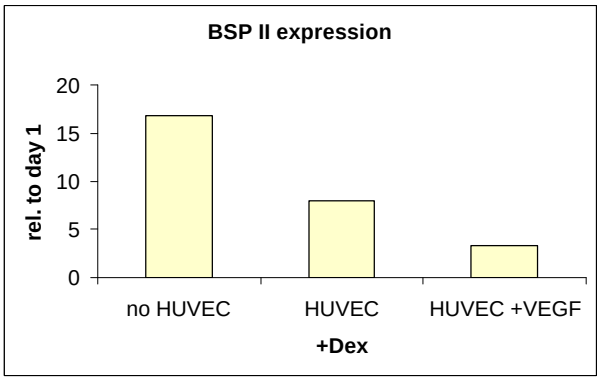

Figure 2: Bone Sialoprotein II expression by BMSC at day 12

DISCUSSION & CONCLUSIONS: These results support the thesis that EC may potentially affect bone precursor cell proliferation and the rate at which BMSC differentiate into osteoblasts. The effect of VEGF suggests that this modulation is dependent on the maturational stage of the endothelial cells.

ACKNOWLEDGEMENTS: We would like to thank Dr. Claude Jaquiery and Dr. Ivan Martin for bone marrow stromal cell supply.

INTRODUCTION OF A THREE-DIMENSIONAL CO-CULTURE MODEL FOR IN VITRO ANGIOGENESIS IN BONE TISSUE ENGINEERING

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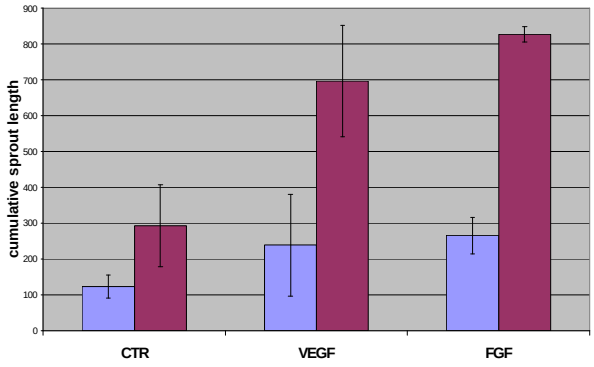
INTRODUCTION: One of the major limitations to the clinical application of tissue engineered bone substitutes remains vascularization of the transplant. The challenge of integrating an ordered capillary tree into tissue engineered constructs *ex vivo* still has to be solved [1]. We have developed a three-dimensional collagen-based co-culture system to assess interactions between human endothelial cells (hECs) and human osteoblasts (hOBs) *in vitro*. In fracture healing as well as in bone tissue engineering angiogenesis represents a crucial step in the formation of new bone. Our model serves as a tool for investigating the effect of heterotypic cell-cell interactions and angiogenic stimulation upon the formation of tube like structures by hECs in an osteoblast environment.

METHODS: Human umbilical vein endothelial cells (HUVECs) were grown as three-dimensional multicellular spheroids and seeded in a collagen matrix to assess sprouting of the spheroids. Sprouts emerging from endothelial cell spheroids form tube like structures resembling early capillaries [2]. Cell contact between hOBs and HUVEC spheroids was established by either adding hOBs into the collagen gel as a single cell suspension or through direct incorporation of hOBs into the EC spheroids thus forming heterogenous co-spheroids. Spatial organization of co-spheroids and sprout configuration was assesed using in gel immunohistochemical wholemount staining techniques and confocal laser microscopy. Cumulative sprout length of spheroids was quantitatively analyzed using digital imaging planimetry.

RESULTS: We were able to demonstrate that HUVECs and hOBs form heterogenous co-spheroids with distinct spatial organization. HUVEC sprouting upon angiogenic stimulation with VEGF and bFGF is suppressed in heterogenous HUVEC/hOB co-spheroids. In our model direct contact with hOBs inhibits formation of tube like structures by hECs, whereas co-culture arrangement of hOBs and hECs without direct ontact does not affect formation of lumenized structures.



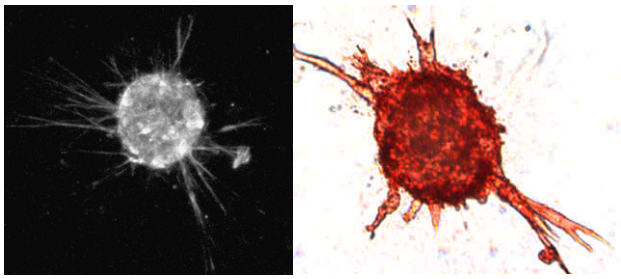


Fig. 1: Confocal laser microscopy image of HUVEC / hOB co-spheroid showing osteoblast filopodia protrusions from the co-spheroid (left) and immunohistochemical wholemount staining for CD31-positive tube like sprouts emerging from HUVEC spheroid in a collagen matrix (right)
Fig. 2: HUVEC spheroid sprouting in direct contact to hOBs (blue) compared to HUVEC spheroid sprouting in absence of hOBs (red)

DISCUSSION & CONCLUSIONS: This study introduces a novel model for investigating *in vitro* angiogenesis in an osteoblast environment. We have demonstrated that contact or proximity between hOBs and hECs modulates capillary formation in a three-dimensional collagen-based spheroid sprouting assay. The model systems introduced in this study will be useful in elucidating the mechanisms involved in regulating angiogenesis in bone formation and will allow further investigations into the character of heterotypic cell-cell interactions for applications in bone tissue engineering.

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OSTEOBLASTS GENERATE AN OSTEOGENIC MICROENVIRONMENT WHEN GROWN ON SURFACES WITH ROUGH MICROTOPOGRAPHIES

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INTRODUCTION: Osteoblast attachment to materials, proliferation, and differentiation are sensitive to the microtopography of the surface (1). On rougher surfaces, osteoblasts assume a more differentiated morphology. Attachment is reduced and those cells that attach exhibit reduced proliferation and increased expression of markers of osteoblastic differentiation. Moreover, they exhibit responses to hormones and growth factors in a manner typical of a differentiated osteoblast. Osteoblast-like cells cultured on osteoclast-resorbed bone surfaces showed similar behavior (2) demonstrating the importance of microtopographical features to bone remodeling in vivo. These studies suggest that microrugosity may also modulate the ability of osteoblasts to synthesize and secrete factors that enhance osteogenesis and at the same time, control osteoclast formation and activity.

METHODS: To test this hypothesis, we examined the response of several cell models at various states in the osteoblast lineage to titanium surfaces with smooth and rough microtopographies. Cells were cultured on Ti disks fabricated by Institut Straumann AG (Waldenburg, Switzerland) to have one of three different surfaces: pretreatment (PT) surfaces were smooth (Ra<0.02 μm); grit blasted/acid etched surfaces (SLA) had a mixed morphology with larger pits 30 to 100 μm in diameter overlaid with small pits 1 to 3 μm in diameter, resulting in an average Ra of 4 μm; and titanium plasma sprayed (TPS) surfaces had surfaces covered with irregular projections, resulting in an average Ra of 5 μm. Cell models included osteoblast-like human osteosarcoma cells (MG63), fetal rat calvarial cells (FRC), mouse osteocyte-like cells (MLO-Y4), and normal human osteoblasts (NHOst) isolated from a female patient. In all experiments cells were cultured on the three surfaces as well as on tissue culture plastic. There were 6 separate cultures per variable and all experiments were repeated a minimum of two times to ensure validity of the observations.

RESULTS: All cells examined exhibited increased levels of local factors associated with osteoblast differentiation in their conditioned media when cultured on SLA and TPS, including TGF-β1, PGE_2, and NO. In addition, responsiveness to steroid hormones associated with osteogenesis and matrix calcification, $1\alpha,25(OH)_2D_3$ and 17β-estradiol, was also increased, resulting in synergistic enhancement in local factor production. Inhibition of prostaglandin production reduced the effects of surface microtopography, indicating that the mechanism involved prostanoid synthesis. Both constitutive cyclooxygenase-1 and inducible Cox-2 played roles based on specific inhibition of each enzyme. Integrin signaling was also important since anti-beta-1 integrin antibody enhanced osteoblastic differentiation. This observation supported other studies in our lab showing that reduced access to RGD-binding sites increased osteocalcin production (collaboration with M. Textor and S. Tosatti, ETHZ). Cells on SLA and TPS also exhibited elevated levels of osteoprotegerin (OPG) in their conditioned media, which was correlated with increased OPG mRNA. In contrast, RANK ligand mRNAs were present in very low levels and no soluble RANKL was detected in the media. OPG was upregulated by $1\alpha,25(OH)_2D_3$ and MG63 cells produced higher levels of $1\alpha,25(OH)_2D_3$ on these surfaces.

DISCUSSION AND CONCLUSIONS: These studies show that the microtopography of the surface modulates growth and differentiation of osteoblast-like cells at various states of maturation in the osteoblast lineage. Part of the effect is due to integrin signaling, suggesting that changes in cell morphology imposed by surface architecture may be a contributing factor. Altered surface energy may also influence the conformation of adsorbed proteins, which may affect integrin binding to the substrate. Once the cells attach, they produce

autocrine and paracrine factors, including steroid hormones that can act back on the osteoblasts further affecting phenotypic expression. Both $1\alpha,25(OH)_2D_3$ and 17β-estradiol enhance osteoblastic differentiation and $1\alpha,25(OH)_2D_3$ is needed for mineral deposition in the extracellular matrix. TGF-β1 and PGE_2 increase differentiation by increasing alkaline phosphatase specific activity. Although TGF-β1 retards terminal differentiation, PGE_2 promotes this process. Indeed, prostaglandin is needed for the increase in TGF-β1 (3). NO increases osteoblast growth and is anti-apoptotic. Together, these factors provide an environment that is osteogenic.

Bone remodeling depends on osteoblast initiated activation of osteoclasts via presentation of RANKL to its receptor, RANK, on the osteoclasts. By increasing OPG levels, the osteoblasts provide a decoy receptor for RANKL, preventing its binding to RANK. In addition, by not releasing soluble RANKL into the media, OPG is not occupied and can bind to RANKL on the osteoblast membrane. Thus, growth on microrough Ti resulted in cells that exhibited a more osteoblastic phenotype and produced local factors and hormones that maintained and enhanced that phenotype, while at the same time producing factors that decreased osteoclast formation and activity.

ACKNOWLEDGEMENTS: This research was funded by a grant from the ITI Foundation, the Georgia Research Alliance, and the Georgia Tech/Emory Center for the Engineering of Living Tissues. Culture disks were a generous gift of Institut Straumann AG. Dr. Lossdorfer is recipient of a postdoctoral fellowship from the German government. The authors thank their collaborators Drs. Adele Boskey (Hospital for Special Surgery, NYC, NY), Marcus Textor (ETHZ, Zurich, Switzerland), Lynda Bonewald (University of Missouri at Kansas City, Kansas City, MO), and J. Edward Puzas (University of Rochester, Rochester, NY).

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INFLAMMATORY REACTIONS TO IMPLANT MATERIALS & BONE RESORPTION: OBSERVATIONS AND MECHANISMS

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INTRODUCTION: Bone resorption is a complex process with a multitude of regulatory steps at cellular and systemic levels. Under physiological conditions bone is continuously remodeled to allow for adjustments to changing environmental requirements and for the repair of microfractures. During remodeling, resorption and formation are in equilibrium, ensuring the maintenance of bone mass. If local or systemic resorption increases over formation, bone mass decreases, as is the case in postmenopausal osteoporosis, in conditions of inflammatory arthritis, or in the formation of osteolytic lesions in periimplant bone.

The recruitment and activation of the bone resorbing cells, the osteoclasts (OC), are modulated by a multifactorial haematopoietic microenvironment, which includes the extra-cellular matrix (ECM), cells of various origins and functional types (osteoblasts, marrow and endothelial cells), and local and systemic growth factors and cytokines (1). Two growth factors were found to be necessary and sufficient for the development of functional OC, colony-stimulating factor-1 (CSF-1) and receptor activator of NF-κB ligand (RANKL). CSF-1, binding to the receptor tyrosine kinase *c-fms*, belongs to the family of lineage-specific haematopoietic growth factors, inducing haematopoietic precursor cells to develop to the monocyte/macrophage (MO/MΦ) lineage (2). RANKL is a member of the membrane-bound TNFα family of growth factors. It binds to the specific receptor RANK and its activity is, at least in part, controlled by an inactivating soluble decoy receptor, osteoprotegerin (OPG) (3).

Besides CSF-1 and RANKL, there are numerous growth factors, which exert profound effects on the recruitment, activation and survival of OC. One of these factors is TNFα, a major product of MO and MΦ. TNFα was suggested to be the causative agent in osteoporosis, to be contributing to the bone loss in inflammatory forms of arthritis, and to be critically involved in aseptic loosening of orthopaedic implants. Furthermore, the spectrum of TNFα activity in bone resorption is gaining versatility through its capacity to synergise with other growth factors and cytokines, such as RANKL.

Within the present study, we describe 1) that metal ions induce the release of TNFα in MO and MΦ, and 2) some aspects of the effects of the cytokine on the formation of OC, which may contribute to the loosening of orthopaedic implants.

METHODS:

Release of TNFa by monocytes. Mononuclear cells from peripheral blood were purified by Ficoll/Hypaque gradient centrifugation. The cells were seeded into culture dishes and incubated with either metal particles or salts. After 40 h, the culture supernatants were collected and the levels of TNFα were determined by ELISA.

TNFa in osteoclastogenesis. The recruitment of OC was studied *in vitro* in two culture systems. (1) Murine bone marrow cells were grown in the presence of RANKL and CSF-1. In the presence of the two growth factors, haematopoietic precursor cells develop into multinucleated, $TRAP^+$ OC-like cells (OCL). (2) Bone marrow cells were grown in a co-culture together with primary osteoblasts. Upon addition of $1,25(OH)_2D_3$ to the culture, OCL form within 6 days (4). Using these culture systems, it is possible to investigate the effects of growth factors and cytokines on the formation of OC. It is possible to differentiate between direct effects on haematopoietic cells or indirect effects through accessory cells. Furthermore, these culture systems serve as tools to investigate the expression of growth factors, receptors, and other components of the haematopoietic microenvironment during OC recruitment *in vitro*.

TNFa in ovariectomy. Given the importance of TNFα in the regulation of bone resorption, it was investigated, whether the cytokine occupies an essential role in bone loss induced under estrogen deficiency. For this purpose, mice deficient in TNFα or in the p55 receptor for TNFα were ovariectomized (OVX). Bone mass was followed in weekly intervals over 3 weeks by pQCT.

RESULTS:

Release of TNFa by monocytes in response to the exposure to metal particles and salts. Cells of the MO/MΦ lineage responded to the exposure to metal particles (cpTi of various sizes, AlOx, stainless steel) by releasing TNFα. The release, however, was primarily dependent on the size of the particles, rather than on the material that was used. When MO were exposed to metal ions (Co^{2+}, Ni^{2+}, Ti^{3+}, Cr^{3+}), they reacted by releasing osteoclastogenic cytokines. The patterns of released cytokines varied among MO from different donors and were dependent on the metal ions the cells were treated with. Co^{2+} induced a significant

release of TNFα in most cell preparations, while the expression of other cytokines (IL-6, IL-1α and –1β) was affected to a lesser extent. Ni^{2+} induced a broader response than did Co^{2+}, the response, however, was less uniform among the cell preparations. Ti^{3+} and Cr^{3+} were considerably less efficient in inducing the release of osteoclastogenic cytokines.

The effects of TNFa on OC formation in vitro. When TNFα was added to cultures of bone marrow cells grown in the presence of CSF-1 and RANKL, the growth factor had no effect on the formation of OCL. Addition of the same amount of TNFα to co-cultures of marrow cells and osteoblasts caused a significant decrease in the formation of OC. If the osteoblasts were derived from $p55^{-/-}$ animals, the inhibitory effect of TNFα was not only blocked, but the growth factor caused an increase in OC formation.

The role of TNFa in bone loss induced by estrogen deficiency. Deficiency in estrogen caused bone loss in mice deficient in TNFα or in the p55 TNF receptor. The extent of bone loss in the ko strains was not different than the OVX-induced bone loss in wt animals.

DISCUSSION: TNFα is a major inflammatory cytokine and has been shown to be a potent stimulator of bone resorption *in vivo* (5). The growth factor has been implicated in aseptic loosening of prosthetic implants, in bone loss after estrogen deficiency and in the increase in bone resorption accompanying inflammatory diseases.

In the present study it was demonstrated that MO and MΦ are induced to release elevated levels of TNFα upon exposure to metal particles or ions. Elevated levels of the cytokine, either in the periprosthetic tissue or systemically, may contribute to the formation of osteolytic lesions and subsequently lead to loosening of the implant.

While TNFα is paramount in the regulation of bone resorption, it acts in concert with other growth factors and cytokines, such as CSF-1, IL-1, and IL-6. In contrast to previous studies, and underlining this concept, we describe here that TNFα is not necessary of OVX-induced bone loss. Other factors can compensate for the loss of TNFα, or, the effect of the cytokine on bone cell biology is more complex than anticipated.

Most interesting was the finding that TNFα *in vitro* exerts dual effects on the formation of OCL. While TNFα stimulated the development of OCL through a direct action on marrow cells, it inhibits the formation of OCL indirectly via osteoblasts.

In inflammatory diseases such as psoriatic arthritis, serum TNFα levels are increased and concomittantly the pool of circulating OC precursors is expanded (6). Circulating TNFα will act directly on the OC precursors, with no accessory cells around mediating a potential inhibitory effect. In the bone microenvironment, however, the effects of TNFα may be quite to the contrary.

Many aspects of the effects of TNFα on bone cell development and function have been elucidated in the past. However, in particular the inhibitory effects of TNFα on the formation of OC are little investigated and will be the topic of further studies.

ACKNOWLEDGEMENTS: The authors wish to thank Prof. C. Müller (Department for Pathology, University of Bern) for providing the $TNF\alpha^{-/-}$ and $p55^{-/-}$ mice, and Drs M. Wimmer and C. Sprecher (AORI, Davos) for providing the sub-µm cpTi particles for our studies. The work was supported by grants from the AO-Foundation, the Synos-Foundation and the SNF to WH.

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A PROPOSED ROLE FOR EXERCISE IN ERODIBLE IMPLANT INCORPORATION

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INTRODUCTION: Tissue engineered scaffolds require vascularization to 1) enhance nutrient exchange and 2) provide cells needed to build new tissue. Cell-seeded scaffolds—bioreactors-- require rapid penetration of vessels or enhanced fluid percolation to keep their contents alive until normal nutrient exchange can be established. Bone fluid flow (BFF) depends on a pumping system which drives percolation through its own matrix. Recent interest in the pumping mechanism has resulted in BFF models which link the pumps to bending of bone by muscle contraction and compression-tension cycles from weight-bearing during locomotion. We have proposed that capillary filtration, the source of the percolating fluid, is sufficiently enhanced by soliton pressure waves in blood driven by the muscle pump during exercise to provide a significant hydraulic pressure component to bone fluid percolating through bone and any bone-implanted scaffold. We present here a proposal and some preliminary results from a pilot project suggesting enhancement of capillary filtration by the muscle pump.

METHODS: Optical bone chambers were implanted in adult NZW female rabbits. Chamber construction and implantation were as usual[1]. At the third week post-op chamber ends were exposed and weekly intravital microscopy commenced. Transcutaneous electrical stimulation was administered with a ToneATronic® TENS at 85V, 80mA and 2Hz. The stimulator was applied externally over the gastrocnemius muscle. A fluorescence digital image was obtained before 30 minutes application of TENS after injection of FITC-D70. After stimulation RITC-D70 was injected. Blood samples were obtained from an aural vein in the ear opposite that being injected with the fluorescent dye after each injection. Blood concentration of dye was determined with a SPEX Fluoromax-3 spectrofluorometer for both serum (absolute concentration) and whole blood (to detect differences which would make fluorescence in vessels an inaccurate indicator of concentration due to RBC color contamination). For analysis 4 vessels were chosen and the average dye concentration profiles before and after 30 minutes of stimulation were obtained.

RESULTS: Results are shown in Figure 1. Extravasated dye levels in TENS rabbits were markedly higher than those in controls. Analysis of profiles using an erfc-based diffusion-convection discrimination model[2] showed that extravasation was convective.

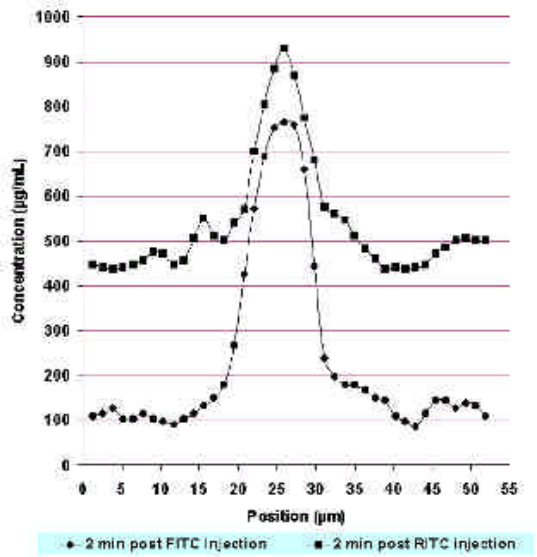

Fig. 1: Extravasation of fluorescent dyes pre- and post- 30 min. TENS. Peaks represent vessels.

DISCUSSION & CONCLUSIONS: These data are consistent with significant contribution to convective percolation of bone fluid through implanted scaffolds by muscle pump-driven extravasating fluid. They do not, however, answer two critical questions: 1) Is the magnitude of this convection a major component of flow through the scaffold? 2) What are the relative contributions of skeletal muscle-generated intravascular pressure solitons and incompressible fluid transmission of bone bending pressure to the convective flow observed? Additional studies with released gastrocnemius muscles are in progress.

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NUMERICAL SIMULATION OF PERI-IMPLANT TISSUE DIFFERENTIATION IN A BONE CHAMBER

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INTRODUCTION: Although it is well known that mechanical loading interacts with tissue differentiation processes, the exact mechanisms are not yet fully understood. A combined approach, consisting of animal experiments and numerical modeling can contribute to a better understanding of the mechano-regulation of tissue differentiation. For the interpretation of the experimental results, one must pay attention to the fact that the bone adaptive response seems to be site-specific and species-specific. To overcome these problems, a repeated sampling bone chamber was developed (figure 1). The goal of the present study is twofold: (1) the numerical modelling of tissue differentiation inside the bone chamber.
(2) the assessment of the feasibility of the mathematical model for mechanically induced tissue differentiation developed by Prendergast *et al.* [1] and Huiskes *et al.* [2] in situations besides fracture healing.

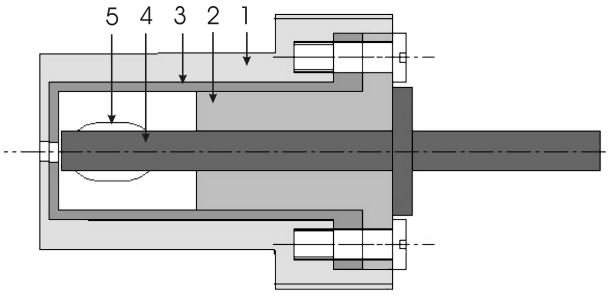

Fig. 1: Assembly drawing of the bone chamber. After insertion of the outer bone chamber (1, outer diameter = 10 mm), there is a healing period of six weeks to assure rigid fixation. After six weeks the inner bone chamber (3) together with the teflon bearing (2) and the test implant (4) are inserted and the experiment can start. Tissue can grow in the chamber via three perforations (5).

METHODS: A 2D axisymmetric and a simplified 3D finite element model were created of the tissue inside the bone chamber (figure 2). At the beginning of the differentiation simulation, the entire chamber was assumed to be filled with granulation tissue. The tissues were modelled as biphasic tissues, their properties are summarised in table 1. Two types of loading regimes were applied in a displacement controlled manner: a sine (frequency 1 Hz) with an amplitude of 50 µm and 160 µm respectively. These correspond to the loading conditions of the animal experiments.

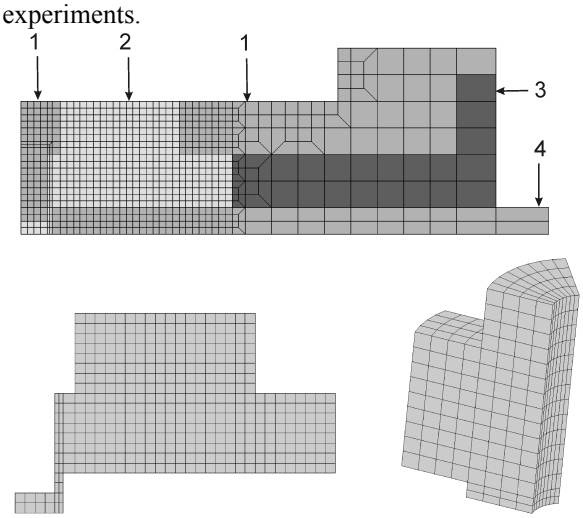

Fig. 2: Axisymmetric finite element model of the entire bone chamber (upper). The outer and inner chamber (1), the tissue inside the chamber (2), the teflon bearing (3) and the test implant (4). Both a 2D and 3D model of the tissue inside the chamber are shown (under).

Table 1. Material properties of the biphasic tissues used in the simulation.

	fibrous tissue	cartilage	bone
E-modulus [MPa]	1	10	1000
Poisson ratio	0.17	0.17	0.3
Permeability $[m^4(Ns)^{-1}]$	10^{-14}	$5 \cdot 10^{-15}$	10^{-13}

For the simulation of the tissue differentiation, the model of Prendergast *et al.* [1] and Huiskes *et al.* [2] was implemented. From the FE model, the maximal distortional strain and relative fluid velocity were calculated. Depending on these stimuli a tissue phenotype for each element was predicted. To avoid numerical instabilities during the simulations, a smoothing procedure proposed by Lacroix *et al.* [3] was implemented.
At the beginning of the differentiation process, mesenchymal cells enter the bone chamber through the perforations. Their differentiation into fibroblasts, chondroblasts or osteoblasts was assumed to be entirely dependent on the local mechanical stimulus. The migration of mesenchymal cells was modelled by means of the diffusion equation (n representing the cell density, t the time and D the diffusion coefficient):

$$D\tilde{N}^2 n = dn/dt \qquad (1)$$

The diffusion coefficient was chosen in such a way that there is a complete coverage of the chamber by mesenchymal cells in six weeks. The results of the diffusion analysis were read before every loading cycle and a rule of mixtures [3] was applied to calculate the average material properties, based on the local mesenchymal cell concentration. Figure 3 shows the entire modelling scheme.

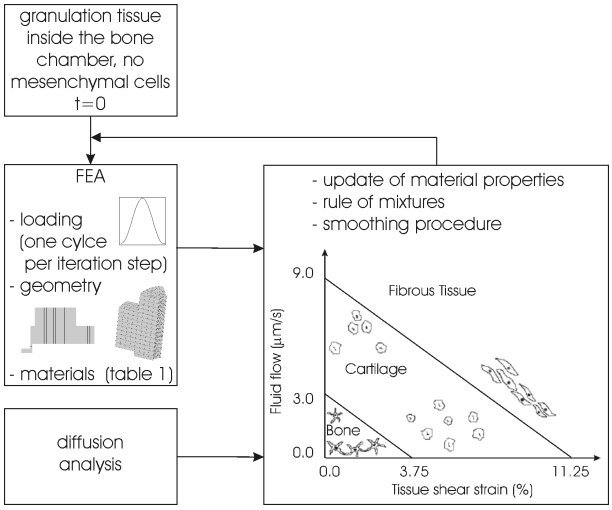

Fig. 3: Simulation scheme. Overview of the entire modelling procedure.

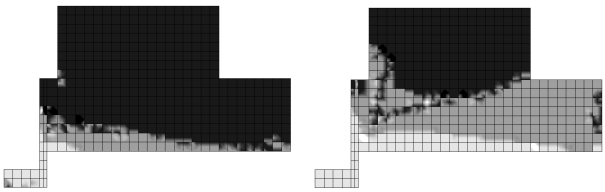

Fig. 4: Tissue types inside the bone chamber after six weeks for the loading regimes with a displace-ment of 50 mm (left) and a displacement of 160 mm (right). Black represents bone, gray represents cartilage and white is fibrous tissue.

RESULTS: Figure 4 shows the situation in the bone chamber after six weeks of loading for both of the loading conditions. Under a displacement of 50 mm, the granulation tissue was able to differentiate into bone in a large volume of the chamber. At the interface between the tissue and the implant, the conditions were such that the formation of cartilage was favored. At the bottom of the chamber, high strains and fluid velocity inhibited any tissue differentiation beyond fibrous tissue. This observation corresponds with the results of the animal experiments. Moreover, the predicted ingrowth pattern resembles the ingrowth pattern observed during the animal experiments (figure 5). The second loading condition (160 µm) has not yet been experimentally investigated. The simulations predict that the tissue will only differentiate into bone in the perforations of the chamber. The rest of the chamber is filled with fibrous tissue and cartilage.

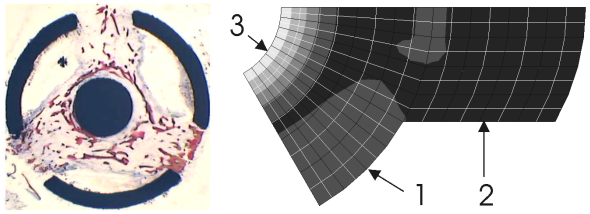

Fig. 5: Tissue ingrowth in the bone chamber. Comparison of a histological section and the result of the numerical simulation. There is a qualitative resemblance in the ingrowth pattern. (1 - immature bone, 2 - mature bone, 3 - fibrous tissue & cartilage)

DISCUSSION & CONCLUSIONS: When comparing the results of the 2D and 3D simulations with the experimental results, a qualitative resemblance in tissue phenotype is seen, in spite of the fact that some simplifications were made. These simplifications comprehend the geometry of the models, the boundary conditions, the differentiation model that was implemented and the presumed behavior of the mesenchymal cells. Due to the limited number of animals in the pilot experiment, only a qualitative comparison was possible. Expansion of the mathematical model with more biological factors, such as cell proliferation, cell apoptosis and the influence of growth factors might allow the simulation of the observed inter-animal differences.

In conclusion, this study is a first step in the simulation of the process of peri-implant tissue differentiation inside a bone chamber. The implemented model succeeds in predicting this process in a qualitative way.

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CO-CULTURE OF OSTEOBLASTS AND OSTEOCLASTS ON RESORBABLE MINERALISED COLLAGEN SCAFFOLDS: ESTABLISHMENT OF AN *IN VITRO* MODEL OF BONE REMODELING

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INTRODUCTION: Bone tissue is characterized by permanent reconstruction (remodeling), triggered by osteoblasts and osteoclasts. To study this process we developed an *in vitro* model that includes osteoblasts - which are responsible for bone regeneration - and osteoclasts which cause bone degradation. Since the biopolymer collagen I and the mineral hydroxyapatite (HAP) are the main components of bone matrix *in vivo*, both cell types are co-cultured on scaffolds of mineralized collagen. By that the main characteristics of bone (synthesis, breakdown, mineralized matrix) are brought together to form a representative model. Our first studies using this model treat the influences of osteocalcin presence and mechanical stimulation on the remodeling process.

METHODS: Mineralized collagen I scaffolds have been prepared after a biomimetic process[1]. The bone cells have been seeded onto two-dimensional (2D) membranes ("tapes") or sucked into three-dimensional (3D) porous sponges, both made from mineralized collagen I. The pores of the 3D scaffolds showed diameters up to 200 µm which is sufficient for the ingrowth of cells. Murine osteoblastic cells (ST2, obtained from DSMC) were seeded 24 hours before human monocytes (purified from peripheral blood buffy coats), which differentiated to osteoclasts during culture. The cells were cocultivated in a perfusion cell culture system for up to 6 weeks. The cell culture medium contained dexamethasone, 1,25-dihydroxy vitamin D3, M-CSF (macrophage colony stimulating factor), RANK-L (receptor activator of NF-$_K$B ligand) and bovine and human serum[2].

Osteocalcin was added to 2D tapes by incubating the scaffolds in medium containing 10 µg/mL osteocalcin, using the strong interaction of this protein with hydroxyapatite[3].

Mechanical stimulation was carried out indirectly by ultrasound (intensity of 2 W/cm^2; frequency of 1 MHz; exposure time of 20 min per day) coupling of the culture medium.

Morphology, growth and differentiation state of osteoblasts and monocytes/osteoclasts were examined with different microscopical methods like SEM and LSM and quantitative RT-PCR.

RESULTS: Co-cultures of osteoblasts and osteoclasts showed typical differentiation over culture periods of 6 weeks. Both 2D and 3D scaffolds have been seeded with the cells. A uniform colonisation of the porous sponges could be obtained by a pressure gradient which permits cell seeding at 4-5 mm depth in the 3D sponges. SEM as well as LSM using fluorescent labelling revealed the morphology and the differentiation state of osteoblasts and monocytes and moreover also the genesis of osteoclasts. Furthermore, typical osteoblast marker like alkaline phosphatase (ALP) and osteoclast marker like tartrate-resistant acid phosphatase (TRAP) could be measured (Fig. 2). Newly synthesised collagen could also be observed using SEM and indirect immunofluorescence.

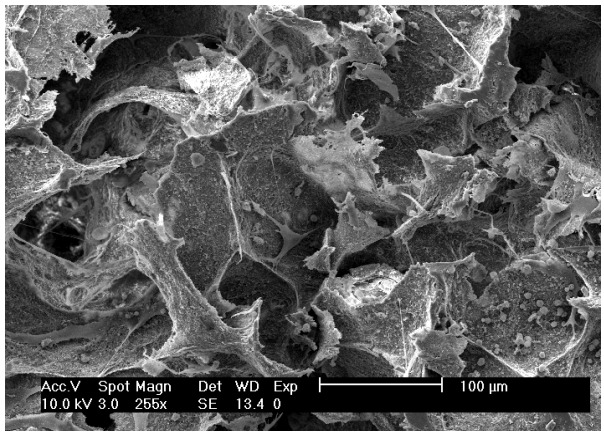

Fig. 1: co-culture of murine osteoblasts (ST2) and human monocytes on 3D-sponge, made from mineralized collagen I, after 5 d in dynamic culture, SEM

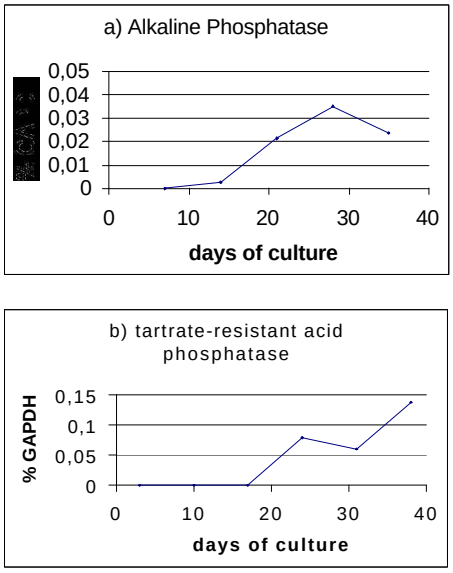

Fig. 2: Expression of mRNA of a) ALP and b) TRAP in co-cultures of osteoblasts and osteoclasts over 6 weeks.

Additionally, a system for mechanical stimulation of the *in vitro* model, based on ultrasound, was tested. Therefore, it is now possible to stimulate the cells during perfusion culture.

The functionalization of the scaffolds with osteocalcin resulted in dramatic changes of gene expression.

DISCUSSION & CONCLUSION:

Using a coculture of osteoblasts and osteoclasts on a bonelike synthetic extracellular matrix, it is possible to establish an *in vitro* model for the remodeling of bone. An additionally mechanical stimulation serves to approach the biological example.

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BONE BIOMECHANICS

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INTRODUCTION: The multi-billion orthopaedic device industry is churning out every year millions of devices, which are fitted to fractured bones to mend them or to bone ends to replace disfunctional joints. Forty years ago Charnley brought about one of the most visible revolutions in the medical profession and the industry catering to it by devising "bone cement" – as we got used to call room-temperature curing resins of polymethyl methacrylate, in its bulk form better known by its trade name Plexiglas (Lucite in the US). Thanks to Charnley, total joint replacement became a household item, admittedly much appreciated by millions of patients grateful to wear (!) one or more of them following, usually, years of suffering from joint pain.

TROUBLES: Not surprisingly, not everything is quite right with cementing a piece of metal in a reamed-out bone cavity by a quickly setting mass of plastic – the so-called revision burden (percentage of surgical procedures done to replace a failed component) is an impressive 20%. Industry did not help -- in spite of decades of R&D on cementless devices -- their overall performance is still inferior to those cemented. Sadly, it seems that all of the efforts to decrease the revision burden have in fact led to its almost dramatic increase. So-called high-demand cementless prostheses are also high-cost items – greatly favored and promoted by the industry. Yet, on average, they appear to loose their anchorage to bone at rates higher than Charnely's.

MOTIVATION: Does industry have anything to learn from biomechanics of bone? Admitting or not, it sure seems in need. This presentation is intended as an encouragement for those who would like to try to research the problems -- no lack here of either the problems or the will to solve them -- and then, hopefully, teach the results to industry. Medical professionals, surgeons in most instances, need just as much, if not more help (to find their way through the maze of offerings). I will present a small collection of hypotheses, some of which have been supported by limited analysis or experimentation, developed by my collaborators and myself at the AO/ASIF Research Institute, Davos, in early nineties. None of it has been published. In a modest way, some of these concepts have been put to use in a total hip prosthesis for dogs, now in some 1'800 dogs, some with 10 years of follow-up, mostly encouraging. But, by and large, these proposals await thorough studies to confirm or to disprove them.

BONE: Even in its most compact, cortical form, bone is a strongly anisotropic composite -- strength of the cortical bone is typically an order of magnitude higher in the longitudinal than in the transverse axes -- stiffness by a factor of three. This difference arises from the osteonal level of organization, itself the result of a continuous process of bone remodeling through coordinated actions of osteoclasts and osteoblasts, the former in the driver's seat. Should the clasts loose their way, bone would quickly loose its load-supporting function. Where is the compass?

BIOMECHANICS: Bone is poroelastic, i.e. its deformation entails fluid transport in all, but most special load/deformation states. Fluid saturates, percolates and convects. All of the bone is affected, its cells not the least. Biomechanics of bone has paid some attention to the impact of fluid movement on mechanical properties of bone, but the effects here, due to stiffness strongly dominating viscous drag (in contrast to, e.g. cartilage), there is not much to write home about. Streaming potentials fared much better, interest in them driven by the potential utility of electrical / magnetic stimulation. And then, there is all of the molecular biology side, almost to exclusion dealing with what molecules are produced and where and, at best, in response to what. But how do they get to the site of action? Through the fluid, no doubt. Perhaps there are bridges to be built here between biology and mechanics – biomechanics is still in need of defending its name (my spelling checker does not like it either). And not only the compass of the clasts is at stake.

COMPASS LOST: Osteoclasts tunnel through the bone at the apex of so-called cutting cones – followed by blasts producing new bone to fill the tunnel. To remove the mineral and digest the bone, the clasts pump H^+ ions through their ruffled border, sealed at the perimeter, causing a strongly acidic environment in which the bone mineral is dissolved. We have proposed that the tunneling process maintains its orientation by the fluid movement in and out of the resorption cavity, driven by the volumetric strain of the surrounding bone. As it turns out, with the boundary conditions approximately representing Haversian bone, the fluid movement at the equator of the cavity is at its strongest and is nearly zero at the apex (pole). Thus, the clasts can do it at the apex because there they *can* maintain their seal and low pH – ions they produce are trapped near the ruffled border, escaping by diffusion only. As it happens, poles of the resorption cavities are (usually, but subject to the boundary conditions) on the main tensile trajectories! Now, what about interfaces to, universally impermeable implants? There can be no flow across these interfaces, hence no dilution of H^+ ions. At and near this enforced no-flow boundary, clasts are left to digest as they please -- the blasts may have difficult time to orient themselves and repair the damage. How about a draining interface? How about neutralizing H^+ ions? Is our ability to provide for an

indefinite, stable to no fault anchor for bone implants fundamentally limited by their (in most cases) impermeable nature?

SMALL SCALE ORIENTATION: A couple of structural levels down from the osteon, collagen fibers and whatever organic part of the matrix is associated with them, are the scaffold of the composite, its reinforcement and its dominant mechanical determinant – deproteinased bone is of no use as a load-carrying material. Collagen in bone, as in other connective tissues, is oriented. By what mechanism? We have studied cartilage and have discovered a plausible mechanism – collagen fibrils can get oriented by oscillating fluid flows, dragging the short fibrils (prior to their incorporation and cross-linking into fibers) against the gel like network of proteoglycans. If at play as well in forming bone (osteoid), there will be a definite problem getting this mechanism to work unperturbed near impermeable interfaces. Again, opening up our implants for fluid flow, at least partially, may be useful.

CONVECTION: Bone is ready and able to fill in a defect such as that created by our preparations of bone to insert a prosthesis. Or to bridge a gap at a fracture site. It seems very plausible that molecular signals driving the process originate at extant bone. How do they move out? Diffusion can of course provide for some of it, but there is a race between bone and other tissue types, which can do the same – grow into defect. If the task is to anchor a prosthesis, bone is at a premium. We have observed in animal studies reluctance of bone to approach our implants – new bone growth seems to readily start out from the extant cancellous bed, but then at a millimeter or so from the implant, to slow down to a crawl, failing to bridge the gap. If convection is at work, it sure cannot work near the impermeable, closed surface of the implant. We have thus tested a perforated nail, as a model, albeit on a millimeter scale, of a hydraulically open "interface". Since bone now readily grew through these perforations, the term interface is rather a misnomer – bone simply engulfed the implant. There are now some 1800 acetabular total hip components implanted in dogs, demonstrating the potential of such hydraulically open anchors.

STABILITY: Some of these have failed to integrate – most likely due to lack of mechanical stability – there is no excuse here – bone will not touch anything that moves. Once a fibrous layer sets in – there is no way to get a satisfying function. Years ago, after numerous failures to provide for a reliable, solid bony integration of cementless total knee prosthesis, a concept of "floating" anchorage was promoted as acceptable – as long as it was not progressive – perhaps it was for undemanding humans, but dogs would not have any of it. Fibrous tissue has no solid matrix to take the load – upon loading its intrastitial fluid is pressurized, much as in cartilage, blocking off any blood supply to the underlying bone. Originally of low density, cancellous bone gradually turns into something very much resembling subchondral bone, sclerotic if not completely dead. Just where the pain originates, is unclear, but it certainly does with any near-normal activity.

GAP PUMP: We have done a large number of fracture healing experiments, addressing design of implants and the issues of treatment protocols, but also the basic mechanisms of bone healing. One of the studies involved gap healing in the context of free space created on purpose around the fracture. The fastest strength recovery was observed in the group with a relatively large inter-fragmentary gap (about a millimeter) and a large space produced on purpose between the bone and the surrounding muscles – the worst results (close to a non-union) were with the same gap size but no space. Intuitive? Perhaps. To help interpret this outcome, we have shown that compressive loading of the early tissue in the gap leads to a net outflow of fluid from the adjacent bone fragments – this would be forced convection bringing out all the good signals; and that mineralization permissive strain distribution closely resembles the conversion of bone precursors to mineralized bone. A statistical outlyer was also detected – a fracture fully healed by three weeks … how about a cluster centered on that outlyer?

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BIOLOGICAL INTERNAL FIXATION INTERFACE BETWEEN BIOLOGY AND BIOMECHANICS

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INTRODUCTION: Biology and biomechanics of treatment and healing of fractures interact and are closely linked. The model of biological internal fixation serves to highlight some of these links.

The fracture disrupts the stiffness of the bone and results in painful loss of limb function. Soft tissue complications such as reflex dystrophy are attributed to spontaneous or iatrogenic immobilization of articulations with consecutive dysfunction of blood supply, patchy bone loss and pain. Internal fixation (surgical stabilization using implants) restores continuous stiffness, abolishes pain and allows early mobilization. Mobilization of the articulations prevents soft tissue complications that were the rule after extensive external splinting of articulations by plaster cast. Still, internal fixation, especially when carried out without care, produces damage to the vascular support of bone and soft tissues.

Internal fixation has recently undergone a basic evolution, which sets a new balance between requirements of stabilization and of damage to blood supply. On the one hand damage to the blood supply is minimized, on the other hand induction as well as tolerance of a certain degree of instability as a result of a more flexible type of fixation is an objective. In the following some aspects of biological internal fixation are discussed based on today's understanding of biology, biomechanics and mechanics. The design of implants and even more so their surgical application are important players with a potential to support striving for a better and safer fracture treatment.

EVOLUTION OF INTERNAL FIXATION: The means of early internal fixation were selected to provide absolute immobilization of the fracture with the goal of immediate restoration of mobility of the articulations in mind[1]. "To allow the fracture to heal in a mechanically neutral environment" precise reduction and application of compression across the fracture plane were the rule. A new type of fracture healing consisting of direct internal remodeling without scar (callus) tissue formation has been observed. It was called "primary bone healing[2]. This type of healing was seen after absolutely stable fixation. It consists, in respect to timing and pattern, of only the last phase of natural healing i.e. the final internal remodeling restoring the original integrity of bone. A prerequisite of primary healing is close and stable adaptation of the fracture planes maintained throughout the period to solid bridging. As long as the applied compression locally exceeds the traction produced e.g. by bending, the fracture plane remains in uninterrupted contact. To achieve such contact the fracture planes were stabilized using the technique of interfragmentary compression[1]. Plates applied as splints across the fracture protected the screw fixation from excessive functional load. Combinations of the two techniques were the rule, pure splinting by plate without compression was considered to be inappropriate.

The observation of callus after internal fixation, which aimed at absolute stability of fixation, was considered to indicate that the goal of stabilization was not achieved. Callus fell into disrepute though it was often callus that ensured the healing in difficult situations.

Beside screw and plate fixation alternative methods of fracture fixation consist in application of either an intramedullary rod[3] or of an external rod connected to the bone by means of transcutaneous pins. Nail or external fixator result in pure splinting that is they reduce but do not abolish mobility at the fracture. The usual pattern of healing after splinting is indirect healing with formation of callus followed by internal remodeling.

Any surgical intervention results in either periosteal (with plate fixation) or endosteal (with nail fixation) damage to the blood supply to bone. The damage to blood supply is in part the result of the surgical preparation (periosteal stripping, medullary reaming etc.) in part the damage is produced by the contact of the implant, a contact, which impedes the inflow and/or outflow of blood to and from bone. Beside the advantages of restoring immediate function of the articulations the shortcoming of absolute stability of fixation is that no relevant callus is induced. Callus appears earlier than internal remodeling and its location outside the bone provides better leverage than the direct connection from bone to bone.

BIOLOGICAL INTERNAL FIXATION: is a kind of surgical stabilization of the fracture that addresses the two shortcomings mentioned[4].The surgical procedure is geared towards minimizing damage to blood supply by avoiding excessive surgical approach abstaining from perfect reduction and absolute stability of fixation. Flexible splinting without interfragmentary compression then provides the natural stimulus for callus production.

The biological internal fixation using the internal fixator principle demonstrates an indirect healing pattern and a low infection rate, which was solidly proven with a very high follow up rate of 97%[5]. The fact that the ends of fracture fragments are not closely adapted allows observing the progress of union. The radiological pattern of healing is similar to the one seen after external fixator treatment. The rate of mechanical failure of screws and plates is exceptionally low in spite of the fact that pure titanium was used as implant material. Pure titanium is known for its low corrosion rate and exceptionally good biological tolerance, but its limited ductility has surprised surgeons that were accustomed to steel, which deforms markedly before breaking occurs. The use of internal fixators with screws that lock within the "plate" alleviates the screw thread from torque and axial tension. The reason for this behavior is that the Morse cone seat locks tightly upon minimal axial traction applied.

The low infection rate observed in clinical studies is backed by animal research testing the local resistance to infection with different designs, materials and procedures of internal plate fixation[6]. The amount of colony forming human pathogenous staphylococci aureus to produce clinical infection was assessed. Between conventional steel DCP (dynamic compression plate) and PC-Fix (point contact fixator) made of pure titanium the latter resisted a 450 times higher load of staph. aureus.

The radiological follow up of adapted and compressed fractures does not allow detecting the process of healing within the fracture but is based on the absence of signs of disturbed healing such as cloudy callus indicating that absolute stability was not achieved[7]. The radiological pattern of healing after non adapted and non compressed splinting appears to be slow at first glance. Still, the successful very early removal of the internal fixator in clinical animal studies seems to prove the quick recovery of load bearing capacity of the fracture after flexible internal fixator splinting[8].

In conclusion biological internal fixation demonstrates a fascinating link between biology and biomechanics after surgical fracture stabilization. It seems to open a promising effect of a newly adapted balance between requirements of stability for painless function, those of minimizing biological damage and taking advantage of callus induction by a small degree of elastic instability.

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BIOPHYSICAL STIMULATION ON BONE REPAIR & REMODELING

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INTRODUCTION: Bone defect and material weakness may lead to fracture even under minor trauma. There are drugs, biologic factors, and biophysical stimuli, which may provide effective fracture repair enhancement. However, fixation stability, extent of surgery, and bone reduction are prerequisites for successful bone healing. Delayed union, mal-union, or non-union will affect patient's quality of life and health care cost. This paper describes the potential augmentation effect of biophysical stimulation on bone fracture repair and an implementation system using a hierarchical approach and knowledge-based execution concept.

PEMF ON OSTEOTOMY HEALING: Canine mid tibia osteotomy was used to study the effect of PEMF on bone union (Fig. 1). Significant increase in new bone formation and mechanical strength was achieved using different dose of stimulation. The contra-lateral intact limb exposed to the PEMF also increased bone strength significantly. The initial biologic effect was cell mediated but the bone strength enhancement was primarily due to increased vascular activity that enhances callus maturation and prevents bone resorption.

CORTICAL DEFECT REPAIR: Cortical defect was used to study the effect of loading on bone repair and remodeling (Fig. 2). Weight bearing was a significant permissive factor on the initial defect repair. Using micro corrosion casting technique, increased vascular formation was found to associate with local tissue strain energy density. At 16 weeks, bone structural strength recovered but not its morphology. This is an ideal creeping substitution model to investigate various bone repair enhancement factors and their dose effect.

KNOWLEDGE-BASED STIMULATION: A computer-aided and knowledge-based stimulation implementation system was conceptualized (Fig. 3). Signal transducer capable of delivering focalized stimulation to assure efficacy and safety will be developed. An ultrasound imaging device is used to monitor bone strength non-invasively in order to adjust stimulation signal and dose to optimize tissue response. An Expert System utilizing basic science and clinical knowledge to customize stimulation protocol with intermediate adjustment will be incorporated. This technology should be regulated to restrict its use and reimbursement only to licensed physicians and bioengineers.

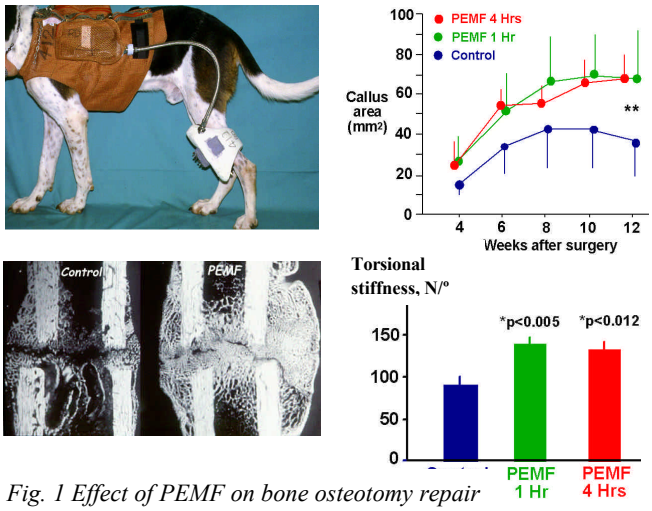

Fig. 1 Effect of PEMF on bone osteotomy repair

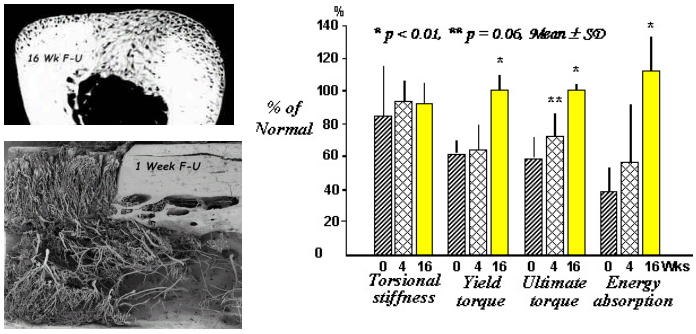

Fig. 2 Cortical defect repair

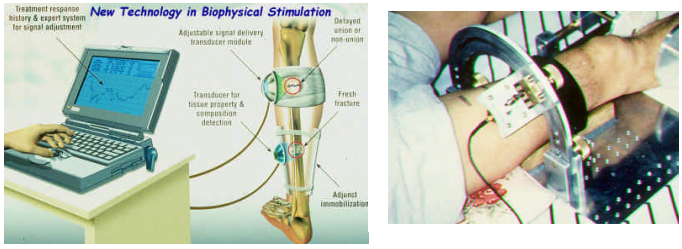

Fig. 3 Computer-aided biophysical stimulation

DISCUSSION & CONCLUSIONS: The basic science of biophysical stimulation is well-established but the technology for its application lags far behind. The lack of treatment control and effective signal delivery was only one of many limiting factors. If a reliable system can be developed with efficacy assurance, biophysical stimulation could become a tool for bone "Tissue Engineering" non-invasively. Bone remodeling, however, remains a biomechanical event that cannot be substituted by biophysical stimulation.

ACKNOWLEDGEMENTS: Many staff and fellows of the Biomechanics Lab contributed to this work. The studies presented here were supported by EBI and AO Foundation.

MECHANICAL STIMULATION & CELL DEATH-SHAPING OUR BONES.

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Bone is the ultimate "smart" structural material. It is self-designing, adapting as it does to the prevailing mechanical needs of the organism by adding new bone in regions that require it and undertaking targeted sculpting of each bone internally and externally to remove redundant material. It is also self-repairing, sensing, removing and replacing damaged or mechanically insufficient volumes of bone. The effector cells for this process are the osteoclasts, which share the early part of their lineage with monocyte/macrophages. The mechanism by which cells are precisely targeted to areas requiring resorption is poorly understood.

The osteogenic effects of load engendered strains have been evident for some time, as has the resorption inducing effect of under-loading our skeletons, but the cell types responsible for orchestrating the targeted function of the effector cells is still in question. It is generally considered that the osteocyte is the most likely candidate for this role due to their distribution throughout the bone matrix, their responsiveness to strain and their existence as part of a syncitial network. However, until recently, it has not been possible to identify osteocyte-specific behaviour that is related spatially to the damage / strain environment and is associated with localised remodelling activity.

Some years ago we noted that osteocytes death by apoptosis is over represented in bone tissue which is for a variety of reasons undergoing rapid remodelling. We proposed that the marked apoptosis of osteocytes observed in women and female rats subjected to acute oestrogen withdrawal provides a targeting mechanism for inducing the well-documented removal of bone by osteoclasts under osteoporotic conditions. Since then a number of reports have confirmed and extended our findings and a relationship between load engendered, targeted osteoclast activity and osteocyte apoptosis has been noted.

These observations raised the exciting possibility that the targeted removal of bone which is under loaded or contains microcracks might also involve the apoptotic death of the osteocyte. I shall discuss work undertaken which would strengthen this possibility. We will consider the possible mechanisms by which controlled cell death might contribute to the signals for bone removal and repair in the light of work involving cells in other tissue systems.

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ELUCIDATION OF MECHANICALLY-REGULATED SIGNALLING PATHWAYS IN BONE AND THEIR APPLICATION TO BONE TISSUE ENGINEERING

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INTRODUCTION: It has long been established that mechanical loading is an important stimulus in maintaining bone mass *in vivo*. The removal of normal mechanical forces applied to the skeleton causes bone loss however, very short periods of defined mechanical stimuli are all that is required to prevent this loss or even to induce bone formation. The mechanisms by which mechanical signals are propagated in bone are not fully understood but the identification of mediators of mechanical signal transduction may allow the mechanical component of the osteogenic signal to be bypassed. This approach is clearly of benefit both to bone pathologies where the skeleton is too weak to resist osteogenic forces and to bone tissue engineering where the mechanical environment of the bone is compromised by culture conditions. Previously we have used osteogenic stimuli to identify genes regulated in osteocytes by mechanical loading *in vivo*[1]. One gene found to be down regulated by mechanical loading was the glutamate/aspartate transporter, GLAST-1. This led to the intriguing possibility that excitatory amino acids such as glutamate, known to be important signalling molecules in the CNS, may mediate mechano-responsive pathways in bone.

In the central nervous system (CNS) glutamate is the major neurotransmitter at excitatory synapses. Excitation of presynaptic neurons causes release of glutamate into the synaptic cleft where it binds to receptors on the postsynaptic neuron to propagate the signal. GLAST-1 is expressed within the plasma membranes of glial cells surrounding these synapses and rapidly binds and transports glutamate into the cells causing termination of the excitatory signal. Whilst other workers have shown that various classes of glutamate receptors are expressed and functional in osteoblasts and osteoclasts, we aimed to determine what the function of GLAST-1 might be in bone and whether it represents a suitable target for modulation of bone cell phenotype.

METHODS & RESULTS: We have cloned the entire open reading frame (ORF) of GLAST-1 from bone and shown that the bone-derived mRNA encodes a protein identical to that expressed in brain[2]. Immunohistochemistry demonstrated that GLAST-1 protein is expressed in osteocytes and osteoblasts and western blotting has revealed a protein of ~70kDa expressed in bone and brain indicating that GLAST-1 mRNA is translated in bone and glycosylated similarly in both tissues[2].
Northern blots have revealed GLAST mRNAs of various sizes indicating that splicing of this gene may occur[1]. We have cloned a novel splice variant of this gene, called GLAST-1a, in which exon 3 is excised[2]. Exon 3 encodes 46 amino acids and GLAST-1a is expressed both in bone and brain *in vivo*.
To determine whether GLAST-1 and GLAST-1a may operate as transporters in bone cells we have investigated their intracellular localisation by transfecting MLOY4 osteocytes and SaOS-2 osteoblast-like cells with GFP-tagged GLAST-1 or GLAST-1a. Scanning confocal microscopy has revealed that GLAST-1 is expressed within the plasma membrane consistent with a glutamate uptake function[3]. Transfection of MLOY4 osteocyte-like cells with GFP-tagged GLAST-1a has revealed a more internalised expression pattern of this variant when compared with GLAST-1[3]. Interestingly both the expression pattern and relative abundance of GLAST-1 and GLAST-1a appeared to be responsive to extracellular glutamate concentration[3].

Recently we have been investigating the function of GLAST-1a by microinjecting *Xenopus* oocytes with cRNA encoding the ORF of GLAST-1a. These experiments have revealed that GLAST-1a can also transport glutamate (Mason, Huggett and Daniels unpublished data).

DISCUSSION & CONCLUSIONS: Other workers have shown that osteoblasts constitutively release glutamate by exocytosis and respond to

glutamate through activation of various ionotropic and metabotropic receptors[4]. Inhibition of glutamate signalling using receptor antagonists can modulate both osteoblast and osteoclast phenotype[4]. In the CNS glutamate transporters such as GLAST-1 are critical regulators of extracellular glutamate concentrations and their activity is controlled by post-translational modifications (oxidation, phosphorylation) as well as by protein trafficking and gene expression. A better understanding of how the activity of these transporters is controlled in bone cells may allow us to mimic the osteogenic effect of mechanical stimuli. This is particularly relevant to bone tissue engineering where modulation of glutamate signalling may ultimately be used to enhance the bone forming capacity of osteoblasts and improve the quality of the matrix they produce.

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ACKNOWLEDGEMENTS: We thank Lynda Bonewald for donating MLO-Y4 cells and Steve Daniels for *Xenopus* oocyte studies. This work was funded by Cardiff University, Welsh Office for Research and Development and The Wishbone Trust..

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BONE TISSUE ENGINEERING - BIOMIMETIC SCAFFOLDS AND ENGINEERED HUMAN OSTEOPROGENITORS

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OVERVIEW:

Given the demographic challenge of an ageing population, the requirement for new bone to replace or restore the function of traumatised, lost or degenerated bone is a major clinical and socio-economic need. Bone formation strategies, although attractive, have yet to yield functional and mechanically competent tissues and clinical imperatives to augment and facilitate osteogenesis have brought mesenchymal and osteoprogenitor cell biology to centre stage. This is due largely to the observation that bone has a tremendous capacity to regenerate from cells with stem cell characteristics. These cells, mesenchymal stem cells can differentiate into fibroblastic, osteogenic, chondrogenic, myoblastic, adipogenic and reticular cells. These primitive progenitors exist postnatally, at low incidence, display considerable plasticity as well as extensive renewal potential. Delivery of these stem cells in appropriate matrices, through the incorporation of signal recognition ligands, growth factors or coupling of appropriate cell adhesion molecules to create biomimetic environments to mediate the molecular and cellular response of the bone cells, offers new approaches to promote osteogenesis and, ultimately, bone repair.

Generation of biomimetic environments is attractive on a number of fronts - Bone has a remarkable capacity for growth, regeneration, and remodelling. This capacity is largely due to the induction of osteoblasts that are recruited to sites of new bone formation. While the process of recruitment remains unclear, the immediate environment of the cells is likely to play a role via cell-matrix-osteoinductive factor-cell interactions.
Thus biomimetic environments within a tissue engineering strategy may provide alternative solutions for skeletal tissue reconstruction.

The challenge facing material scientists, cell biologists and clinicians will be the development of protocols, new tools and above all multidisciplinary approaches for *de novo* bone formation both in terms of healthcare costs and, more importantly, improved quality of life.

Current approaches and challenges to be presented from work in Southampton include:

i) the ability of a synthetic 15-residue peptide, P-15, related biologically to the active cell binding domain of type I collagen to promote adhesion, proliferation, differentiation and osteogenesis of human osteoprogenitors on 3-D scaffolds (fig 1),

ii) manipulation of the developmental potential of human osteoprogenitors on modified PLA polymer structures and biomimetic structures with bone morphogenetic factors, and

iii) the examination of bone formation using biomimetic scaffolds *in vivo*.

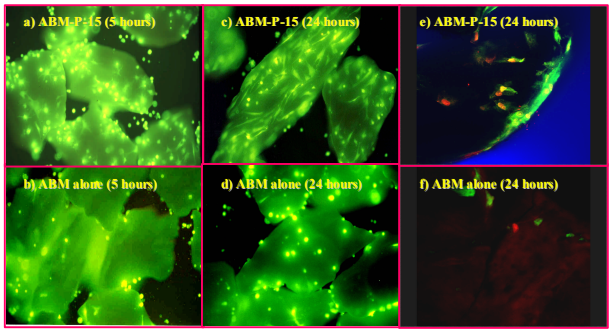

Fig. 1: Stimulation of HBM cell adhesion and proliferation on P-15 adsorbed ABM scaffolds (a, c, e) compared with ABM alone (b, d, f). Green fluorescence indicates live cells marked with CFMDA. e) & f) confocal 3-D images.

Ultimately clinical evaluation will be required to determine whether biomimetic microenvironments that promote osteoprogenitor differentiation and facilitate the exploitation of extracellular matrix cues for osteogenesis offer exciting prospects for de novo bone formation and skeletal regeneration for an increasing ageing population.

ACKNOWLEDGEMENTS: Funding from the BBSRC and EPSRC is gratefully acknowledged. The work presented and many useful discussions is derived from all at University Orthopaedics, Daniel Howard, Xuebin Yang, David Green, Kris Partridge, Trudy Roach and Nicholas Clarke as well as fruitful collaborations with Professor Shakesheff and Professor Howdle (University of Nottingham) who provided the PLA growth factor encapsulated scaffolds and Professor Song Li and Professor Bhatnagar (University of California, Berkeley) who provided and collaborated on the P-15 studies.

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SCULPTURING THE ARCHITECTURE OF MINERALIZED TISSUES: SOLUBLE, INSOLUBLE AND GEOMETRIC SIGNALS

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INTRODUCTION: Nature relies on common and limited molecular mechanisms tailored to provide the emergence of specialized tissues and organs. The bone morphogenetic and osteogenic proteins (BMPs/OPs) family is an elegant example of Nature's parsimony in programming multiple specialized functions deploying molecular isoforms with minor variation in amino acid motifs within highly conserved carboxy-terminal regions. [1-4] BMPs/OPs, pleiotropic members of the transforming growth factor-β (TGF-β) superfamily, are soluble mediators of tissue morphogenesis and powerful regulators of cartilage and bone differentiation in embryonic development and regeneration in postnatal life. A striking and discriminatory feature of BMPs/OPs is their ability to singly induce *de novo* endochondral bone formation in extraskeletal heterotopic sites as a ricapitulation of embryonic development. [1-4] Strinkingly this prerogative, originally solely assigned to BMPs/OPs, has been extended to the TGF-β isoforms themselves with a marked site and tissue specificity of endochondral bone induction yet remarkably and strictly in primates only. [3,4] The presence of several molecular forms endowed with osteogenic activity poses important questions about the biological significance of this apparent redundancy, additionally indicating multiple interactions during bone tissue formation and tissue morphogenesis of disparate organs and tissues. The menu for enunciating the rules of architecture that sculpt cortico-cancellous structures of the bone and regulate bone regeneration and bone tissue engineering in clinical contexts lists additional complex interactions but requires three key components: an osteoinductive soluble signal; an insoluble substratum which delivers the signal and acts as a scaffold for new bone formation; and host cells capable of differentiation into bone cells in response to the osteoinductive soluble signal. [1,4] The signals responsible for osteoinduction are conferred by the osteogenic members of the TGF-β superfamily. This communication describes site-specific modifications of bioactive biomimetic matrices endowed with the striking prerogative of initiating bone formation by induction even in absence of exogenously applied osteogenic members of the TGF-β superfamily. [4-6]

METHODS: The heterotopic and orthotopic models of tissue morphogenesis by osteoinductive and osteoconductive biomaterials have been described in detail [4,5] and height clinically healthy Chacma baboons (*Papio ursinus*) were implanted heterotopically in the *rectus abdominis* muscle with four different types of resorbable and non-resorbable sintered porous hydroxyapatite (SPHA) discs, 20 mm in diameter with a series of concavities prepared on both planar surfaces. Discs 25 mm in diameter of porous hydroxyapatites were also implanted orthotopically in non-healing calvarial defects. Tissue specimens were harvested on day 30, 90 and 180 and processed for histological, biochemical and molecular analyses. Additional sections were used for immunohistochemical staining of OP-1 and BMP-3, markers of bone formation. Total RNA was extracted and probed for γ-actin, OP-1, BMP-3, TGF-β_1 and collagen type IV cDNAs.

RESULTS: Morphological analyses on day 30 showed mesenchymal collagenic condensations within concavities of the substratum with vascular invasion and capillary sprouting within the invading tissues with capillary elongation in close contact with the implanted hydroxyapatite biomatrix and bone formation by induction (Fig. 1). There was attachment and differentiation of mesencymal cells at the hydroxyapatite/soft tissue interface of the concavities and expression of BMP-3 and OP-1 mRNA in differentiating osteoblast-like cells resident within the concavities of the *smart* biomimetic matrices which was followed by immunolocalization of the secreted proteins BMP-3 and OP-1 at the interface of the sintered hydroxyapatite biomatrix with the invading mesenchymal tissue. Signal intensity of mRNA expression differed between the different types of SPHAs with marked variation of mRNA expression of BMP-3, OP-1 and type IV collagen. Non-resorbable SPHAs orthotopically implanted induced 32.9∀6.5 and 36.8∀8.8 bone volume on day 90 and 180 respectively. Resorbable SPHAs 27.1∀5.2 and 27.5∀11.3, respectively.

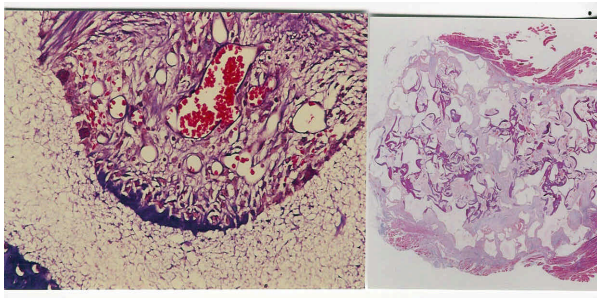

Fig1: Vascular invasion and bone differentiation by induction within concavities of highly crystalline sintered hydroxyapatie biomatrices.

DISCUSSION & CONCLUSIONS: Morphological, biochemical and molecular evidence has been harnessed in our laboratories to guide the incorporation of specific angiogenic and osteogenic activities into biomimetic matrices of sintered highly crystalline hydroxyapatites. Sintered hydroxyapatites implanted heterotopically in *Papio ursinus* induce the reproducible spontaneous differentiation of bone. The geometry of the insoluble signal is a critical parameter for bone induction to occur: concavities of a specific dimension bind OP-1 and BMP-3 and then initiate a sequential cascade of events driving the emergence of the osteogenic phenotype and the morphogenesis of bone as a secondary response. We have now shown that the spontaneous induction of bone differentiation initiates even in concavities of resorbable biomimetic matrices. Additional and complementary data were deduced by Northern blot analyses to study the mRNA expression of specific gene products induced by responding cells within the concavities of the sintered biomimetic matrices and showing critical differences in expression of mRNA markers of bone formation according to the type of implanted biomatrices. Results indicate that the geometry of the substratum is not the only driving force since the structure of the insoluble signal dramatically influences and regulates gene expression and the induction of bone as a secondary response. We now propose the following cascade of molecular and morphological events culminating in the induction of bone initiating within concavities of the *smart* biomimetic matrices: 1) vascular invasion and capillary sprouting within the invading tissue with capillary elongation in close contact with the hydroxyapatite biomatrix; 2) attachment and differentiation of mesenchymal cells at the interface of the *smart* concavities; 3) expression of TGF-ß and BMPs/OPs gene products in osteoblast-like cells resident and differentiating within the concavities; 4) expression and synthesis of specific BMP/OP proteins from transformed and differentiated osteoblasts onto the sintered crystalline hydroxyapatite; 5) intrinsic osteoinduction depending on a critical threshold of endogenously produced BMPs/OPs initiatiating bone formation. Soluble signals induce morphogenesis, physical forces imparted by the geometric topography of the insoluble signal dictate biological patterns, constructing the induction of bone and regulating the expression of selective mRNA of gene products as a function of the structure. In the primate only, heterotopic bone induction is initiated by naturally-derived BMPs/OPs and TGF-ß1, recombinant hBMPs/OPs and TGF-ßs and sintered hydroxyapatite biomimetic matrices with a specific geometric configuration. This indicates that in primates bone tissue develops as a mosaic structure in which members of the TGF-ß superfamily singly, synergistically and synchronously initiate and maintain the developing morphological structures and play different roles at different time points of the morphogenetic cascade. In the primate only TGF-ßs and BMPs/OPs are sculpturing tissue constructs that help to engineer skeletal tissue regeneration in molecular terms: morphogens exploited in embryonic development are re-exploited and re-deployed in postnatal tissue regeneration.

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EFFECTS OF GEOMETRICAL FACTORS ON THE RESORPTION OF CALCIUM PHOSPHATE BONE SUBSTITUTES

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INTRODUCTION: Calcium phosphate ceramics have proved their adequacy and efficiency as bone substitute materials. Despite their widespread use, there is a growing demand for faster resorbable calcium phosphate bone substitutes. The resorption rate of a bone substitute depends on many factors such as the patient (sex, age, metabolism, social habits, etc...), the implant location, the composition of the bone substitute and its geometry. For a surgeon or an engineer, it is difficult to control the first factors, but possible to control the composition and geometry of the bone substitute. Here, we propose to use a new approach to determine the effect of geometry on the resorption rate of bone substitutes.

THEORETICAL: The theoretical approach was based on four main assumptions: (i) the pores are spherical and ordered according to a face-centered cubic packing; (ii) the resorption is surface-controlled; (iii) the resorption is only possible if the surface can be accessed by blood vessels of 50 µm in diameter (= 2 r_i); and (iv) the resorption time of a given amount of calcium phosphate is proportional to the net amount of material. Two separate cases were considered: granules and blocks. Based on these assumptions, the calculations showed that the determination of the optimum pore size depends on two main factors: the time required for bone ingrowth and the time required to resorb the ceramic assuming that all surfaces are resorbed simultaneously. The rate of bone ingrowth, t_I, depends on the smallest one-dimensional distance of the implanted material, W, the minimum interconnection radius required to allow bone ingrowth, r_i (= 25 µm), the radius of the pores, r_p, the distance between the pores, d_p, and the linear resorption rate, R_r (Eq 1). The time required to resorb the ceramic, t_R, depends on the radius of the pores, the distance between pores, and the linear resorption rate (Eq 2).

$$t_I = \frac{\sqrt{r_i^2 + \left(2r_p + d_p - \sqrt{r_p^2 - r_i^2}\right)^2} - r_p}{R_r\left(4r_p + 2d_p\right)} \cdot W \; ;$$

$$t_R = \frac{r_p\left(\frac{\sqrt{3}}{\sqrt{2}} - 1\right) + d_p\left(\frac{\sqrt{3}}{2\sqrt{2}}\right)}{R_r}$$

Therefore, the maximum time required to resorb a ceramic is given by the addition of the ingrowth time, t_I, and the resorption time, t_R. The plot of this total time versus the pore radius indicates that there is a minimum of resorption time in the range of 150 to 300 µm. The position of this miminum depends on the block size (Fig 1). In the range of 150 to 300 µm, there is an optimal combination of bone ingrowth, ceramic resorption and porosity. The model can also be used to assess the time required to resorb granules. The volume of the granules, $V_{g,t}$, is a function of the initial volume, $V_{g,0}$, the linear resorption rate, R_r, the time, t, and the initial granule radius, $r_{g,o}$.

$$V_{g,t} = V_{g,0}\left(1 - \frac{R_r}{r_{g,0}}t\right)^3$$

This equation indicates that the granules are resorbed faster when they are smaller. Taking into account that bone ingrowth between the granules should be possible, the granules should have a relatively small diameter, typically around 100-200 µm. It might be adequate also to have an irregular shape that can on one side increase the specific surface area and on the other side increase the size of the gaps between particles The comparison of the predictions of the model with experimental data shows a very good agreement, i.e. more than 80% of the results were explained with the model. In conclusion, the model is a useful tool to define adequate geometries for bone ingrowth and resorption of the bone substitute.

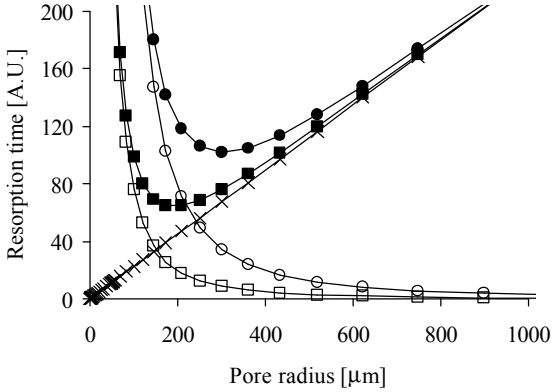

Figure 1: Time to (x) fully resorb one unit cell of the block (= t_R) or to (o, ?) open all the pores of the block to enable bone ingrowth (= t_I). Conditions: d_p = 0; Block thickness: (?) 5 mm; (o) 20 mm. The combined curves (addition of the two curves) are represented by black symbols. Block thickness: (?) 5 mm; (?) 20 mm.

BIORESORBABLE SCAFFOLDS PREPARED BY SUPERCRITICAL FLUID FOAMING

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INTRODUCTION: With orthopaedic implants, bone grafts represent a large part of bone surgery. Tissue engineered scaffolds can serve as 3D-templates to guide and enhance bone regeneration, by facilitating cell migration, proliferation and differentiation [1]. Supercritical CO_2 foaming of polymers has been reported to create highly porous structures [2], and start to be used for bioresorbable polymer scaffolds [3]. It avoids the use of organic solvents. This simple and flexible technique allows also to add fillers to reinforce the structure, or solvent-sensitive biological factors. After saturation of the polymer, the reduction of pressure induces a thermodynamic instability, which causes nucleation and growth of gas pores in polymer matrix. Varying foaming conditions allows to control foam morphology [4].

METHODS: Two commercial bioresorbable polymers were used, without further purification: a poly L-lactic acid PLA (Boehringer Ingelheim, Germany) and a copolymer of D,L-lactic and glycolic acids PLGA 85/15 (Purac, Netherlands) characterized respectively by intrinsic viscosity of 1.8 and 2.5 dL/g. PLGA 85/15 discs were obtained by compression moulding. PLGA preforms or PLA pellets were placed in the high pressure chamber (Autoclave France). Amorphous PLGA 85/15 was heated to 40°C, and semi-crystalline PLA to 190°C. Both were saturated by supercritical CO_2 (pure >99.995%; SL gas, CH) under different pressures, before depressurizing, and additional water cooling in case of PLA. Foam morphology was characterized by SEM (Philips XL30) observations of fracture surfaces. Apparent density and compression modulus (Universal Tensile Testing machine; 0.5 mm/min) were also evaluated.

RESULTS: Several parameters (saturation pressure and time, depressurization and cooling rates) were varied to study their effects on foam morphology, porosity and mechanical properties and to determine which conditions lead to a suitable scaffold for bone tissue engineering (Fig1). Partially interconnected pores with a diameter ranging from 200-1000 µm can be manufactured (Fig2).

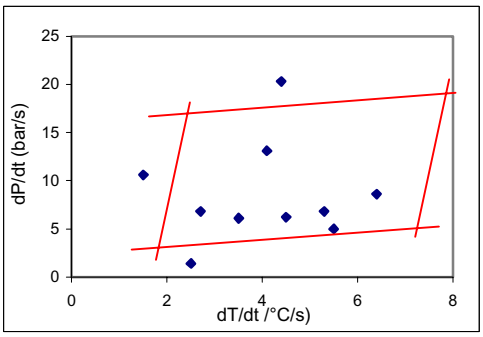

Fig 1. Processing window for PLA

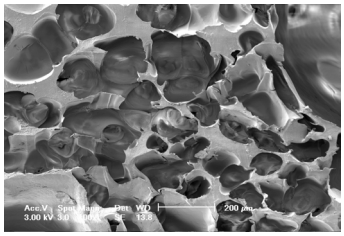

Fig. 2. PLA morphology (2.5 bar/s; 2.7 °C/s)

DISCUSSION & CONCLUSIONS: Foaming conditions, polymer composition and viscosity have a significant effect on the 3D-matrices' porosity and properties. Increasing saturation pressure will increase the potential number of pores, whereas a slow cooling (in case of melt foaming) will promote coalescence and thus larger pores but also interconnections. Porosity and pore size of bioresorbable structures obtained by supercritical fluid process will likely be adequate for bone tissue engineering. Flexibility of the technique can also allow to integrate reinforcing fillers into the polymer matrix to improve its mechanical behavior.

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SYNTHETIC RHBMP-2 DELIVERY SYSTEMS: FROM SURFACE EROSION TO CELL TRIGGERED RHBMP RELEASE

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INTRODUCTION: In 1988 the first cDNAs coding for bone morphogenetic proteins (BMPs) were cloned with the primary goal to use the bone inducing principle in the clinic for fracture healing especially for non-unions and the augmentation of bone. Meanwhile a lot of knowledge about the BMPs and their diverse roles has accumulated but the initial goal to use BMP routinely as inducer for bone formation in clinical situations has not been reached due to the lack of suitable carrier materials. Products, which came just recently on the market, use natural materials as delivery system. However, concerning their potential immunogenicity, batch variability, and complex purification procedures the clinical need for synthetic biomaterials is still large. Towards this end, we have designed slow release rhBMP delivery systems termed foamspheres. Foamspheres are based on poly (lactide-co-glycolide) acids, are mechanically more stable than hydrogels, and proved useful for the augmentation of bones. For the treatment of bone defects, we molecularly engineered synthetic hydrogels that contain a combination of adhesive and matrix-metallo-protease MMP-sensitive oligopeptides. Using these hydrogels in combination with rhBMP-2 to heal a critical size defect in the rat cranium, we demonstrate that they are an excellent delivery system for rhBMPs and can be applied in bone defects.

MATERIALS AND METHODS: PLGA based foamspheres were created by a double emulsion-solvent evaporation technique. Branched PEG's (Shearwater Polymers, USA; 4arm, Mw: 10, 15 and 20kD) were end-functionalized with vinylsulfone. Crosslinker peptides with different enzymatic activity (kcat/Km) were designed bearing a cysteine on both ends of a matrix metalloproteinase (MMP) substrate sequence, e.g. GCRD-*GPQGIA-GQ*-DRCG. Hydrogel disks were implanted in critical size calvarial defects in rats (N>3). After 3, and 5 wk, animals were sacrificed, and the defect regions explanted, radiographed, and histologically processed.

RESULTS AND DISCUSSION: In vitro assays showed that over a period of 10 days all rhBMP is released from the foamspheres. When 12 µg rhBMP-2 was administered via foamspheres the bone height of the calvarial bone increased significantly to 2.88 ± 0.39 mm ($P < 0.003$) compared to the control untreated calvarial bone (0.90 ± 0.1 mm). Application of 12 µg rhBMP via a collagen based hydrogel increased bone height to 1.5 ± 0.8 mm, significantly less than achieved by the application of the same amount of rhBMP via foamspheres. Synthetic polyethylenglycol based hydrogels linked by MMP sensitive peptide designed for the application as rhBMP delivery systems in bone defects showed in vitro an initial rhBMP release of 10%. Following 60 h of continuous washing, 90% of the rhBMP was still entrapped in the gel compared to 60% entrapped in a collagen gel. An instant release of the rhBMP from the synthetic PEG gel could be achieved by the application of MMPs. In vivo at a dose of 5 µg rhBMP-2, bone formation in the rat cranium defect was observed. By 5 wk, implant materials were fully resorbed, and new bone covered the defect area. The healing response was critically depen-dent on the proteolytic sensitivity and the hydrogel structure. Control materials made with an MMP-insensitive peptide, showed no cell infiltration and significantly less bone formation around the still intact gel implants. Therefore, entrapped rhBMP is biologically inactive and needs cell triggered release by MMPs for activation.

CONCLUSION: The clinical use of rhBMP demands for delivery systems optimized for specific applications. For bone augmentation, the foamspheres seem to be an appropriate slow rhBMP delivery system. For bone defects, a PEG based material, which imitates the natural ECM in its ability to undergo cell-triggered proteolytic degradation and rhBMP release proved to be a very suitable material. Due to the intrinsically non-adhesive/passive character of the major gel component PEG, cell-material interactions can be engineered de novo in an elegant and well-defined way. We believe that this new class of biomaterials is an alternative to existing natural biomaterials for the reconstruction of bone but also for various other tissues.

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POTENTIAL AND LIMITATIONS OF BONE ANABOLIC THERAPY WITH PARATHYROID HORMONE.

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INTRODUCTION: Therapy of osteopenic states consists mainly of treatment with anti-resorptive agents, such as hormone replacement therapy (HRT), selective estrogen receptor modulators (SERMs), various calcitonins and bisphosphonates (BPs). While anti-resorptive agents are powerful tools for preventing or retarding bone loss, their impact in patients with established osteoporosis is less impressive. Owing to our difficulties to identify patients at risk at an early stage, osteopenia remains often undetected until substantial amounts of bone have been lost or even until fractures occur. Anabolic drugs like PTH might be of particular value for treating the large group of osteopenic patients detected at a late stage and should show significant advantages over anti-resorptive agents with regard to the reduction of fracture rates. This abstract addresses the potential and limitations of bone anabolic therapy with PTH.

METHODS & RESULTS: PTH can exert both anabolic and catabolic effects on bone depending on the pattern of exposure to the hormone [1]. The optimal duration of the PTH pulses in rats to create the pulsatile pattern was investigated by Dobnig and Turner [2]. Results demonstrate that the exposure of bones to supraphysiological levels of hPTH exposure should not exceed 6 to 7 hours in total. Similarly, in osteoblast-like cells isolated from newborn rat calvaria [3], intermittent exposure to PTH for the first 6h of each 48h cycle stimulated osteoblast differentiation, while continuous exposure to PTH during the 48h incubation-cycle strongly inhibited osteoblast differentiation. Both, cAMP/PKA and Ca2+/PKC signaling pathways, appear to be involved independently in the bone response to PTH but activation of the former is mandatory for the bone anabolic effect.

Initially, PTH-induced bone gain appears to result from direct activation of bone lining cells on virtually all cancellous and cortical surfaces (activation of modeling drifts) [4;8]. Bone apposition on cancellous surfaces leads to trabecular thickening *(Fig 1)* a decrease in trabecular separation [5;6] with their number remaining unchanged. Endocortical and periosteal bone apposition are also observed.

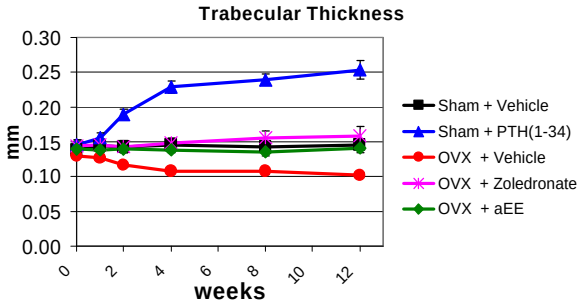

Fig 1: Trabecular thickness measured in the proxim. tibia metaphysis in OVX-rats by microCT.

The anabolic action of PTH and presumably most other bone forming agents is critically dependent on the existing template [7; 8]. Severely osteopenic bones with a poor template will show less dramatic bone gains when therapy is initiated, and *de novo* formation of trabecular bone has not been observed following the administration of therapeutically relevant doses of PTH.

PTH is anabolic for bone when administered less than daily, an important finding when considering that parenteral (s.c.) administration is required for at least 18 months to achieve the desired therapeutic results. Several studies [8;9] demonstrated the anabolic action of PTH in rats and primates [5] even when administered every 3 or 4 days only.

Bone gain is not infinite and indeed is limited by a feed-back mechanism (mechanical sensor?) and the bone mass strength achieved in the plateau is dependent on the PTH-dose *(Fig 2)*.

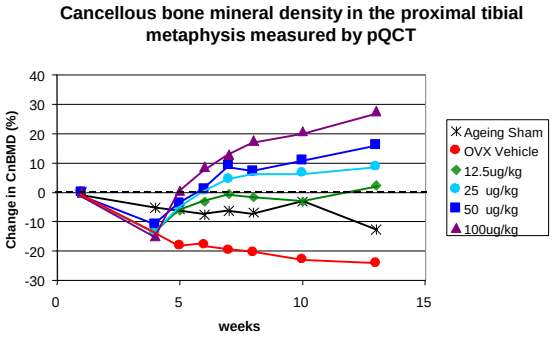

Fig: 2: PTH: Dose dependent increase in cancellous BMD measured by pQCT in the prox. tibia metaphysis in rats. OVX-intervention study.

Upon cessation of treatment, bone mass and strength must be maintained either through antiresorptive therapy (SERM, HRT, BP, CT) or continuous anabolic treatment with PTH or else the bone will be lost again [10]. Continuous PTH for maintenance may not be a good choice since it would keep bone in a state of high turnover.

One of the greatest concerns with PTH is indeed the potent activation of Haversian and cancellous bone remodeling seen in primates (11), dogs and rats (*Fig 3)* [12], which is also evident in clinical trials, especially in the ultradistal radius [13]. The good

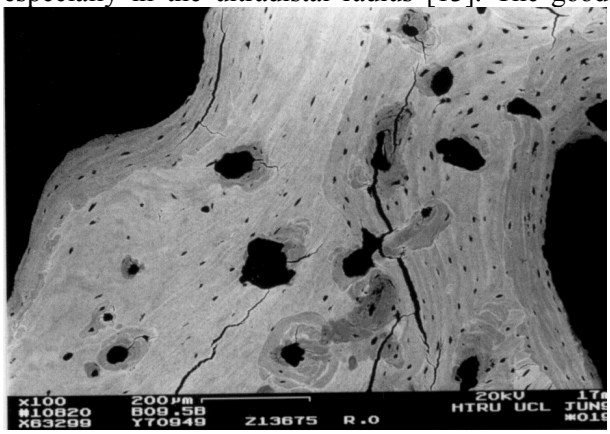

Fig 3: BSE-image of rat vertebra showing strong bone remodeling after long-term treatment with PTH(1-34).

news about the Haversian remodeling is, that this bone loss appears to represent a transient phenomenon and that the remodeling space will fill in after cessation of anabolic treatment [14]. Clinical data also suggests that co-treatment with antiresorptive agents may prevent some of the remodeling associated with PTH-therapy [15].

By the time PTH will become widely available to patients as a bone anabolic agent, a considerable number of them will have been exposed to bisphosphonates. BPs are rapidly integrated into the superficial layers of the mineralized matrix and thus it was important to study possible interactions of such a pretreatment on the response to PTH. Pretreatment of old rats with a low dose of 28µg/kg of alendronate (s.c., 2 inj/week) blunted the anabolic response to the PTH-analog SDZ PTS-893 administered at 100µg/kg (s.c. daily) as measured by DEXA and pQCT [16]. In agreement with bone mass data, biomechanical tests showed reduced gain in bone strength after treatment of rats with the analog in BP pretreated animals. Serial pQCT measurements indicated that the PTS-893 induced activation of bone formation was delayed by roughly two weeks and that in addition, pretreated rats failed to achieve the same bone gain in the plateau as their placebo pretreated controls. The blunting of the anabolic response may result from a physicochemical interaction of the matrix embedded BP with the first responding cell i.e. the bone lining cell. However, the benefit of adding an anti-remodeling agent to PTH-treatment, especially with regard to the reduction in Haversian remodeling, may outweigh the worries about the blunted anabolic response.

DISCUSSION & CONCLUSIONS: At this time, PTH is the only approved bone anabolic treatment for postmenopausal osteoporosis. PTH activates bone lining cells into bone forming osteoblasts within 6 hours after transient exposure. The peptide increases trabecular thickness but is unable to generate a new bony template in severely depleted areas. Endocortical and periosteal bone formation is also induced by PTH. Upon cessation of treatment, bone will be lost unless it is protected by antiresorptive therapy. Co-treatment regimens (PTH + antiresorptive agent) appear to offer some protection against PTH-induced Haversian and cancellous bone remodeling. However, some data indicate, that BPs can blunt the initial response of the bone lining cells to PTH, possibly through some physicochemical interaction. Despite of some limitations, PTH is a promising novel therapy for the treatment of OP.

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DIFFERENTIAL ROLES FOR SMALL LEUCINE RICH PROTEOGLYCANS IN BONE FORMATION

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INTRODUCTION: The small leucine-rich proteoglycans (SLRPs), decorin and biglycan have been identified as matrix components in many connective tissues. Within soft connective tissues, such as skin, these macromolecules are primarily substituted with a dermatan sulphate (DS) glycosaminoglycan (GAG) chain, whilst in mineralized matrices of bone, the chondroitin sulphate (CS) substituted form predominates. The ability of the protein and GAG moieties to interact with collagen and growth factors have led to proposed functions in matrix assembly and modulation of cellular behaviour. Studies have also indicated that these macromolecules are also capable of interacting with calcium and hydroxyapatite surfaces, suggesting roles in either inhibiting or regulating mineral crystal growth and controlling crystal morphology. In order to further assess the role of SLRPs during bone formation, the present paper discusses the temporal expression of decorin and biglycan during bone formation, examining the biochemical nature and potential modification to structure during synthesis of the premineralised and mineralising matrices.

METHODS: A model system of primary bone cell culture models derived from marrow stromal cells and alveolar bone supporting teeth was used. We have identified periods relating to cell proliferation and development of the osteoblast phenotype associated with matrix deposition, remodelling of the osteoid, prerequisite to mineral deposition. Temporal expression of decorin and biglycan was assessed by RT-PCR. SLRP components were chaotrophically extracted from the matrix synthesized at various time points described above. SLRPs were separated by immunoprecipitation using the monoclonal antibody CS56 (Sigma), immunoreactive for CS. The GAG moiety within each fraction was examined by cellulose acetate electrophoresis whilst the proteoglycan species were identified by Western blot analysis using polyclonal antibodies against decorin and biglycan.

RESULTS: From these studies we have shown that decorin and biglycan exhibit differences in their patterns of expression and the nature of their glycosylation. Biglycan is expressed in two distinct phases relating to cell proliferation, after which it is removed from the matrix and appears to be re-expressed at a time point relating to the onset of mineralization. Of significance, biglycan expressed during cell proliferation was substituted with DS chains, whilst biglycan synthesized during mineralization carried CS chains. Decorin was expressed later than biglycan, associated with early matrix deposition, with significant mRNA levels continuing to the mineralization stages. Again, DS-decorin prevailed with osteoid, whilst CS-decorin predominated within the mineralizing matrix. During remodeling of the osteoid, the GAG chain appears to be selectively removed from decorin, with the protein core persisting within the matrix.

DISCUSSION: The nature of the GAG chain conjugated to SLRP and the timing of its expression would seem to dictate the function decorin and biglycan play in bone formation. The role of DS-SLRP may correlate with those present in soft tissues such as skin. The role of biglycan is unclear but it has been implicated in regulating cell attachment, migration and angiogenesis (1,2). Within bone, our results would suggest a role in controlling cell proliferation. Conversely, expression of decorin arrests the growth of tumour cells (3) and the delayed expression within our bone culture model may indicate a role in down-regulating cell proliferation. Decorin also has potentially differing roles in collagen fibrillogenesis during matrix formation. DS-decorin appears to inhibit fibrillogenesis (4), whilst CS-decorin promotes fibrillogenesis (5) and biglycan would seem to display no effect (5). Both CS-decorin and CS-biglycan are capable of binding hydroxyapatite and influencing crystal growth, even when bound to collagen (5), suggesting roles in regulating mineral deposition. Overall the varied regulatory activity of decorin and biglycan is an important consideration for bone formation and tissue engineering.

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ACKNOWLEDGEMENTS: Dr LW Fisher for the provision of antibodies and the Medical Research Council, UK for financial assistance.

SUPPRESSION OF BONE RESORPTION BY BISPHOSPHONATE FOLLOWING INTRAMUSCULAR ECTOPIC BONE FORMATION INDUCED BY rhBMP-2

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INTRODUCTION: Autogenous bone, allograft and artificial bone substitutes are frequently used in orthopaedic, plastic and reconstructive surgery. Autogenous bone graft has the greatest potential for bone conduction and induction with no risk of microorganism transmission and immune reaction. However, in some cases, autogenous graft is not adequate for large defect reconstruction. Bone graft availability and donor site morbidity are other concerns. In our previous study [l], generation of intramuscular autogenous bone tissue was achieved by using recombinant human bone morphogenetic protein-2 (rhBMP-2) with ß-tricalcium phosphate (ßTCP) as a carrier. However, absorption occurred early after bone induction through normal bone remodeling mechanism. This study was conducted to investigate whether simultaneous administration of bisphosphonate would enable the maintenance of induced bone tissue.

METHODS: Eighty 12-week-old female Sprague-Dawley rats were used. Experiment 1: Single administration of rhBMP-2 (17µg/30µl) solution was applied to the ßTCP disc 5mm in diameter and 2mm in height. The disc was inoculated into the quadriceps of rats. Specimens were harvested at 1, 2, 3, 4 weeks (n=5 in each group). Experiment 2: Simultaneous administration of Bisphosphonate (Minodronate YM529) with rhBMP-2 (17µg/17µl) solution at different YM529 concentrations (10^{-4}, 10^{-5}, 10^{-6}, 0M/13µl) was applied to the ßTCP disc. The discs were implanted similarly to the previous experiment. Specimens were harvested at 2 and 4 weeks (n=5 for each concentration). The specimens were soft X-rayed, H&E and TRAP (spell out) specific stained. Ratio of induced bone area to the total area of ßTCP disc was measured by Scion image (NIH image). The number of TRAP positive cells was counted at 2-4 visual fields of each sample, and then averaged. Compressive strength test was performed on the samples at 4 weeks in Experiment 2 specimens. Plain ßTCP discs without any drug addition of were used as controls.

RESULTS: Experiment 1: The ratio of bone tissue area and the number of TRAP-positive cells reached maximum after 2 weeks and then declined. Experiment 2: The percentage of bone tissue area was well preserved even at 4 weeks (Fig.1).

Addition of YM529 decreased the number of TRAP-positive cells after 2 weeks of inoculation (Fig.2). We also observed increased bone tissue strength in the groups of 10^{-4}M and 10^{-5}M YM529 concentrations.

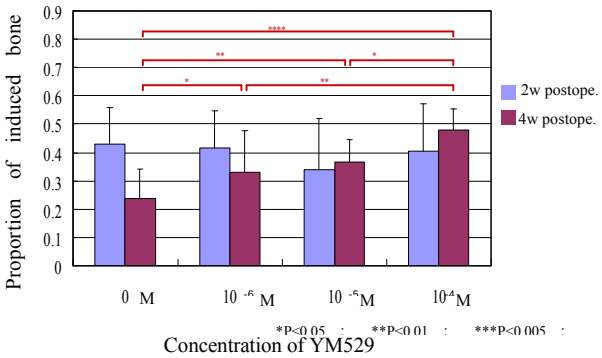

Fig. 1: Experiment 2: Simultaneous administration of YM529. Induced bone area.

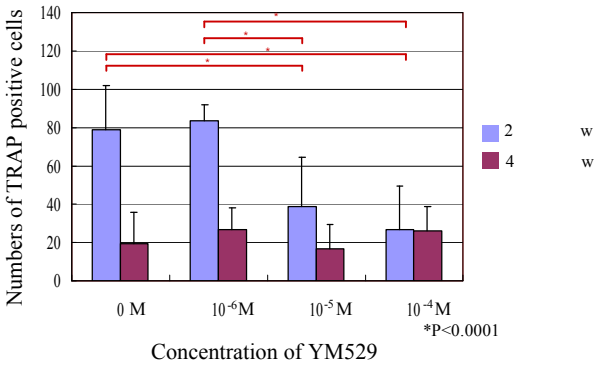

Fig. 2: Experiment 2: Simultaneous administration of YM529. Number of TRAP positive cells.

DISCUSSION & CONCLUSIONS: The concurrent use of bisphosphonate prevented bone absorption attributed to osteoclastic activity inhibition after bone induction by rhBMP-2. The compressive strength also increased without rapid bone absorption in the newly induced bone. The combination of rhBMP-2 with bisphosphonate in ßTCP may have potential in clinical use for bone tissue transplantation.

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OSTEOGENESIS IMPERFECTA - CLINICAL AND MOLECULAR DIVERSITY

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INTRODUCTION: Osteogenesis imperfecta (OI) is a heritable medical disorder characterized by bone fragility due to impairment in bone quantity and quality, which may present with a wide range of disease severity. The term osteogenesis imperfecta has been used to describe the clinical features of this disorder since the nineteenth century, long before any underlying gene mutations were described. Many individuals with OI are now known to possess mutations in one of the two genes for type I collagen (COL1A1 and COL1A2), though others do not. However, while OI exhibits both clinical and molecular diversity, it is not possible to clearly distinguish clinical phenotypes on the basis of their gene mutation.

CLINICAL DIVERSITY: The most widely used clinical classification of OI is that proposed by Sillence, which divides OI into four types. Type I OI describes individuals with the mildest form of the disease. Fractures are not common at birth, incidence remains low throughout life, and there is little skeletal deformity. Type II OI describes individuals with the most severe form of the disease, who die in utero or soon after birth. Multiple fractures are present in the fetal bones, which are severely deformed. Type III OI represents the most severe phenotype compatible with post-natal life. Individuals have multiple fractures and show severe and progressive skeletal deformity. Type IV OI encompasses those individuals who fall between the type I and III categories, and represents the most phenotypically heterogeneous group. It has now been recognized that type IV OI can be subdivided based on radiographic, histologic and metabolic characteristics and this has led to expansion of the classification system. Type V OI describes individuals who show distinctive hypertrophic callus formation at healing fracture sites and calcification of interosseous membranes in the forearm. Type VI OI describes individuals with signs of a bone mineralization defect, but in the absence of metabolic abnormalities associated with rickets/osteomalacia. Type VII OI describes individuals with characteristic rhizomelic shortening of the femur and humerus and a recessive inheritance. Other categories of OI undoubtedly exist and await formal identification.

MOLECULAR DIVERSITY: About 70% of all individuals who can be classified with OI possess a mutation in either their COL1A1 or COL1A2 gene. Those who do not possess such a mutation include all individuals with types V, VI and VII OI. These newer forms of OI are definitely not due to mutations in the genes encoding type I collagen. In the case of type VII OI, the existence of large pedigrees has enabled the causative gene to be localized to chromosome 3p22-24.1, a region in which no collagen gene or osteoid protein gene has yet been described. The sporadic nature of types V and VI OI has so far precluded identification of the locus of the causative gene. The absence of any detectable COL1A1 or COL1A2 mutation also occurs in some individuals with classical types I, III and IV OI, albeit a minority of individuals. Of those individuals with a COL1A1 or COL1A2 mutation, the majority involve point mutations perturbing a glycine codon. In our experience such mutations represent about 80% of those detected in the COL2A1 gene, but 40% in the COL1A1 gene. The other mutations in the COL1A1 gene reflect deletions or insertions giving rise to a frameshift, point mutations at donor or acceptor sites involved in splicing, or point mutations generating a premature stop codon. The majority of individuals with these latter types of mutation have relatively mild disease. For the glycine mutations, disease severity shows regional variation in relation to the location or type of amino acid substitution, rather than showing discrete trends.

DISCUSSION: Because of the molecular heterogeneity of OI, disease diagnosis should be based on clinical presentation. This is particularly true at present, as current medical treatment of the disease is applicable to all forms of OI. Until specific gene therapy approaches are developed and accepted, medical intervention is aimed at increasing the quantity of bone by bisphosphonate therapy. This can lower fracture incidence, improve quality of life, and make orthopaedic intervention less problematic.

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GENETIC CONTROL OF BONE REMODELING

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Bone remodeling is the physiologic process used by vertebrates to maintain a constant bone mass between the end of puberty and gonadal failure.

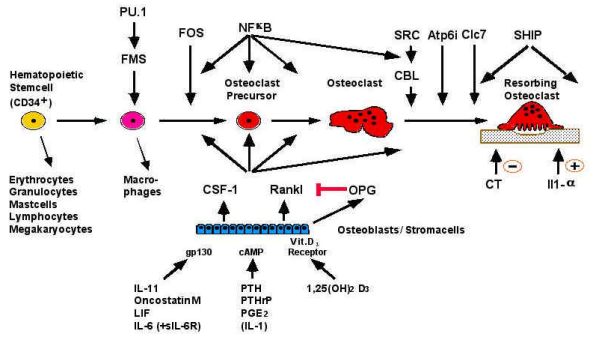

Fig. 1: Bone Resorption. The osteoclast is the only cell capable of resorbing bone. This cartoon presents the essential from the hematopoietic stem cell to the functional active multinucleated osteoclast.

Bone mass is of critical importance for skeletal integrity and skeletal function. A sufficient bone stock is required for locomotion, for protection of inner organs, as a reservoir of vital ions, and as the scaffold for skeletal repair and osteosynthesis. A molecular understanding of this process is therefor of paramount importance for almost all aspects of skeletal physiology and many facets of bone diseases. Besides the well characterized and critical local regulation of bone remodeling, recent genetic studies have shown that there is a central control of bone formation, one aspect of bone remodeling[1,2,3,4,5].

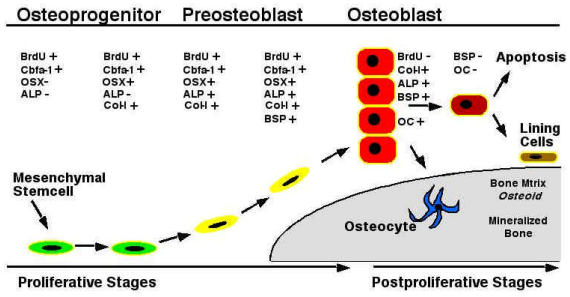

Fig. 2: Bone Formation. New bone is synthezised by osteoblasts that differentiate from the mesenchymal lineage.

This central regulation involves leptin, an adipocyte secreted hormone that controls body weight, reproduction and bone remodeling following binding to its receptor located on hypothalamic nuclei. This novel physiologic concept may shed light on the etiology of osteoporosis and help to identify new therapeutic strategies for osteoporosis and its associated clinical problems like delayed fracture healing.

The overall goal of this review is to show how the dialogue of medicine and mouse genetics helped to uncover a new concept in skeleton physiology that in turn opens a new direction of research and offers potentially novel therapeutic avenues.

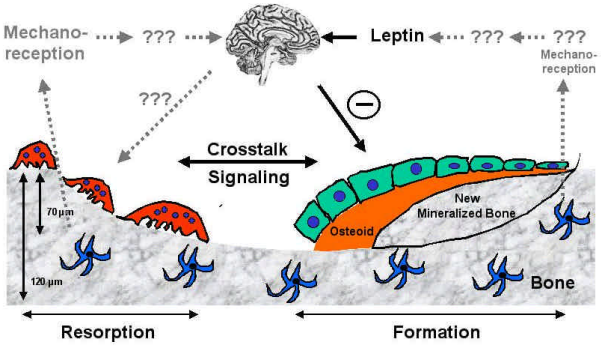

Fig. 3: Bone Remodeling. Presentation of the different levels that control bone remodeling.

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FLUOROCHROME LABELLING OF BONE DYNAMICS

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INTRODUCTION: During growth, bone continually undergoes changes of shape and structure. Accretion in length and thickness, modeling, and drift activities lead to different morphologies and determine the relative position between various skeletal parts. After completion of growth, pathological processes, aging, and degeneration are responsible for structural alterations, and healing processes and adaptation to new functional demands require new structural characteristics. Certain stains are able to bind to sites, which are calcifying. These stains stay there for a long time and serve as markers, allowing the identification of tissues, which were mineralized at the time of dye administration.

METHODS: The yellow Tetracyclines [1], Xylenol orange [2], Alizarin red derivatives [3], or green fluorescein derivatives like Calcein or DCAF [4] can be easily distinguished in bone by the color of their fluorescence. The fluorochromes are administered systemically. Filter sets for fluorescence microscopy can either be chosen for selective excitation of single fluorochromes, or broad band filters may be used for simultaneous visualization of several fluorochromes.

RESULTS: The fluorochromes, with their active iminodiacetic acid groups, form chelate complexes with apatite. Though these substances are primarily used for labeling of bone formation, this process is not specific for bone formation alone, but is rather depending on the availability of binding sites. In addition to the mineralization process, sites of mechanical injury of a mineralized tissue, lacuna where osteoclastic resorption has exposed binding sites, or regions of pathologic mineralization may present bone seeking fluorochromes as well. Bone formation, however, represents by far the majority of labeled sites.

DISCUSSION & CONCLUSIONS: Bone seeking fluorochromes provide a useful tool for analyzing changes of bone morphology. The presence of the fluorochromes indicates site, time and amount of bone deposition, and enhances the information contained in bone specimens. The administration of several fluorochromes, distinguishable by color, offers distinct advantages over single labels.

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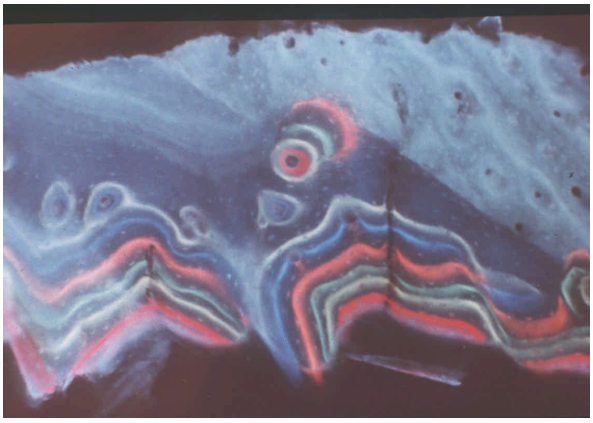

Fig. 1: Labeling of cortical bone drift by consecutive intravital administration of labels of different color.

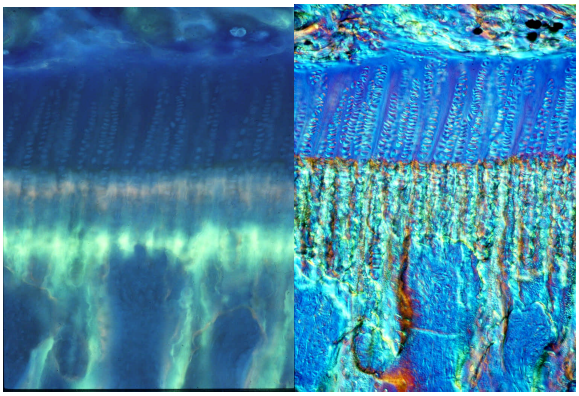

Fig. 2: Progress of mineralization in a growth plate. The pink fluorescent line shows cartilage, which was mineralized two days prior to harvesting of the specimen, the yellow band mineralized one week earlier.

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IMMUNOHISTOLOGICAL IDENTIFICATION OF THE OSTEOCLAST FORMATION REGULATOR OSTEOPROTEGERIN LIGAND (OPGL/RANKL/TRANCE) IN HUMAN BONE TISSUE

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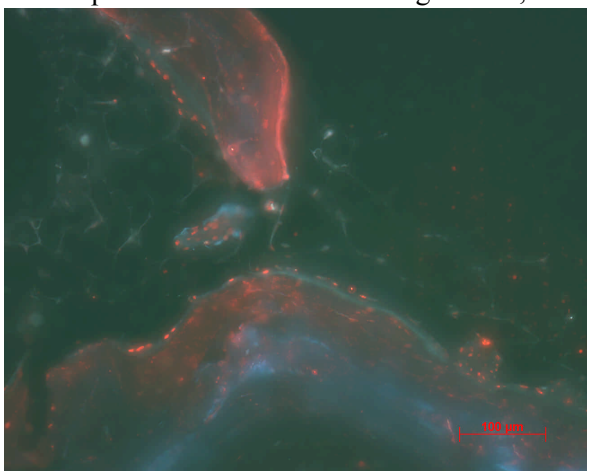
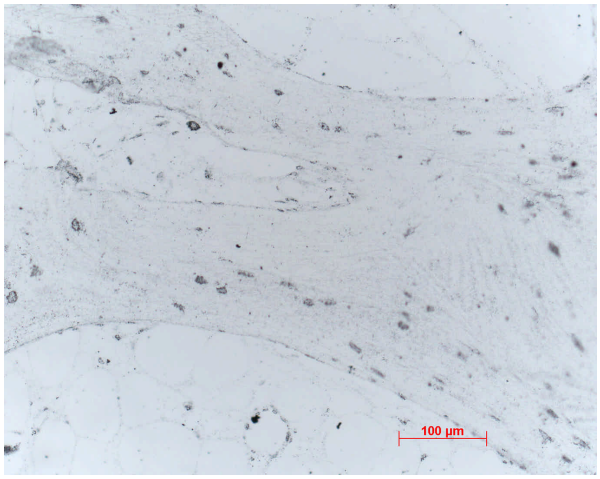
INTRODUCTION: Receptor activator of NF-κB ligand (RANKL, also called ODF/TRANCE/ OPGL) is an essential factor during differentiation of osteoclast progenitors of the monocyte/ macrophage lineage into osteoclasts. RANKL is produced by osteoblasts and exerts its biological effects through binding, in either a soluble or a membrane-bound form, to its receptor RANK, which has been determined to be the signalling receptor essential for RANKL-mediated osteoclastogenesis. The activity of RANKL is neutralised by binding to osteoprotegerin (OPG), which also is secreted by osteoblasts. The biological importance of this system is underscored by the induction of severe osteoporosis in mice by targeted ablation of OPG and ny the induction of osteopetrosis by targeted ablation of RANKL or overexpression of OPG Oteoclastogensis may thus be determined principally by the relative ratio of RANKL to OPG in the bone marrow microenvironment, and alterations in this ratio may be a major cause of bone loss in many metabolic and immunologic disorders. All three suspects RANKL, RANK and OPG are members of the tumour necrosis factor (TNF) superfamily. Although multiple hormones and cytokines regulate various aspects of osteoclastogenesis, the final effector is RANKL. In order to get a better insight into this very complex regulatory system, this study aimed to determine where RANKL protein is located in human bone tissue.

METHODS: The type II transmembrane member of the TNF superfamily was stained immunohistochemically using a monoclonal anti-RANKL mouse IgG2B, that was detected by a CY3 conjugated secondary antibody for immunofluorescence or by a 5 nm gold conjugated secondary antibody, which was silver enhanced for bright-field visualisation. Human bone tissue was freshly obtained (with permission #18/02 from Graubünden Kantonale Ethikkommission), ethanol fixed and embedded in Technovit. The stained bone samples were investigated in comparison with human cartilage tissue which acted as a negative control. In addition to bright-field microscopy, the stained samples were also analysed with a field emission scanning electron microscope (FESEM).

RESULTS: RANKL was clearly labelled in the membrane of osteoblasts and osteocytes (Fig 1). Furthermore, staining of RANKL protein was seen on osteoclasts and in the matrix surrounding the Howship lacunae. In human cartilage tissue, which

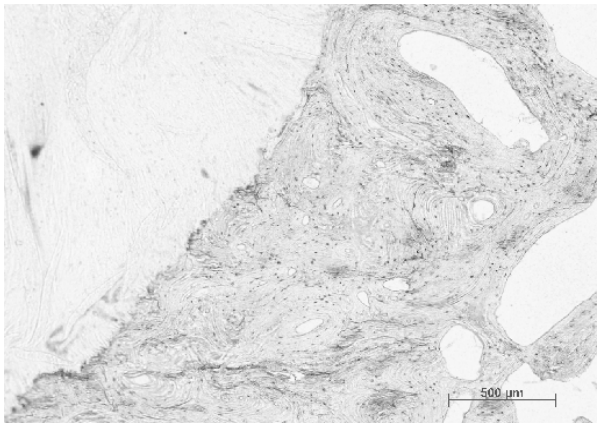


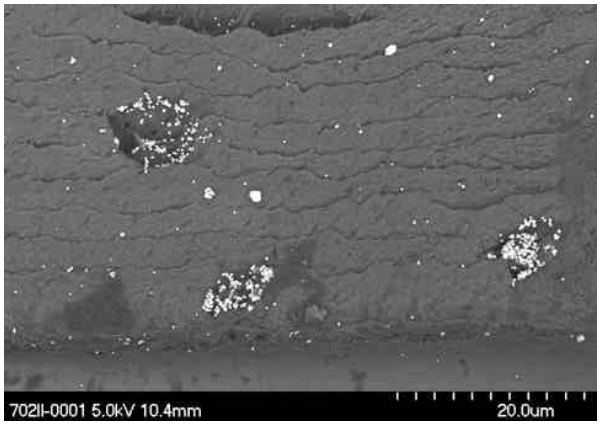


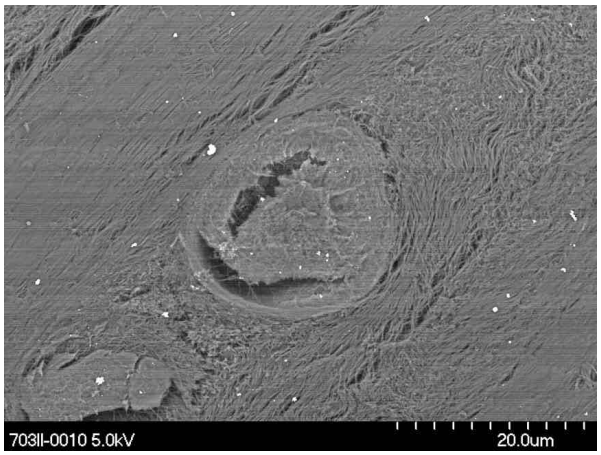
was used as a negative control, chondrocytes were not stained (Fig 2).

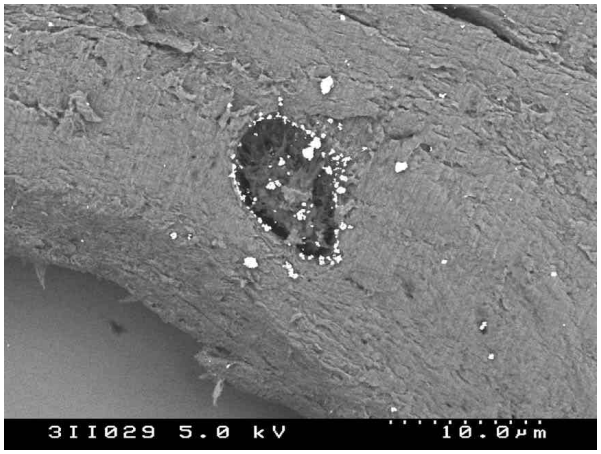
Fig 1: RANKL protein staining in osteoblasts and osteocytes, a) CY3 immunofluorescence, b) 5 nm gold stained in bright-field microscopy

Fig 2: Comparison of human bone and cartilage tissue, a) cartilage/bone boarder, b+d) RANKL labelled osteocytes, c) chondrocyte not labelled









DISCUSSION & CONCLUSIONS: The presence of RANKL protein in the membrane of osteoblasts and also the secretion of RANKL by osteoclasts has been hypothesised in earlier studies. In this study RANKL protein was observed for the first time in the membrane and in the long processes of osteocytes. This result strongly indicates the crucial involvement of osteocytes in orchestrating bone remodelling by influencing differentiation and activation of osteoclasts.

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ACKNOWLEDGEMENTS: Professor Berton Rahn, Christof Sprecher, Nora Goudsouzian of Tissue Morphology group Catrin Davies and the whole Interface Biology group of the AO Research Institute. Dr. med. Thomas Perren (Co-Chefarzt) from the Dept. of Orthopaedic Surgery in the Hospital Davos for human samples.

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3D MICRO-TOMOGRAPHIC IMAGING OF BONE AND BIOMATERIALS

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INTRODUCTION: Quantitative endpoints have become on important factor for success in basic research and the development of novel therapeutic strategies in biology and biomedicine. Biomedical imaging of three-dimensional (3D) biological structures has therefore received increased attention for it is often the basis on which both qualitative (i.e. visualization) and quantitative (i.e. morphometry) image processing is performed. Especially for the assessment of microstructural properties in small animals (i.e. mice), a number of new imaging modalities have been introduced in recent years. These techniques can typically be used to image a variety of different biological materials raging from soft to hard tissues. Whereas soft tissue imaging is actually a much larger market and is nowadays regularly used as a standard procedure to image and visualize biological structures, hard tissue imaging (i.e. bone) has made large progress with respect not only to qualitative imaging but also the direct 3D quantitative analysis of microstructural images in both human and animal bone and biomaterials.

In this article, strategies for new 3D approaches of quantitative image processing in the study of bone and biomaterials will be presented. The focus will be on the bioengineering and imaging aspects of research. With the introduction of microstructural imaging systems such as desktop micro-computed tomography (μCT), a new generation of imaging instruments has entered the arena allowing easy and inexpensive access to the 3D microstructure of bone and biomaterials, thereby giving researchers a powerful tool for the exploration of bone failure and reconstruction.

3D MICRO-CT IMAGING: Quantitative bone morphometry is a method to assess structural properties of the trabecular bone. Trabecular morphometry has traditionally been assessed in two dimensions, where the structural parameters are either inspected visually or measured from sections, and the third dimension is added on basis of stereology [1]. Micro-computed tomography is an alternate approach to image and quantify trabecular bone in three dimensions. Where early implementations of 3D micro-tomography [2,3] focused more on methodological aspects of the systems and required equipment not normally available to a large public, a more recent development [4] emphasized the practical aspects of micro-tomographic imaging. The project aimed to enlarge the availability of the technology in basic research and clinical laboratories. This and similar types of systems, now also commercially available, are typically based on a compact fan- or cone-beam type of tomograph, also referred to as desktop μCT, providing nominal resolutions ranging from 5 to 50 μm. Specimens with diameters of a few millimeters to around 80 mm can be measured. Desktop μCT has been used extensively for different research projects involving microstructural bone [5] but are especially useful for the assessment of quantitative endpoints in small animals; i.e. the mouse [6,7]. Since the introduction of these systems, there has been an increasing demand for micro-tomography throughout the world.

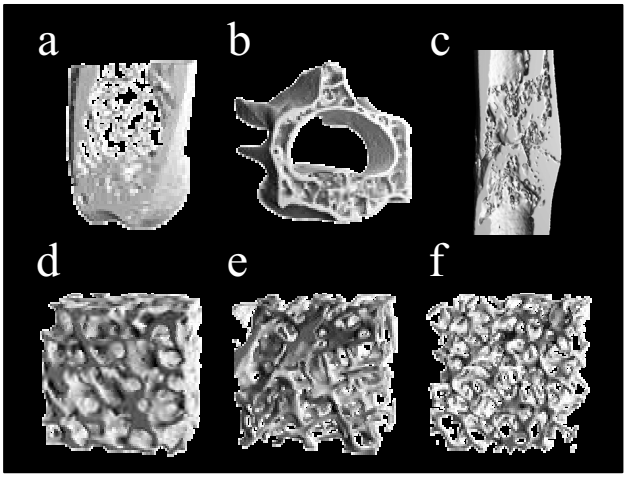

Fig. 1: Micro-tomographic images from a) mouse distal femur; b) mouse lumbar vertebra; c) mouse femur diaphyseal fracture; d) human femur; e) human iliac crest; f) human lumbar spine.

HIGH-THROUGHPUT PHENOMICS: Desktop μCT has been used extensively for different research projects involving quantification of the human skeleton (Fig. 1d-f) but the technique is equally useful for the assessment of quantitative endpoints in small animals; especially the mouse (Fig. 1a-c). Today, the mouse is the most important animal model in molecular biology and the genetics of bone. Because of the recent deciphering of the mouse genome and the high homology between mouse and human genome, the mouse is a ideal model to study the influence of different genes on skeletal phenotypes. Since the number of investigated animals per study can be very high (> 1000; Fig. 2), analytical methods need to be fully

automated and user independent to efficiently assess bone structure and function.

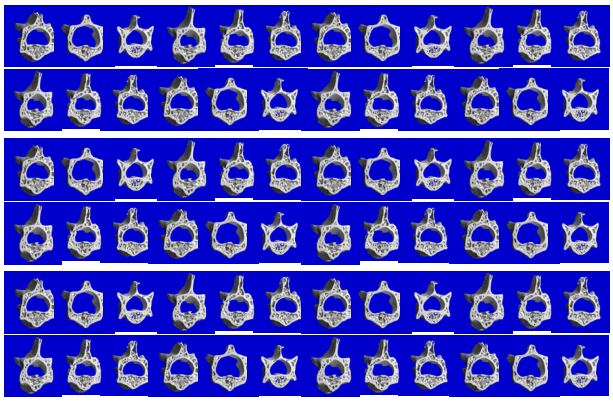

Fig. 2: High-throughput phenomics for genetic studies in mice: up to one hundred spines per day can be measured and automatically analysed with a resolution of 10 µm in three dimensions.

Despite the enormous data volume on the order of terabytes, high-throughput phenomics holds high promises. In a recent study using this technology, we were able to demonstrate that bone architecture and strength are influenced by different genes on different chromosomes and that in different bones different sets of genes are active [8].

IMAGE-GUIDED FAILURE ASSESSMENT: Although structural bone analysis can give interesting insight into how bone develops and changes over time, it can only provide estimates on a statistical basis on how bone fractures are actually related to these changes in bone architecture. For this reason, we have developed an image-guided technique [9] that utilize micro-compression in combination with micro-computed tomography and allows direct three-dimensional visualization and quantification of fracture progression on the microscopic level (Fig. 3). In order to compute local displacements and strains in the compressed structures novel image analysis approaches have been developed [10]. In that study, we found that the average computed local strain corresponded well with the applied global strain. However, local strains between nodes were found to be as high as eight and six times the global strain for compressive and tensile local strains, respectively, providing further evidence for a band-like, local failure behavior of trabecular bone (Figure 3). These strains were found in rod-like elements that were aligned with the main strain axis. Some of these elements bend, others buckle, and some are compressed. Inter-node strains can indicate active structure elements, but they cannot distinguish the different behaviors of these elements and how much energy is absorbed. They also do not correlate well with the amount of deformation in an element. Therefore, inter-node strains alone cannot fully explain the failure mechanisms of trabecular bone.

Micro-mechanical bone failure evaluation provides a novel experimental approach to monitor fracture behavior of trabecular bone. With upcoming synchrotron radiation based µCT systems we are now even able to extend micro-tomographic imaging to the nanometer scale opening up exploration of cellular and compositional analysis of normal and pathological tissues in the context of bone fracture and fracture healing. Future studies will investigate how failure behavior of pathologic bone distinguishes itself from normal bone and how this behavior can be linked to genetic factors.

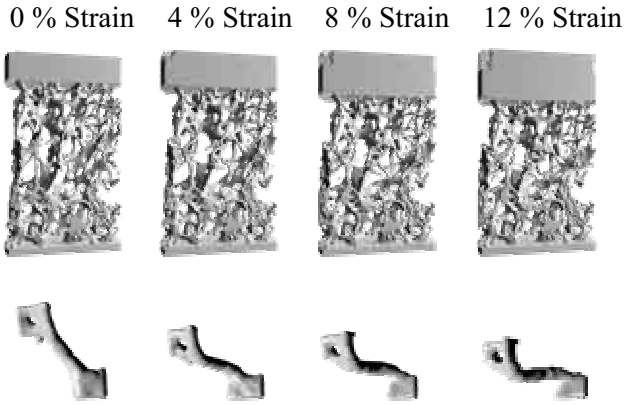

Fig. 3: Failure assessment in human spine using time-lapsed tomographic imaging. Upper row shows a specimen that is compressed and imaged in steps of 4% strain. The lower row shows how micro-compression can be used to non-invasively monitor the deformation of individual elements.

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ROLE OF THE EXTRACELLULAR MATRIX IN THE BONE-MATERIAL INTERFACE

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INTRODUCTION: The formation of direct bone-material contacts are the generally accepted prerequisite for successful skeletal implants. Maintenance of a material interface to living, properly mineralized bone under functional loading conditions is then called osseointegration. It is the aim of this review to show that not the bone cells, but the extracellular matrix of bone plays the important role in the beginning of bone ongrowth and ingrowth to the different materials surfaces, as well as during the whole lifetime of the respective implants in interaction with material specific degradation and, of course, design specific mechanical load transfer.

METHODS: Interactions of material specific properties and/or degradation with the adjacent bone matrix will be shown from experimental studies of the three major classes of implant materials: corrosion of a metallic material[1], degradation of calcium phosphate ceramics and glasses[2], and certain properties of polymers[3]. After intravital polyfluorochrome labelling, the implants have been embedded with surrounding tissues in polymethylmethacrylate, undecalcified ground sections prepared, surface-stained with Paragon- or Giemsa-solution, and evaluated by transmitted light and fluorescence microscopy. Mineralization was assessed on the same sections from corresponding microradiographs (MR) and back scattered electron (BSE) microscopic images.

RESULTS: Figure 1 shows the tip of a broken dental drill (manganate-steel) 16 weeks after "implantation" in the distal sheep tibia[1]. Deposition of secondary corrosion product iron-oxide caused demineralization of the surrounding bone (arrows) and rust-staining (see Fe-profile).

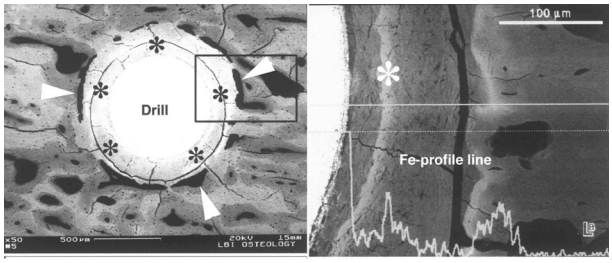
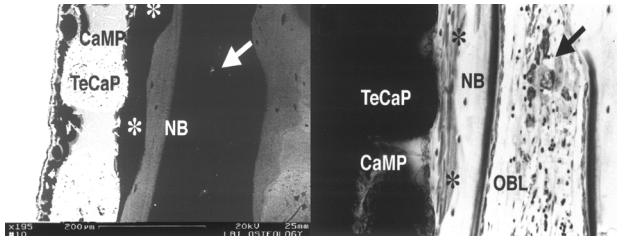
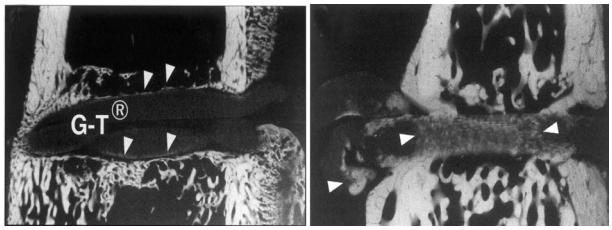


Figure 2 shows the pelvic bone interface of an implant coating (tetracalcium-phosphate granules/ calcium metaphosphate-glass), 16 weeks after implantation[2]. The seemingly void spaces on the BSE-image(*) are composed of demineralized new bone matrix, as can be concluded from the corresponding surface-stained section (right), and from fluorochrome labelling.



In Figure 3, the interfaces of strands of Gore-Tex® in rat tibial bone, 2 weeks (left) and 8 weeks (right) after implantation are shown[3]. Endosteal bone formation had started on mineralized matrix depositions (arrows) within the surface pores of this polymer, and had then virtually impregnated the implant with living bone (arrows).



DISCUSSION & CONCLUSIONS: As pointed out in a recent review[4], in vitro experiments and in vivo observations have shown that not only the "bioactive" ceramics and glasses, but also "bioinert" metal oxides, and even polymers allow for apposition of mineralized bone matrix. In course of time, however, material degradation and ion release may negatively influence mineralization.

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AN EX VIVO BONE CHAMBER FOR BONE TISSUE ENGINEERING

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OVERVIEW: The first publication on the use of mesenchymal stem cells on resorbable scaffolds for bone tissue engineering was in 1987 1. This German patent was based on a series of studies investigating the development of bovine mesenchymal stem cells and small slices of tissue cut from the germanitive zone of the juvenile porcine growth plate grown in the skin pouch of a juvenile nu/nu mouse (2). In the case of the bovine mesenchymal cells innoculation of 1 million cells which had been attached to collagen were scraped off the petri plate an injected under the skin. Figure 1 shows a section of a nodule about 2mm across after 4 weeks. The cells proliferate, but only those within about 100µm of the surface are forming bone. Underneath this bone layer is a zone of vital cartilage, and most of the tissue has is dead. By 6 weeks blood vessels have penetrated the dead cartilaginous tissue and new bone strats to form. The bony nodule is characterized by a disorganized growth. In the case of the 1mm slice of the gorowth plate, after 4 weeks a small rudiment had grown containing epiphyseal cartilage, a growth plate and trabecular bone (figure 2). As in the case of the mesenchymal cell experiment this newly formed bone was of mouse origin, suggesting that with the blood supply new stem cells forming bone were transported to the site. The growth plate and cartilageremained pig tissue however. These results showed that in addition to the growth and differentiation, some organizing factor was required to give shape to the new tissue. However the shape forming material should , if implanted, be able to resorb. We investigated a number of materials and one especially:- poly-l-Lctic acid with varying percentages of tricalcium phosphate (4,5) was investigated closely.

Clearly, in the absence of blood vessels the proposed scaffold should allow a good diffusion path. Krogh's Nobel prize is as applicable now as it ever was. The thickness of trabeculae seem to follow his limit of how far a cell can exist from a surface. In a tissue such as trabecular bone, the number of cells per volume of tissue is far lower than in other tissues, thus the oxygen usage per volume of tissue is low and cells might live by diffusion alone.

In these models an important factor was missing, mechanical forces. Using the flow chamber techniques we developed and adding this to a design for mechanical load we never realized, we built a loading chamber to apply and measure forces and deformations in trabecular bones or scaffolds: the zetos (fig 3). The results of these experiments conducted by us, the AO group of R.Geoff Richards, Everett Smith and Laurence Vico show, in brief, that the response to amplitude and frequency of loading is similar qualitatively to the responses found in vivo.

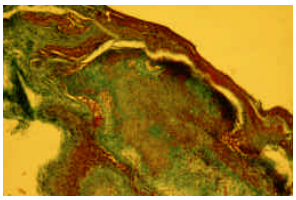


Figure 1

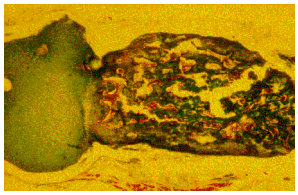
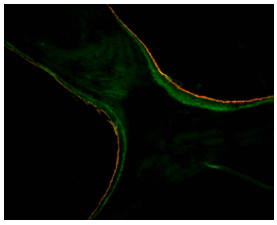


Figure 2

Masson Golder stained tissue grown in the skin pouch of nu/nu mice. Fif. 1 shown after 4 weeks injection of 1 million bovine stem cells. Fig. 2 shows the placement of a 1mm x 1mm x2mm slice of tissue. Both figures show tissue approx 3mm thick at the smallest dimension



F*ig 3. Fluophore staining of bone growth using Rahn's method of human trabecular bone grown in a Zetos chamber*

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ACKNOWLEDGEMENTS: We gratefully acknowledge support from AORF grants (2000-72 ;02-J7) and ESA (AO-99-122) for Zetos development.

LONG TERM PERFUSION LOADING OF TRABECULAR BONE CORES AND FORMATION RATE

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The purpose of this study was to determine human femur head trabecular bone core (5mm thick and 10 mm in diameter) response to mechanical loading at, 0, 650 and 1000 με, stimuli in a culture loading chamber and loading system. The computer-controlled high-voltage piezoelectric crystal stack permits bone specimens to be precisely loaded while force and compression were simultaneously measured. Static and dynamic histomorphometry responses of the loaded bone cores were investigated.

Twenty trabecular bone cores were obtained from a human femur head of a 68-year-old male patient who had hip replacement do to coaxarthrosis. The perfusion medium was Dulbecco Modified Eagle Medium (DMEM) containing: 10% fetal calf serum (FCS), 72.2mg/l Ca and 28.5mg/l P, 2 mM glutamine, 5 mM ß-glycerophosphate, streptomycin and penicillin G at 50,000 U/l each, vitamin C 10 ? g/ml, 0.12g/l of NaHCO3 and 10 mM Hepes. All bone cores and medium were maintained at 37 o C, in a controlled temperature room, and a pH of 7.2-7.3 throughout the study periods. The cores were perfused at a rate of 3.2-3.4 μl/hr using an Ismatec pump. The medium was changed at 48-hour intervals. Three groups were randomly formed, control none loaded (N=6), loaded at 650 me (N=7) and at 1000 me (N=7). All bone cores were loaded in the loading chambers, for 300 cycles/day, at 1 Hz for 49 days. Bone cores were label on days 19, and 32 with Calcein for two hours, and on day 44 with Alizarin red for 4 hours.

There were no differences among groups for MAR detected between control (0.425 μm/day), and loaded bone cores 650 με (0.534 μm/day) and the 1000 με (0.486 μm/day) over the 49 day study by ANOVA, therefore results are reported as mean (SD) for all groups. The relative bone volume of the cores averaged 26.6 (9.8)% of total volume. Alizarin red label on day 44 was extensive with 79 (23)% labeled surface. Double label surface from day 34 and 44 averaged 15 (14)% and MAR averaged 0.48 (0.18) ? m/day. Only two sections had double calcein labels (day 19 + 34). Stained sections show abundant osteoid, osteoblasts, and scattered osteoclasts indicating continued bone turnover.

We concluded that while the level of mechanical loading was not adequate to increase the rate of bone formation, all bone cores showed MAR and resorption over the 49 days study as demonstrated by the presents of both double and triple labeled trabeculae. It is also clear from the histological sections that there are active osteoblast and osteoclast at the 49th days of the study.

In addition these ex vivo bone cores demonstrate mineral apposition rates similar to those from in vivo double labeled iliac crest biopsies in 11 females (65-74) of .477 ? m/day (Recker 1988) and in 22 males (27-72) of .517 (.075). This study also indicates that 650 and 1000? ? were not sufficient to alter bone formation in the human femur bone cores using this loading regiment. Future studies will examine different loading cycles, magnitude of loading strains, and loading waveforms.

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PERFUSION CULTURE FOR CORTICAL BONE

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INTRODUCTION: Cell monolayers or organ cultures are still the most widely used tools in studying tissues in vitro. However, since both of these methods have limitations, the technique of perfusion chambers has widely spread and is in use by more than 100 research groups for a broad spectrum of studies. The advantages of ex vivo perfusion systems are obvious, with respect to reducing the amount of test animals used for biocompatibility studies and to overcome the drawbacks of in vitro cell and organ cultures. However, to study cortical bone in a set up that is suitable for investigations of material, bone and bacteria interactions we had to develop a new technique [1].

METHODS: Tibial bones from Swiss Mountain sheep's were acquired from the local slaughterhouse. With a sterile preparation process the bone was exposed stripped from periosteum and discs with a diameter of 10.6 mm were prepared with a saline cooled, diamond edged trephine (STRATEC). Bone cores were washed in medium containing antibiotics for 30 minutes before placed into the chambers.

We used custom-made bone chambers that consist of polycarbonate, which could be steam sterilized and reused several times. With an inlet at the lower and an outlet at the upper side of the chamber the in-between lying bone-disc is perfused in a perpendicular flow pattern with fresh media with the aid of a minimal dispersing (1 ml/ hr) computer controlled roller pump.

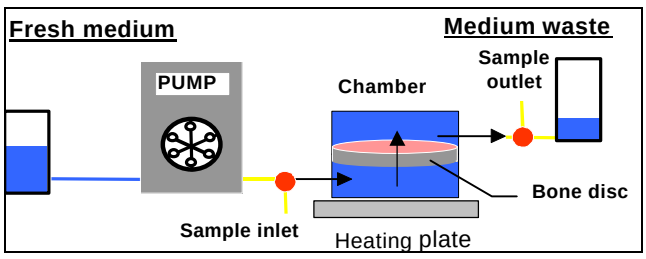

Fig. 1: Diagram of experimental setup

The system is a closed semi circuit system with cooled sterile media supply, a roller pump, valves for sampling before and after the chamber a warming plate at 37°C with isolation hood and disposal bottles for the used culture media. To use the system outside an incubator we also adapted the commercially available culture media BGjB [2] with HEPES buffer so that a carbon dioxide independent constant pH was maintained. To proof the viability and stability of the culture media we analyzed the in- and outflow daily for sodium, potassium, calcium, magnesium, glucose, lactate and pH. Cultures were run up to 21 days.

Fig. 2: Cart with warming plate, roller pump, laptop PC, refrigerator and waste bottles (left), close up of warming plate with 8 chambers (right).

Daily drawn 1 ml samples were analyzed in the laboratory of the local hospital. At the endpoint of every experiment the samples were perfused for 30 minutes with procion red and subsequently fixed in 4% formalin and embedded in resin for histological analysis.

RESULTS: To maintain a stable pH of 7,4 a flow of 2 ml per hour and a supplementation of 15 mM HEPES proved to be sufficient. However, a drop in pH during the first two days corresponded with the increase in lactate of up to 1 mmol/l. Potassium levels were higher in the beginning, the other electrolytes remained stable during the experiments. The culture medium was oxygenated by diffusion through the gas permeable silicon tubing. Portion red staining showed that there is a distinctive flow pattern across the perpendicular perfused bone discs. Histological evaluation revealed viable tissue in the center of the discs, but some destruction at the edges.

DISCUSSION & CONCLUSIONS:

In the recent years new models were developed to study bone ex vivo with rather sophisticated tools as in vivo loading [3]. However, to study

osteomyelitis only animal models and bacterial in vitro models so far are available. The bone disc in this model serves as a barrier between two compartments, thus resembling the inner and outer part of cortical bone. The system demonstrated to maintain ovine bone cells (osteoblasts, osteoclasts, osteocytes) viable up to 21 days. In pilot experiments we already used this two-compartment model in conjunction with different implant metals and bacteria. Since the interactions of osteosynthesis materials, bone and bacteria are essential in the course of an infection; our model seems capable of filling this gap in-between the above-mentioned methods, thus possibly reducing further the need of using living animals as test objects.

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FRACTURE HEALING IN VITRO

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INTRODUCTION: Since the early works of Fell in 1929 [1] it is known that femoral rudiments, isolated from newborn fowls are capable of undergoing considerable histological and morphological development when cultivated in vitro. Other researchers from those early days of tissue culturing already used Fells model for studying fracture repair *in vitro* [3, 4] with loosely attached fractured bone ends. However, a current literature search revealed only a few publications using isolated bones in respect to fracture healing [2]. Using modern cell culture technologies (sterile laminar flow, defined culture media, incubator) the following study was designed to elucidate the prospective of using isolated complete rat femurs as a model for studying fracture repair in vitro.

METHODS: Wistar rats aged 6 days were used in three groups, 10 animals each, with the endpoint of one, two or three weeks. Both femora of every animal were explanted under sterile conditions in a laminar flow with the use of microsurgical instruments and a stereomicroscope. All bones were intact macroscopically after explantation and stripping of the periosteum. The remaining tissue at the bone surface was mildly digested in growth media with 0,02% Collagenase A for 45 minutes, followed by washing in Tyrode's Balanced Salt Solution (TBSS). Custom-made plates (0.5 x 1.5 x 3 mm) from high-grade silicone (Angst und Pfister AG, Zurich, CH, Cat.No.AP1010192503) were steam sterilized and used as an external fixation frame. Four sterile acupuncture needles with a diameter of 0,3 mm (C-Type, No. 8, 0.30 x 50 mm SEIRIN, Germany) were inserted in a rectangular fashion through a gauge with four evenly spaced 0.3 mm openings into the bone and the silicone plate. The artificial fracture was made by a cut in the middle of the diaphysis with a scalpel on the surface of a sterile glass slide. The fixed bones were submersed in 6-well culture dishes with 5 ml media of BGJb (Biggers, Gwatkin, Judah with Fitton Jackson Modification; Life Technologies 22591-010) with 50µg/ ml sodium ascorbate. No antibiotics were used. Culture media was changed every 72 hours and inspected for infections. At the endpoint of every experiment the femurs were x-rayed with contact micro radiography, subsequently fixed in 4% buffered formalin and embedded in LR-White resin for further histological evaluation. The Movat pentachrome staining I was chosen to investigate the fracture gap and possible healing process.

RESULTS: Development of the rudiments in all groups continues to progress normally compared to uninjured, unfixed controls, i.e. increase in length and breadth, with normal histogenesis of cartilage and bone. During enlargement the epiphyses retained their characteristic shape, their histological differentiation continued and appeared similar to the in vivo pattern. The maximum increase in length during cultivation was about 30% after 3 weeks. No antibiotics were used, however there was only a dropout rate due to infection of 10% evenly distributed among the groups. After one week a fibrous tissue filled the fracture gap, which was partly replaced in the later course with osteoid, however the replacement by osteoid tissue in the epiphyseal regions continued normally. In cases of misalignment of the fracture gap larger than 0,5 mm calcification was only partly.

Fig. 1: View from above (left) and side view (right) of external fixed rat femur with four stainless steel acupuncture needles (0,3mm). Scale and white silicone frame below.

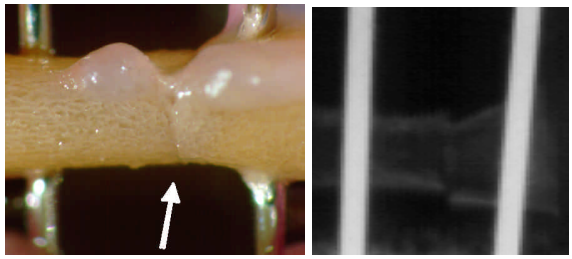

Fig. 2: Fractured femur, after 2 week in vitro culture, close up view (left), micro radiography (right).

DISCUSSION & CONCLUSIONS: The mammalian epiphysis is capable of continuing its characteristic histological differentiation even when deprived of nerve and blood supply and cultivated in a heterologous medium outside the animal body. In this experiments femora of newborn rats were used which still expresses embryological growth pattern but are on a higher stage of development compared to the rudiments used by Niven [4] and Krull [3], therefore external fixation was possible, resembling an osteosynthesis. The observed calcification of the fracture gap was also very dependent on the precise alignment of the fracture ends and was only partly in cases of misalignment larger than 0,5 mm. The author would like to acknowledge the authors of the listed references who described basic methods and ideas of our experiments 70 years ago, however only few studies were published ever since which made use of this old knowledge. Since the cultures are easy to handle and the setup is inexpensive, more sophisticated experiments involving growth factors could profit from this old, newly reinvented in vitro model.

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STANDARDISED CHARACTERISATION OF PERI-IMPLANT BONE STRUCTURE USING CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

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INTRODUCTION: The histologic evaluation of bone adjacent to dental implants is mostly done using the sawing and grinding technique according to Donath and Breuner and the evaluation of stained thin sections by light microscopy. A less time and resources consuming method would be desirable.

METHODS: 190 dental implants (6 different types) which were derived from two different bone types (mandible and maxilla) of beagle dogs were used for this study. The implants were embedded in PMMA (Technovit) and cut along their long axis. The non-decalcified, unstained blocks were evaluated by CLSM (Leica) in the reflection mode (488nm). With a 10x objective images of edge length 1,5 mm, 1024 * 1024 pixel were obtained. An automated biomapping technique with an automated sample stage generated consistent images of the whole implant. Serial scans in the z-axis were obtained to overcome difficulties of obliquities of the sample surface. The maximum projection images of these image staples produced the highest contrast for further qualitative and quantitative evaluation of the periimplant hard tissue.

RESULTS: In all cases high contrast images for the qualitative histologic evaluation were obtained. For the histomorphometric analysis images of the unstained samples it was possible in all cases, after applying a median filter, to process segment using a simple threshhold technique.

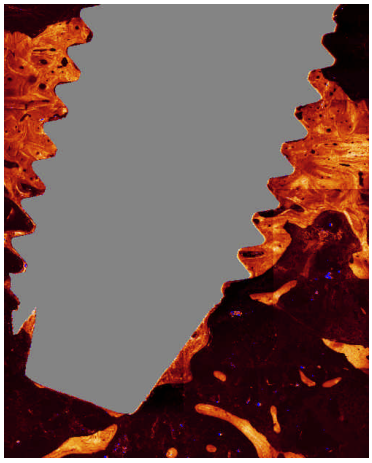

*Fig. 1: CLSM reflection image of the total implant 9*6 mm (merging of 6*4 images)*

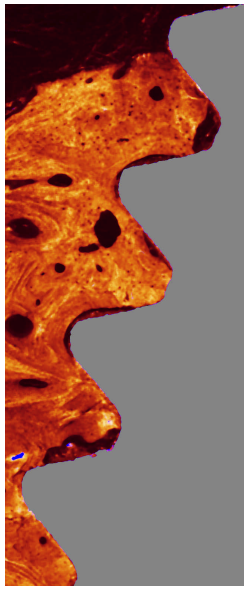

*Fig. 2: CLSM reflection image of the bone implant border 1*3 mm*

DISCUSSION & CONCLUSIONS: The CLSM technique in the reflection mode can be used a semi-automatic, standardised technique for the evaluation of the peri-implant bone. The evaluation of unstained, non-decalcified bone blocks minimises possible influences of the grinding and staining process.

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BIOACTIVE GLASSES MODULATE TISSUE-SPECIFIC GENE EXPRESSION IN RAT NASAL CHONDROCYTE CULTURES

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INTRODUCTION: The replacement of bone tissue is a common problem in medecin and dentistry. Autografts are known to be the ideal bone replacement solution for cases of bone loss in both orthopedic and periodontal surgery. However, problems such as limited donor supply have led us to think about other types of bone substitutes such as alloplastic materials. One category of these biomaterials is the bioactive glasses tested in this study. Previous studies have shown that this material promotes cellular differentiation and can be entirely integrated to bone by forming a chemical bond with the host tissue. However most of these studies are made on bone cells, for instance the studies realized in our laboratory[1, 2], have demonstrated *in vitro* a stimulation of osteogenesis.
It is of interest that during long bones formation, the process is preceded by a stage of endochondral ossification, in other words the formation of a cartilaginous tissue that is resorbed and replaced later by bony tissue. This process also occurred during bone healing following trauma or placement of implants. For this reason we decided to evaluate the effect of 45S5® bioactive glasses on the behavior of chondrocytes *in vitro*. To achieve our goal we used primary cell culture, that mimics in the closest way, what happens during endochondral ossification *in vivo*, from the stage of cell proliferation till the stage of hypotrophic chondrocyte and finishing by matrix mineralization[3]. We analyzed the phenotypic expression of different cartilaginous markers such as type II collagen, aggrecan, Cbfa1, Sox 9, Indian Hedgehog (Ihh), alkaline phosphatase and type X collagen.

METHODS *Biomaterial tested* : the biomaterials used were 45S5® bioglasses composed of (in weight %) : 45 % SiO_2, 24,5 % CaO, 24,5 Na_2O, and 6% P_2O_5. 60S granules were used as control in our experiments.

Culture model : chondrocyte were enzymatically isolated from nasal septum of 21 day- old fetal Sprague Dawley rats, as previously described[3]. Briefly, nasal septum were aseptically dissected and fragments incubated in phosphate buffered solution with collagenase (400 U/ ml) and hyaluronidase (750 U/ ml), for 2 hr at 37°C. Then cells were dissociated from the cartilage fragments, and plated at 3.5 x 10^4 cell/cm^2. The glass granules were added on day 1 of culture. All cultures were incubated in DMEM supplemented with 10% foetal calf serum, ß-glycerophosphate, and ascorbic acid, in a humidified atmosphere of 5% CO_2 in air at 37°C.

Transmission electron microscopy : The cells cultured with bioglass particles, were fixed on day 10 in Karnovsky solution (4% paraformaldehyde, 1% glutaraldehyde) for 1 hour. After several rinses in 0.2M sodium cacodylate buffer (pH 7.4), cell cultures were post-fixed for 1 hour in osmium tetroxide diluted in 0.2M sodium cacodylate buffer. The cells were then dehydrated in graded series of ethanol and left overnight in a mixture of absolute ethanol and epon (1:1). The next day the cells were embedded in Epon Araldite and incubated at 60°C for 1 day. Ultrathin sections were performed, collected on copper grids, and stained with 2.5% uranyl acetate in absolute ethanol for 4 minutes and lead citrate for 2 minutes. The sections were then examined under a TEM (Philips CM-12).

Alkaline Phosphatase activity : The specific activity of alkaline phosphatase was assayed in cell layers as released p-nitrophenol from p-nitophenolphosphate. The optical density was read at 410 nm, and the enzyme activity was expressed as unit per milligram of total proteins (estimation of protein content was carried out using the Pierce BCA protein assay kit).

Total RNA isolation and real-time RT-PCR : total RNA was extracted at day 5, 9, 12, 16, and 20 using a phenol/chloroform method (Tri reagent®). The concentration and purity of RNA were determined by light absorbance at 260nm and by calculating A_{260}/A_{280} ratio respectively. The integrity was confirmed by electrophoresis on an agarose ethidium bromide gel. Two micrograms of total RNA from each sample was used for Reverse Transcriptase (superscript II®), using oligo(dT) primer. Then cDNA obtained were used for quantitative PCR (light cycler, Roche diagnostic®) using forward and reverse primers with sybrgreen method. Each RNA sample was analyzed in triplicate for Aggrecan, Sox9, Type II and X collagen, Cbfa1/Osf2 and Ihh mRNA, and was corrected for GAPDH mRNA levels, used as a reference.

RESULTS: Phase contrast microscopy showed multiple zones of mineralization, but more abundant in cultures with 45S5 when compared to 60S. Transmission electron microscopy showed the establishment an intimate contact between the cells and the granules, a collagen-rich matrix was also observed in both cultures. Alkaline phosphatase activity measures at day 5, 8, 12, 15 and 18, showed that the enzyme gradually increased up to day 15 and then decreased on day 18. The levels of ALP activity were higher at all times, in cultures with 45S5 bioglasses when compared to control. Furthermore analysis of the expression of specific chondrogenic markers was determined at day 5, 9, 12, 16, and 20 using real-time RT-PCR. Chondrocytes in both cultures expressed the specific makers but at a higher level in 45S5 cultures when compared to control. Finally, the tissue-specific gene expression coincided with the temporo-spatial pattern observed during *vivo* chondrogenesis.

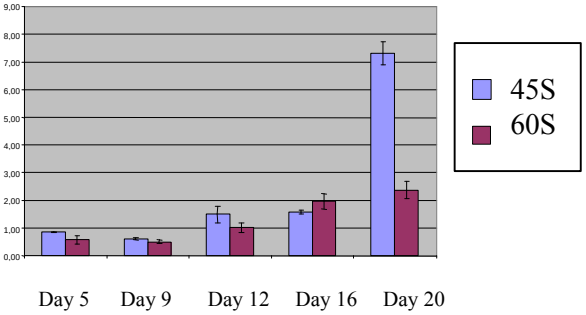

Fig. 1 Effect of 45S5 granules on type X collagen expression, in primary chondrocyte culture.

DISCUSSION & CONCLUSIONS: Our results have shown that bioactive glasses cannot only support the proliferation of chondrocytes *in vitro*, but can also stimulate their differentiation. Alkaline phosphatase activity, increased in cultures of 45S5. The stimulation of the expression of various early and late chondrogenic markers, as well as transcriptional factors such as Sox 9 or Cbfa-1, indicates that bioactive glasses can modulate the expression of these genes, and promotes cellular differentiation. For these reasons, bioactive glasses appeared to be an ideal scaffold that supports and promotes cell proliferation and differentiation in the domain of tissue engineering.

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ACKNOWLEDGEMENTS: The 45S5® bioactive glasses and the bioinert 60S glasses, were kindly provided by US Biomaterials Corporation - Nova bone (Alachua, FL, USA).

CHARACTERIZATION OF FETAL TRABECULAR BONE CELLS FOR TISSUE ENGINEERING

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INTRODUCTION: Each year, 450,000 bone grafts are performed in the USA alone. Tissue engineering to replace diseased tissues with living substitutes has been attracting significant interest in the area of medical technology. Three elements are central in bone tissue engineering: 1) scaffold, b) cells, and c) proteins. In our laboratory we have been interested in fetal cell tissue engineering as bone cells originating from fetal tissue have the advantage that they are less differentiated, possess a vast capacity for multiplication and due to their immaturity can more easily pass the immunological incompatibility seen with adult cells. By associating fetal bone cells with a biodegradable scaffold, it is possible to obtain a cellular tissue where the cells are protected/resistant and that could easily be transplanted for tissue repair.

METHODS: Bone biopsies: Bone samples were obtained from a 30 year old patient during hip surgery and from fetal tissue following voluntary abortion (16 weeks) and tissues were obtained with accordance with the University Hospital Ethics Committee. Osteoblast culture: primary osteoblast cultures were established by rinsing tissue in PBS (containing penicillin and streptomycin 3 times). Trabecular bone was mechanically dissociated with a scalpel blade and medium was added (DMEM + 10% FCS). Medium was changed every two days. Growth curves: Cells were seeded at 1 x 10^4 cells. At each time point, cells were detached with trypsin/EDTA, centrifuged and counted.(triplicate). Alkaline Phosphatase activity: The alkaline phosphatase activity was measured using a kit (Sigma chemicals Co.). Treatment: Cells were treated with vitamin D3 10^{-8}M and dexamethasone 10^{-8}M. The activity was normalized to total cellular protein, wich was determined by a Bio-Rad protein assay. 3-D culture on scaffold: Biodegradable scaffolds (Baxter Tissue Fleece, native equine collagen) were used as a support for fetal bone cells. Cells were seeded into scaffolds and following 5-15 days, scaffolds were mounted in O.C.T. and frozen in isopentane cooled in liquid nitrogen. Frozen sections were cut and colored with H&E

RESULTS and DISCUSSION: We have seen that fetal bone cells can be isolated and have alkaline phosphatase activity after differentiation treatment (Fig.1). They have very high dividing potential when compared to cells of the 30 year old patient. In addition, fetal bone cells grow extremely well in association with a biodegradable collagen scaffold (unlike adult cells) which results in the creation of a 3-D tissue, which is easily manipulated.

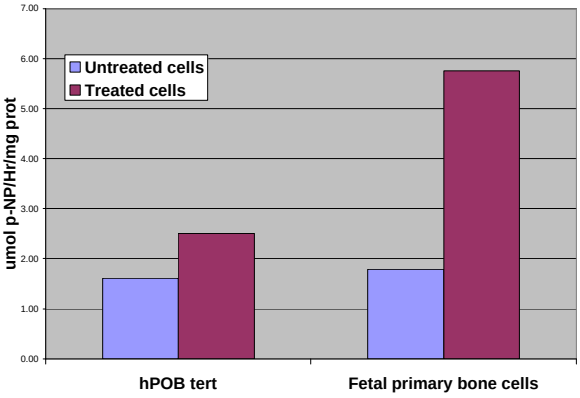

Fig. 1: Alkaline phosphatase expression after 1 week culture treatment with vitamin D3 and dexamethasone in comparison with an immortalized cell line.

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DISTINCT EXPRESSION OF APO-1 & CASPASE-8 IN HUMAN GROWTH PLATE

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INTRODUCTION: Enchondral ossification, the replacement of cartilage by bone in the human growth plate (physis), is the basis of longitudinal bone growth. Former studies showed that chondrocytes in the growth plate are assumed to undergo apoptosis, a form of programmed cell death. Apoptosis is an active, biological process, occurring in several tissues, predominantly when cells reach the end of their lifespan. Apoptotic cells start a cascade of self destroying mechanisms and are morphologically characterized by nuclear chromatin condensation, cell shrinkage, decay in apoptotic bodies and phagocytosis by immuno-competent cells. Before growth plate chondrocytes are removed by apoptosis, they pass through several stages of differentiation and maturation. This fact is expressed by four separate histological zones of the physis, including the resting zone (RZ), proliferating zone (PZ), hypertrophic zone (HZ) and degenerative zone (DZ). The first three mentioned zones are areas of protein synthesis, cell increase and formation of collagen type X, while the DZ is characterized by collagen decay, vascularisation and massive calcium release into the matrix. Although the morphological features of apoptotic chondrocytes are well known, there is little information about the mediators which regulate these complex cellular mechanisms in the growth plate. APO-1, also termed FAS or CD-95, is a member of the tumor necrosis factor (TNF) family and was already proven in rabbit growth plates. Caspase-8, former called FLICE, belongs to the interleukin-1ß converting enzyme (ICE) family of cysteine proteases. Caspases are synthesized as inactive precursor forms and are activated by proteolytic cleavage. The aim of our study was to investigate the role of APO-1 and Caspase-8, two pro-apoptotic proteins, in chondrocyte maturation and differentiation in the human physis by the technique of immunohistochemistry (IHC).

METHODS : We used the human growth plates of resected polydactylic fingers of five infants (f:m=1:4; mean age: 16,6 months; range: 13-22 months). For immunohistostaining, by the biotin-avidin staining method, the material was fixed in 10% formalin for three days and then embedded in paraffin. After deparaffination through xylene and graded alcohol, the material was cut into slices 4µm thick. For removal of endogenous peroxidase, the specimens were treated with 3 per cent H_2O_2, followed by antigene retrieval in the microwave (10', 150 W). The sections were then incubated either with the anti-APO-1 antibody or with the anti-Caspase-8 antibody at the dilution of 1:100 at 4°C for 18 hours, followed by incubation with the biotin-labelled antibody solution for 30 minutes, respectively. The reaction products were developed with Triton and 3,3' diaminobenzidine and counterstained with haematoxyline.

Control section were incubate with anti-D antibodies. Quantification of APO-1 and Caspase-8 was performed by light microscope according to the calculation method of Ueda et al. or obtaining the positive ratio of the Ki-67 index. In each zone (RZ, PZ, HZ) of one section positively and negatively stained cells in five fields, respectively, containing the largest number of positive cells, were counted three times at a magnification of x200. The mean number of positive chondrocytes was divided by the total number of cell in every single field. Statistical analysis was done by the Kruskal-Wallis test, p values given as H for corrected ties ($p<0,05$ was considered significant). All data are given as mean ± SD.

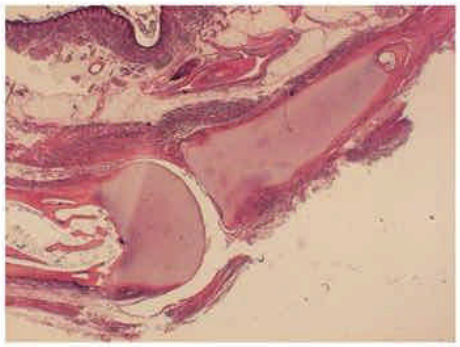


Fig. 1
Growth plate stained with hematoxylin-eosin

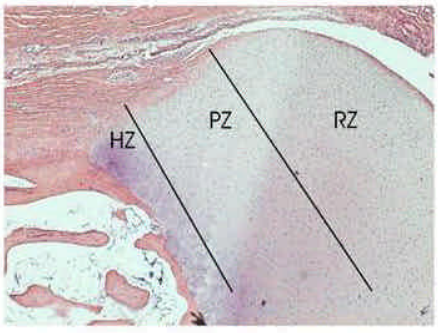


Fig. 2
Different zones of the growth plate

RESULTS: APO-1 positive and Caspase-8 positive chondrocytes were found in all three investigated zones (RZ, PZ, HZ) of the physis. The expression of proteins increased from the RZ to the HZ near to closure of the growth plate. 13,17±1,8% cells in the RZ were positive for Caspase-8, 19,33±2,91% in the PZ and 28,22±2,13% in the HZ ($p<0,001$). APO-1 was expressed in 17,4±6,174% in the RZ, in 22,1±6,2% in the PZ and in 33,067±8,39% in the HZ ($p<0,001$).

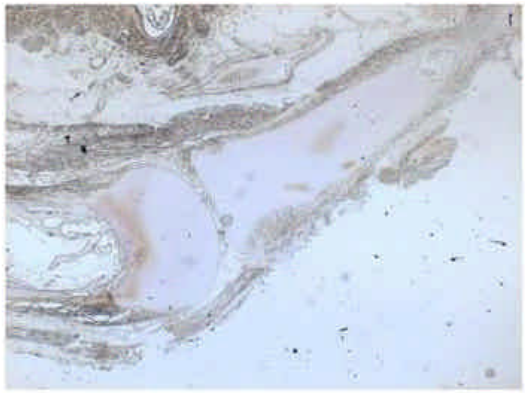


Fig. 3
APO-1 staining of the growth plate

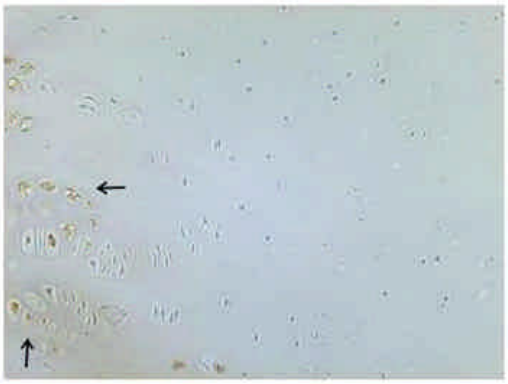


Fig. 4
↗ Positive cells for APO-1 in the HZ

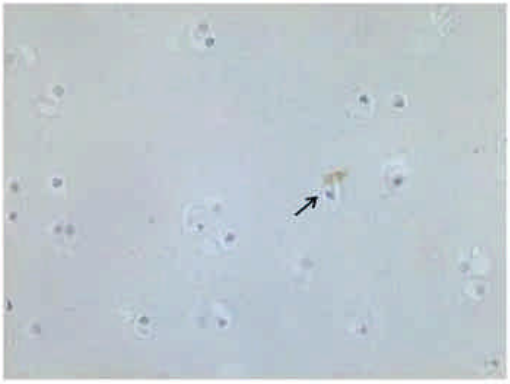
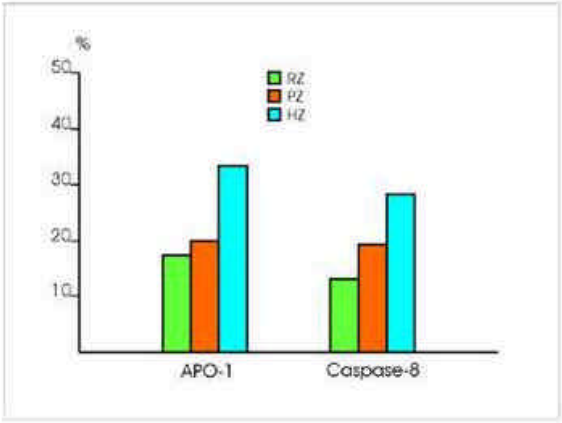


Fig. 5
↗ Caspase 8-positive cell in the RZ



Tab. 1 : Quantitative distribution of APO-1- and Caspase-8- positive cells in the different zones of the physis

Conclusion: Physiological cell decay and cellular regeneration is essential for tissue homeostasis in many mammalian organisms. This steady state of cells is, maintained by apoptosis, a physiological way of cell death. Apoptotic elimination of chondrocytes in the growth plate is the basis for secondary ossification through vascular ingrowth and calcification in the cartilaginous bony anlage and thus facilitates longitudinal bone growth. Chondrocytes in the RZ are ordered single or paired and represent the cell pool for the following maturational processes. In the PZ chondrocytes undergo rapid cell division and are arranged in vertical columns in the HZ.

The findings of our study indicate that both APO-1 and Caspase-8 have a distinct expression pattern during chondrocyte differentiation and further a significantly increasing expression in the HZ. Our data suggest that pro-apoptotic proteins are involved in the growth plate regulation of human chondrocyte maturation. Cartilage tissue engineering made great progress in the recent years, so therefore apoptotic regulation mechanisms should be considered in this promising technique. Our results may also be helpful in the knowledge of incorporation of transplanted autologous chondrocytes and the origin of skeletal disorders.

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THE MOLECULAR AND CELLULAR CHANGES UNDERLYING INCREASED BONE FORMATION IN A MOUSE MODEL WITH GENERALIZED PROGRESSIVE HYPEROSTOSIS

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INTRODUCTION: Schmid metaphyseal chondrodysplasia (SMCD) is an autosomal dominant disorder caused by mutations in *COL10A1* gene, characterized by drawfism, *coxa vara* and flaring of the metaphysis. The majority of the mutations reported are clustered within the carboxy-terminal NC1 domain. These mutations are though to impair trimer assembly that initiates from the NC1 domain. To study the pathological and molecular consequences underlying this disease, we have produced transgenic mice expressing a SMCD mutation in the mouse collagen X gene, a 13-bp frame-shift deletion in the NC1 domain. The phenotypes of the mice showed characteristics of SMCD such as drawfism, shortened limbs (figure 1) and growth plate abnormalities.

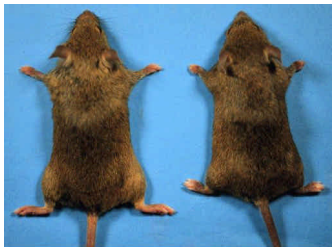

Fig. 1: collagen X gene mutant mouse (right) show characteristics of SMCD patients such as drawfism and short limbs when compare with a wildtype mouse (left).

However, we observed an additional phenotype of generalized progressive hyperostosis, which is not seen in SMCD patients, with thickening of bones (figure 2), suggesting deregulated bone growth and/or remodeling. Bone remodeling is important in maintaining the quality and quantity of bone and mineral homeostasis. It involves two tightly-coupled but opposing events, bone formation and bone resorption which are carried out by osteoblasts and osteoclasts, respectively. Normally, bone formation is in balance with bone resorption to maintain a constant bone mass.

Altered balance between these two processes can lead to hyperostosis (too much bone) or osteoporosis (not enough bone).

Fig. 2: X-ray shows the bone density of the entire skeleton in transgenic mouse (right) is higher than non-transgenic littermate (left).

METHODS: Using *in vivo* and *in vitro* approaches, we systematically analyze parameters for osteoblast and osteoclast activities in this transgenic mouse model.

RESULTS: Osteoclast analysis indicated that the number and activity are not reduced, suggesting that altered resorption is not a major contributing factor. However, in situ hybridization showed that osteoblasts are more active in producing Collagen I, a major bone matrix component. Cultured osteoblasts supplemented with serum derived from transgenic mice showed elevated Collagen I synthesis compared to that of non-transgenic serum, strongly suggesting that a humoral factor is simulating osteoblast function in this transgenic mouse.

DISCUSSION & CONCLUSIONS: Work is currently underway to identify this systemic factor. Uncovering the molecular basis for bone overgrowth in these mice will provide insight into the regulation of bone formation and the potential for discovering new drugs that can stimulate bone formation. This will be clinically relevant for the treatment of osteoporosis and allied conditions.

ACKNOWLEDGEMENTS: This project is supported by the Research Grants Council of Hong Kong.

CALCIFICATION AND OSSIFICATION IN OSTEOBLASTIC CELL CULTURES.

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INTRODUCTION: When cultured in appropriate osteogenic media, osteoblastic cells form a calcified extracellular compartment[1-3]. These cultures are often used as reference models to study biomaterials on their osteoinductive capacity, including the formation of a mineralized matrix. To be equivalent to bone extracellular matrix, this compartment has to contain collagen I fibers on which bone hydroxyapatite crystals are deposited. Although many papers describe mineralization in cultures of osteoblastic cells, no clearcut correlations between the origin of the cultured cells and the observed mineralization patterns are described. We evaluated and compared mineralization in cultures of UMR-106 osteosarcoma cells and freshly obtained osteoblastic cells derived from different tissue sources.

METHODS: *Cell cultures* -UMR-106 osteosarcoma cells were purchased from ECACC. Osteoblastic cells were obtained from fetal rat calvaria and long bones (enzymatically and by explant) and adult rat long bones, periost (explant) and bone marrow. To increase the number of cells standard culture medium was used (DMEM, 10%FBS, 100µM L-ascorbic 2-phosphate). To induce differentiation, cells were cultured in osteogenic medium (α-MEM, 10% FBS, 4mM L-glutamine, 100 µM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate). As a reference culture UMR-106 osteosarcoma cells were used.

Assays for osteogenic differentiation

Alkaline phosphatase activity (a parameter for early osteoblast differentiation) was measured spectrophotometrically (using p-nitrophenylphos- phate as a substrate and absorbance measurements at 405 nm) and histochemically (using BCIP/NBT as a substrate and aceton fixed cells).

Mineralization of the extracellular matrix was evaluated by histochemical methods (Von Kossa and Alizarin Red S staining), TEM and infrared spectrometry.

RESULTS: Data concerning ALP-activity and mineralization in the different cell cultures are summarized in Table 1.

Table 1. ALP activity (mM pNP/mg protein) and mineralization patterns in different cell cultures

	ALP	mineralization
UMR	> 45	dispersed
fetal long bones/exp	> 10	dispersed
fetal long bones/enz	> 15	dispersed
adult long bones/exp	> 10	dispersed
fetal calvaria/exp	5	absent
fetal calvaria/enz	> 15	nodules
periost exp	> 45	nodules
bone marrow	> 45	nodules

(exp = explant / enz = enzymatically)

Despite the differences in mineralization patterns, infrared analysis showed that the mineral formed in all cell cultures was hydroxyapatite . Electron microscopy showed the presence of needle like structures in the extracellular compartment, representative for hydroxyapatite crystals. In calvaria, periost and bone marrow derived cell cultures, the extracellular compartment contained collagen I fibers and matrix vesicles in which crystal formation was observed. Collagen fiber formation in UMR and long bone derived cultures was not obvious.

DISCUSSION & CONCLUSIONS: To investigate biomaterials on their osteogenic potential, meaning the stimulation of osteoblastic cells to form an organic matrix and the consequent mineralization of the matrix and to be sure that the biomaterials not just are associated with dystrophic calcification, it is important to use the right cell cultures. Osteoblastic cells derived from long bones and commercially available osteosarcoma cell lines should not be used, because in these cell culture models ossification is hard to distinguish from non-osteoblastic calcification. Freshly obtained cell cultures from either periost, calvaria or bone marrow are preferred.

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OSTEOBLASTIC CELLS TO STUDY CELL/BIOMATERIAL INTERACTIONS: A COMPARITIVE STUDY

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INTRODUCTION: To study interactions of osteoblastic cells with biomaterials, osteoblast-like cell lines from different species are often used. Although cell lines can be used for biocompatibility testing, it is more convenient to use freshly isolated osteoblastic cells to study cell adhesion and the osteoconductive and -inductive properties of the biomaterials. Consequently a sufficient amount of easily obtained and well-characterized osteoblastic cells capable of differentiating is a useful tool to study biomaterial/cell interactions essential for bone tissue engineering.

Osteoblastic cells were derived from adult and fetal rat via different isolation techniques. The isolation and in vitro proliferation of primary cultures and the osteogenic potential of subcultures were compared.

METHODS: Osteoblastic cells were isolated (enzymatically or explant cultures) from fetal (21 day) Wistar rat calvaria and long bones (trabecular bone) and from adult (3 month old) Wistar rat long bones (trabecular bone and periosteum) [1, 2].

Explant culture. Bone pieces (from both fetal calvaria and long bones and adult long bones) and periosteal explants (from adult long bones) were cultured in L-ascorbic acid 2-phosphate containing medium.

Enzymatic isolation. Bone pieces (from fetal calvaria and long bones) were digested sequentially in a trypsin II-S-collagenase IA solution at 37°C for 10, 20, 30, 50 and 70 minutes. Rat calvaria cells and long bone-derived cells obtained from the last three respectively two digestion steps were pooled and plated at a concentration of 20000 cells/cm². They were cultured in L-ascorbic acid 2-phosphate containing medium.

Subculturing. After confluence , the cells derived from fetal calvaria, long bones and adult long bones were plated (20000 cell/cm²) in osteogenic medium (containing L-ascorbic acid 2-phosphate and β-glycerophosphate) and cultured for 21 days. After 4, 14 and 21 days, they were analyzed for their osteoblastic markers.

Periosteum-derived cells were passaged for up to 4 months and at each passage, they were analyzed for their osteogenic differentiation.

Analysis. Osteogenic differentiation was evaluated based on alkaline phosphatase activity (early marker), morphology, nodule formation and mineralization of a collagen containing extracellular matrix (late marker).

RESULTS: Calvaria cells were easier to obtain and the amount of cells released by enzymatic isolation was higher than for the long bone cells. The expansion of the cells in primary culture was highest for fetal calvaria cells and adult periosteum-derived cells compared to fetal and adult long bone cells.

All cultures expressed high alkaline phosphatase activity in secondary culture except for calvaria cells obtained by spontaneous outgrowth and periosteum-derived cells. Periosteum-derived cells had initially a negligible alkaline phosphatase activity. Little cuboidal cells could be observed in 25 % of the cultures already at early passages. These cultures showed an increasing alkaline phosphase acitivity starting at P1 until P7.

Enzymatic isolation of fetal calvaria and long bone cells favoured the osteogenic differentiation. Enzymatically isolated calvaria cells formed well defined three-dimensional nodules which mineralized restricted to this area. In contrary, cultures derived from fetal as well as adult long bones mineralized in ill defined deposits throughout the culture and only formed occasionally nodular-like structures. Periosteum-derived cells at passages 3-5 formed numerous nodules. From that passage on, nodule formation capacity decreased until completely absent. The mineralization pattern of these subcultured cells became diffuse and comparable to the cultures derived from fetal and adult long bones.

DISCUSSION & CONCLUSIONS:

The present study demonstrates that considering the isolation method, proliferative capacity and the osteogenic potential, the enzymatically released

fetal calvaria cells are most satisfactory to study cell/biomaterial interactions.

However, disadvantages of this type of cells are related to their advanced differentiation stage. Consequently, it is difficult to expand them into large amounts or to keep them in culture for long periods (> 21 days). The latter renders these cells less suitable for long-term in vitro test systems in which the biomaterial only degrades over a period for up to 3 months. The use of progenitor cells may circumvent these problems. Periosteum-derived cells are in a less differentiated stage, which can be favourable to expand them more easily and to keep them for longer periods in vitro.

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IN VITRO DIFFERENTIATION CAPABILITY OF CORD BLOOD DERIVED UNRESTRICTED SOMATIC STEM CELLS ALONG THE OSTEOGENIC AND CHONDROGENIC LINEAGE

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INTRODUCTION:. Human umbilical cord blood contains hematopoietic precursor and stem cells. In recent years it has become an important alternative source for transplantation of hematopoietic stem cells. Here we describe a novel CD45-negative stem cell termed USSC (unrestricted somatic stem cell). It is derived from human umbilical cord blood, placental blood and/or the blood of a newborn child. It is capable of differentiating into mesenchymal stem or progenitor cells, hematopoietic lineage stem or progenitor cells, neural stem or progenitor cells or endothelial stem or liver progenitor cells[1]. These cells represent the progenitor of the hematopoietic lineage as well as that of the mesenchymal lineage as well as that of the neural lineage. Our studies describe the differentiation of USSC towards the osteogenic and chondrogenic lineage.

METHODS: USSC were generated from human umbilical cord blood and expanded as adherent cell layers. Monolayers were harvested by trypsinization, washed and resuspended at a concentration of $1x10^6$/ml in PBS.

Osteogenic differentiation

Cells are plated at 8000 cells/cm^2 in 24 well plates. At 70% confluency the expansion medium is supplemented with 10^{-7}M Dexamethasone, 50µM **A**scorbic acid-2 phosphate and 10 mM ß-**G**lycerol phosphate (**DAG**) to induce osteogenic differentiation. Medium changes are performed every 3 to 4 days.

Suspension culture of cell aggregates

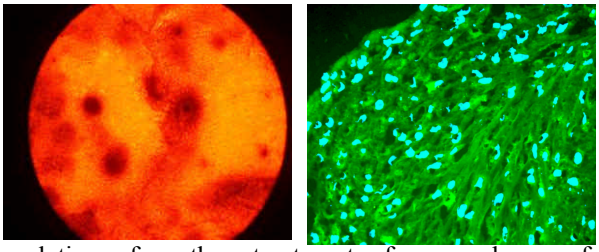
Cells were cultured in defined medium containing DMEM high-glucose supplemented with antibiotic (1:100 dilution), 100 nM dexamethasone, 35 µg/mL ascorbic acid-2- phosphate, 1 mM sodium pyruvate, and ITS+ (1:100 dilution), 10 ng/ml TGFß1. Aliquots of $2x10^5$ cells in 0.5 ml of medium were then centrifuged at 150g in 15 ml polypropylene conical tubes. The pelleted cells were incubated at 37°C and 5% CO_2. Cell aggregates were cultured for 21 days with medium changes every two to three days

RESULTS: Two weeks of osteogenic stimulation result in the deposit of calcium phosphate which can be visualized by Alizarin Red staining and quantitated by a Calcium assay (Sigma). Osteogenic differentiation is further confirmed by visualization of alkaline phosphatase activity and RT-PCR analysis of distinct osteogenic marker genes like Osteopontin, Osteocalcin alkaline phosphatase and bone sialoprotein.

In vitro micromass cultures demonstrate that USSC cultured in the presence of chondrogenic induction factors are capable of differentiating towards the chondrogenic lineage. The detection of both type II collagen and proteoglycan confirm chondrogenic differentiation. RT-PCR analysis reveal a chondrogenic differentiation as early as day 7 following stimulation.

Fig. 1: *Alizarin Red staining and collagen type II staining as characteristics of USSC differentiating along the osteogenic and chondrogenic lineages*

DISCUSSION & CONCLUSIONS: In summary, the adherent USSC population described here provides a novel and clinically advantageous pluripotent cell



population for the treatment for numbers of degenerative disorders. The establishment of a panel of HLA-typed, preferentially allogeneic USSC, fully tested for virological and microbiological parameters, will be a biological and logistical advantageous source of cells for tissue regeneration.

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ACKNOWLEDGEMENTS: This study was supported by the Wirtschaftsministerium NRW, GER.

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CONTROLLED RELEASE OF NOVEL FGF VARIANTS FOR BONE FRACTURE HEALING

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INTRODUCTION: Bone formation, remodeling and healing require orchestration between cellular activity, matrix production and mineralization, all of which are under the delicate control of communication networks of growth factors and other signaling molecules.

Bone formation and regeneration through tissue engineering is now regarded as a major alternative strategy to restore and maintain the function of the human skeleton. Cells, growth factors and biomimetic scaffolds may be used individually or combined to generate 'smart' therapeutic agents that can mimic and enhance the bone's natural capacity to regenerate and heal. Bone-forming agents should ideally have osteoinductive potential, to help the body actively recruit and produce bone forming cells and osteoconductive characteristics to provide a physical scaffold onto which bone can grow. We have developed a novel synthetic bone substitute with potent osteoconductive properties that can also serve as a biocompatible delivery system for growth factors and osteoprogenitor cells.

The FGF System that comprises Fibroblast Growth Factors (FGFs), their receptors (FGFRs) and signaling networks, is directly involved in every stage of skeletal development where its role has been actually validated by nature. Multiple inherited human skeletal disorders, including short-limbed dwarfism and craniosynostosis syndromes, are caused by point mutations in FGF receptors ([1]). Moreover, FGFs have been found to stimulate bone healing and systemic administration of FGFs induces new bone formation and increases bone mass implicating a therapeutic potential of FGFs in osteopenia and bone repair. We have developed novel therapeutic products composed of specific, tailor-made **FGF variants** to accelerate bone formation and fracture healing. These unique variants are selected for higher receptor avidity, stability, tissue specificity and regenerative potential than the native protein. We have combined these novel receptor specific FGF variants with our bone substitute to induce bone formation and fracture healing.

METHODS:

Release of FGF variant from the modified apatite bone substitute: FGF2 variant was bound to the apatite derived bone substitute by adsorption. The release rate was estimated by incubating the bone substitute in tissue-culture medium and collecting fractions at fixed intervals. The amount of released ligand was assayed by ELISA assay and by cell proliferation assay.

FGFR-Transfected FDCP Proliferation Assay In order to determine the release of FGF variants from the synthetically modified bone substitute a biological cell-based assay was utilized. The FDCP cell line transfected with FGFR cDNA, exhibit a dose-dependent proliferative response to FGF. The cells response to various ligands is quantitated by a cell proliferation assay with XTT reagent (Cell Proliferation Kit, Biological Industries Co.).

Rat bone defect tibia model:The model is based on the creation of a defect in the cortex of the diaphysis in the rat tibia. Animals are anesthetized according to a standard protocol and a defect is created in the proximal tibial methaphysis, 3-4 mm below the collateral ligament insertion, by drilling a hole of 2 mm diameter and 2-3 mm in depth. Modified apatite bone substiute is implanted at the defect site and allowed to heal for 3-8 weeks. Healing was monitored by x-ray and histology.

RESULTS*: FGF ligands can be released in a controlled manner from the modified apatite bone substitute:*

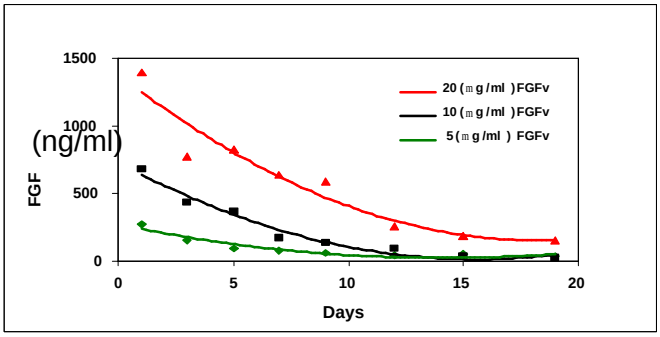

Fig.1 Controlled release of FGF from modified apatite bone substitute after binding different doses of FGF variant

The amount of FGF variant released from the bone substitute is plotted in Fig. 1. The rate of release of the bioactive agent varies accordingly to the amount bound and may be optimized for different applications and tissue types. For example, endochondral bone formation may benefit from a different release pattern of FGFs that intermembranous bone formation.

Biocompatibility of bone substitute:

In *in vivo* experiments in which the bone substitute was implanted in a bone defect performed in rat tibia, the biocompatibility of the substance could be observed. Histological examination (Fig.2) shows the formation of new bone surrounding the partially resorbed apatite bone substitute crystals, with hypercellularity composed primarily of osteoblast-like cells lining the surface as well as the development of bone-marrow (BM) rich matrix and multiple blood vessels (BV) filling the spaces in between the newly formed bone islets.

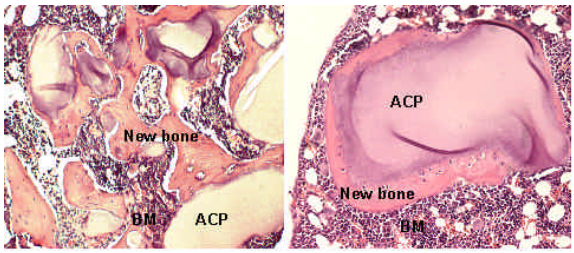

Fig.2. ProChon's bioapatite induces new bone formation. Cross section in a rat tibia bone formation model.

DISCUSSION & CONCLUSIONS: Autogenous cancellous bone, considered the gold standard for bone grafts, provides osteoinductive growth factors, osteogenic cells, and osteoconductive structural scaffold. However, autografts present significant disadvantages, including procurement morbidity, increased operative time, potential complications, and limited availability. When an autograft is not feasible, allograft is the most commonly used alternative. However, use of allogenic bone carries certain disadvantages since it requires harsh processing to minimize the risk of disease transmission and host immune response, which partially depletes its mechanical strength and biological properties. Although autografts and allografts are used most frequently, synthetic composite bone substitutes that combine an osteoconductive matrix with osteoinductive growth factors and osteogenic cells may ultimately replace both.

We have shown that the novel apatite bone substitute provides a biocompatible matrix that can be formulated to release controlled amounts of bioactive agents, such as FGF ligands to enhance and support bone formation in the lesion area.

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EFFECTS OF 45S5 BIOACTIVE GLASS PARTICLES ON TITANIUM PERI-IMPLANT BONE HEALING. A HISTOMORPHOMETRIC STUDY IN RATS.

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INTRODUCTION: Many situations in orthopedic and oral and maxillofacial clinical practice require metallic implants to be combined with bone substitutes eg, bioactive glasses (BGs). The ability of BG particles to promote osseous healing has been demonstrated in several experimental models. In a previous study, we performed histologic and microchemical determinations by scanning electron microscopy (SEM) and energy-dispersive x-ray analysis (EDX) of newly formed bone tissue surrounding titanium (Ti) implants and bioactive glass particles used as bone filling material[1]. The aim of the present study was to perform a histomorphometric evaluation of bone healing around titanium implant placed simultaneously with bioactive glass particles in hematopoietic bone marrow of rat tibia.

METHODS: Thirty male Wistar rats weighing 90 ± 5 g were employed throughout. Under anesthesia by intraperitoneal injection of 8 mg of ketamine hydrochloride (Ketalar, Parke-Davis, Morris Plains, NJ) and 1.28 mg of xylazine (Rompun, Bayer, Leverkusen, Germany) per 100 g of body weight, the skin was disinfected, shaved, and a longitudinal incision of 1.5 cm was made along the frontal aspect of both tibiae. Subcutaneous tissue, muscles, and ligaments were dissected to expose the external surface of the tibiae in the area of the diaphyseal bone. An end-cutting bur (1.5 mm in diameter) was used to drill a hole reaching the bone marrow.
Overheating and additional bone damage were prevented by using manual rotating impulsion.
A commercially pure titanium laminar implant (5x1x0.1mm; Implant-Vel, Buenos Aires, Argentina) was introduced gently into the hole in each tibia and placed inside the medullary compartment, parallel to the long axis of the tibia (Ti group)[2]. In the contralateral tibia (Ti/BG group) a titanium laminar implant and 15 mg of melt-derived BG 45S5 particles (nominal composition by weight: 45% SiO_2, 24.5% Na_2O, 24.5% CaO, 6% P_2O_5; 90-710 µm, PerioGlas® US Biomaterials, Alachua, Fl) were implanted. The wounds were carefully sutured.
The animals were housed in plastic cages and maintained on a 12:12 hour light:dark cycle. They were fed rat chow and water *ad libitum*.
The guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication Nº 85-23, Rev.1985) were observed.
The animals were sacrificed by ether overdose in groups of 10 at 14, 30 and 60 days after implantation. The tibiae were resected, fixed in 20% formalin solution, and radiographed.

Histologic Processing: The tibiae were processed for embedding in methyl-methacrylate resin. The samples were then sectioned using a saw, and three slices were cut at approximately 500 µm, perpendicular to the implant. The cross sections were ground using a grinding machine and finished manually with sandpaper to obtain sections about 35 µm thick. The sections were stained with 1% toluidine blue for histologic and histometric evaluation by light microscopy (Zeiss Axioskop 2 MOT, Carl Zeiss, Jena, Germany).

Histomorphometry: Histomorphometric determinations based on standard stereologic methods were performed with an image analyzing system (Kontron KS300 v.2, Kontron Elektronik, Munich, Germany). Tracings of the projection of the sections were made at x160 magnification. The peri-implant bone area and the percentage of bone-to-metal contact were evaluated.

The results were statistically analyzed by Student´s *t* test. Data were reported as mean ± SD at a significance level of $P<.05$.

RESULTS: Uncomplicated healing post-implantation in all rats was determined by radiographs .

Histologic Findings: Light microscopy of the histologic sections showed that observed. All implants remained *in situ* as a large proportion of both biomaterials was surrounded by newly formed bone. The rest of the surface was in contact with the bone marrow.

There was no occurrence of macrophages or related inflammatory cells in any of the interface regions of either of the groups.

Ti group: 14 and 30 days after implantation, lamellar bone tissue was observed on most of the implant surface (bone-to-metal contact). Additional bone growth was observed after 60 days.

Ti/BG group: 14 and 30 after implantation, lamellar bone tissue bridges between Ti and BG particles were observed (*Figure 1*).
Areas of bone formation, unrelated with BG particles, were detected around titanium implants. Additional bone growth was observed at 60 days.

Histomorphometric Analysis
Both groups (Ti and Ti/BG) exhibited a statistically significant increase in peri-implant bone area as a function of time ($P<.05$). Compared to Group Ti, Group Ti/BG showed a statistically significant ($P<.05$) increase in peri-implant bone area at all the experimental times (*Table 1*).No statistically significant differences were found between groups when comparing percentage of bone-to-metal contact (*Table 2).*

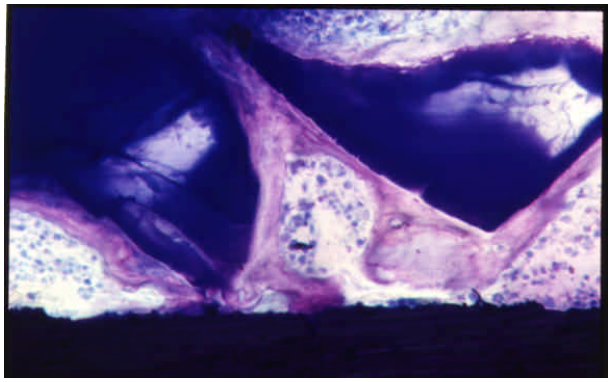

Fig. 1:Newly formed bone tissue lies between the Ti implant and bioactive glass particles 14 days postimplantation (toluidine blue; original magnification x400).

Table 1. Peri-implant bone area (mm^2)

	Days postimplantation		
	14 (n:10)	30 (n:10)	60 (n:10)
Ti	1168±114 *	1444±109 *	2554±84 *
Ti/BG	1600±102	1793±103	2698±75

Mean ± SD *$P<.05$ mm^2 of projection

Table 2. Bone-to-metal-contact (%)

	Days postimplantation		
	14 (n:10)	30 (n:10)	60 (n:10)
Ti	31±4 *	38±3 *	39±3 *
Ti/BG	35±3	41±2	42±4

Mean ± SD *$P>.05$

DISCUSSION & CONCLUSIONS: The results of the present study evidence that simultaneous implantation of 45S5 BG particles around a titanium implant caused an increase in osteogenic activity in the peri-implant micro-environment.
In a previous study using this experimental model a transient appearance of Si at 14 and 30 days postimplantation and a rise in the Ca:P ratio in peri-implant bone tissue when BG particles were employed were observed[1].
Bioactive glass dissolution ions (Si, Ca, P, and Na) plays an important role in the formation of a bone-like apatite on its surface *in vitro* and *in vivo*[3], and have been shown to exert a genetic control over the osteoblast cell cycle, leading to diferentiation and proliferation of bone cells and the expression of genes that regulate osteogenesis and production of growth factors[4].These findings would explain the increase in osteogenesis reported herein.

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SOL-GEL BIOGLASS STIMULATE OSTEOGENESIS IN MOUSE PRIMARY CELL CULTURE

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INTRODUCTION: During the last decade, studies have been directed to find a substitute that can replace not only the structure of bone but also its functional capacities, in other terms to promote repair and regeneration by the host tissue itself. Bioactive glasses developed in the early 1970s [1], are of particular interest due to their unique property of being osteoconductive and osteoproductive at the same time, thus they can induce the differentiation of osteoblasts and stimulate the formation of bone tissue both in vivo and in vitro [2,3]. This bioactivity is related to their capacity of forming a direct chemical bond with the host tissue via an intervening layer of carbonated hydroxyapatite which is chemically and structurally similar to the mineral of bone. Although their ostegenic capacity is widely documented and their use in various clinical cases of bone loss have shown successful results, yet the mechanism behind their bioactivity remains largely unknown, and the cellular responses to these glasses have not yet been clearly elucidated. The aim of the present study was to evaluate at a molecular level the effects of bioactive glasses on the differentiation of osteoblastic cells, by studying the effect on the expression of major markers of the osteoblast phenotype.

METHODS: *Biomaterial tested*: The 58S bioglasses had a composition (in weight %): 60% SiO_2, 36% CaO and 6% P_2O_5. 60S bioinert granules were used as control in our experiments.

Culture model: osteoblasts were enzymatically isolated from calvaria of 18 day old fetal swiss mice, as previously described by Nefussi et al. [4]. Briefly, calvaria were aseptically dissected and fragments incubated in phosphate buffered solution with 0.25% collagenase for 2 hr at 37°C. Then cells were dissociated from the bone fragments, and plated at 4x 10^4 cell/cm^2. The glass granules were added on day 1 of culture. All cultures were incubated in DMEM supplemented with10% fetal calf serum, ß-glycerophosphate, and ascorbic acid, in a humidified atmosphere of 5% in air at 37°C.

Totan RNA isolation and Northern Blot: total RNA was extracted at day 6, 12, 18, using a phenol/chloroform method (Tri reagent®). The concentration and purity of RNA were determined by light absorbance at 260nm and by calculating A_{260}/A_{280} ratio respectively. The integrity was confirmed by electrophoresis on an agarose ethidium bromide gel. Twenty micrograms of total RNA from each sample was denatured, and loaded on a 10% formaldehyde gel for electrophoretic separation. Total RNA on the gel was transferred onto Hybond+ nylon membranes with 20X SSC solution for 24 hr, and fixed at 80°C for 2 hr. The hybridization was performed at 42°C using ^{32}P-dCTP labelled probes. Hybridized membranes were exposed to Biomax films and the mRNA profiles analyzed with visiomic program.

RESULTS: Phase contrast microscopy showed that 2 hours later, cells started to attach and spread on culture dishes. On day 3 to 4 of culture, the cells were confluent and began to form multicellular layers. Furthermore, the cells showed morphological changes one of which was the appearance of cellular condensations, these were the areas in which future bone nodule formation were to be seen. The first zones of mineralization were noticed around the granules especially in the cultures with the bioactive granules, at more advanced stages, true mineralized bone nodules were formed in both cultures.

The expression of characteristic osteoblastic markers was determined at day 6, 12, and 18 using Northern blot techniques. The mouse osteoblasts in both cultures, expressed the major phenotypic markers namely Cbfa1, PAL, BSP, Osteopontin and Osteocalcin. All the markers were detected at day 6 of cultures, while Cbfa1 decreased with time and the rest of markers increased or remained at the same level at advanced stages of the culture. An interesting finding was that the levels of mRNA of all these markers especially osteocalcin, was higher at all times, in cultures of sol-gel glasses when compared to control.

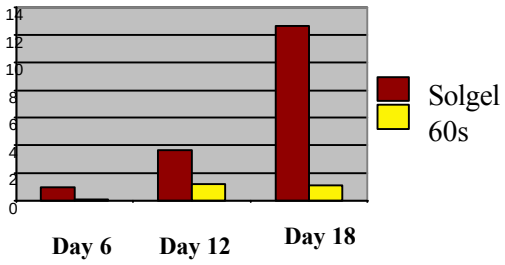

Fig. 1: Effect of sol gel biogalsses on the expression of ostecalcin, in primary mouse cultures

DISCUSSION & CONCLUSIONS: A line of evidence suggests that bioactive glasses can promote osteogenesis *in vitro*[5] and *in vivo*, however little is known on the cellular and molecular mechanisms underlying this effect. Our findings demonstrated that the cells maintained their phenotype in cultures of sol gel glasses, this was outlined by the expression of all phenotypic markers in a similar temporal pattern when compared to control. Furthermore the fact that the major osteoblastic markers were enhanced in cultures with bioactive glasses, indicates that these glasses has the capacity to stimulate the growth and osteogenic differentiation, by upregulating the major osteoblastic markers and the key transcription factor of bone (Cbfa1).

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BONE FORMATION IN GLASS-CELLULOSE COMPOSITE IMPLANT IN RAT FEMORAL DEFECT FOLLOWS WOLFF'S LAW

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INTRODUCTION: Different methods have been described to create, fill and follow bone formation in osseous defects using biomaterial implants. For practical reasons (large number of samples, economical tests, etc.) small animals such as rats are favourable despite of the fact that a critical size defect is difficult to achieve. We developed a standardised rat femur model to study bone formation in various biomaterials. The method allows biological (histology), biochemical (protein and DNA content, Northern hybridization and immunohistochemistry) and biomechanical (bending tests, etc.) analyses[1,2].
Using this model we detected that the femur reacted by producing new bone opposite to the defect site.

MATERIALS AND METHODS: Young adult male Sprague-Dawley rats were used in these experiments. A full-thickness cortical defect in the antero-lateral aspect of *trochanter major* was made by a series of dental drills and extended to 2.4 x 8 mm using a special device. After evacuation of debris and bone marrow the implants were inserted and bone formation was followed up to 24 weeks.
Some defects were also left empty to follow spontaneous healing. Tetracycline and calcein fluorescence were used to monitor the growth of mineralized bone by subcutaneous injections (tetracycline 25 mg/kg and calcein 7.5 mg/kg) at three different time points (*Figure 1*). The labelled animals were killed at 1, 4, 8, 12 and 24 weeks, femurs were prepared and fixed either in 4% paraformaldehyde, embedded in hydroxyethyl-methacrylate (HMA) and 6 µm sections were stained by van Gieson or in ethanol, embedded in isobornyl-methacrylate and sectioned to 20 µm slices by a cutting-grinding method. Several other animals were kept until 60 weeks for routine histology.

Fig. 1: Scheme for labeling with calcein and tetracycline.

RESULTS AND DISCUSSION: Histological sections showed that bone growth started from the implant ends, progressed into the matrix as a cancellous bony bridge, which was remodelled gradually to the centre of the implant and formed an almost complete tubular bone (*Figure 2*). There was still implant material left in the defect at 60 weeks after implantation. Thus, the defect never healed completely and was observed as a depression in the bone (*Figure 3*). Tetracycline and calcein labelling demonstrated new bone growth endosteally into the implant.

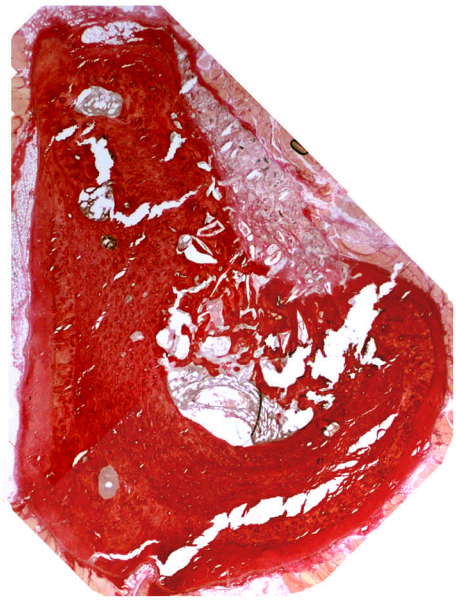

Fig. 2: van Gieson stained cross section of bone at 24 weeks.

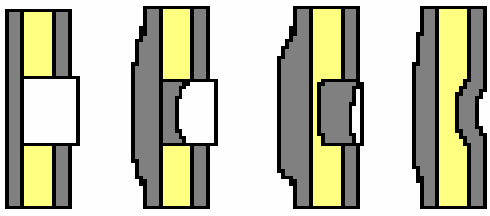

Fig. 3: Schematic picture of bone healing in the cortical defect model.

There was also a strong periosteal thickening of bone mainly opposite to the defect that supported the tubular bone structure (*Figure 4*). This bone

growth was active during the first four weeks and did not progress much thereafter. The Wolff's law determines how much and where bone will be formed to maintain bone strength. This fact and the availability of periosteum, which is active in bone healing, explains why new bone appeared first periosteally opposite to the defect.

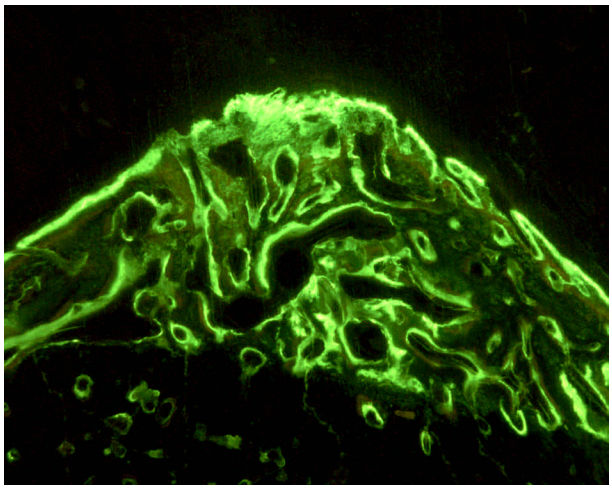

Fig. 4: Fluorescence labelling of the periosteal growth of bone.

This method produces sufficient sample size to allow histology, scanning electron microscopy, biochemical methods and biomechanical tests. Bending strength measurements of the bone containing the implant showed no differences between femurs with or without the implants. The bone was strengthened according to Wolff's law throughout the periost but especially at the opposite site already before new bone was formed into the implant. This response was rapid and might explain why appearance of new bone in the implant occurred at a rate less then expected.

CONCLUSIONS: We developed a method to evaluate implant materials in rat femur defects. The healing of the defect occurred according to Wolff's law. Much of the strength was achieved by thickening of bone opposite to the defect and later also by growth of new bone through the implant. Periosteal thickening thus allows achievement of normal bone strength quicker than new bone growth into the implants. This effect allows a clear-cut comparison of osteoconductive/ osteopromotive properties of the implant materials.

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COMPARISON OF HUMAN BONE MARROW STROMAL CELLS SEEDED ON CALCIUM-DEFICIENT HYDROXYAPATITE, ß-TRICALCIUM PHOSPHATE AND DEMINERALIZED BONE MATRIX.

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INTRODUCTION: Repair of large bone defects represents a challenge to orthopedic surgeons since autogenous grafts are not available in large amounts and its removal causes morbidity at the donor site.[4] Numerous animal studies demonstrated that seeding periosteal cells or bone marrow stromal cells (BMSCs) on suitable matrices prior to implantation can improve healing of large bone defects.[3] A new tissue-engineering approach is to cultivate BMSCs on matrices to further increase the bioactivity of the cell-matrix composite before implantation.[1] Both, synthetic and allograft materials, are available at present. Among the resorbable ceramics are materials such as ß-tricalcium phosphate (ß-TCP), and a recently emerging group of ceramics called low-temperature apatites (LTA), such as for example Calcium-deficient hydroxyapatite (CDHA). These LTA are produced from reactive calcium phosphates at room temperature. In contrast to most apatite compounds, these LTA are not sintered. As a result, LTA possess a very large specific surface area (20-100m^2/g), similar to that observed in bone apatite crystals. The aim of this study was to compare three resorbable biomaterials regarding seeding efficacy with human bone marrow stromal cells (BMSCs), cell penetration into the matrix, cell proliferation and osteogenic differentiation.[2]

METHODS: Calcium-deficient hydroxyapatite (CDHA), ß-tricalcium phosphate (ß-TCP), and demineralized bone matrix (DBM) were seeded with human BMSCs and kept in human serum and osteogenic supplements for 3 weeks. To evaluate the seeding efficiency the cells that did not adhere to the matrix were counted on the culture dish on day 1 after harvesting the cell-matrix composite. For light microscopy, samples were fixed, embedded in paraffine and stained with hemalaun-eosine. To assess cell penetration into the matrix representative slices of the upper, the lower (each 200 µm below the surface) and the middle of the matrix were selected for evaluation. These slices were randomly examined with regard to the number of cells per view field in a 10x magnification. The average cell number of 3 random fields was calculated. Three categories were established: (I) no cells per view field, (II) 1-3 cells, and (III) more than 3 cells per view field. The total amount of protein was measured according to the method of Lowry. Alkaline phosphatase (ALP) activity was assessed by incubation with p-nitrophenyl phosphate and measuring the conversion to p-nitrophenol for 3 minutes. Osteocalcin (OC) was quantified using a one-step ELISA called N-MID™ (Osteometer Biotech, Harlev, Denmark).

RESULTS: The allograft DBM and CDHA exhibited both an excellent seeding efficacy while the performance of ß-TCP was lower when compared (tab.1). There was a significant difference between DBM and β-TCP on the first day.

Table 1. Adhesion of cells after seeding:

	Non-adherent cells	SD
CDHA	4.5 %	± 9 %
TCP	41.5 %	± 33 %
DBM	0.0 %	0

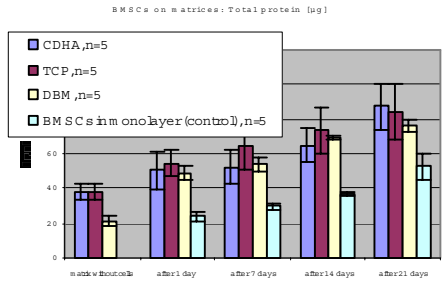

Fig. 1: The total protein content increased significantly on all matrices (CDHA, ß-TCP, and control p=0.002 c^2= 15; DBM p=0.029 c^2= 9) and the control group (BMSCs in monolayer). There were no significant differences between the groups.

BMSCs in monolayer had a significant increase of protein, but not of ALP.BMSCs were found inconsistently within the synthetic materials, whereas in DBM they were found more homogeneously distributed throughout the matrix.

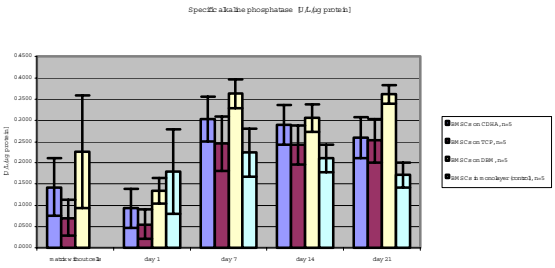

Fig 2: The values for specific ALP increased on all matrices (CDHA p=0.014, c^2=10.68; ß-TCP p=0.015, c^2=10.469; DBM p=0.042, c^2=8.2) and no significant difference was found between the groups.

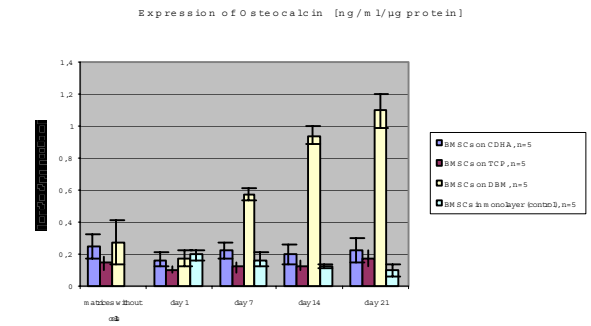

Fig 3: Osteocalcin values increased significantly higher for BMSC in cultures on DBM when compared to CDHA and ß-TCP (DBM p=0.029, c^2=9; ß-TCP p=0.014, c^2=10.68). The OC levels decreased significantly in the BMSC monolayer culture (p=0.004, c^2=13.08).

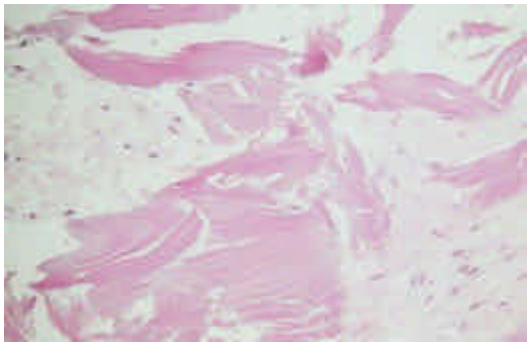

Fig 4A

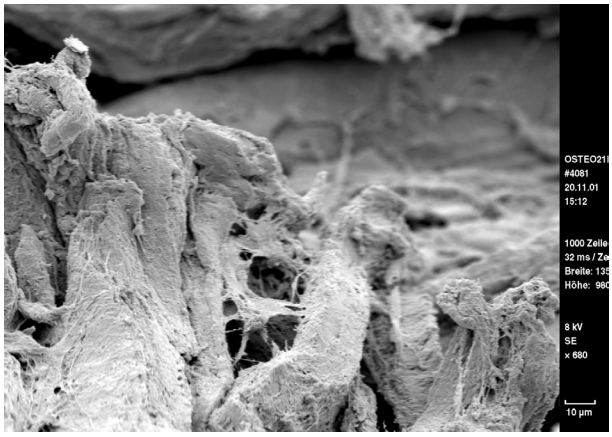

Fig 4B: (A) Histology and (B) REM pictures of BMSCs on DBM after 21 days. BMSCs were found throughout the whole matrix

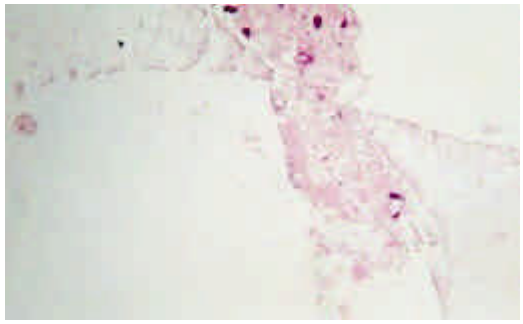

Figure 5: Histological evaluation of CDHA: Cells were found mainly on day 1 after seeding.

DISCUSSION & CONCLUSIONS: All three matrices promoted BMSC proliferation and differentiation to osteogenic cells. DBM allografts seem to be more favorable with respect to cell ingrowth tested by histology, and osteogenic differentiation ascertained by an increase of OC. CDHA with its high specific surface area showed more favorable properties than ß-TCP regarding reproducibility of the seeding efficacy.

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BIOMIMETIC BONE GRAFT MATERIAL – BIODEGRADABLE, MINERALIZED 3D-COLLAGEN-SCAFFOLDS

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INTRODUCTION: The aim of our research is the development of biodegradable synthetic bone graft materials which mimics the composition and structure of natural bone tissue. For this purpose, a process called synchronous biomineralization has been established. During this proceeding, the collagen fibril assembly and the mineralization of the fibers with hydroxyapatite take place simultaneously. The product can be described as mineralized collagen: a composite material, consisting of the main protein and mineral phases of natural bone [1]. The lyophilization of suspensions of mineralized collagen led to porous, three-dimensional scaffolds. Additionally, the chemical crosslinking with EDC stabilizes the porous structure of the composite material. Their pore structure allows the cultivation of osteoblasts and osteoclasts inside the sponges - the co-cultivation of these two bone cell types in a synthetic mineralized extracellular bone matrix can be used as an in vitro model for bone remodelling [2]. Furthermore, the porous scaffolds have been modified with growth factors such as the osteoinductive rhBMP-2 (recombinant human bone morphogenetic protein) and the angiogenic factor rhVEGF (recombinant human vascular endothelial growth factor).

METHODS: By using the above mentioned biomimetic biomineralization process, which requires physiological conditions and precise reaction parameters, samples of different shape and dimensions could be generated easily. In order to characterize and optimize the porous mineralized collagen-scaffolds several physical (SEM, LSM, BET, DSC), mechanical as well as biochemical methods (ELISA) have been applied.

Fig. 1: Porous mineralized collagen-scaffolds in different shapes and size (left), SEM-picture of the microstructure of the mineralized collagen-I fibrils, magnification: 20000x (right).

RESULTS: The porosity of the mineralized collagen scaffolds adjusted by the freeze drying procedure was investigated utilizing the BET-method and SEM. The optimized scaffolds with a pore diameter of about 200 µm admitted the smooth ingrowth of the described cell culture. Even after swelling in buffer solution or cell culture medium the obtained porous macrostructure could be maintained.

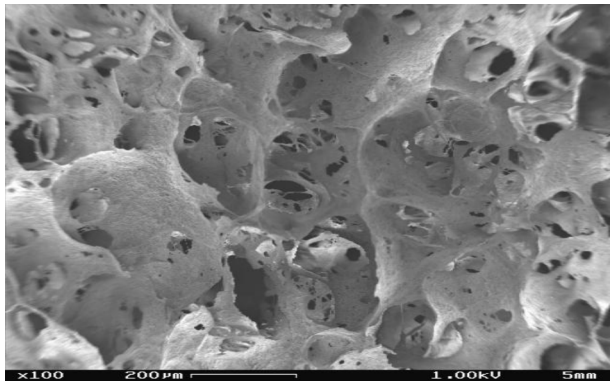

Fig.2: Microstructure of a porous 3D scaffold, made of mineralized collagen I, frozen at -26°C (SEM-picture, magnification 100x)

Mechanical compression tests have shown the high stability and elasticity of swollen 3D-scaffolds, which have been keeping their cylindrical shape during the experiment.

Regarding the improvement of the *in vivo* bone formation and regeneration the implantable mineralized collagen-scaffolds have been functionalized with rhBMP-2 and VEGF. The investigation of the release kinetics of these growth factors was the main subject of our study concerning the medical use.

DISCUSSION & CONCLUSIONS: Our developed scaffolds seem to be suitable materials for the hard tissue reconstruction useful for the therapy of large bone defects as well as for promoting the spine fusion process.

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HISTOMORPOLOGICAL QUANTIFICATION OF BONE GROWTH USING AN *EX-VIVO* LOADING AND CULTURING SYSTEM FOR HUMAN BONE

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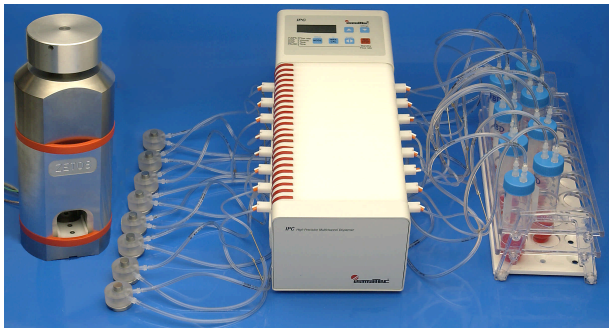
INTRODUCTION: The ZETOS system used in the following experiment is a novel *ex-vivo* culturing and loading system for trabecular bone cores[1]. In the following experiment human trabecular bone was cultured and loaded using two different medias: DMEM and BGJb-Fitton Jackson modified media. DMEM media is known to induce osteoblast proliferation, BGJb media stimulates osteoblast differentiation[2].



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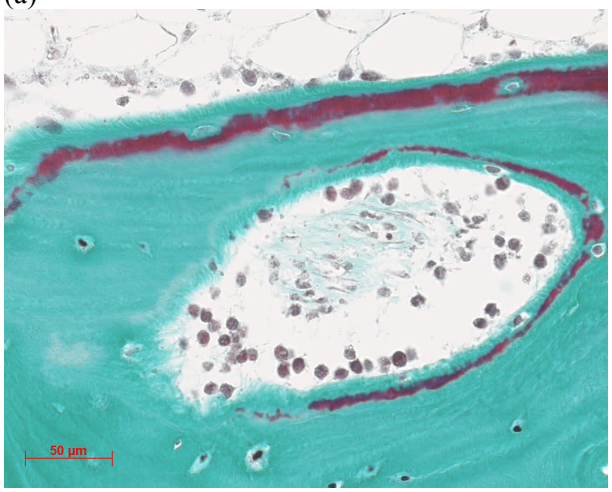
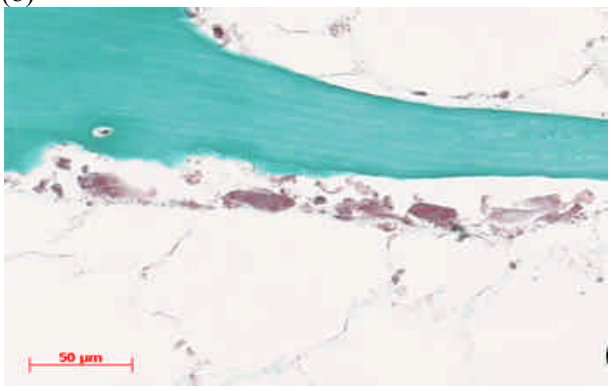


(b)



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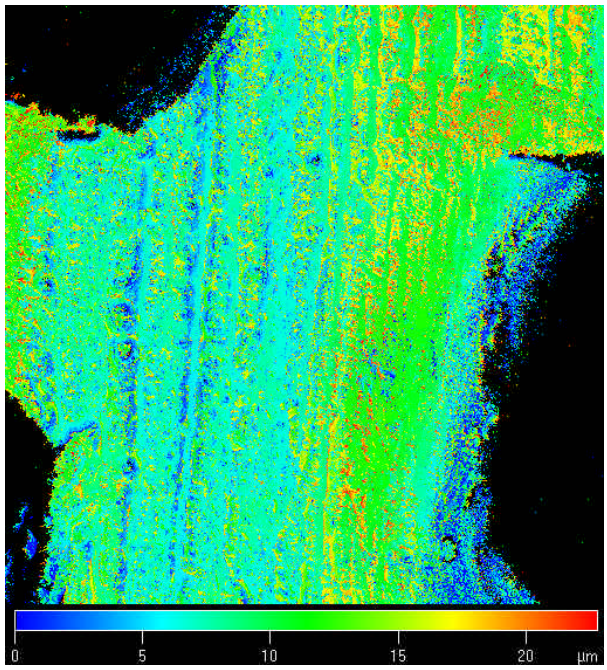

Fig. 1: (a), ZETOS system (b) Cultured bone with Osteoid seam,(c) osteoblasts, osteocytes and osteoclasts (c) Confocal image of saw marks on bone surface after preparation.

METHODS: 25 bone cores (5mm height, 10 mm diameter) from a male 81 year old patient receiving a hip prosthesis were prepared as described previously[1,3]. Work was carried out with approval of the Ethic Commission of Graubunden, CH (18/02), with patient consent. The femoral head was cut into 7 mm thick sections with an Exakt 300 band saw. Cores, 10 mm in diameter, were drilled from the sections with a specially designed diamond tipped hollow fluted drill. The cores were cut closer to parallel with an Exakt band saw, to the height of 5 mm. Throughout the cutting procedure the bone was irrigated with sterile 0.9% NaCl at 4°C to limit the amount of damage caused by heat, remove bone chips and to stop the bone from drying out. Bone cores were washed in Earls x1 (50 ml Earles 10x, 447 ml H_20, 3 ml 7,5% $NaHCO_3$) for 3x10 min and then in Earls + antibiotics (375 IE Streptomycin, IE Penicillin, 4 µg Amphotericin B per 1 ml Earles 1x) for 30 min before being inserted into the chambers.

The assembled system was placed into a 37°C room and left to settle for 36 hours. Cores were split randomly into 5 groups with 5 cores each: DMEM mechanically loaded, DMEM not-loaded, BGJb mechanically loaded and BGJb not-loaded and a fresh control. Bones of the control group were fixed for histological processing right after the preparation with 70% ethanol. The circulation rate was set to 7ml/h, media was changed every day. Culturing time was 26 days. Loaded bone was loaded for 21 days with 3000 µStrain (15µm displacement), 5µm preload, 300 cycles per day, 1 Hz with a wave form recorded from a person jumping. After culturing the bone cores were fixed in 70% ethanol and embedded in Technovit 9100 New at -20°C. 7µm microtome sections were cut and stained with Masson Goldner, Toluidine blue or Giemsa. Sections were evaluated using a macro produced for the ZEISS KS400 software. Statistics were calculated using Mann Whitney Test (p=0,05). For confocal analysis of the cutting parallelity of the EXAKT saw, bovine trabecular bone was scanned after 1 day of loading using a ZEISS LSM 150 confocal microscope.

RESULTS: In all groups no sign of necrosis was observed after 26 days of culturing, Ostecytes, osteoblasts, osteoclasts, adipocytes and bone marrow cells were found in all groups (Fig 1). Nevertheless only few osteoid seams were observed. Relative osteoid area (O.Ar/B.Ar) was increased significantly in bone cores cultured in BGJb media compared to ones cultured in DMEM. No difference was observed between loaded and non-loaded bone cores cultured in DMEM media, though there was a difference between loaded and non-loaded bone cores cultured in BGJb media (Fig. 2). On the test bovine bone, regular surface saw marks with a depth of about 10µm could be measured (Fig. 1).

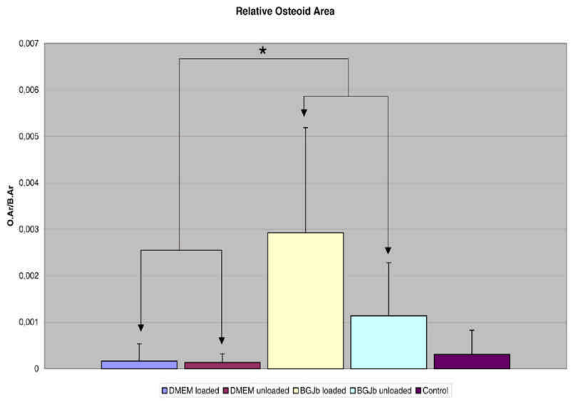

*Fig. 2: Results of measurements of Relative Osteoid Area, significant different results are marked with *.*

DISCUSSION & CONCLUSIONS: The bone cores of the four groups survived the culture period in the ex-vivo system, but only the osteblasts cultured in BGJb media changed their former growth habit. Loading did not induce significant osteoid formation. This result urged us to take a look at the bone surface, using bovine bone cut with the EXAKT saw. Regular deep saw marks from the preparation could be found which are likely to be absorbing some of the compression. Ongoing experiments are being conducted using a higher preload and a better method of final preparation of the bone cores using a Leica annular saw to reduce surface artifacts and increase parallelity.

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ACKNOWLEDGEMENTS: The authors would like to thank Dr. Thomas Perren (Davos Hospital) for supplying several hips and knees for the bone culture system development. Thanks to Prof. Berton Rahn (ARI) for helpful microscopy discussions, Christoph Sprecher for technical assistance with the confocal microscope. Thanks to Torsten Pohl, Eckhard Bröckmann and Marita Kratz (Marburg) for help with the continual development of the Zetos culture system. The work was partially funded by the 3R Foundation 78/01 and from the RAETIA stipendium from the AO Research Institute.

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QUANIFICATION OF BONE MATURITY USING SCANNING ACOUSTIC MICROSCOPY

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INTRODUCTION: During growth, bone undergoes continuing changes of its structure, which are accompanied by changes in mechanical properties. Treatment strategies may be influenced by both components. Skeletal maturity of an individual is clinically assessed using radiographs of the hands. This site often does not correspond to the site of interest, mechanical characteristics are not addressed by this method, and the technique includes the hazards of radiation. Using ultrasound it seems possible to bypass such drawbacks. It was thus the goal of the present study to investigate ultrasound as a possible alternative to the radiological technique.

METHODS: Buccal cortical bone of the lower jaw of three age groups of pigs (2-3, 4-6, 18-24 months) was compared in three planes. Acoustic impedance, transmission velocity, stiffness, and bulk modulus of each pixel (30µm)[1] were assessed using a reflection scanning acoustic microscope. Microradiographs were used for quantifying the density of pores.

RESULTS: The average conduction velocities, acoustic impedance, stiffness and bulk modulus increased with age. In the frontal plane the values were significantly higher than in the other planes, and group differences were most significant in this plane. Microradiographically, major porosities were found in the youngest age group, which caused a high amount of backscatter from different depth levels. This significantly influenced microscopic ultrasound-reflection and resulted in non-realistically high values of ultrasound velocity.

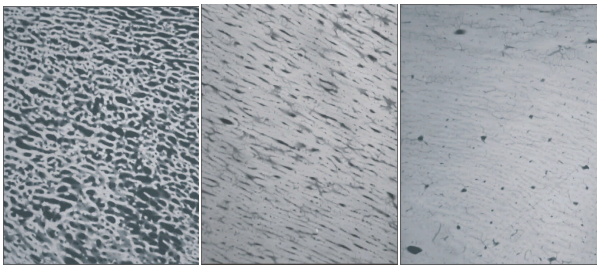

Fig. 1: Increasing bone density with age (left to right) The high porosity of young bone disturbs the ultrasound reflection characteristics.

DISCUSSION & CONCLUSIONS: In young bone high porosity leads to major scatter of ultrasound, which makes the method less precise than in the more compact structures of advanced age groups. Impedance is correlated with bone mineral density[1,2,3], while the conduction velocity shows mainly information on bone structure[4,5,6]. For assessment of bone maturation, transmission velocity is best measured parallel to and within osteons (frontal plane). Backscatter could be used for quantification of bone architecture, and thus bone maturation, when analysing the reflection spectrum for extremely fast conduction speeds. Transmission as well as backscatter measurements do not seem to be sensitive enough for verifying bone maturation in vivo.

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ACKNOWLEDGEMENTS: The histology team of the AO Research Institute helped with sample preparation, and D. Pfluger provided statistical support.

MULTI-LAYER SCAFFOLD FOR CULTURING BIPHASIC JOINT CARTILAGE IN VITRO

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INTRODUCTION: Tissue engineering technique nowadays is limited to culturing single tissue, while in clinical application, tissues to be repaired are often composite tissues such as the human articular surface is a typical biphasic composite tissue. In our previous study, we found that the partly digested cartilage fragments could reconstruct cartilaginous tissues *in vitro*. For reconstructing two layer tissue in an orderly pattern, a specific multi-layer porous scaffold that containing of an upper cavity were prepared. Partly digested cartilage fragments were injected into the cavity, thereafter the bone marrow cells were seeded in the multi-layer scaffold, wherein cartilage tissues fragments and bone marrow cells, by taking advantage of their disparity in volume, are to be distributed into different layers. The specific multi-layer porous scaffold with two kinds of cells was cultured to form a biphasic composite tissue *in vitro*.

METHODS: A multi-layer of porous scaffold with an upper hollow cavity was prepared by solvent/merging particular leaching method[1]. Articular cartilage was harvested from a one-week old New Zealand rabbit and was cut and sieved to particles of size 520-860 μm. 50 mg of tissue fragments were partly digested in 0.1 % type II collagenase solution for 2 hours at 37°C. A straight 18-gauge needle on a 1 mL syringe was used to inject the partly digested tissue into the cavity of the scaffold. Bone marrow cells were harvested from a New Zealand rabbit weighted 2 kg. 5×10^5 bone marrow cells were seeded into the scaffold after injecting the cartilage tissue fragments. The scaffolds were placed in a 500 ml spinner flask to incubate for 2 weeks and 1 month. The samples were fixed in PBS solution containing 4% formalin, sectioning with the paraffin-embedded section method, and finally, staining with hematoxylin-eosin and safranin-O.

RESULTS: Neocartilage is observed to grow around the seeded tissue fragments after 2-week incubation inside of the hollow cavity. The cells of the newly formed tissue exhibit the typical spherical morphology of chondrocytes and were in lacunae, surrounded by the newly formed of cartilaginous matrix. A layer of bone marrow mesenchymal cells can be observed at the inner side of the porous structure. Figure 1 shows a histological examination of a tissue after 4-week incubation. The neocartilage tissue has already fused together with seeded tissue fragments, with the size of the whole tissue block being 5.2 mm in width and 1.8 mm in depth. The underside of tissue block has grown from upper hollow cavity into the lower porous structure, forming an interface similar to that formed between cartilage and subchondral bone. At porous structure on the lower end of scaffold, the pores of porous structure are filled up with the proliferated bone marrow mesenchymal cells.

DISCUSSION & CONCLUSIONS: In the study two types of tissues can be cultured separately and reconstructed in vitro by taking advantage of their disparity in volume and the materials of the scaffold. The multi-layer porous scaffold can be utilized to culture multi-layer tissue. In the future, by taking only few amounts of cartilage tissue and bone marrow from patient, and incubating subsequently with the invented multi-layer porous carrier, one is able to grow large amounts of tissues that can be used as implants in transplantation surgery to treat the wide damaged area and full thickness defect.

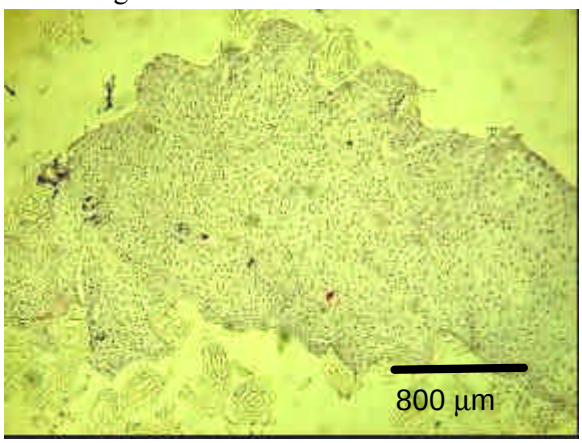

Fig.1 shows histological examination of biphasic tissue after 4-week incubation.

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ACKNOWLEDGEMENTS: The authors would like to thank the Ministry of Economic Affairs (Taiwan) for financially supporting this research.

BIOCOMPATIBILITY OF SCAFFOLD COMPONENTS AND HUMAN BONE FETAL CELLS

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INTRODUCTION: Bone is the most commonly replaced tissue of the body, with over 450'000 bone repair procedures performed per year in the United States alone[1]. For clinical bone transplantations, tissue engineering techniques based on the delivery of cells to the defect through the use of 3-D scaffold materials, are currently investigated.

As part of the bone tissue engineering project developed in Lausanne, two bioresorbable polymers (PLA, Boerhinger Ingelheim and PLGA 85/15, Pürac Biochem) were used to create a structure that could guide bone formation by facilitating cell migration, proliferation and differentiation. The 3-D foam morphology could be modified varying the processing conditions. In addition, the possibility of integrating ceramic materials into the polymer matrix might improve its mechanical properties. Hydroxyapatite (HA) and β-tricalciumphosphate (β-TCP) were chosen for this purpose due to their capacity to stimulate natural bone repair and for their stability[2].

For this project, we choose to use fetal bone cells that, in comparison to adult cells, show a higher proliferation capacity, are less differentiated into mature osteoblasts and present less immunological compatibility issues if used as tissue gafts[3]. The aim of this study was to investigate the possibility of associating fetal bone cells with a biodegradable scaffold for tissue repairing *in vivo*.

METHODS: Bone fetal cells were cultured in 75 mm^2 flasks, and collected by trypsinization and centrifugation just before use. Cells were seeded in the presence of PLA, PLGA, HA, β-TCP on 6 cm Petri dishes at density of 10^5 cells/dish. In parallel, skin fetal cells were tested to check for a possible difference in behavior. During two weeks, cells were observed for their ability to grow in the immediate neighborhood of the compounds The medium was changed once, after the first week of culture. At this intermediate time and at the end of the experiment, pictures from each well were taken.

DISCUSSION & CONCLUSIONS: During the two weeks of culture, bone and skin fetal cells were able to proliferate in all groups. The copolymer with PLGA 85/15 was totally surrounded by cells that seem to anchorage to it (Fig. 1).

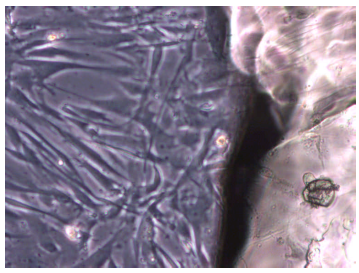

Fig. 1: Proliferation of bone fetal cells around the PLGA 85/15 copolymer

In the presence of ceramics, the cells proliferation was not disturbed (Fig. 2). Further investigations will be necessary to understand if these particles are ingested by the cells or if they are stuck on the membrane.

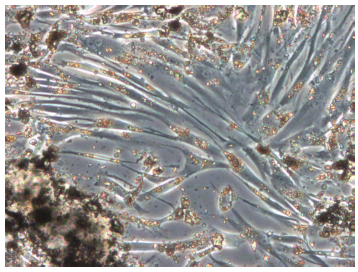

Fig. 2: Proliferation of bone fetal cells in the presence of b-TCP

In this preliminary study, we show that the chosen compounds are biocompatible with bone fetal cells. We are currently further investigating the behavior of the cell-scaffold construct.

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Preliminary results of enhanced osteogenesis by Fibrogammin and mesenchymal stem cells on chronOS cylinders.

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[4]*Aventis, Marburg,D,* [5] *AO Research Institute, Davos, CH*

INTRODUCTION: Bone tissue engineering represents one of the most promising concepts in orthopedic surgery for bone repair and reconstruction [1]. Several attempts of combining osteoconductive and osteoinductive agents seem promising. A key point of this work is the seeding of ovine mesenchymal stem cells (MSC) on a combination of two materials, chronOS and Fibrogammin. chronOS is a resorbable ceramic bone substitute for orthopaedic defects with excellent osteoconductive properties [2]. The osteoinductive influence of factor XIII (plasmatransglutaminase, Fibrogammin®, Aventis, Behring) has been reported by several other authors [3-4]. In this approach the advantages of both materials are combined with the use of MSC.

METHODS: MSC were isolated from bone marrow aspirates by centrifugation over a Ficoll histopaque layer (20 min., 800g, density 1.077). MSC were collected, washed, diluted in DMEM and seeded into monolayer flasques. Before seeded on the chronOS discs (8,5x20mm) the cells had been grown up for proliferation for one week. chronOS cylinders were soaked with 800 µl of a Fibrogammin 1250U/1ml solution and dried at 37°C for one hour. MSC were seeded on the discs in a concentration of $2x10^6$ cells per disc. DMEM containing 10% fetal calf serum, 10 nM dexamethasone and 10 nM ascorbic acid-2-phosphate was used. To initiate mineralization, the medium was supplemented with 1% of a 500 mM phosphate solution. The cultivation time of the MSC was 17 days. Disc sections were cut into small pieces and used for microscopical, histological analyses. Alkalic phosphatase (AP) activity was measured.

RESULTS: MSCs showed a good adherence on the chronOS disc surface. After 17 days of incubation, the porous discs had been overgrown by cells. Preincubated discs with Fibrogammin showed a higher density of cells than without labelling. Most of the outer macropores became sealed off by a continuous layer of cells (Fig. 1). Histological sections confirmed that the discs were invaded by cells. Fibrogammin labelled cells showed a more intense extracellular matrix staining by toluidine blue than unlabelled discs (Fig. 2). AP activity (Fig. 3) was also increased in Fibrogammin labelled chronOS discs.

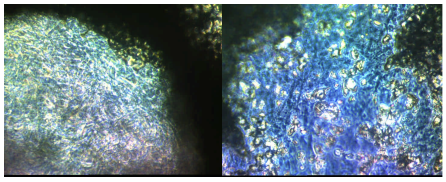

Fig. 1: Phase contrast microscopy of MSC layers without (left) and with Fibrogammin (right).

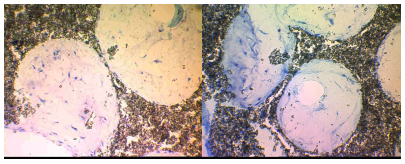

Fig. 2: Toluidine blue staining of MSC layers without (left) and with Fibrogammin (right).

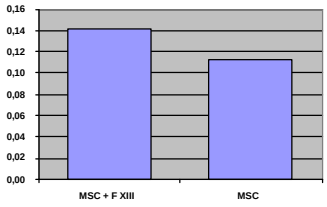

Fig. 3: AP activity of MSC in chronOs cylinders.

DISCUSSION & CONCLUSIONS: The combination of Fibrogammin and chronOS showed a clear and positive effect on cell growth of MSC. AP activity was increased and histological stains showed a dense extracellular matrix production. Further studies should give more precise information on type I collagen and osteocalcin production. A combination of chronOS and Fibrogammin can improve bone and fracture healing.

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PROLIFERATION OF HUMAN FETAL CELLS IN FIBRIN GEL

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[1]Center for Orthopaedic Research, EPFL, 1015 Lausanne, CH; [2]Lab of Fetal Medicine, Dept of Obstetrics, CHUV, 1011 Lausanne, CH; [3]Lab of Polymer & Composite Technology, Materials Institute, EPFL, 1015 Lausanne, CH; [4]Hôpital Orthopédique de la Suisse Romande, 1005 Lausanne, CH

INTRODUCTION: For clinical bone transplantations, engineered tissues are being studied with great interest by many researchers. However, there are still no totally satisfying grafts because of possible immuno-rejection and/or mechanical weakness of the scaffold. If the grafts are large, cells in its central part tend to die because of nutrition shortage. To solve these problems, we are developing a new bone tissue engineering. The idea is to use cells for facilitating matrix production, gel for nutrition storage and cell carrier and polymer scaffold for the mechanical support. We use fetal cells that have advantage in immunological tolerance[1], fibrin gel that is biodegradable and may induce vascularization[2], and Poly-lactite-acid reinforced scaffold. In this study, we investigate the proliferation of human fetal cell in a fibrin gel.

METHODS: Fetal cells are cultured on 75 mm^2 culture flask, collected by trypsinization and centrifugation just before use. Cell suspension is adjusted to 3 different concentrations (3.2×10^6 cells/ml, 6.4×10^6 cells/ml, 12.8×10^6 cells/ml). Tisseel® fibrin sealant (Baxter AG) is used as gel material. Fibrinogen and Thrombin solutions are made referring to manufacturer's instruction. Aprotitonin solution is added to the fibrinogen powder and diluted with equal volume of PBS. $CaCl_2$ solution is added to thrombin powder for slow solidification (4 IU/ml) and mixed with equal volume of each cell suspension. 30 µl of fibrinogen solutions are poured on to each well of 96 well plate. Then 30 µl of thrombin-cell suspensions are poured on the fibrinogen. It takes about 5minutes to obtain a gel. Fibrin–cell gels are then removed from the 96 well plate and cultured in 24 well plate with 1 ml of culture. At day 0, 3, 6 of culture, 4 samples from each group are taken for measurement of proliferation using CellTiter (Promega). The same samples are used for image acquisition of viable cells. Samples are immersed in 100 µl of fluorescein diacetate to stain live cell into green and 100 µl of propidium iodide to stain dead cells into red. The fluoroscent images are taken with confocal microscope.

RESULTS/DISCUSSION: During the 6 days of culture, the fetal cells in fibrin gel proliferate in all groups (Fig. 1). With the lower cell density, the proliferation was faster probably due to the fact that cells have more space to proliferate than with the higher cell concentrations.

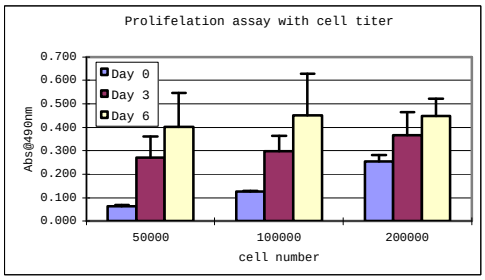

Fig. 1: Proliferation of different density of fetal cells in fibrin gel (mean ± SD, n=4)

Fluorescent images show elongated fetal cells in gel after 6 days in comparaison to round shape cells just after seeding (Fig.2). The fetal cells are then able to grow in the fibrin gel.

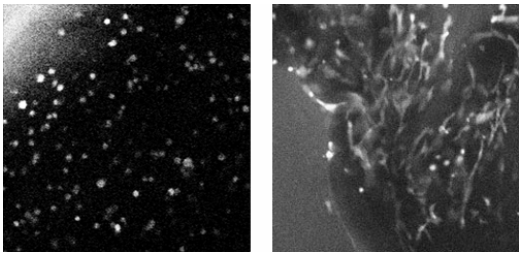

Fig.2: Live cells image in fibrin gel (Left: Day 0, Right: Day6 of 0.8×10^6 cells/ml)

In this preliminary study, we show that fibrin gel can be used as carrier for fetal cells at least from the proliferation point of view. We need to determine now the influence of fibrin gel on the gene expression of fetal cells, especially for osteoblastic markers.

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ADDITION OF OSTEOCALCIN TO HA/COLLAGEN COMPOSITES

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INTRODUCTION: The filling of large osseous defects still poses a major problem in reconstructive surgery. While the ostoeconductive properties of hydroxyapatite (HA)-collagen composites are well recognised [1], the goal of the present study was to investigate, if the addition of osteocalcin, an extracellular matrix protein, enhances bone healing around such implants. Osteocalcin is expressed by osteoblasts and is thought to play a role in the early stages of bone healing. It is crucial in regulating osteoblast activity and binding of hydroxyapatite.

METHODS: Cylindrical implants of 2.5 mm diameter containing 97.5% HA and 2.5 vol% collagen type I were introduced into the tibial head of male adult Wistar rats. 10µg/g of osteocalcin (OC) was added to one group. 6 animals of each group were killed at 2, 7, 14, 28 and 56 days. Specimens were fixed with 4% buffered formalin, washed, decalcified in EDTA, dehydrated and embedded in paraplast. For Immunohistochemistry 2? m sections were dewaxed, dried, washed and incubated with primary antibodies that were detected with biotinylated secondary antibodies followed by a streptavidin/biotin-peroxidase complex. Peroxidase activity was visualised with 3´-3´-diaminobenzidine. We used antibodies against the prevalent intermediate filaments of mesenchymally derived cells (vimentin), bone-specific extracellular matrix products (osteopontin, bone sialoprotein), cell adhesion molecules (CD44s), macrophages (ED1 Cathepsin D) and osteoclasts (TRAP).

RESULTS: 7 days after implantation the interface around the OC implants was rich in mononuclear cells, while around the controls there was a more fibrous interface. Small newly formed osteoid seams were observed around both implants. At 14 days newly formed osteoid reached the implant surface in both groups, the contact area being greater in the OC group. At 28 days the newly formed woven bone around OC implants was replaced partly by lamellar bone (Fig. 1). Inflammatory cells, fibroblasts and mesenchymal stromal cells were observed abundantly around both implant types at day 2 and decreased more rapidly around the OC implants than in the controls. Staining for CD 44, indicating interaction of fibroblasts and osteoblasts, was seen as a small seam around the implants at day 2 and increased until day 14, more markedly around OC implants. Macrophages, specified with Cathepsin D and ED 1, were abundant at day 7 through 14 and decreased thereafter around the OC implants, while the reaction for ED1 and Cathepsin D increased until day 28 in the controls. Total cell numbers per low power field differed significantly at days 7, 14 and 28 ($p<0.05$). Both osteogenic progenitor cells and osteoblasts, staining for osteopontin and bone sialoprotein, were observed earlier (day 7) at the interface around OC implants than around the controls (day 14).

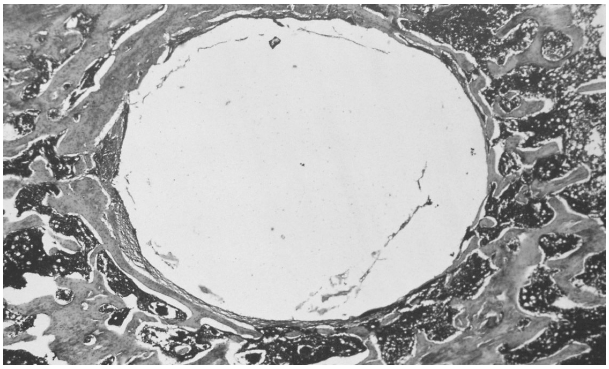

Fig. 1: Newly formed lamellar and woven bone around OC implant at 28 days (HE, x10).

DISCUSSION & CONCLUSIONS: The addition of osteocalcin enhances bone formation around HA-collagen composites in the rat tibia. The earlier observation and increased number of mononuclear phagocytozing cells at the interface around the OC implants suggests an earlier onset and higher rate of bone remodeling. The higher osteogenic capability of the bone-implant interface was demonstrated by the earlier and increased expression of bone-specific matrix proteins (osteopontin, bone sialoprotein) around the OC implants. The addition of further matrix components to HA-collagen composites appears to be an attractive step in the attempts to achieve a better osteogenetic potential of bone substitute materials to enhance defect healing.

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LONG-TERM AGING OF BRUSHITE CEMENTS IN PHYSIOLOGICAL CONDITIONS : AN IN VITRO STUDY.

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INTRODUCTION: An in vitro aging experiment in simulated physiological conditions has been undertaken, with the aim of understanding the physico-chemical aspects of the long term in vivo degradation of brushite cements.

A statistical design of experiment has been established in order to evaluate selected parameters of the initial cement composition on their long term in vitro aging. Four compositional factors were selected : (A) nature of rheological additive (hyaluronic acid–HA vs hydroxyproprylmethyl cellulose–HPMC, appr. 1 %wt), (B) source of sulfate ions ($CaSO_4 \cdot 0.5H_2O$–plaster of Paris vs sulfuric acid, atomic ratio S/Ca = 0.013), (C) magnesium phosphate addition (0 %wt vs appr 8.5 %wt $MgHPO_4 \cdot 3H_2O$) and (D) final cement porosity (35 %vol vs 45 %vol). Thus, 16 different cement compositions were prepared and left for aging in physiological conditions from 1 day up to 16 weeks (37°C, modified Hank's solution free of glucose and Mg ions, renewed every 2–3 days).

Elemental chemical analyses (P, S, Ca, Mg) and pH measurements were used to calculate the state of saturation of the recovered aging solutions with respect to various phases, such as brushite ($CaHPO_4 \cdot 2H_2O$, DCPD), hydroxyapatite ($Ca_5OH(PO_4)_3$, HAp), octocalcium phosphate ($Ca_8H_2(PO_4)_6 \cdot 5H_2O$ OCP) and newberryite ($MgHPO_4 \cdot 3H_2O$, DMPT). The phases present in the aged cement specimens were determined by X-ray diffraction.

Already available results show that brushite cement aging is mainly affected by the presence of Mg and by the nature of the rheological additive (HA vs HPMC). Thus, the simultaneous presence of HA and of Mg visibly promotes faster degradation of brushite cements ; with HA and Mg, extensive degradation occurs between 8 and 12 weeks, whereas all the cements containing HPMC keep their full integrity beyond 16 weeks. Solubility calculations confirm the higher solubility of DCPD in brushite cements containing HA and Mg simultaneously. In that case the specific area of brushite is increased. The simultaneous presence of HPMC and Mg increases the supersaturation of the recovered physiological solutions with respect to HAp ; up to 8 weeks, only the aging solutions brought into contact with brushite cements containing HPMC and Mg are supersaturated with respect to OCP.

In conclusion, this study shows that the degradability of brushite cements in physiological conditions is markedly increased by small amounts of additives such as DMPT and HA, which increase the solubility of DCPD in physiological liquids. On the other hand, their stability is strongly increased by the presence of HPMC. Simultaneous addition of HA and Mg to these cements promote OCP and HAp precipitation in surrounding physiological liquids.

A second study is underway to focus on the Magnesium effect. The effects of Magnesium salt nature and granulometry will be investigated.

ACKNOWLEDGEMENTS: The financial support of the Robert Mathys foundation and of Stratec Medical is gratefully acknowledged. Special thanks are due to Dr M. Bohner ant to C. Frei for stimulating discussion and suggestions.

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IN VITRO EVALUATION & SURFACE CHARACTERISATION OF BIORESORBABLE POLYLACTIDE FOAMS SURFACE COATED WITH FIBRONECTIN & COLLAGEN

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INTRODUCTION: Synthetic biodegradable polymers are ideal for their application in tissue engineering owing to their mechanical strength, easy manipulation into desired shapes, and optimal degradation kinetics, creating the needed (micro) stress environment for the growing tissue [1]. However, their hydrophobic nature, lack of cell-recognition signals, and poor surface structure and chemistry for cell attachment, proliferation, and differentiation limits their application as supportive three-dimensional matrices for cells [2].

Coating surfaces with extracellular matrix (ECM) proteins such as fibronectin, vitronectin, and collagen, provides an 'adhesive interface' between the scaffold material and the cells that resembles the native cellular milieu, whose organization and production modulates and enhances cell adhesion through transmembrane integrin receptors [3]. The reason for selecting complete ECM proteins as opposed to peptide sequences is that fibronectin and collagen not only provide the cell binding sequence for cell adhesion but also provide secondary interactions with other ECM proteins and interactions with growth factors that stabilise the binding of the cells thus strengthening cell adhesion, which results in enhanced cell growth and maturation. Furthermore ECM proteins do not cause any harmful side effects, as they are the natural ligands found *in vivo*.

In the present work highly porous poly(D,L-lactide) (PDLLA) scaffolds were prepared by thermally induced phase separation (TIPS) process of polymer solutions and subsequent solvent sublimation. The scaffolds were characterised in terms of their surface properties, before and after protein surface modification.

METHODS: Purasorb® PDLLA with inherent viscosity of 1.62 dl/g was purchased from Purac biochem (Goerinchem, The Netherlands). Dimethylcarbonate (DMC, 99% in purity), used for the fabrication of PDLLA foams was purchased from Sigma Aldrich. The polymers and solvent were used without further purification.

The polymer was dissolved in dimethylcarbonate to give a polymer weight to solvent volume ratio of 5 %. The mixture was stirred overnight to obtain a homogeneous polymer solution. The resulting solution was transferred to a lypholisation flask, which was immersed in liquid nitrogen and maintained at -196°C for 2 h. The frozen mixture was then transferred into an ethylene glycol bath at -10°C and connected to a vacuum pump (10^{-2} mbar). The solvent was sublimed at -10°C for 48 h and then at 0°C for 48 h. The sample was completely dried at room temperature in a vacuum oven until reaching a constant weight. More details about the preparation of the foam scaffolds have been presented elsewhere [4].

Surface characterisation of the porous PDLLA scaffolds was carried out pre- and post-modification of the surfaces with human plasma fibronectin purchased from Chemicon Europe (Hamsphire, UK) and bovine collagen type I purchased from Sigma Aldrich (Dorset, UK). To determine the biocompatibility of the scaffold surface, murine fibroblast cultures were established to evaluate cell adhesion and proliferation.

The porous morphology of the samples was examined with scanning electron microscopy (SEM) working at 20 kV. Specimens were cut with a razor blade to enable examination of longitudinal and transverse sections.

Time and pH-dependent Zeta potential measurements in 1 mM KCl supporting electrolyte solution were performed to characterise the material

swelling in water and the changing surface chemistry. Wettability measurements using the capillary-rise method and scanning electron microscopy were used to characterise the samples as a function of surface modification and time in culture. Zeta potential, as well as wettability measurements allow the changes of the polymer surface character to be followed in a non-destructive manner.

RESULTS: The foams possess two distinct pore sizes, as shown in Figure 1 i.e., macroprores with diameters > 100 μm interconnected by micropores of 10-50 μm diameter. The pores are preferentially orientated in the cooling direction, which is typical of the TIPS process [4].

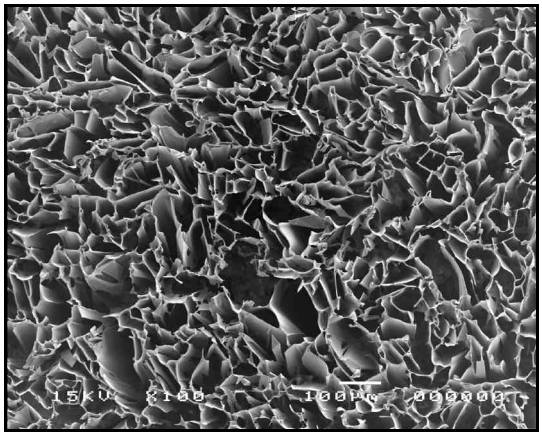

Fig 1: SEM micrograph perpendicular to the tubular pore direction in a PDLLA scaffold.

As determined by previous studies [4], the apparent density of the PDLLA foams is 0.06 g/cm^3; porosity of the scaffolds is > 93%, with a total porous volume of 9.5-11 cm^3/g.

The measured Zeta potential of the pure polymer scaffold is negative over the whole pH range and the isoelectric point (i.e., where Zeta potential is zero) is in the remit of pH 2.2. The zeta potential plateau value is –25 mV. The results of the Zeta potential measurements clearly indicate that the polymer contains acidic functional groups. The polymer is quite hydrophobic prior to protein adsorption as shown by the wettability measurements.

DISCUSSION & CONCLUSIONS: The objective of the current work was to characterise the PDLLA scaffold in terms of porosity, pore size, wettability, and electrokinetic behaviour by zeta potential measurements.

The porous morphology of the PDLLA scaffolds as examined with SCM supports their application in tissue engineering. The high porosity, void fraction, interconnected pores and continuous channels provide sufficient space for cell adhesion, distribution, and ECM regeneration. However, the application of such polymers for cell adhesion, proliferation, and differentiation may be compromised owing to their hydrophobic nature.

Surface characteristics of polymers for biomedical application are highly important. Further work entails optimisation of these parameters through surface chemistry modification and protein surface coating, resulting in higher seeding density of murine fibroblast cells in comparison to the unmodified scaffolds and generating an environment that promotes normal physiological cellular behaviour.

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THERMALLY OXIDISED TI6AL4V ALLOY ENHANCES OSTEOBLASTIC CELLS BEHAVIOUR

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INTRODUCTION: Ti6Al4V is among the most commonly used implant materials due to its high corrosion resistance, good mechanical properties and low toxicity. Thermal oxidation can offer thick, highly crystalline oxide films with very good protective performances of both wear and corrosion properties. Surface characteristics of materials play an essential role in osteoblast adhesion on biomaterials. The initial interaction of bone cells with the biomaterial influence the cell capacity to proliferate and to differentiate itself on contact with the implant [1]. In our previous study, we have demostrated that thermal oxidation treatment of Ti6Al4V at 500ºC and 700ºC for 1 hour protect the material and do not decrease the high in vitro corrosion resistance of the Ti6Al4V alloy [2]. The purpose of the present study was to evaluate the biocompatibility of surface modification after thermal oxidation treatments at two different temperatures in comparison to the polished as-received state in primary culture of human osteoblastic cells.

METHODS: The material used in this research was Ti6Al4V (TiAlV) (wt%) alloy. The oxidation treatment was performed at 500 ºC (TiAlV500) and 700 ºC (TiAlV700) for 1 hour, in air. Human osteoblastic cells were derived from fresh trabecular bone explants from knee obtained during arthroplasty procedures in patients aged 75±5. Kinetic of cell attachment was determinated for quantification by spectrofluorimetry using BCECF-AM. Actin cytoskeletal organization was examined by labeling with rhodamine phalloidin. The morphology of cells was tested using acridine orange which simultaneous stain for DNA and RNA. Alamar blue staning was used to determine the cell viability while Hoechst 33342 staining was used to evaluate cell proliferation as a correlation of DNA content.

RESULTS: Temporal profile of the osteoblastic cell attachment showed that the percentage of bound cells increased with time until 3 hours on all tested substrates. The percentage of adhesion after 1 and 2 hours was significantly greater on TiAlV700 than time matched TiAlV500. To evaluate the actin cytoskeletal organization we have used the criteria of Sinha which classified the degree of cytoskeletal organization into three classes [3]. Each class represents a different stage in degree of cytoskeletal reorganization, with Type I the least organized, Type II intermediate and Type III the most organized. The relative percentage of each morphologic cell type was determined as a function of time and substrate. The percentage of Type III cells increased with time on all substrates tested. After 3 hour, thermal oxidation significantly increased the percentage of Type III cells. Thus, TiAlV700 showed maximal number of this type of cells when compared to TiAlV500 and TiAlV untreated. Data of cytoskeletal organization correlate well with the enhanced cell attachment observed for TiAlV700 when compared to TiAlV500. Microscopical examination of cell morphology after 24 hours in culture revealed well spread, confluent and viable osteoblasts on all substrates tested. No obvious differences were visually found between the different substrates.

Hoechst staining showed that, in all cases, the cells proliferated between day 3 and day 7. After 3 days in culture, a statistically significant increase in proliferation was measured for osteoblasts on TiAlV and TiAlV500 as compared to TiAlV700. At 7 days there was no significant difference in the proliferation on any of the material. This pattern was repeated for cell viability measured by Alamar Blue staning.

DISCUSSION & CONCLUSIONS: Thermal oxidation treatments do not affect the material biocompatibility. Moreover, our data on early adhesion events such as cell attachment and cytoskeletal organization indicate that TiAlV700 improves osteoblast response as compared to TiAlV500. Experiments aimed to study cell proliferation and viability indicate that TiAlV700 do not alter the osteoblast long term properties. Taken together, these results indicated that thermal oxidation improves osteoblast behaviour in comparison to the polished as-received state.

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MORPHOLOGICAL CHANGES IN THE OSTEO-CARTILAGINEUS ENDPLATE AND ANULUS FIBROSUS OF CERVICAL AND THORACIC SPINE AFTER TRAUMA – CASE REPORTS.

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INTRODUCTION: The aim of the study was to evaluate typical morphological changes in the endplate and the AF in relation to time after trauma. Animal study group: The anulus fibrosus (AF) and the endplate of lower cervical spine from healthy 6 to 8 months old pigs was used to evaluate vertebral bone and disc architecture as well as cell death by necrosis. Patients study group: Specimens of the AF and endplate from the lower cervical and thoracic spine of four traumatically injured patients were taken during operation. Anterior fusion was performed in between four months after trauma. Recent investigations have shown that chondrocytes undergo apoptosis to regulate cell proliferation in the hypertrophic zone of the growth plate in the maturation process 1. Evidence has been presented so far that apoptotic cell death occurs in the AF of degenerative discs 2+3. The possibility that necrosis is even more important in traumatic injured discs, has not yet been published.

METHODS: Animal study group: Specimens from AF were investigated after one, twelve and twenty four hours after death, to study necrosis in chondrocytes. Endplates were collected and embedded in different positions to evaluate different angles of cutting planes (sagital to horizontal planes). Patient study group: Specimens from four patients (17 – 58 a) have been investigated so far. One taken at the day of trauma, the other three at one week, three weeks and four months. Tissue samples were taken during operation and fixed immediately (less than 15 minutes) for histological and ultrastructure evaluations. Investigations were done by light microscopy (LM) and transmission electron microscopy (TEM). The specimens were fixed for LM investigations with Schaffer solution for two days followed by dehydration with Ethanol and embedded in Methylmethacrylat. The tissue blocks were sectioned at 4-6µm. Staining was performed with Goldner and Methylenblue. LM sections were examined on an AX70 Olympus-Microscope.

If possible specimens were taken for TEM. From outer AF tissue samples were diced in one millimetre cubes. They were fixed in 0,1 M Glutaraldehyd in Cacodylat buffer for twelve hours. The specimens were rinsed with Cacodylat buffer and postfixed with Osmiumtetroxyd. Embedding was procedured in the routine manner with Araldit. Semithin (6µm) and ultrathin (0,90µm) sections were cut and stained with Uranyl acetat and lead nitrat (Reynolds). Sections on 200µm mesh grids were examined in a TEM 10 (Zeiss). Five different areas of each patient and each specimen of the pig were studied.

RESULTS, DISCUSSION & CONCLUSIONS: Animal study group (LM and TEM): In TEM morphological changes could be detected after 12 hours, starting with swelling of the organelles and sometimes also with leaking in the cell membrane. At 24 hours same lesions became more intense. Osmiophilic cell detritus was identified in the disc tissue same time. In LM horizontal planes of sections gave a better overview, than sagital sections. Morphological changes in LM have not been as obvious as in TEM in the time range of twenty four hours.

Patient study group (LM and TEM): Not surprisingly, in specimens obtained on the day of trauma (patient 1) ample signs of massive bleeding and cracks within both, hard and soft tissue components of the samples were the predominant features. In TEM cells appear swollen, next to osmiophilic cells.

At one week after the event (patient 2), irregularities especially at the bone-(fibro)cartilage interface including first signs of vascular proliferation and bone remodelling, bleeding due to vascular damage and dead chondrocytes were conspicuous. Electron micrographs showed that cell death is evident as well as swollen cells with enlarged organelles.

Cartilage cell clusters as a sign of focal remodelling was evident three weeks after trauma (patient 3) in both, at the osteochondral junction and AF.

Four months after trauma (patient 4) vascular ingrowths into bone marrow, endplate and AF was paralleled by interposition of fibrous connective tissue containing large amounts of cellular components. New areas of fibrocartilage were seen next to damaged tissue. Ultrastructural investigations demonstrated strongly osmiophilic

chongrocytes and on the other hand sign of necrosis.

In preliminary results, necrosis seems to be more important in the first two weeks after trauma. Cartilage cell clusters could be seen first three weeks after trauma. Four months after trauma vascular ingrowths into bone marrow and anulus fibrosus is evident.

We expect with our morphological investigations of the posttraumatic period to show the specific reaction of the chondrocytes in discs and endplate to comparable traumata. This might influence the clinical procedure in some cases.

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HISTOLOGICAL AND BIOLOGICAL ANALYSIS OF TISSUE DIFFERENTIATION DURING BONE HEALING AROUND TITANIUM IMPLANTS

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INTRODUCTION: Due to the insertion of an implant, a cascade of bone healing events occurs such as blood clot formation, inflammatory reaction eventually followed by the formation of a fibrous callus [1]. The latter will be replaced by woven bone, that in turn will be remodeled into lamellar bone. At the end, this leads to direct bone-to-implant contact coined "osseointegration". However, detailed information on the cellular processes and tissue differentiation that occur early during bone healing around endosseous implants is very limited. In the perspective of immediate implant loading, this insight becomes important. The aim of the present study was to investigate these healing processes surrounding a titanium implant and mainly during the first days and weeks after implant placement.

METHODS:
Animals: Five mature New Zealand White rabbits were selected. Both in trabecular bone of the epiphysis and in cortical bone of the diaphysis of the tibia, implants were placed at different time points, respectively 42, 28, 14, 7, 3 and 1 day before sacrifice.
Implants: Cp wrought titanium implants, with a diameter of 1 mm and lengths between 6 and 10 mm, according to the thickness of the bone, were placed press-fit.
Specimen preparation and analyses: The samples (implants with surrounding bone) from 2 rabbits were fixed in 2 % paraformaldehyde, whereafter the implants were removed and the bone samples decalcified and embedded in paraffin. The samples from the 3 other rabbits were fixed in an 4% buffered formaldehyde solution, after which the implants were removed from the samples in 2 out of 3 rabbits only. The samples were dehydrated in a graded series of ethanol and embedded in polymethylmetacrylate (VWR International, Leuven). The removed implants were screened with a scanning electron microscope (FEI-Philips XL30 ESEM FEG) to ensure that no tissues were ripped off during implant removal.
The following stainings were performed:
Paraffin sections: hematoxylin–eosine staining for a general overview, TRAP staining (tartrate resistant acid phosphatase) for the visualisation of osteoclasts and safranine O-Fast Green staining for the visualisation of cartilage.
Polymethylmetacrylate sections: toluidin blue staining for a general overview, Von Kossa staining for the visualisation of mineralised bone and Goldner staining for the visualisation of osteoid.

RESULTS & DISCUSSION: Presently we are analysing the histology. 12 implants per animal were introduced according to an inverse time scheme, limiting the subject-dependent variation. Time points of 1, 3 and 7 days were chosen to focus on the early events of bone healing around the implants, with special attention to the different cell types that allow correct healing. 42 days was taken as maximum time point because the remodeling cycle takes 6 weeks in rabbits. The implants were removed in 4 out of 5 rabbits to allow embedding in paraffin and/or production of micro-thin sections resulting in a higher number of sections per sample. Histological sections up to 4 µm (polymethyl-metacrylate) or 7 µm (paraffin) were cut when the implants were removed. Cutting the samples with the titanium implants still present resulted in a limited number of sections per sample and in thicker sections up to 20 µm.
In future experiments, histomorphometric and molecular analyses will be performed as well. As mentioned earlier, there is no detailed information on the types of cells that sequentially are present during the first steps of healing of bone around an implant. This study is part of a project on, bone healing and bone response around loaded implants.

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OSTEOCONDUCTIVITY OF INJECTABLE COMPOSITES IN RABBITS

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INTRODUCTION: Thermoplastic composites of bioactive glass S53P4 and Poly(ε-caprolactone-co-DL-lactide) were developed as injectable bone filler material. Composites with different amount and different area/volume ratio of bioactive glass (BAG) were earlier characterized *in vitro*[1]. Aim of this study was to evaluate composites with different amount of BAG for their osteoconductive properties and overall biocompatibility in bone.

METHODS: Bioactive glass S53P4 (Abmin Technologies, Finland), granule size 90-315 µm, was blended in P(CL/DL-LA) matrix as described by Rich et al [1]. Wt-% composition of the glass is 53.0 SiO_2, 23.0 Na_2O, 20.0 CaO, 4.0 P_2O_5. Three composites and neat copolymer as control were used in the study (Table 1). Sucrose as *in situ* porosity creating agent was blended to one composite to enhance bone ingrowth. Initial compressive strength of the composites was determined using PC programmed testing machine.
The study was approved by the Ethical Committee for the Animal Experiments at the University of Turku and it was carried out according to national guidelines for laboratory animal care. Operations were performed under general anesthesia and aseptic surgical standards were followed. Trephine bur was used to drill a round defect (depth 5 mm, diameter 6 mm) in the medial condyle of the distal femurs of 12 female NZW rabbits. Materials were heated and injected to fill the bone defect (n=24).
At 4 and 8 weeks 6 animals were killed, specimens obtained, fixed and dehydrated in ethanol and embedded in resin. Cut&grind –method was used to produce thin sections for light microscopy. Van Gieson's and Masson-Goldberg trichrome stains were used. Direct bone contact at the biomaterial-tissue interface was measured from the sections with computerized histometric analysis system.

Table 1. Composites of bioactive glass S53P4 (BAG) and P(CL/DL-LA) *used in the study.*

	C40	C50S	C60	C0
Wt-% of BAG	40	50	60	-
Wt-% of sucrose	-	20	-	-

RESULTS: Composites had good handling properties and adjusted well to the cavitary bone defect. They were injectable at 47-50°C and hardened within 30 seconds. Initial compression strength of the composites C60 and C50S was 7(±1) MPa. All materials had low foreign body cell reaction and they were considered biocompatible in bone.

Results of the histometric analysis are shown in *Fig.1.* New bone formation occurred on all composite specimens whereas copolymer without BAG was always surrounded by thin and dense fibrous tissue capsule. Both ongrowth and ingrowth of bone was regulated by the amount of glass granules in the composite. C40 had only few glass granules in contact with host tissue and showed almost no bone ingrowth as well as poor bone contact. Composites C50S and C60 showed good integration into trabecular bone and especially C60 conducted bone growth along the composite surface. Bone ingrowth occurred around the glass granules in outermost part of the composites. Addition of sucrose did not result in significantly higher bone ingrowth.

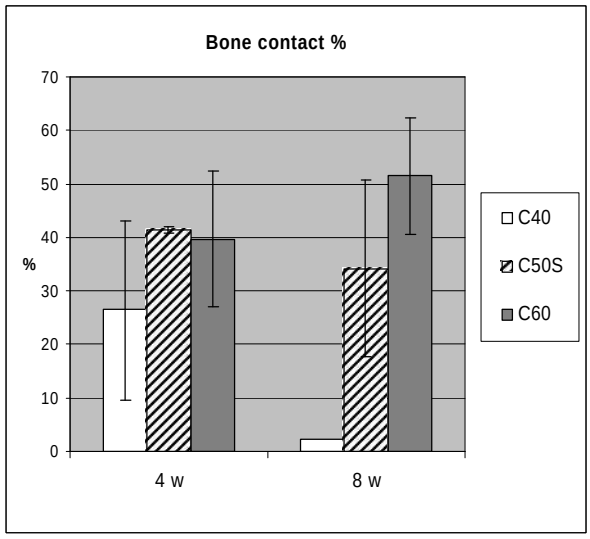

Fig.1.Direct bone contact % (average, SD) at the composite-tissue interface at 4 and 8 weeks measured as percentage of the whole interface.

DISCUSSION & CONCLUSIONS: Injectable composites of bioactive glass S53P4 and P(CL/DL-LA) were biocompatible and integrated into host bone. Resorption of the copolymer *in vivo* seems to be slower than expected and may need to be adjusted for better clinical performance. Growth of bone into the composite occurred via glass granules but ingrowth rate was modest. Addition of sucrose as an *in situ* porosity creating agent did not result in significant increase in bone ingrowth in 8 weeks. Composites with 50 and 60 wt-% of bioactive glass showed good osteoconductivity, had good handling properties and initial mechanical properties corresponding to those of human cancellous bone. In conclusion, they fulfill several properties of ideal injectactable bone filler substitute.

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Phenotypic Osteoblasts for *in vitro* Testing of Bone Implants and Substitutes

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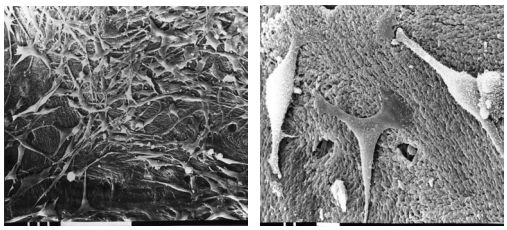
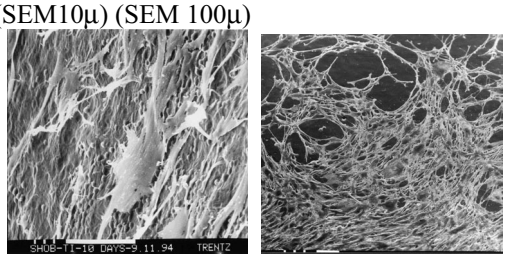
INTRODUCTION: The treatment of osseous defects remains an unsolved problem. Autogenic bone grafts represent the state-of-art treatment of bone defects. However, disadvantages of autogenic bone grafts include limited availability, harvesting morbidity, and insufficient biomechanical properties. These problems have initiated the development of several allogenic, xenogenic, and synthetic bone graft alternatives. Still, related cell-mediated immune responses, as well as synthesis and resorption processes by osteoblasts and osteoclasts are not yet fully controllable. Bone remodelling is a result of the balance between osteoclastic resorption and osteoblastic synthesis, clinically representing a crucial issue as to long term mechanical stability. Furthermore, vascularisation and involvement of neuronal fibres and neuropeptides are very important for the development, growth, and differentiation of bone cells and matrix [1]. In the present *in vitro* study we investigated the feasibility to culture osteoblasts of various origins on synthetic, xenogenic, and metallic materials.

METHODS: Cells: Mouse embryonic cells (MC3T3-E1), Primary Human Osteoblasts (hOB)

Implant Materials: Metals: Titanium (c.p. Titanium ISO TC150 3832/2), Stainless steel (ISO TC150 3832/1). Bone Substitutes: Xenogenic Solvent Dehydrated Cancellous Bone, Synthetic Bone Substitutes Hydroxylapatite. Methods of Analysis: MTT-Test [2], Alkaline phosphatase (ALP) and Osteocalcin (OC) level were measured on day 1, 3, and 7 after culture. Additionally Scanning Electron Microsocopy (SEM) was performed on day 7.



MC3T3-E1+Hydroxylapatite (SEM10µ) hOB + Hydroxylapatite (SEM 100µ)



hOB + Titanium (SEM 100µ) hOB + stainless steel (SEM 100µ)

Fig 1: SEM of in-vitro osteoblasts on different biomaterials.

RESULTS: MTT-Test: Cytotoxicity and cell proliferation did not show significant differences between the various cells and implant materials. ALP level was significantly lower regarding human osteoblasts cultured on stainless steel and Hydroxylapatite (Table 1). OC production of hOB was significantly lower on Hydroxylapatite [3]. MC3T3-E1 cells showed comparable growth on all investigated materials. Primary human osteoblasts (hOB) showed different growth on all biomaterials. Best results were obtained on titanium.

MC3T3-E1	24 hours	72 hours
Titanium	89.7 ± 2.7	82.0 ± 3.8
Steel	*77.7 ± 1.5	*58.3 ± 2.0
HA	81.7 ± 4.1	*27.7 ± 1.2
hOB	24 hours	72 hours
Titanium	104.7 ± 7.7	108.7 ± 9.8
Steel	*77.7 ± 1.5	*75.7 ± 1.8
HA	*80.3 ± 2.3	*40.3 ± 3.2

Table I: ALP 24 & 72 hrs after culture * $p < 0.05$

CONCLUSION: Transplantation of cultured bone cells grown on biomaterials represents a promising concept for trauma and orthopaedic surgery. However, appropriate materials are necessary for efficient cell scaffolding. The present results suggest that primary human phenotypic osteoblasts represent the most appropriate model system to evaluate the biocompatibility of bone substitutes *in vitro* [4]. Moreover, the related osteoblast markers (Osteocalcin, ALP, ProCollagen Type I) are expressed more adequately compared to human osteoblast cell lines.

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CYTOKINES SECRETION IN RESPONSE TO WEAR PARTICLES OF THERMALLY OXIDISED TI6AL4V ALLOY

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INTRODUCTION: Aseptic loosening of orthopaedic implants is a serious problem that leads to pain, loss of function, and the need for revision surgery. This problem is caused by a multistep process that includes generation of wear particles from the implants, activation of macrophages and other cells by wear particles, production of cytokines that stimulate osteoclast differentiation and or osteolysis due to the increased number of osteoclast [1]. IL-6 (interleuquin-6) and TNF-α (tumour necrosis factor-α) are the main cytokines that provoke local bone loss and modulate the release of bone resorbing factors from other cells [2]. Titanium (Ti) alloys are well known for their superior mechanical properties as well as for their good biocompatibility, making them desirable as surgical implant materials. However, these alloys have been proven to behave poorly in friction since wear particles were often detected in tissues and organs associated with Ti implants [3]**.** Thermal oxidation treatments of TAValloy aimed to obtain "in situ" ceramic coatings, mainly based on rutile, can offer thick, highly crystalline oxide films with very good protective performances. When this Ti alloy is implanted long term wear results in the accumulation of rutile particles from the surface [4]. The purpose of the current study was to evaluate the inflammatory effects of wear particles released from the oxide layer produced on TAValloy in primary culture of human osteoblasts and macrophages.

METHODS: Mean size of comercially pure Ti and rutile (TiO_2) particles (Johnson Matthey, Ward Hill, MA) was 1 μm. Human osteoblastic cells were derived from fresh trabecular bone explants from knee obtained during arthroplasty procedures in patients aged 69±5. Human peripheral blood monocytes were obtained from healthy donors and isolated by Ficoll sedimentation and adhrence to plastic. Osteoblast and macrophages (200000 cells/well) were seeded in six-well plates in the absence of particles (control group) or with different concentrations of Ti and TiO_2 particles (4.5×10^7, 4.5×10^8, 4.5×10^9 particles/well) for 24 hours. Medium was collected and used to determinate IL-6 secretion for osteoblastic cells and macrophages and TNF-α for macrophages. Cytokines secretion was measured by a solid phase sandwich Enzyme Linked-Inmunosorbent Assay (ELISA; IL-6 Biosource, Camarillo, CA; TNF-α, CLB, Amsterdam, The Netherlands). Aliquots of cell lysates were used for protein determinations by BCA protein assay kit (Pierce, Rockford, IL). Cytokines secretion was corrected by protein total amounts for all the samples tested. Cell toxicity was stimated by the lactate dehydrogenase (LDH) release assay using the Cyto Tox 96 citotoxicity kit (Promega, Madison, WI). Experimental medium as well as different particle solutions were tested for endotoxin using the limulus amoebocyte assay (Sigma, St. Louis, MO).

RESULTS: Osteoblast exposed to Ti particles at a concentration of 4.5×10^9 particles/well produced significantly more IL-6 than osteoblast exposed to TiO_2 particles or unexposed. Macrophages exposed to Ti particles produced much more IL-6 than TiO_2 at the three concentrations tested, being this increase maximum at 4.5×10^9 particles/well. No differences were observed when macrophages were exposed to TiO_2 particles compared to control group. Exposure of macrophages to Ti particles highly induced TNF-α secretion at the three concentrations tested, being this increase maximum at 4.5×10^9 particles/well. Exposure of cells to TiO_2 or Ti particles was not toxic, as reflected by release of the intracellular enzyme lactate dehydrogenase.

DISCUSSION & CONCLUSIONS: The developed surface thermal oxidation treatment seems to increase TAVbiocompatibility. This is highlighted by the lower impact of this treatment on the release of proinflammatory and pro-resortive cytokines. This study demonstrates that wear debris derived from thermal oxidation treatments of prosthetic materials can elicit different biological responses. Understanding these responses may help to identify materials better suited for prostheses.

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BIOMECHANICAL DESIGN OF POROUS STRUCTURES FOR BONE GROWTH STIMULATION

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INTRODUCTION: The ideal bone substitute should integrate bone growth stimulating factors like bioactive materials, cell biology and biomechanical stimulation. The former two factors are generally translated into a porous construct loaded with cells and/or growth factors [1]. In order to allow this structure to promote bone ingrowth a particular porous architecture is required, related to the design of the bone substitute. This structure should mimic the biomechanical properties of the replaced bone at both the macroscopic and microscopic level. For the macroscopic level the apparent stiffness should mimic that of bone and thus impair stress shielding in the surrounding bone. On the microscopic level the porous structure should generate strains that stimulate the bone cells to form new bone. The question can be raised whether the biomaterial and biomechanical requirements can be combined.

METHODS: High-resolution finite element models of three different manufactured porous architectures (fig. 1) were generated using micro-CT images. The bulk mechanical properties of the models were based on those of potential biomaterials. The parameters used for the evaluation of the biomechanical behaviour of these structures were the apparent stiffness (E_{app}) and the histograms of the major principal strains (ε) and stresses (σ) within the porous construct, the former in relation to bone mechanosensing and bone stimulating strain signals [2] and the latter in relation to material strength (σ_{max}).

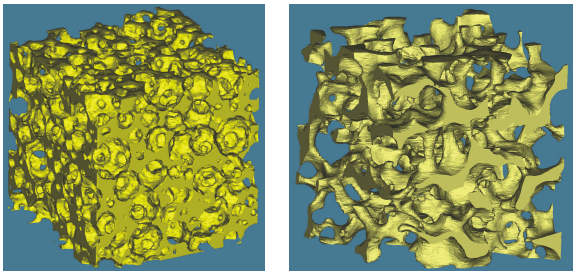

Fig. 1: Architectures 1 and 2 used for the evaluation of these biomaterials for bone growth stimulation.

As a proof of principle three different architectures were combined with different loading conditions (*in vitro* and *in vivo*) and different materials (HA, Al_2O_3, Ti, PMMA and HAPEX^{TM}) to demonstrate the various possibilities of the approach. Figure 2 shows the flowchart of the applied procedure. For the *in vitro* loading condition a vertical frictionless compression test was simulated. For the *in vivo* loading condition a vertical physiological pressure with confined compression was used.

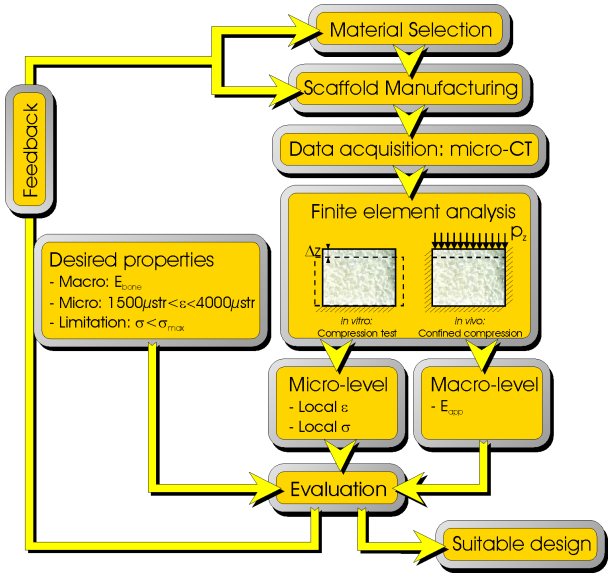

Fig. 2: Flowchart of the procedure to design biomechanically active porous structures.

RESULTS:

Table 1 shows the results for 3 different biomaterials under *in vitro* loading, each with a different architecture. Table 2 compares the results for the *in vitro* and *in vivo* loading of Al_2O_3. Table 3 shows the results for architecture 1 under *in vitro* loading with three different assigned material properties.

The histogram of the principal strains in porous HA (architecture 1) under *in vitro* loading can be seen in figure 3, with the colours indicating the strain regions of bone resorption, homeostasis and stimulation [2].

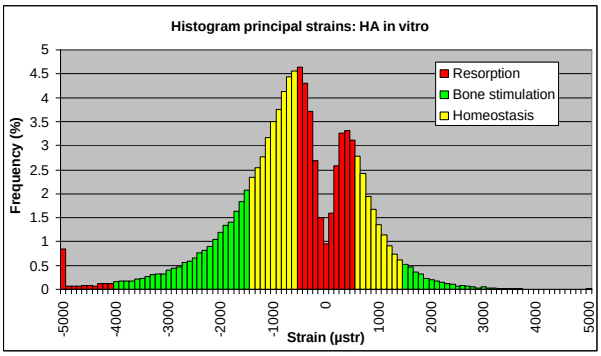

Fig. 3: Histogram of principal strains in HA (architecture 1) under in vitro loading.

Table 1. Bulk mechanical properties and biomechanical properties of hydroxyapatite, Al_2O_3 and titanium, respectively for architectures 1, 2 and 3, under in vitro loading.

	HA	Al_2O_3	Ti
E (GPa)	100	400	110
σ_{max} (MPa)	150	400	450
Porosity (%)	78.9	73.6	84.5
E_{app} (GPa)	3.41	15.9	1.79
% $\sigma > \sigma_{max}$	20.6	34.8	1.3
% $\varepsilon_{bone\ stimulation}$	21.8	21.3	15.5
% $\varepsilon_{resorption}$	33.4	36.5	46.6
% $\varepsilon_{homeostasis}$	44.7	42.1	37.8

Table 2. Biomechanical properties of porous Al_2O_3 (architecture 1) under in vitro and in vivo loading.

	Al_2O_3	
Loading condition	In vitro	In vivo
E_{app} (GPa)	13.653	14.812
% $\sigma > \sigma_{max}$	36.5	6.4
% $\varepsilon_{bone\ stimulation}$	21.8	2.2
% $\varepsilon_{resorption}$	33.4	76.6
% $\varepsilon_{homeostasis}$	44.7	21.1

Table 3. Biomechanical properties of architecture 1 with different material properties under in vitro loading.

	HA	HAPEX™	PMMA
E (GPa)	100	9	2
σ_{max} (MPa)	150	90	30
E_{app} (GPa)	3.41	0.307	0.068
% $\sigma > \sigma_{max}$	20.6	0.04	0.0
% $\varepsilon_{bone\ stim}$	21.8	21.8	21.8
% ε_{resorp}	33.4	33.4	33.4
% $\varepsilon_{homeost}$	44.7	44.7	44.7

DISCUSSION & CONCLUSIONS: Al_2O_3 has the highest bulk stiffness and with architecture 2 the lowest porosity, which results in a very high apparent stiffness (table 1). In contrast, the porous HA and Ti have an apparent stiffness that is in the range of that of cortical bone. Hence stress shielding will be much higher in such an Al_2O_3 scaffold. Due to the high strength/stiffness ratio of Ti, only a very small amount of stresses (1.3%) in the porous Ti structure exceed the strength (σ_{max}) and the Ti structure will withstand the imposed load. However the amount of favourable strains is the lowest of the three structures. Bone formation will be more stimulated in the other two structures.

When comparing the *in vitro* and *in vivo* loading of Al_2O_3 (table 2), a stiffening of the structure due to the confined compression can be noted. The imposed loads *in vivo* are smaller than *in vitro* and the structure is very likely to withstand the *in vivo* loads. However, the strains generated are much less osteogenic than under *in vitro* loading.

When comparing different materials in the same architecture (table 3), the same influence of the strength/stiffness ratio on the strength of the structure can be noted. HAPEX™ and PMMA have a much better strength behaviour than HA. Since the same architecture is used, and the *in vitro* loading consists of a compression by an imposed *displacement* of the top surface, the strains are only defined by the displacement and the architecture, and independent of the bulk material properties.

The approach demonstrated here allows evaluation of different architecture-material combinations under different loading conditions. By providing feedback to material selection and scaffold manufacturing (fig. 2), and using patient-specific desired properties, an optimal individualised bone replacement structure can be obtained. Thus a valid design procedure for tailoring scaffolds to specific applications through feedback to materials and manufacturing is realised.

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QUANTIFICATION OF THE ROLE OF MECHANICAL PARAMETERS ON THE BONE RESPONSE AROUND LOADED TITANIUM IMPLANTS.

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INTRODUCTION: Bone has the ability to alter its mass and structure with changing mechanical usage. It is well established that strain magnitude [l], rate and frequency [2-3] are variables that affect bone. However, questions remain with respect to the actual nature of the mechanical stimulus and the mathematical model that best describes and predicts bone adaptive phenomena. An important factor that hampers the interpretation of animal experimental results is the fact that the bone adaptive response seems to be *site-specific* and *species-specific*. This makes the validation of mathematical theories of bone adaptation an even greater challenge. The influence of several loading parameters on the peri-implant bone adaptive response will be investigated. The aim of this study is to describe the experiments guided by poro-elastic finite element modeling.

METHODS: Highly controlled loading experiments are designed for a c.p. titanium cylindrical implant that is osseointegrated within a bone chamber (Figure 1) installed into the proximal tibia of adult New Zealand white rabbits. The bone chamber model allows to investigate the effect of well controlled loading parameters and protects the tissues inside the chamber from external (uncontrolled) mechanical loading conditions. Data derived from pilot studies on the bone chamber model within our group [4] has shown its validity. The outer bone chamber (∅ 10 mm) (a) is in close contact with the inner bone chamber (b) and both are perforated (c). After osseointegration, the test implant (d) is subjected to specific loading conditions (applied at point 'e') in a displacement-controlled manner by means of an external loading device. During loading, the test implant slides in a Teflon bearing (f). All components are held together by means of screws (g). For every considered loading mode (compression, tension, shear) the bone response will be investigated for 3 different strain rates. For every strain rate two different combinations of amplitude and frequency will be investigated. By combining different load parameters in the animal experiment, the relative importance of strain magnitude, frequency, and rate can be investigated.

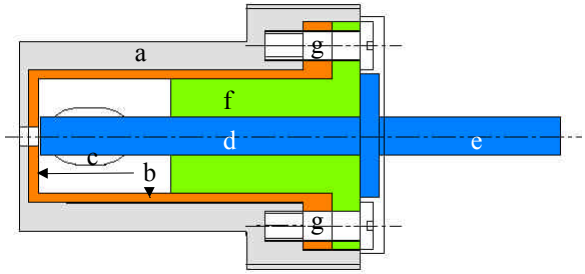

Figure 1: Scheme of bone chamber with test implant.

Before the actual animal experiment starts, a poro-elastic finite element analysis was performed to define the implant displacement that induces the required strains in the tissue surrounding the loaded implant. Frost's mechanostat (Frost 1987) was used as a guideline for the different loading conditions to be applied, resulting in strain levels corresponding to different bone modeling and remodeling activities at the bone-implant interface.

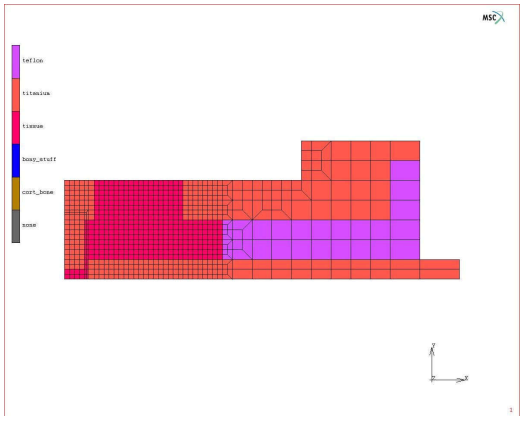

Figure 2: 2D (axisymmetric) model of the bone chamber. The following materials are present: teflon (purple), titanium (orange), tissue (pink).

An axisymmetric finite element model of the chamber and the tissue inside the chamber was created (Figure 2). Poro-elastic material properties were assigned to the tissue inside the chamber and

corresponded to immature (trabecular) bone (Table 1). In order to determine the displacements that have to be applied in the animal experiments a cylindrical volume of interest was defined around the implant, which considered the tissue within a distance of 0.5 mm from the implant surface. The average (equivalent Von Mises) strain within this volume was used as a criterion to determine the displacement to be applied.

Table 1. Material properties used in the simulations.

Young's modulus [MPa]	Poisson's ratio	Permeability [$m^4(Ns)^{-1}$]
500	0.32	10^{-13}

The bone response to the mechanical stimuli will be investigated by use of microfocus computer tomography and will be analysed histologically and histomorphometrically on undecalcified sections after harvesting the test implant and the inner bone chamber containing the tissue inside the chamber.

RESULTS: Results of the poro-elastic finite element analysis defining the implant displacement which induces the required strains in immature bone tissue surrounding the loaded implant are shown in Table 2.

Table 2. Results of the poro-elastic finite element analysis (loading frequency 1 Hz). Average (equivalent Von Mises) strains (+ standard deviation) within the volume of interest are presented.

Required strain	± 100 με	1800 με	4000 με
Displacement	1 μm	7 μm	14 μm
Real strain	297 ± 130 με	2082 ± 913 με	4161 ± 1826 με
Reaction force	10 N	71 N	141 N

Figure 3 shows the equivalent Von Mises strain distribution for the '4000 με' loading regime.

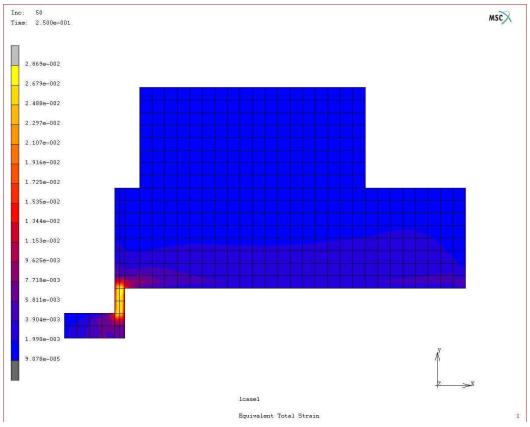

Fig. 3: 2D-analysis for the loading regime of 1 Hz-4000 με, requiring a displacement of 14 μm. The equivalent Von Mises strain distribution is shown here.

DISCUSSION: The originally created finite element analysis served as input for defining the loading conditions of the animal experimental part. The results of these experiments will in return be used as input for the optimalisation of the finite element analysis. The study of different loading parameters within the same site of the same animal by means of a repeated sampling bone chamber eliminates variability due to site-specific and species-specific influences. At this moment the animal experimental part is in progress. Due to the assumptions made for the composition of the computer model and its boundary conditions, the results from the finite element analysis are inherently limited and will be evaluated and optimized when the results of the experimental part are available.

The gained knowledge will be relevant for the biomechanical optimization of skeletal implant fixation and for the application of biomechanical principles within the field of skeletal tissue regeneration.

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TECHNOVIT 9100 NEW AS A LOW-TEMPERATURE METHYL METHACRYLATE EMBEDDING METHOD FOR IMPROVED UNDECALCIFIED TRABEBULAR BONE IMMUNOHISTOCHEMISTRY

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INTRODUCTION: To date few resins combine well-preserved undecalcified bone morphology with reliable immunohistochemistry. Standard methyl methacrylate embedding techniques yield excellent bone morphology but produce unreliable immunohistochemistry since methyl methacrylate has highly exothermic polymerisation. Technovit 9100 New is a low temperature methyl methacrylate embedding system that is believed to significantly improve tissue antigenicity by allowing –20°C polymerisation. In this study, Technovit-embedded undecalcified trabecular bone yielded immunolabelling with 6 bone matrix markers and preserved morphological features in semi-thin 7 µm sections stained with Masson-Goldner, von Kossa, and Toluidine Blue.

METHODS:

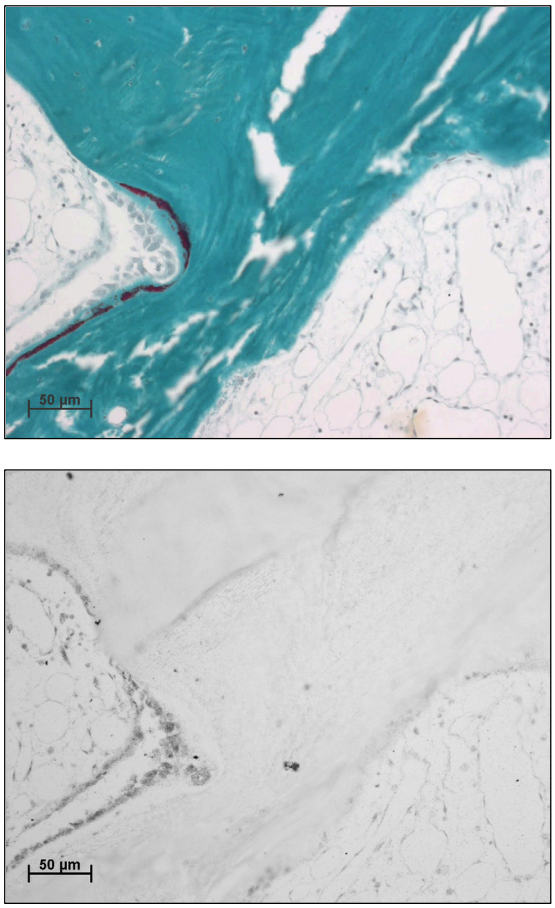
Human (Knees) and bovine (metacarpal) trabecular bone cores were used in the study. Work with human bone was carried out with approval of the Ethic Commission of Graubunden, CH (18/02), with patient consent. Bovine bone was obtained from the local slaughter house (3-4 month old males). Excised bone cores were fixed in 70% ethanol or 4% paraformaldehyde, dehydrated through an ethanol series, defatted in xylene and embedded in Technovit 9100 New. 7 µm serial sections were immunolabelled with monoclonal antibodies against alkaline phosphatase (ALP), Pro-collagen type I aminopropeptide (PINP) and osteonectin from the Developmental Studies Hybridoma Bank (Iowa, USA), and polyclonal antibodies against osteocalcin, osteopontin and bone sialoprotein from the NIH (MD, USA) using immunogold labelling with silver enhancement with no counterstaining. Other sections were also stained with Masson-Goldner, von Kossa and Toluidine Blue.

RESULTS:

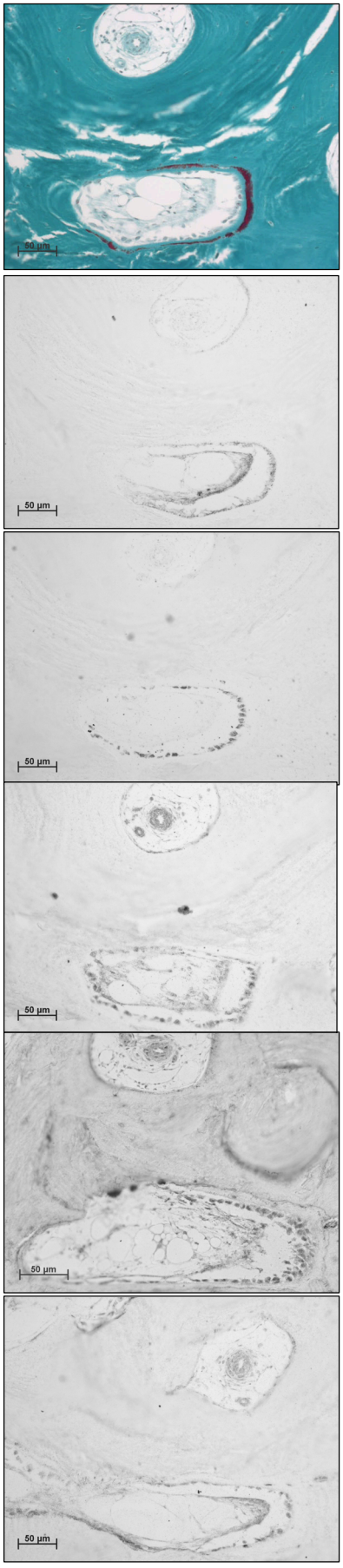
Osteoblasts showed immunoreactivity for osteocalcin, ALP, PINP, osteonectin, osteopontin, and bone sialoprotein (Fig. 1, 2). Cartilage was not immunoreactive for these markers. Antibodies for osteocalcin, osteonectin, PINP, and osteopontin recognized epitopes in both human and bovine bone. Antibodies for ALP and bone sialoprotein recognized epitopes only in human bone.

Bone morphology was well-preserved in 7 µm sections stained with Masson-Goldner, Toluidine Blue, and von Kossa (fig.2). All major cellular constituents of the bone were well-differentiated.

Fig. 1:



Immunolabelling in human bone (73 year old female, knee). A) Masson-Goldner section B) serial section from A labelling osteocalcin. *On the following page- C) Masson-Goldner followed by serial sections D)-H), D) ALP, E) PINP, F) osteonectin, G) osteopontin, H) bone sialoprotein.*



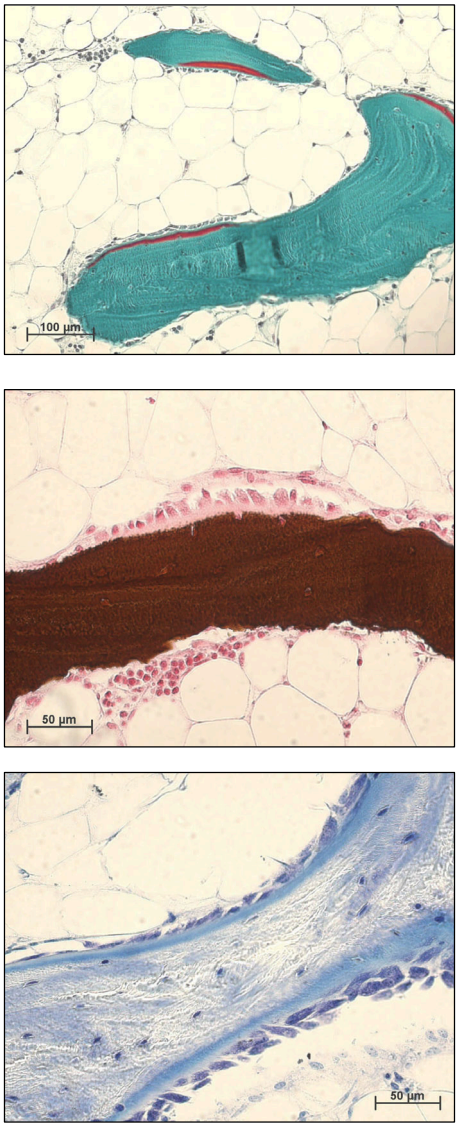


Fig. 2: General histology. A) Masson-Goldner, B) von Kossa, and C) Toludiine Blue.

DISCUSSION & CONCLUSIONS: Successful immunolabelling with various bone matrix markers using human and bovine trabecular bone demonstrates significant antigenic preservation using the low temperature embedding method. Technovit 9100 New has shown to be useful for both routine undecalcified bone morphology and immunohistochemistry. This level of specific labelling was not observed with LR White (results not shown) and was totally absent with methyl methacrylate.

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PERFORMANCE OF A NEW CANCELLOUS BONE EXPLANTS TISSUE CULTURE-LOADING SYSTEM

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INTRODUCTION: A new apparatus "ZETOS" that allows cultivating adult cancellous bone explants under controlled culture and loading environment is tested. Suitable culture conditions over a three week period were determined and the effects of a loading regimen were analyzed.

METHODS Bovine cylindrical (10mm diameter) bone biopsies from 3 sternums were precisely machined with a diamond trephine from 1cm thick bone slices, then strictly plan parallel surfaces were cut in order to obtain 5mm height disks (±1µm). These samples fitted in chambers of similar dimensions and were provided with culture medium (DMEM, 10% FCS, ascorbic acid 10^{-8} M). We determined an optimal medium flow rate from the peristaltic pump at 6ml/h which ensured a uniform diffusion within the bone sample and fluorochrome labellings. Nine samples were immediately processed for histomorphometry and were used as basal control (B Ctr). Twenty six were maintained in culture over 3 weeks, 14 were loaded (L) at 4000µS, 1Hz, 300 cycles per day and 12 were unloaded (U).

RESULTS: Bone mass and Young Modulus (E) were similar in B Ctr, L and U. Trabecular thickness was higher in L and U compared to B Ctr (20%). Bone formation parameters (osteoid parameters, mineral apposition rate and bone formation rate) were increased in L versus U (by 48, 30, 48%, respectively).

Table 1. Bone histologic parameters.

Group	BV/TV (%)	Tb.N (mm-1)	Tb.Th (µm)	Os/BS (%)	MAR (µm/day)	BFR/BS (µm2/µm3/day)
B Ctr.	13.4±3.43	2.51±0.38	67.95±16.85	8.61±1.65		
L	14.44±2.3	2.26±0.30	80.18±13.42	14.38±2.98	0.93±0.17	1.17±0.46
U	13.31±2.10	1.68±0.23	79.49±8.12	9.72±3.67	0.7±0.17	0.79±0.31

DISCUSSION & CONCLUSIONS: In conclusion, a 3-week culture period did not alter mechanical properties and osteoblastic cell reactivity. This model proved to be sensitive to loading conditions. The exercise-induced osteoblastic stimulation might lead to structural adaptation after longer experiments.

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