In vitro study of osteoclastogenesis under simulated bone augmentation

The effects of bone-conditioned medium and saliva on osteoclastogenesis.

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**Dedicated to all the people that have been
by my side during this adventure,
especially to my family**

A journey of 1000 miles starts with the first step
– Lao Tsé

*Vivir no es sólo existir,
sino existir y crear,
saber gozar y sufrir
y no dormir sin soñar.
Descansar, es empezar a morir.*
- Gregorio Marañón

Research environment

The research leading up to the thesis here presented started in July 2013. Collaborators of this PhD were affiliated with:

- Department of Oral Surgery and Stomatology, School of Dental Medicine, University of Bern, Switzerland.

- Department of Oral and Maxillofacial Surgery, School of Dental Medicine, Universitat Internacional de Catalunya, Barcelona, Spain.

- Laboratory of Oral Cell Biology, School of Dental Medicine, University of Bern, Switzerland.

- Robert K. Schenk Laboratory of Oral Histology, School of Dental Medicine, University of Bern, Switzerland.

- Department of Cranio-Maxillofacial Surgery, Bern University Hospital, Inselspital, Bern, Switzerland.

- Department of Oral Biology, Medical University of Vienna, Vienna, Austria.

- Department of Implant Dentistry, Federal University of Santa Catarina, Florianopolis, Brazil.

- Department of Conservative Dentistry and Periodontology, Medical University of Vienna, Vienna, Austria.

Summary

The present PhD thesis is a compendium of four publications broadening the knowledge on osteoclastogenesis under simulated bone augmentation, more especially about the effects of saliva and bone-conditioned medium on osteoclastogenesis. Resorption of bone grafts and host bone, can be a challenge especially when a bonny defect has to be regenerated or there is a lack of host bone due to a trauma, pathology, aging or tooth extraction among others. In the oral cavity, saliva is present and can reach mineralized surfaces, however, the relationship between saliva and bone resorption is yet unknown. Herein, we examined whether saliva affects the process of osteoclastogenesis in vitro, possibly affecting bone healing and bone regeneration. Bone regeneration is a common procedure in traumatology, periodontology, oral and maxillofacial surgery that involves the use of bone fillers. Bone autograft is considered to be the gold standard bone substitute due to its trinity of properties: osteoinductivity, osteoconductivity and osteogenesis. Paracrine factors released from bone autografts might contribute to the overall process of graft consolidation, however the underlying mechanisms are unknown. Here, we determined the protein spectrum released from porcine bone chips into the conditioned medium (BCM) to mimic the paracrine environment of cortical bone grafts. Some of the factors released by bone autografts could maybe influence on the autograft resorption and therefore explain why osteoclasts rapidly form on the surface of bone chips at augmentation sites. The underlying molecular mechanism, however, is unclear. Soluble factors released from bone chips in vitro have a robust impact on mesenchymal cell differentiation. Here we determined whether these soluble factors change the differentiation of hematopoietic cells into osteoclasts, still unknown.

Based on the in vitro results here presented, it can be observed that saliva suppresses osteoclastogenesis and leads to the development of a phagocytic cell phenotype, therefore affecting function of osteoclasts, the bone resorbing cells. Resorption of bone autografts could be attributed to some of the proteins detected on the secretions of bone autografts, termed bone conditioned medium (BCM). Proteomic analysis showed that BCM contains more than 150 proteins, among which, 43 were categorized into "secreted" and "extracellular matrix". We discovered growth factors that are not only detectable in BCM, but potentially also target cellular processes involved in bone regeneration e.g. pleiotrophin, galectin-1, TGF-β-induced gene (TGFBI), latency-associated peptide forming a complex with TGF-β1, and TGF-β2. Results here presented on the influence of BCM on osteoclastogenesis demonstrated that activated BCM by heat is able to stimulate

osteoclastogenesis in vitro. These in vitro results support the notion that the resorption of autografts may be supported by as yet less defined regulatory mechanisms. Moreover the presented protocols on the use of BCM should encourage to further reveal the paracrine effects of bone grafts during bone regeneration and open a path for translational research in the broad field of reconstructive surgery.

Taking everything together, it can be concluded that saliva affects bone resorption towards the development of a phagocytic cell line, and that not only saliva affects bone resorption but also the secretions from autologous bone grafts. There is enough evidence to conclude that bone autografts not only have three properties, but one more: a regulation property, the fourth dimension of autologous bone grafts.

Table of contents

Abbreviations

BCM: Bone Conditioned Medium

BCMh: Bone Conditioned Medium heated

DBM: Demineralized Bone Matrix

DCM: Conditioned Medium from Demineralized Bone Matrix

TGF-beta: Transforming Growth Factor beta

RANKL: Receptor Activator of Nuclear Factor Kappa-B Ligand

M-CSF: Macrophage Colony Stimulatory Factor

TRAP: Tartrate-resistant acid phosphatase

BM: Bone Marrow

List of papers

This thesis is based on the following papers, which will be referred in the text by their Roman numerals:

Paper I
Saliva Suppresses Osteoclastogenesis in Murine Bone Marrow Cultures
Jordi Caballé-Serrano, Barbara Cvikl, Dieter D. Bosshardt, Daniel Buser, Adrian Lussi, Reinhard Gruber.
Journal of Dental Research 2015;94:192-200. doi: 10.1177/0022034514553977
Impact Factor: 4.1 (according to JCR)

Paper II
Proteomic Analysis of Porcine Bone-Conditioned Medium
Jordi Caballé-Serrano, Dieter D. Bosshardt, Daniel Buser, Reinhard Gruber
International Journal of Oral and Maxillofacial Implants 2014;29:1208–1215. doi: 10.11607 /jomi.3708
Impact Factor: 1.9 (according to JCR)

Paper III
Conditioned Medium from Fresh and Demineralized Bone Enhances Osteoclastogenesis in Murine Bone Marrow Cultures
Jordi Caballé-Serrano, Guenther Schuldt, Dieter D. Bosshardt, Jordi Gargallo-Albiol, Daniel Buser, Reinhard Gruber
Clinical Oral Implants Research 2015. doi: 10.1111/clr.12573
Impact Factor: 3.8 (according to JCR)

Paper IV
Bone Conditioned Medium: preparation and Bioassay
Jordi Caballé-Serrano, Kosaku Sawada, Guenther Schuldt, Dieter D. Bosshardt, Daniel Buser, Reinhard Gruber
Journal of Visualized Experiments, accepted January 2015
No Impact Factor. Indexed in PubMed/MEDLINE, SciFinder and Scopus

Background

Reflexions as a PhD candidate

PhD or *Phylosophiae Doctor* does not refer solely to the field of philosophy, but is used in a broader sense in accordance with its original Greek meaning, which is "love of wisdom" (Wikipedia.org).

Doing a PhD implicates and has to implicate a broad sense overpassing the threshold of science. It comes, as the original name says, to a higher philosophical level, a lover of wisdom. It refers to any kind of wisdom, scientific and personal wisdom. It implicates another way of thinking, to interact with your environment, with the people that surround you.

On bone cells

Osteoclasts
Osteoclasts are the unique bone resorbing cells. Osteoclasts originate from hematopoietic stem cells, when the key factor, receptor activator of nuclear factor kappa-B ligand (RANKL), is present (1). Osteoclastogenesis also requires the M-CSF receptor (c-fms) (2). Osteoclasts are characterized by their multinucleated morphology and the expression of tartrate-resistant acid phosphatase (TRAP), cathepsin k (CatK) and the calcitonin receptor (CTR). Osteoclasts express co-stimulatory molecules activating the immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway (3). Osteoclast-associated receptor (OSCAR) and triggering receptor expressed in myeloid cells (TREM2) are receptors that are associated with the respective adaptor molecules Fc receptor common gamma chain (FcRγ) and DNAX-activating protein 12kDa (DAP12), respectively. Downstream signaling pathways culminate in the increased expression of transcription factors c-fos and nuclear factor of activated T cells c1 (NFATc1), both master regulators of osteoclastogenesis. Moreover, microphthalmia-associated transcription factor (MITF) and PU.1 can modulate osteoclastogenesis (4). Also relevant are genes that regulate cell fusion i.e. dendritic cell–specific transmembrane protein (DC-STAMP) and the ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2 (Atp6v0d2) (5, 6).

When osteoclasts are developed, activation can occur. Activated osteoclasts are polarized and form distinct and unique membrane domains, including the sealing zone, the ruffled border and the functional secretory domain.

(7) Osteoclasts polarization includes rearrangement of F-actin fibers from the cytoskeleton adopting a ring-shape that consists on a dense continuous zone of highly dynamic podosomes (8). These podosomes help osteoclasts to move around the mineralized surface creating resorption grooves or tunnels. Osteoclasts are not only important to remodel bone, but also to release calcium phosphates and growth factors contained in the bone matrix, to the environment. (9) Osteoclast numbers are controlled through the regulation of the formation process and the lifespan. Normally osteoclasts die by apoptosis, however in some conditions, osteoclast survival can be extended in some pathological conditions like in Paget's disease. (10) Osteoclast formation, activity and survival can also be regulated by hormones, like calcitonin - acts directly upon osteoclasts to inhibit their activity or oestrogen - acts indirectly, via the regulation of several cytokines. (11).

Osteoblasts and osteocytes
Osteoblasts are the bone forming cells. Osteoblasts belong to the mesenchyme linage and their formation and development is controlled through all life locally and systemically. (12) Osteoblast function can also depend on different proteins, like the bone morphogenetic protein (13). Bone morphogenetic proteins for example, can be used clinically to stimulate bone formation during fracture repair or bone regeneration (14). Osteoblasts produce a range of different molecules, like cell-cell adhesion proteins, particularly cadherins, which affect osteoclast differentiation and function. (11) Other proteins like Connexin 43 (11) allow connection between osteoblasts, very important to facilitate exchange of ions and small molecules like ATP, nitric oxide and prostaglandins.

Osteocytes, that are the most abundant cells in the bone, are osteoblasts that have freed apoptosis at the end of their cycle and have been trapped in the bone matrix. Contrary to osteoblasts, osteocytes can live decades entombed in the bone matrix. Osteoblasts undergo profound morphologic changes, losing cytoplasm organelles and acquiring a stellar shape with numerous extensions that will connect to other osteocytes through a network inside the mineralized bone, called canalicular network. (15) Osteocytes can transmit signals through the canalicular network like a neuron would do, influencing osteoclasts and osteoclasts. It was initially thought that osteocytes had only a mechanical transduction function (16), but has been shown that not only can transduce mechanical stimuli but also release paracrine signals to the environment to control from the distance osteoblasts and osteoclasts (15, 17).

Paracrine factors

Bone cells can communicate with themselves and with their environment. Different cross-talks exist between the cells to regulate bone formation and resorption via the RANK-RANKL (1), but not only to regulate bone formation and resorption. Osteoblasts complete complex communication networks to interact between them for example releasing Parathyroid hormone-related protein to stimulate the activity of mature osteoblasts, oncostatin M, a member of the IL-6/gp130 family of cytokines, to stimulate osteoblast activity or ephrins to regulate osteoblast differentiation. (18) Osteocytes, considered now the master directors of bone, release chemicals and mechanical signals to regulate osteoblasts and osteoclasts. (19) For example, osteocytes can regulate osteoblasts by producing different molecules such as nitric oxide and prostaglandin E2 or sclerostin (20).

Some of the osteocyte secreted signals, like sclerostin, are also found in bone autograft supernatant, also termed bone conditioned medium (BCM). (21) BCM mainly contains proteins released because of mechanical deterioration of the bone matrix and from the remaining viable cells, including osteocytes (21) – but not selectively those being actively released by osteoclasts during bone resorption (22). Autografts presumably release soluble factors distinguished from the endocrine function of orthotropic bone in whole-organism physiology (23). This factors released by bone autografts have the potential to affect the differentiation of mesenchymal cells in vitro. (24) The first convincing evidence for a regulation function of bone is based on the observation that supernatants from murine long bones that were freed from bone marrow as well as periosteal and endosteal cells, support myelopoiesis in vitro (25). In this model, the osteocytes contributed to the production of growth factors that accumulated in the cell culture medium (25, 26). Bone conditioned medium (BCM) therefore contains the complex mixture of secreted proteins from native cortical bone that is commonly termed "secretome" (27, 28). A characterization and quantification of this secreted proteins by means of a proteomic analysis is always a good tool to study secreted factors . Putting everything together, there is increasing evidence that freshly prepared bone chips release bioactive molecules, thereby supporting the hypothesis that autografts have a transient paracrine-like function.

On bone regeneration and graft consolidation

Principles

The process of bone regeneration involves different types of cells and a variety of signalling pathways to ensure the success of the process. (29)

Bone regeneration consists on three steps. The first step includes hematoma formation and inflammatory reaction. This phase, is short compared to the other two phases, and plays an essential role in the bone regeneration process as prepares the environment for the following phases. After the injury, the formation of a blood clot and the activation of platelets occur. The fibrin matrix provided by the blood clot, facilitates the infiltration of immune cells that would clean and help to release growth factors trapped in the fibrin matrix. Moreover these inflammatory cells release signalling molecules and cytokines that would help to recruit cells necessary for the second and third step. (29, 30) At the end of this phase, osteoclasts are present on the surface of the bone defect.

The second step is the repair phase. In this phase the bone callus is formed, connecting different bone fragments. Endothelial cells and blood vessels start to penetrate the defect area with mesenchymal cells, forming the granulation tissue. This step is crucial to enable the mechanical stabilization of broken parts and to initiate bone formation. Mesenchymal cells will create an osteochondrogenic tissue that will turn into bone. At the end of this phase, woven bone is present that will be later replaced by high-quality lamellar bone. (31) The last step is the remodelling phase. During this last step, the callus is gradually removed and new direct connection between bone parts takes over its mechanical function. Finally, woven bone undergoes structural changes and is replaced in a remodelling process by fully functional lamellar bone. (32)
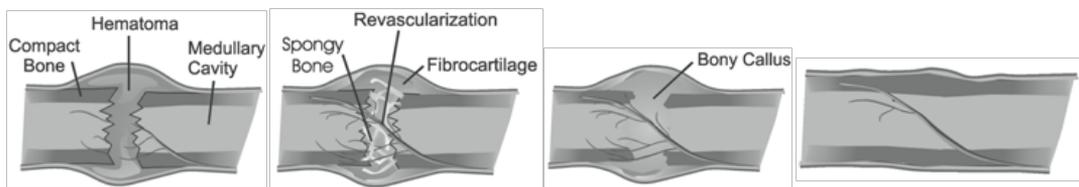
Hematoma formation & Inflammatory Phase   *Repair Phase*   *Remodelling Phase*

From the book: Bone Regeneration and Repair. Biology and Clinical Applications. Lieberman J.R & Friedlaender G.E.; Springer 2005.

Bone augmentation (GBR)
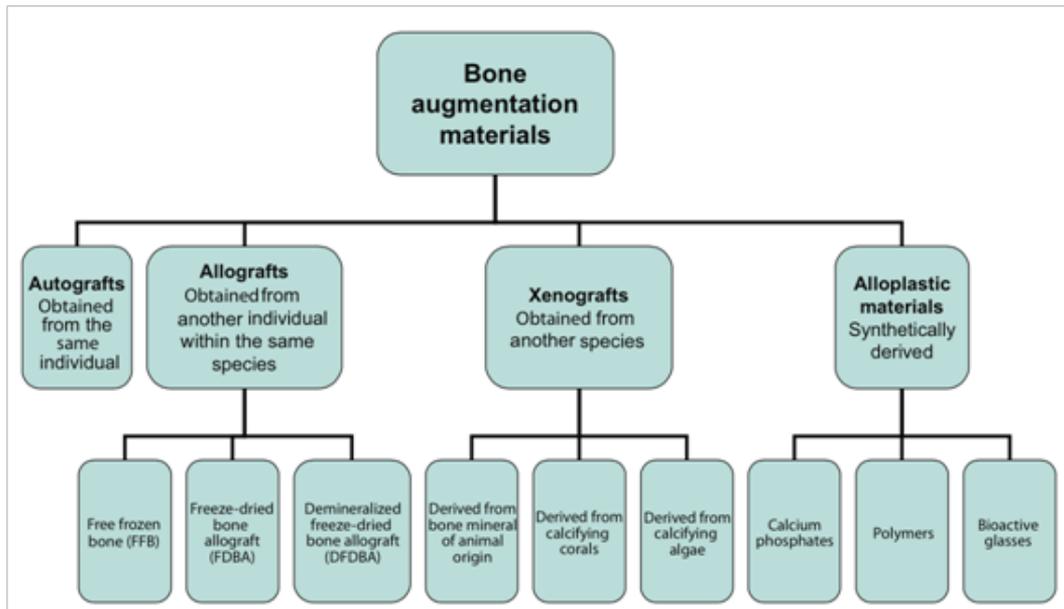Bone augmentation is a common procedure in oral, maxillofacial and orthopaedic surgery (33-35). Malformation, trauma, and dental implant surgery can make bone grafts necessary for reconstruction surgery (34). Bone augmentation by means of guided bone regeneration (GBR) was introduced by Dahlin et al. in 1988 to achieve bone regeneration with the aid of a barrier membrane. (36) Before 1988, a concept to create a closed environment to

promote healing was already in use to regenerate nerves and tendons. (37, 38) In the bone field, in 1957 Murray et. al. (39) reported bone formation under plastic cages in bone defects in a dog model. This study and many others provided first evidence that bone regeneration is enhanced when soft tissue ingrowth into the bonny defect is avoided. With the help of guided tissue regeneration concepts, where regeneration of tissue is achieved by allowing cells with regenerative capacity to populate the defect, GBR strategies started to develop.

GBR surgical protocol mandates the use of an occlusive membrane facing bone surface in order to prevent ingrowth of soft tissue cells and allow ingrowth of osteogenic cells from the surroundings. (36, 40-43) Different types of occlusal membranes can be used, including resorbable and non-resorbable (44). A bone filler is also needed to prevent the membrane to collapse – if the membrane is not rigid enough- and to guide bone regeneration. (45-47) Different bone fillers can be used, being the autologous bone the gold standard due to its properties and the ability to achieve good results in a long term (48). Overall, GBR is a predictable method to lead regeneration in bone defects and to create de novo bone formation recapping intramembranous ossification. (49)

Bone grafts
Bone grafts are needed for different purposes aiming to guide or support the bone regeneration. They can be used to bridge small and large defects, to support a barrier membrane or to avoid resorption of a bone augmentation. It is important that the bone grafts are safe, biocompatible and do not transmit pathogens to the host. Different types of bone grafts can be differentiated according to their origin, or method of production. The following diagram shows the different categories of bone grafts ranging from grafts obtained from the same individual (autografts), obtained from another individual from the same specie (allograft), obtained from other species (xenografts) and bone fillers that are synthetically produced (alloplastic materials).

Bone augmentation materials

- **Autografts** Obtained from the same individual
- **Allografts** Obtained from another individual within the same species
  - Free frozen bone (FFB)
  - Freeze-dried bone allograft (FDBA)
  - Demineralized freeze-dried bone allograft (DFDBA)
- **Xenografts** Obtained from another species
  - Derived from bone mineral of animal origin
  - Derived from calcifying corals
  - Derived from calcifying algae
- **Alloplastic materials** Synthetically derived
  - Calcium phosphates
  - Polymers
  - Bioactive glasses

From the book: 20 Years of Guided Bone Regeneration in Implant Dentistry. Buser D; Quintessence publishing 2009.

These different bone grafts and fillers have different properties. Autografts have unique characteristics which make them a favourable material for bone regeneration; they contain living cells that can contribute to bone formation (50), serve as a scaffold (51), and contain bioactive molecules that are released from the bone matrix upon resorption (22). This trinity of properties is known as osteogenicity, osteoconductivity, and osteoinductivity (52, 53). However, this trinity of properties do not rule out that there may be further properties, as yet poorly defined, which add to the existing knowledge of how autografts modulate bone regeneration. Autologous bone is widely used in the clinical practice involving different specialties like maxillofacial surgery, dentistry or orthopedics. Availability of bone autografts depends on the host, making sometimes suitable the use of other bone grafts with less grafting morbidity. Bone allografts have less availability issues and no grafting morbidity, however have other limitations such as immunologic dissimilarities from the recipient, potential disease transmission, limited availability or high costs (54). Bone allografts also contain growth factors like BMPs (55-57) and can be considered osteoinductive. Resorption of autografts and allografts is similar (58), however it is not understood why these types of grafts are resorbed. It is possible that molecules released by this bone grafts initiate or at least support the process of osteoclastogenesis.

Xenografts and alloplastic bone substitutes eliminate almost all the risk of immune reactions and cross-infection- only few infection cases have been described (59). Xenografts, specially the derived from natural bone, have been well studied. Proteins and organic components are withdrawn intensively by means of heat and chemical action to ensure that the material is inert and still biocompatible. Resorption of xenografts is less pronounced compared to autografts or allografts. Hematopoietic stem cells, are able to proliferate in the surface of the xenografts, differentiating into a osteoclast-like cells and resorbing the surface minimally. (60) Graft resorption however is almost inexistent, considering xenografts clinically nonresorbable. Finally, alloplastic bone substitutes can be made of different materials such as tricalcium phosphate, hydroxyapatite or a combination of both. Alloplastic materials are highly biocompatible and support bone formation having variable rates of resorption (61). Tricalcium phosphates can also be osteoinductive (62-64). Nevertheless, any bone graft or bone filler can get over the properties of bone autografts.

Macrophages & dendritic cells in the context of bone regeneration
Macrophages belong to the myeloid lineage and differentiate from bone marrow (BM) hematopoietic stem cells. (65) Macrophages in bone can reside in periosteal and endosteal bone-lining tissues and increase in number in areas with high bone anabolism. (66-68) Macrophages in bone can be characterized by a weak expression of tartrate-resistant acid phosphatase, CD115 or CD68 among other markers. (66, 67) Resident macrophages can co-exist and interact with inflammatory macrophages derived from monocytes during fracture healing (66, 69), a phenomenon that has not been well described. It is known the role of macrophages in soft tissue regeneration and repair. (70) However, the importance of macrophages on bone regeneration is less known.

The role of macrophages in soft-tissue healing is not comparable to the role they play in bone regeneration and repair. Bone healing, dislike the soft tissue repair, results in a regenerated tissue without the formation of a scar that is able to support load. Bone regeneration or healing starts with an inflammatory phase that will lead to an anabolic phase and later on to a remodelling that will shape the tissue to its final functional morphology. (71) After bone fracture occurs, hard tissue integrity is damaged and a hematoma is formed. After the hematoma is formed, the mesenchymal cells start to excrete signals that would activate innate immune cells including bone resident macrophages. After resident macrophages are activated, other inflammatory immune cells will be recruited that will help to clean and disinfect the fracture area. (71) Fracture healing failure or development of inflamma-

tory diseases can occur if inflammatory macrophages, that have a short life span, remain in the bone tissues. (72) After the inflammatory phase, resident macrophages might play a role in both the catabolic and anabolic phases of bone repair (73-75) suggesting that their action goes beyond the inflammatory phase. Interestingly, resident macrophages can possibly have a cross talk with the osteoclasts also influencing bone remodelling. (76)

Knowing the importance of resident and inflammatory macrophages in bone, it is important to understand the crosstalk between the immune and osseous systems in order to develop new strategies to improve fracture healing, bone regeneration or graft consolidation. Specially is important to elucidate relation and cross-talks of the immune bone system and osteoclasts, the latter ones playing a central role in resorption of bone fillers, for example bone autografts .

On Saliva

Composition
Saliva is produced by the submandibular, parotid, and minor salivary glands, providing a continuous rich source of electrolytes, mucus, antibacterial compounds, enzymes, and growth factors that support lubrication and initiate food digestion. For example, proteomic analysis revealed that saliva contains more than three thousand different proteins including cystatins carbonic anhidrases or proline-rich proteins. (77, 78) Defense proteins involved in both innate and acquired immunity, like salivary immunoglobulins and salivary chaperokine HSP70/HSPAs (70 kDa heat shock proteins), are also included in the list. (79) Saliva proteins also provide the pellicle layer on enamel to support mineralization and the formation of a biofilm. (80) Human saliva has a pro-inflammatory effect on fibroblasts. (81) Cases in which patients suffer from a lack of saliva such as the systemic autoimmune disease Sjögren syndrome (82) or patients receiving head and neck radiotherapy (83) emphasize the physiologic functions of saliva important for the integrity of the oral mucosa. Moreover, saliva has been implicated in the support of oral soft tissue healing, for example, desalivated rodents have impaired healing of extraction sites. (84) Saliva can reach sites where oral hard tissue is present and molecules within saliva such as the protease inhibitor cystatin C can suppress bone resorption (85). So far, however, there has been no clear relationship established between saliva and bone resorption. Nevertheless, saliva can reach the dental hard tissues, so it is possible that saliva may have an impact on the differentiation of local hematopoietic cells that can become osteoclasts but also phagocytic cells.

Context in bone healing and resorption

Not much is known about the role of saliva in bone healing and bone resorption. Different types of cells are involved during bone healing, including cells derived from the hematopoietic linage like phagocytic cells and osteoclasts. Despite the fact that saliva can reach the bone surface and holds soluble factors known to modulate osteoclastogenesis (86), the effect of whole saliva on osteoclastogenesis remains unknown. In physiologic situations, osteoclast activity is required for bone remodeling (such as in the repair of fatigue damage) and modeling (such as where bone adapts to functional loading) as has been demonstrated in alveolar bone (87). In pathologic situations, overwhelming osteoclast activity causes systemic and local bone loss, which is the hallmark of osteoporosis and periodontal and peri-implant disease, respectively. Saliva has also shown to prevent bone loss in desalivated rats with induced periodontitis (88). Moreover, saliva can induce expression of inflammation markers in osteoblasts (89) also shown in fibroblasts (90). It is essential to elucidate how saliva affects bone regeneration, especially bone resorption –present in the first phase of bone regeneration- as saliva can enter easily in contact with bone surface during the routine clinical procedures and may therefore affect the outcome of the bone regeneration.

Taking everything together, clinical knowledge on bone regeneration and graft consolidation is wide but, what is missing to know? We do not know much about the biologic potential of the gold standard graft, the autologous bone. Bone autografts are easily resorbed and promote faster bone formation and graft consolidation, but why? Moreover to which extent saliva is affecting or playing a role to the bone healing or bone regenerations is also unknown. Saliva can enter in contact with bone very easily, specially during dental treatments. There is not still any knowledge about what happens to bone when is exposed to saliva. Therefore in the present PhD thesis we will aim to answer the presented questions to broaden the knowledge in dentistry, specially in the fields of bone healing and regeneration .

Aims

General aim
Study the behaviour of osteoclasts and their progenitor cells subjected to different conditions present in the oral cavity "in vitro".

Secondary aims
1; Compare activity of osteoclasts subjected to different growth factors.
2; Analyse the protein levels and genetic expression through different techniques of molecular biology.

3; Perform phagocitation studies with various cell types under diverse conditions.

4; Analyse composition of stimulation substances used, specially bone conditioned medium and saliva.

5; Test stimulation substances in oral fibroblasts as a proof of principle.

Aim of the papers

Paper I
The aim of the study was to examine whether saliva affects the process of osteoclastogenesis as well as the formation of phagocytic cells in vitro.

Paper II
The aim of the study was to characterize the paracrine factors of bone conditioned medium by means of proteomic analysis.

Paper III
The aim of the study was to analyse the impact of fresh and demineralized bone conditioned medium on osteoclast formation.

Paper IV
The aim of the study was to present how to prepare bone conditioned medium and test its activity in vitro.

Results of each paper

In the following section, results of each paper are presented. Complete results with the figures and tables are included in the papers.

Paper I

Saliva suppresses RANKL-induced osteoclastogenesis in vitro

To investigate the impact of saliva on osteoclastogenesis, murine bone marrow cells were grown in the presence of RANKL, M-CSF, and TGF-β. As expected, progenitors differentiated into TRAP-positive multinucleated cells. Fresh sterile saliva greatly inhibited this process down to a concentration of 10% saliva in the culture medium. Expression of osteoclast genes CTR, CathK, and TRAP were statistically significantly reduced in bone marrow cultures treated with saliva from six independent donors. Overall, freshly prepared saliva suppressed osteoclastogenesis in murine bone marrow cultures.

Saliva suppresses RANK, signaling molecules, and fusion genes

To determine whether the effect of saliva is caused by a decreased responsiveness to RANKL or M-CSF, the expression levels of the respective receptors RANK and c-fms were determined. The addition of saliva led to a substantial decrease in the expression of RANK and to a lesser extent also c-fms. In line with this observation, the expression of PU.1 and MITF, both of which are regulators of RANK (91), was decreased. Saliva also decreased the expression of TRAF6 and the respective downstream master regulators of osteoclastogenesis, c-fos and NFATc1, which are needed for DC-STAMP and Atp6v0d2 expression (92, 93). In agreement, saliva markedly decreased the expression of DC-STAMP and Atp6v0d2. Because saliva substantially increased NF-κB target genes in oral fibroblasts (81), we also examined expression levels in the in saliva-treated murine bone marrow cells. Accordingly, the mRNA expression of CXCL1, CXCL2, CCL2, IL6, PTGS2, and CSF2, known downstream target genes of NF-κB, was increased. Altogether, the addition of saliva led to a decrease in the expression of genes involved in osteoclastogenesis while NF-κB target genes were increased.

Saliva supports the formation of phagocytic cells

Next, the impact of saliva on the co-stimulatory molecules of osteoclastogenesis was examined. Saliva decreased OSCAR, which is considered an osteoclast marker gene regulated by NFATc1, while the three other co-stimulatory molecules DAP12, TREM2, and FcRγ, which are also characteristic for myeloid cells, including phagocytic cells, remained unchanged. This indicates the potential that saliva induces the development of phagocytic cells. To test this assumption, a phagocytosis test was performed. Latex

beads were ingested, consistent with the possibility that phagocytic cells develop in the presence of saliva. Also, multinucleated osteoclast-like cells ingested latex beads, however, much less compared to the cells that formed in the presence of saliva. Moreover, bone marrow cultures exposed to saliva showed increased levels of CD40, CD80, and CD86, all co-stimulatory molecules to prime T-cells. Saliva increased the expression of CD11c, an adhesion molecule that binds to the extracellular matrix (e.g., fibrinogen and ICAMs). Overall, the data point towards the development of a phagocytic phenotype when bone marrow cells are cultivated in the presence of saliva. It was further investigated if the shift of bone marrow cultures towards phagocytic cells was accompanied by increased expression of inflammation-related genes. GM-CSF and IL-18 are interdependent inhibitors of osteoclastogenesis (94) and were shown to be slightly upregulated; meanwhile the expression of IL-4, IFN-□, IL-10, IL-33, and IFN-□ remained similar to the control. Overall, it remains unclear how saliva causes a shift towards the formation of phagocytic cells.

Saliva blocks fusion of osteoclasts

Saliva prevents the differentiation of osteoclasts from their progenitors. The question then arose of whether saliva can reverse this process. To answer this question, osteoclastogenesis was initiated for 4 days, after which filter-sterilized saliva was added to the cultures for another 3 days. Saliva suppressed the formation of the large multinucleated cells with a massive cytoplasm, but did not reverse the TRAP staining of the cells present. Also, the expression of the osteoclast marker genes was not considerably changed by saliva under these conditions. In support of the histology, the expression of the two fusion genes, DC-STAMP and Atp6v0d2, was decreased in the presence of saliva. When mature osteoclasts were seeded onto dentin slides, the addition of saliva allowed resorption pits to become visible, although with a moderately reduced diameter. Thus, saliva hindered fusion of differentiated osteoclasts but did not reverse differentiation or inhibit resorption.

Saliva does not negatively affect cell viability

To assess if saliva could affect cell viability, live-dead staining of cultures supplemented with saliva was performed. Live-dead staining, where green are viable cells and red are dead cells, showed no differences between the groups with and without saliva. To check whether saliva affected proliferation of bone marrow cultures, an expression analysis of the cell-cycle related genes MIKI67, PLK1, and BUB1 was performed. All of these genes showed slight upregulation in the presence of saliva. Taken together, cells remained viable when grown in the presence of 20% saliva.

Paper II

Protein categorization and interaction construction

A total of 175 proteins were detected in BCM with different concentrations and variations among the three batches. Out of the 175 proteins, 43 were described as being secreted or present in the extracellular matrix. The PAN-THER classification system was used to classify these proteins according to their function or to the biological process in which they are involved. PAN-THER classified the proteins into thirteen groups according to their function and into twelve groups according to their biological process.

The largest group according to the protein function was involved in extra-cellular matrix processes (15%) and in signaling functions (15%). A significant number of proteins were also involved in enzyme modulation (12%) or transfer carrier (12%). Two major groups of biological processes - cellular process and metabolic process - were represented by 16% of proteins each. Other biological processes are cell communication (13%), transport (10%), developmental process (10%), and cell adhesion (10%). This wide range of categories indicates that proteins present in conditioned medium of freshly prepared cortical bone chips have diverse cellular functions and they are involved in widespread biological processes.

Protein-protein interaction analysis

To understand the interactions among the 43 proteins, a protein-protein inter-action analysis was carried out. A 72% (34 out of 43) of the proteins could be networked. Six out of the seven possible interactions were described; interactions with neighborhood evidence were not described. Most interactive protein was decorin (DCN). Decorin showed to have fusion evidence, co-occurrence evidence, experimental evidence, text-mining evidence, database evidence and co-expression evidence interactions with biglycan. Moreover, DCN also had two types of interactions with Complement C1q subcomponent subunit A (C1QA). These two interactions were fusion evidence, co-expression evidence. Other strong interactions are for example the ones between decorin and transforming growth factor beta-1 (TGF- β1), TGF- β1 and transforming growth factor beta-2 (TGF- β2) or SPARC with TGF- β1. The interaction with text-mining evidence was the most common among the proteins, which are interactions described in the scientific literature.

Paper III

BCMh supports RANKL-induced osteoclastogenesis in vitro

To investigate the impact of BCM on osteoclastogenesis, the number of TRAP+ MNCs in the presence of RANKL and M-CSF was determined. As

shown in Figure 1A,B, BCM failed to cause any visible changes in osteo-clastogenesis, even though at the RNA level of the marker and fusion genes CTR, TRAP, Oscar, and ATP6 were increased. Since TGF-β can enhance osteoclastogenesis (95), TGF-β is found in BCM (24), and TGF-β can be activated by heat (96). BCM was exposed to 85°C for 10 minutes. Accordingly, BCMh increased the expression of osteoclast markers and fusion genes approximately two-fold. A resorption assay performed on dentin discs confirmed the activity of osteoclasts but no quantification was performed because of the large variations within and between the experiments. Together these findings show that BCMh moderately enhances osteoclastogenesis in vitro.

BCMh increases osteoclast signaling molecule expression

To determine whether the effect of BCMh possibly involves increased responsiveness of cells to RANKL or M-CSF, the expression levels of the respective receptors RANK and c-fms were determined. BCMh increased expression of RANK by around two-fold but did not increase expression of c-fms. BCMh also increased expression of TRAF6 and the respective downstream master regulators of osteoclastogenesis, NFATc1 and particularly c-fos. Expression of PU.1 and MITF was not changed significantly. Taken together, BCMh increased the expression of some but not all osteoclast signaling molecules.

BCMh-induced c-fos expression requires TGF-β signaling

In line with a potential role of TGF-β in mediating the effect of BCMh on osteoclastogenesis, blocking of TGF-βR1 kinase (SB431542) decreased c-fos expression. TRAP staining also showed the suppression of osteo-clastogenesis by SB431542. Further support for a potential role of TGF-β signaling in the bone marrow macrophages comes from observations that BCMh increased the respective target genes IL11, ACTA2 and CTGF, and that BCMh stimulated phosphorylation of SMAD3, p38, and JNK in a macrophage cell line. Together, these data support the assumption that BCMh mediates at least part of its activity via TGF-β signaling.

BCMh does not affect cell viability and proliferation

To assess if BCM could affect cell viability, live-dead staining of cultures supplemented with BCM and genetic expression analysis of cell-cycle related genes was executed. To determine whether BCMh affected proliferation of bone marrow cultures, expression analysis of the cell-cycle related genes MYBL2, BUB1, PLK1 and MIKI67 was performed. Expression of cell-cycle related genes was not affected by BCM. Therefore, cells remain viable but are not forced to expand when exposed to BCMh.

Paper IV

Bone Conditioned Medium was prepared from fresh porcine bone chips. General overview of the process to prepare BCM and to use biomaterials in combination with BCM is shown. During the BCM preparation, it is important to obtain large bone chips with long movements as short movements or very small bone chips can affect the quality of the final BCM. Quality of BCM can be controlled by analyzing the gene expression of BCM target genes: ADM, PTX3, IL11, NOX4 and PRG4. ADM and PTX3 are downregulated down to 0.4-fold and IL11, NOX4 and PRG4 can be upregulated to 200-fold. If oral fibroblasts do not express BCM target genes at the level shown, check the health of the cells or prepare new BCM from new mandibles. Oral fibroblasts stimulated with 20% of conditioned medium from pasteurized bone chips and conditioned medium from demineralized bone chips, showed similar gene expression to cells stimulated with BCM. However, gene expression of oral fibroblasts exposed to conditioned medium from sterilized (121°C) bone chips, was comparable to unstimulated controls.

General Discussion

The oral cavity is a unique organ in the Animal Kingdom. It is where all starts. Food enters the organism and starts a journey through the body starting in the stoma. Is important to preserve the integrity of the mouth, as without intake, life is threatening. It is therefore rational to think that the oral cavity might have some exclusive properties and characteristics that will not be shared with the rest of the body. One of these unique characteristics is for example, the presence of saliva. Saliva is produced by the submandibular, parotid, and minor salivary glands, providing a continuous rich source of electrolytes, mucus, antibacterial compounds, enzymes, and growth factors that support lubrication and initiate food digestion. Saliva seems to play a crucial role in the conservation of the integrity of the oral cavity, for example healing faster soft tissue wounds. (84) But, what happens when a major wound is created and saliva enters in direct contact with bone?

In the results presented in Paper I, saliva inhibited osteoclastogenesis towards the development of a phagocytic cell type. All osteoclast markers were suppressed and those characteristics of phagocytic cells, were up-regulated. In bone, two different types of macrophages can exist: the resident macrophages and the inflammatory macrophages. (66, 69) The resident macrophages interact with the inflammatory macrophages when a fracture or injury in the bone occurs. In the first phase of healing, the inflammatory phase, recruited inflammatory cells clean and disinfect the injured area. (71) In the oral cavity saliva can reach the bone surface in many occasions like in traumas, teeth extraction, periodontal treatment or dental implant placement. In these occasions bacteria and other pathogens present in the oral cavity, can contact and infiltrate the bone. It is therefore rational to think that the response of the resident and inflammatory macrophages will be increased to clean and disinfect the area. Saliva might play a very important role in this first stage of bone healing, not only by preventing an exacerbate bone resorption but also stimulating the immune system and increasing the amount of cells able to clean and disinfect the area. The present study is a good primer to start investigating the connections between saliva, the immune system and bone resorption.

Bone resorption in the oral cavity is not only a matter of concern when saliva enters in contact with bone like in tooth extractions or fractures, but also when bone has to be regenerated artificially to restore its function. Bone can be regenerated using different methods and different bone substitutes, being autologous bone grafts the gold standard. (52, 53) In Paper II, III and IV we chose a clinically inspired approach to study bone autografts where

fresh bone chips were left in culture medium allowing passive release of proteins in a physiologic environment. Using these settings, cells had the ability to continue producing growth factors that might have a function on the course of graft consolidation. In Paper II, the proteomic analysis revealed that BCM contained a big number of proteins like: galectin-1, trypsin, angiogenin, tenascin-c, biglycan, SPARC, decorin, macrophage migration inhibitory factor, annexin A1, lactotransferrin, insulin-like growth factor-binding protein 5, transforming growth factor beta-1, transforming growth factor beta-2, many of them already described in bone supernatants but using other extraction methods (27, 97, 98). There is evidence to suggest that soluble factors released from transplanted bone support bone resorption and osteoclastogenesis, particularly because BCM and DCM contain TGF-β (24, 99), and TGF-β supports in vitro osteoclastogenesis (95).

Resorption of bone autografts is a matter of concern and occurs early during graft consolidation. Large number of osteoclasts rapidly develop when bone chips are placed into bone defects (51), likely explaining why no residual autografts were detectable two years after contour augmentation (100). Resorption occurs independently from the anatomical regions where the bone is harvested and the reception sites, e.g., bone harvested from the iliac crest or ramus and used for sinus floor elevation or ridge augmentation (101, 102). Bone graft resorption is then a clinical principle in bone regeneration that also helps to remove debris and dead bone after injury or disease. The results presented in Paper III supports the notion that bone autografts release factors that affect osteoclastogenesis and therefore bone resorption. However BCM had to be exposed to 85°C to considerably enhance osteoclastogenesis, probably activating by heat latent growth factors like TGF-β (103-106). Based on these results, current clinical concepts can be complemented in order to predict the outcomes of bone augmentation. Finally, in paper IV we showed how by means of the BCM, different treatments of the bone or biomaterials can be studied in order to test their ability to retain and release growth factors liberated by bone autografts therefore present in the regeneration site. Taken together, BCM papers here presented prove a new fourth characteristic of bone autografts: the regulation property.

The clinical relevance of the research here presented is still open for debate. Bone resorption sometimes is a clinical challenge in many situations like after a tooth extraction, fracture or when performing a guided bone regeneration procedure. Results here presented on the effects of saliva on bone resorption and the use of bone autografts, contribute to extend the knowledge on how osteoclastogenesis works under different conditions. Result presented on the innate and even the adaptive immune response

to saliva suggests that bone exposed to saliva is less resorbed. Does this means that if saliva enters in contact with bone during dental and maxillo-facial procedures we will have less bone resorption? At this point, whether the in vitro data translate into clinics is a matter of speculation. In the same direction are the novel observations on bone resorption and autografts. If autografts resorption occurs due to the presence of TGF-β in vivo, remains unknown. Bone autografts are widely used and improve the results of bone regeneration procedures, pioneer knowledge here presented explains some of the properties of autografts and corroborates its especial characteristics compared to other types of grafts. Results here presented can inspire clini-cians to continue or start using bone autografts. Moreover, the in vitro data of this thesis seems to support the clinical observations concerning bone graft resorption. The information presented here can be used to advance current clinical concepts in order to predict the outcome of bone augmenta-tion procedures .

Conclusions

- Saliva shifts the differentiation of hematopoietic cells towards the phagocyte lineage, overcoming the simulated microenvironment of osteoclastogenesis. (Paper I)

- Cortical bone chips release proteins into the culture medium that are partially described to have an impact on bone regeneration, including forty-three different growth factors. (Paper II)

- Soluble factors released from fresh cortical bone affect osteoclastogenesis, increasing osteoclast specific genes and resorption of dentin discs. (Paper III)

- Release of molecules from fresh and processed bone grafts can be tested by the use of conditioned mediums. (Paper IV)

Future perspectives

Life expectancy worldwide is rising, therefore increasing as well the age of our patients. It is common to see elderly patients that lost their teeth and require an oral rehabilitation, including surgical procedures to increase the amount of bone. This bone augmentation and subsequent graft consolidation, is sometimes tricky and requires more than one step. In successful cases, after 4 to 6 months a bone-like tissue is ready to receive a dental implant. But sometimes bone regeneration cannot be achieved due to a lack of host bone or failure of the regeneration.

Future studies and research on bone regeneration should address these drawbacks on bone regeneration such as failure of graft consolidation. Future strategies should be developed to improve procedures and materials to support bone regeneration faster and safer without the need of grafting autologous bone, the gold standard. In the studies here presented, an attempt was made to further characterize autologous bone grafts and explore their activity once placed in the regeneration site. These pioneer data should be the trigger for future studies on the characterization of bone autografts, knowing that a better knowledge on autografts would help to engineer future regeneration materials. Understanding how growth factors contained in BCM play a role in vivo, should be also a matter of concern. In vivo studies placing BCM with a carrier, for example lyophilized, in a bonny defect could help to understand how factors released by bone autografts work. Also, would be interesting to place BCM subcutaneously in naked mice to determine how BCM affects soft tissue. Could be that the 43 growth factors contained in the BCM, also play a role in the survival or proliferation of soft tissue cells. Another aspect that could be studied is the contribution of osteocytes on the BCM composition. BCM contains sclerostin, a molecule that is only secreted by cementoblasts and osteocytes. What would contain a BCM from bone without osteocytes? Will its effect be comparable to the BCM from bone with osteocytes?

The results here presented on saliva and osteoclasts are intriguing. Saliva is present in the mouth of almost every human and every mammal. However not much is known about its effect on bone. Multidisciplinary research teams including immunologists, oral surgeons and scientists should go into deep about the effect here described on osteoclastogenesis and osteoclast function and saliva. Saliva does not seem only to inhibit osteoclastogenesis, possibly affecting bone regeneration and graft consolidation, but also to primer development of immune cells like phagocytes. This interesting finding can be used to design new strategies on the study of xerostomy and

Sjögren's Syndrome as well as the influence of saliva on bone healing and regeneration. Studies with desalivated models –mice, rats or dogs- could investigate how bone healing occurs after a tooth extraction or an exposure of bone to the oral cavity. Moreover, what would happen if saliva is placed intentionally into a bonny defect for example in a lyophilized form? Randomized clinical trials on patients could also be design to study the success of bone regenerations with low saliva flow.

Overall, more research has to be done in both topics: bone resorption on autografts and saliva. In the long future, new biomaterials with the four properties of bone autografts could be developed. Also in the long term, proteins responsible for the effect on bone resorption of saliva could be recognized and isolated in order to use them therapeutically to avoid bone resorption and enhance immune response in the site.

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Biking in Piz Nair at 3.000m   *Research is fun!*   *Abschied Apero, Bern*
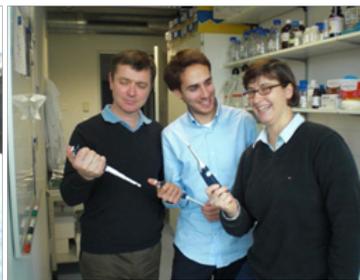
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Paper I

Saliva Supresses Osteoclastogenesis in Murine Bone Marrow Cultures

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Saliva Suppresses Osteoclastogenesis in Murine Bone Marrow Cultures

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Abstract

Saliva can reach mineralized surfaces in the oral cavity; however, the relationship between saliva and bone resorption is unclear. Herein, we examined whether saliva affects the process of osteoclastogenesis in vitro. We used murine bone marrow cultures to study osteoclast formation. The addition of fresh sterile saliva eliminated the formation of multinucleated cells that stained positive for tartrate-resistant acid phosphatase (TRAP). In line with the histochemical staining, saliva substantially reduced gene expression of cathepsin K, calcitonin receptor, and TRAP. Addition of saliva led to considerably decreased gene expression of receptor activator of nuclear factor kappa-B (RANK) and, to a lesser extent, that of c-fms. The respective master regulators of osteoclastogenesis (c-fos and NFATc1) and the downstream cell fusion genes (DC-STAMP and Atp6v0d2) showed decreased expression after the addition of saliva. Among the costimulatory molecules for osteoclastogenesis, only OSCAR showed decreased expression. In contrast, CD40, CD80, and CD86—all costimulatory molecules of phagocytic cells—were increasingly expressed with saliva. The phagocytic capacity of the cells was confirmed by latex bead ingestion. Based on these in vitro results, it can be concluded that saliva suppresses osteoclastogenesis and leads to the development of a phagocytic cell phenotype.

Introduction

Saliva is produced by the submandibular, parotid, and minor salivary glands, providing a continuous rich source of electrolytes, mucus, antibacterial compounds, enzymes, and growth factors that support lubrication and initiate food digestion. Saliva proteins also provide the pellicle layer on enamel to support mineralization and the formation of a biofilm (Siqueira et al. 2012). Human saliva has a proinflammatory effect on fibroblasts (Cvikl et al. 2014). Cases in which patients suffer from a lack of saliva, such as the systemic autoimmune disease Sjögren syndrome (Castro et al. 2013), or patients receiving head and neck radiotherapy (Shiboski et al. 2007) emphasize the physiologic functions of saliva important for the integrity of the oral mucosa. Moreover, saliva has been implicated in the support of oral soft tissue healing—for example, desalivated rodents have impaired healing of extraction sites (Bodner et al. 1991). Saliva can reach sites where oral hard tissue is present and molecules within saliva, such as the protease inhibitor cystatin C, can suppress bone resorption (Stralberg et al. 2013). So far, however, there has been no clear relationship established between saliva and bone resorption. Nevertheless,

saliva can reach the dental hard tissues, so it is possible that saliva may have an impact on the differentiation of local hematopoietic cells that can become osteoclasts but also phagocytic cells.

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A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

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Dental hard tissues—namely, cementum, dentin, and enamel—are resorbed by multinucleated cells referred to as *odontoclasts*, which strongly resemble osteoclasts (Sahara et al. 1998), the major bone-resorbing cell (references in appendix). In physiologic situations, osteoclast activity is required for bone remodeling (e.g., in the repair of fatigue damage) and modeling (e.g., where bone adapts to functional loading), as has been demonstrated in alveolar bone (Huja et al. 2006). In pathologic situations, overwhelming osteoclast activity causes systemic, and local bone loss, which is the hallmark of osteoporosis, and periodontal and peri-implant disease, respectively. Much has been discovered on the role of leukocytes in periodontal disease and that oral fibroblasts can modulate osteoclastogenesis in vitro (references in appendix). Notably, oral pathogens such as *Porphyromonas gingivalis* can provoke T-cell-dependent osteoclastogenesis but can also directly modulate the formation of osteoclasts (references in appendix). Despite the fact that saliva can reach the bone surface and that it holds soluble factors known to modulate osteoclastogenesis (Buduneli et al. 2008), the effect of whole saliva on osteoclastogenesis remains unknown.

Osteoclast development from hematopoietic cells requires receptor activator of nuclear factor kappa-B (RANK) signaling (Hsu et al. 1999), together with the signals coming from the macrophage colony-stimulating factor (M-CSF) receptor (c-fms, MCSF-R, CSF1R). Osteoclasts are multinucleated cells expressing tartrate-resistant acid phosphatase (TRAP), cathepsin K, and the calcitonin receptor. Costimulatory molecules activate the immunoreceptor tyrosine-based activation motif–dependent pathway (Koga et al. 2004). Osteoclast-associated receptor and triggering receptor expressed in myeloid cells (TREM2) are receptors that are associated with the adaptor molecules Fc receptor common gamma chain (FcRγ) and DNAX-activating protein 12 kDa (DAP12), respectively. Downstream signaling pathways culminate in the expression of c-fos and nuclear factor of activated T cells c1 (NFATc1), interdependent master regulators of osteoclastogenesis. Also PU.1 and microphthalmia-associated transcription factor (MITF) integrate downstream signaling pathways during osteoclastogenesis (Sharma et al. 2007). Cell fusion requires osteoclast stimulatory transmembrane protein; dendritic cell-specific transmembrane protein (DC-STAMP); and ATPase, H$^+$ transporting, lysosomal 38 kDa, V0 subunit d2 (Atp6v0d2) (Lee et al. 2006; Silva et al. 2007). Here we determined the expression levels of the respective genes to understand the role of saliva during osteoclastogenesis in vitro.

Phagocytic cells, including macrophages and dendritic cells, originate from hematopoietic cells and are capable of processing foreign material and presenting antigens to immune cells (Savina and Amigorena 2007). A differentiation shift toward the development of macrophages and dendritic cells at the expense of osteoclastogenesis was reported for tetracycline (Kinugawa et al. 2012), IL-3 (Gupta et al. 2010), and IL-33 (Kinugawa et al. 2012) and with the anticonvulsant drug diphenylhydantoin (Koide et al. 2009). Phagocytic cells characteristically express CD40, CD80, and CD86, all costimulatory molecules to prime T-cells (Pletinckx et al. 2011), CD14 (a co-receptor of Toll-like receptor), and the integrin CD11c (Dudley et al. 1989). Since phagocytic cells are required for local defense, they are typically located in tissues in contact with the external environment, including the oral mucosa (Hu et al. 2007), and they play a role in periodontal diseases (Helmerhorst and Oppenheim 2007). Phagocytes such as dendritic cells and, therefore, also their progenitor cells are found in the suprabasal layer of the stratified squamous epithelia of oral mucosa (Lombardi et al. 1993). Thus, saliva can reach phagocytic cells and their respective progenitors. For example, the role of tick saliva on dendritic cell formation and function has been extensively studied because ticks can suppress host immunity by secreting immunomodulatory molecules in their saliva (Heaney 1990). Surprisingly, however, we are not aware of studies examining the impact of human saliva on the formation of phagocytic cells.

We have previously found that fresh sterile saliva causes a strong activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) target genes in fibroblasts from oral soft tissue (Cvikl et al. 2014). NF-κB is also a key regulator of hematopoietic cell differentiation, including osteoclasts (Iotsova et al. 1997) and dendritic cells (Ouaaz et al. 2002). Considering that saliva can reach the bone surface—for example, after tooth extractions, during guided bone regeneration, or following periodontal treatments—it is possible that saliva activates NF-κB signaling in hematopoietic cells and thereby affects osteoclastogenesis and the formation of phagocytic cells. Based on murine bone marrow cultures, the data show that the addition of saliva considerably decreased osteoclastogenesis and led to the development of phagocytic cells.

Materials and Methods

See appendix.

Results

Saliva Suppresses RANKL-induced Osteoclastogenesis In Vitro

To investigate the impact of saliva on osteoclastogenesis, murine bone marrow cells were grown in the presence of receptor activator of nuclear factor kappa-B ligand (RANKL), M-CSF, and transforming growth factor beta (TGF-β). As expected, progenitors differentiated into TRAP-positive multinucleated cells (Fig. 1A, 1B). Fresh sterile saliva greatly inhibited this process (Fig. 1A, 1B) down to a concentration of 10% saliva in the culture medium (Appendix Table 2). Expression of osteoclast genes calcitonin receptor, CathK, and TRAP were significantly reduced
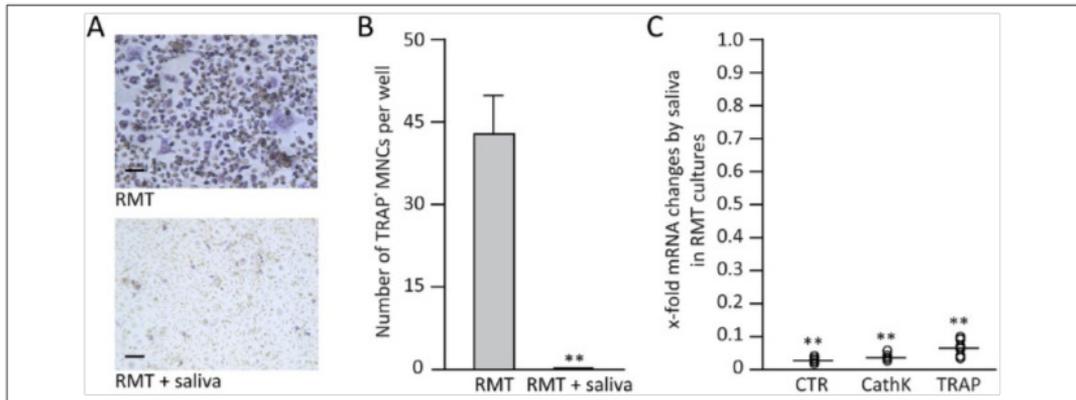
Figure 1. Saliva suppresses RANKL-induced osteoclastogenesis in vitro. Murine bone marrow cells were grown in the presence of RANKL, M-CSF, and TGF-β (RMT). (**A, B**) After 3 d, the RMT group contained TRAP-positive multinucleated and mononucleated cells prone to fusion. Saliva greatly reduced the formation of TRAP-positive multinucleated cells. (**C**) Expression of genes characteristically expressed by osteoclasts: CTR, CathK, and TRAP showed reduced expression in bone marrow cultures treated with saliva from 6 independent donors. Bars represent 20 μm; **$P < 0.01$.

in bone marrow cultures treated with saliva from 6 independent donors (Fig. 1C). The Appendix Figure further shows that saliva also suppressed osteoclastogenesis when RANKL was replaced with TNFα (Appendix Fig. A), when TGF-β was omitted (Appendix Fig. B), and in the presence of 1μM dexamethasone (Appendix Fig. C). Whole salivary cortisol levels were 7.9 ± 1.4 nM (Appendix Table 3). Moreover, nonsterile saliva also blocked the process of osteoclastogenesis (Appendix Fig. D). The cystatin C neutralizing antibody could not reverse the inhibitory effect of saliva on osteoclastogenesis (Appendix Tables 4 and 5). Overall, freshly prepared saliva suppressed osteoclastogenesis in murine bone marrow cultures.

Saliva Suppresses RANK, Signaling Molecules, and Fusion Genes

To determine whether the effect of saliva is caused by a decreased responsiveness to RANKL or M-CSF, the expression levels of the respective receptors RANK and c-fms were determined. The addition of saliva led to a substantial decrease in the expression of RANK and, to a lesser extent, c-fms (Fig. 2A). In line with this observation, the expression of PU.1 and MITF, both of which are regulators of RANK (Ishii et al. 2008), was decreased (Fig. 2B). Saliva also decreased the expression of TRAF6 and the respective downstream master regulators of osteoclastogenesis, c-fos and NFATc1 (Fig. 2B), which are needed for DC-STAMP and Atp6v0d2 expression (Kim et al. 2008; Yagi et al. 2007). In agreement, saliva markedly decreased the expression of DC-STAMP and Atp6v0d2 (Fig. 2C). Because saliva substantially increased NF-κB target genes in oral fibroblasts (Cvikl et al. 2014), we also examined expression levels in
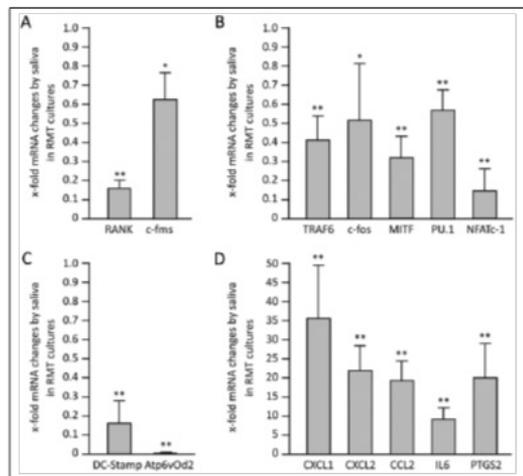
Figure 2. Saliva suppresses RANK, signaling molecules, and fusion genes. Addition of saliva led to a substantial decrease in the expression of (**A**) receptors, (**B**) downstream master regulators of osteoclastogenesis, and (**C**) genes involved in cell fusion. In contrast, saliva increased expression of (**D**) NF-κB target genes. *$P < 0.05$, **$P < 0.01$, compared with osteoclast cultures with RANKL, M-CSF, and TGF-β1 (RMT).

saliva-treated murine bone marrow cells. Accordingly, the mRNA expression of CXCL1, CXCL2, CCL2, IL6, PTGS2, and CSF2, known downstream target genes of NF-κB, was increased (Fig. 2D). Altogether, the addition of saliva led to a decrease in the expression of genes involved in osteoclastogenesis, while NF-κB target genes were increased.
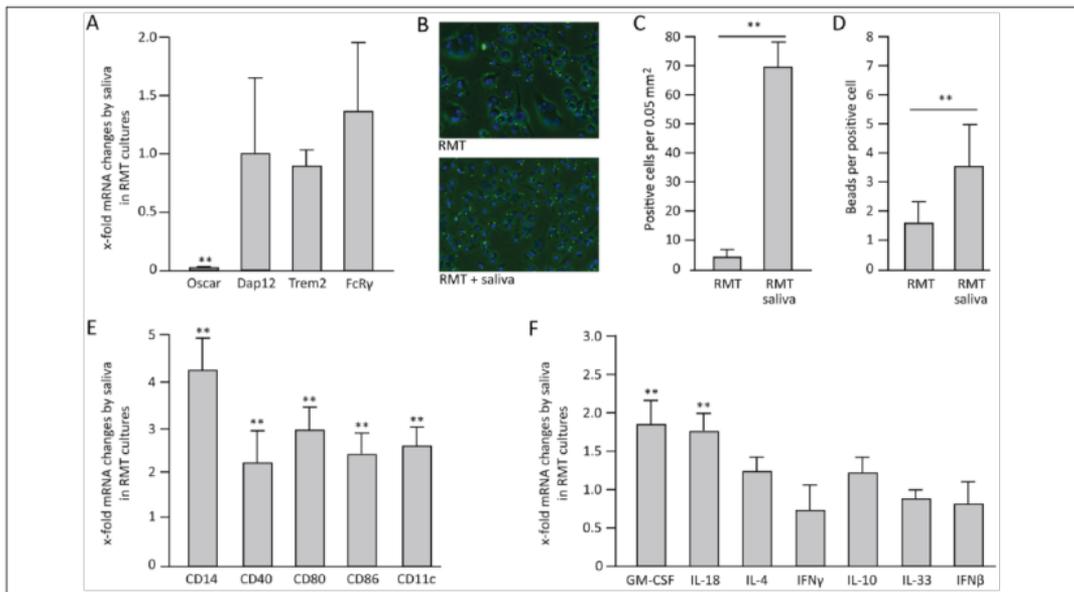
Figure 3. Saliva supports the formation of phagocytic cells. (**A**) The addition of saliva led to the decrease in expression of OSCAR but not the other costimulatory molecules. (**B**) Green fluorescent latex beads were ingested, indicating the presence of phagocytic cells. (**C**, **D**) Positive cells were mainly cells in the saliva group, but osteoclast-like cells also ingest latex beads. (**E**) Genes that are characteristic for phagocytic cells are increasingly expressed when bone marrow is cultivated in the presence of saliva. (**F**) Among the endogenous inhibition of osteoclastogenesis, only GM-CSF and IL-18 were moderately increased in expression by saliva. **$P < 0.01$, compared with osteoclast cultures with RANKL, M-CSF, and TGF-β1 (RMT).

Saliva Supports the Formation of Phagocytic Cells

We next sought to examine the impact of saliva on the costimulatory molecules of osteoclastogenesis. Saliva decreased osteoclast-associated receptor, which is considered an osteoclast marker gene regulated by NFATc1, while the 3 other costimulatory molecules DAP12, TREM2, and FcRγ, which are also characteristic for myeloid cells, including phagocytic cells, remained unchanged (Fig. 3A). This indicates the potential that saliva induces the development of phagocytic cells. To test this assumption, a phagocytosis test was performed. Latex beads were ingested, consistent with the possibility that phagocytic cells develop in the presence of saliva (Fig. 3B). Also, multinucleated osteoclast-like cells ingested latex beads, however, much less compared with the cells that formed in the presence of saliva (Figs. 3C, 3D). Figure 3E shows that CD14, a coreceptor of Toll-like receptor, had increased expression. Moreover, bone marrow cultures exposed to saliva showed increased levels of CD40, CD80, and CD86, all costimulatory molecules to prime T cells. Saliva increased the expression of CD11c, an adhesion molecule that binds to the extracellular matrix (e.g., fibrinogen and ICAMs). Overall, the data point toward the development of a phagocytic phenotype when bone marrow cells are

cultivated in the presence of saliva. It was further investigated if the shift of bone marrow cultures toward phagocytic cells was accompanied by increased expression of inflammation-related genes. GM-CSF and IL-18 are interdependent inhibitors of osteoclastogenesis (Udagawa et al. 1997) and were shown to be slightly upregulated; meanwhile, the expression of IL-4, IFN-γ, IL-10, IL-33, and IFN-β remained similar to the control (Fig. 3F). Overall, it remains unclear how saliva causes a shift toward the formation of phagocytic cells.

Saliva Blocks Fusion of Osteoclasts

Saliva prevents the differentiation of osteoclasts from their progenitors. The question then arose of whether saliva can reverse this process. To answer this question, osteoclastogenesis was initiated for 4 d, after which filter-sterilized saliva was added to the cultures for another 3 d. Saliva suppressed the formation of the large multinucleated cells with a massive cytoplasm but did not reverse the TRAP staining of the cells present (Figs. 4A, 4B). Also, the expression of the osteoclast marker genes was not considerably changed by saliva under these conditions (Fig. 4C). In support of the histology, the expression of the 2 fusion genes, DC-STAMP
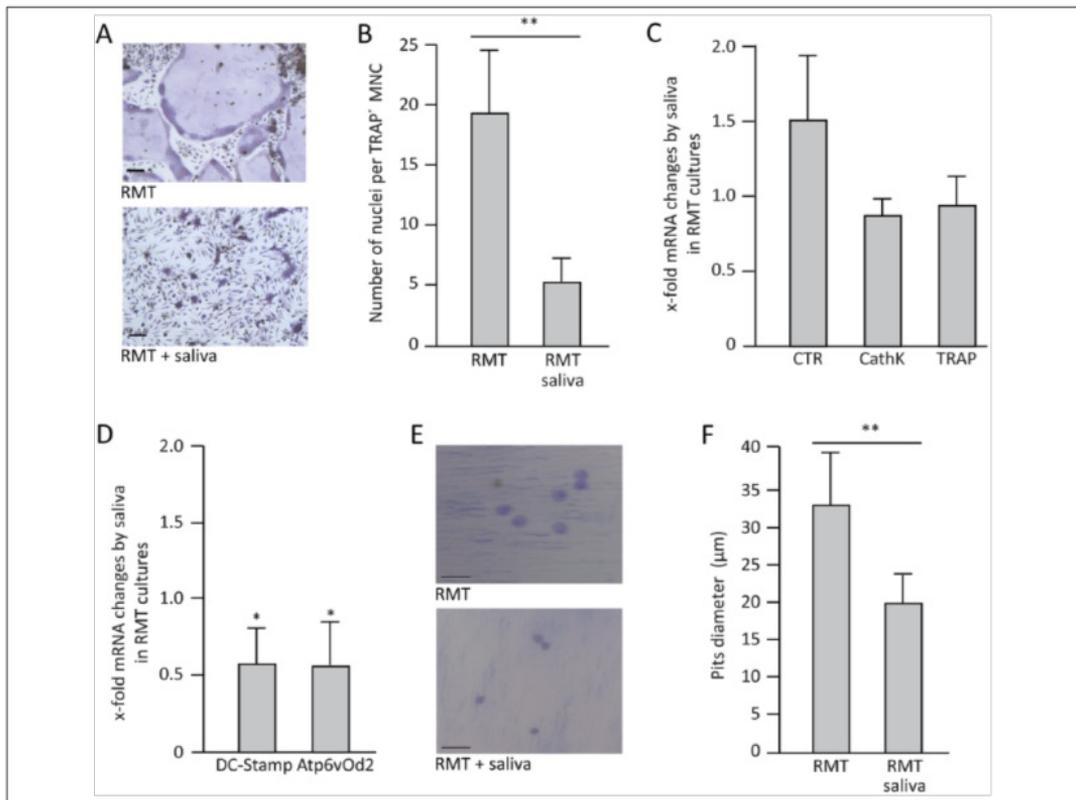
Figure 4. Saliva blocks fusion of osteoclasts. Osteoclastogenesis was induced with RANKL, M-CSF, and TGF-β1, after which saliva was added for another 3 d. The addition of saliva led to the inhibition of cell fusion into multinucleated cells with a massive cytoplasm (**A, B**) but did not reverse the expression of osteoclast marker genes (**C**). Saliva, however, led to decreased expression of the fusion genes (**D**). Osteoclasts seeded onto dentin slides formed resorption pits (**E**) yet with a lower diameter in the saliva group (**F**). Bars represent 20 μm; *$P < 0.05$, **$P < 0.01$.

and Atp6v0d2, was decreased in the presence of saliva (Fig. 4D). When mature osteoclasts were seeded onto dentin slides, the addition of saliva allowed resorption pits to become visible (Fig. 4E), although with a moderately reduced diameter (Fig. 4F). Thus, saliva hindered fusion of differentiated osteoclasts but did not reverse differentiation or inhibit resorption.

Saliva Does Not Negatively Affect Cell Viability

To assess if saliva could affect cell viability, live-dead staining of cultures supplemented with saliva was performed. Live-dead staining, where green are viable cells and red are dead cells, showed no differences between the groups with and without saliva (Figs. 5A and 5B). To check whether saliva affected proliferation of bone marrow cultures, an expression analysis of the cell-cycle related genes MIKI67,

PLK1, and BUB1 was performed. All of these genes showed slight upregulation in the presence of saliva (Fig. 5C). Taken together, cells remained viable when grown in the presence of 20% saliva.

Discussion

Saliva inhibited in vitro osteoclastogenesis and led to the formation of phagocytes in bone marrow cultures. Saliva substantially decreased RANK and, to a lesser extent, also c-fms expression, seemingly lowering the responsiveness of the cells to the essential ligands RANKL and M-CSF, respectively. Accordingly, the expression of the master transcription factors of osteoclastogenesis and the downstream target genes regulating differentiation and cell fusion was diminished. The genes characteristically expressed by phagocytic cells, however, were increasingly expressed,
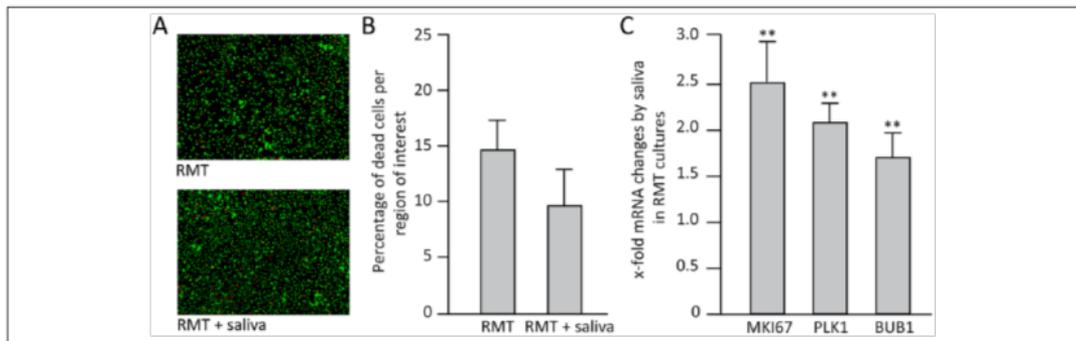
Figure 5. Saliva does not negatively affect cell viability. (**A**) Live-dead staining of bone marrow cultures supplemented with saliva show viable cells staining green with a few dead cells appearing red. (**B**) The negligible number of dead cells was similar in both groups. (**C**) The addition of saliva led to a weak increase in the expression of cell-cycle-related genes MKI67, PLK1, and BUB1. **$P < 0.01$, compared to osteoclast cultures with RANKL, M-CSF, and TGF-β1 (RMT).

and a phagocytosis assay was positive. Together, the data suggest that saliva suppresses osteoclastogenesis in favor of the development of a phagocytic phenotype in vitro.

The data are consistent with the interrelated expression of genes involved in osteoclastogenesis. It is reasonable to propose that reduced RANK expression decreases osteoclastogenesis; however, whether saliva directly targets the RANK gene remains unknown. Alternatively, saliva can decrease c-fos, PU.1, and MITF, which control RANK expression (Arai et al. 2012; Ishii et al. 2008), thus suppressing the feed-forward loop to stimulate osteoclastogenesis. MITF or PU.1 alone activates the osteoclast-associated receptor reporter (So et al. 2003). Considering that all changes cumulate in the excretion of NFATc1, it is also clear that the master gene is suppressed in this scenario. Moreover, c-fos and NFATc1 are required for DC-STAMP and Atp6v0d2 expression (Kim et al. 2008; Yagi et al. 2007), providing further genetic evidence for what is obvious from the histochemical staining—namely, that saliva greatly suppresses osteoclastogenesis. Whether the shift of bone marrow cultures toward phagocytic cells is mediated by increased GM-CSF and IL-18 (Udagawa et al. 1997) is uncertain. Overall, it remains unclear how saliva induces a phenotypic shift in osteoclasts toward the formation of phagocytic cells.

Bone marrow cells exposed to saliva, even in the presence of the strong inducers of osteoclastogenesis, express marker genes of phagocytic cells, including CD14 (a coreceptor of Toll-like receptor); CD40, CD80, and CD86 (all costimulatory molecules to prime T cells); and CD11c (an adhesion molecule highly expressed in dendritic cells). Moreover, the costimulatory molecules DAP12, TREM2, and FcR4, which are also characteristic of myeloid cells, are at least not decreased by saliva. These data, together with the positive ingestion of latex beads, suggest that bone

marrow cells develop phagocytic cell characteristics. Moreover, the live-dead staining data and expression analysis of the cell-cycle-related genes show that saliva can support the expansion of the phagocytic cells. The next step would be to characterize the phagocytic cells in detail, as they likely develop into dendritic cells. The latter cells have great implications in innate and adaptive immunity and are located in the stratified squamous epithelia of oral mucosa (Lombardi et al. 1993).

The finding that saliva decreases osteoclastogenesis, even though saliva increases the expression of NF-κB target genes in murine bone marrow cultures, is surprising, particularly because NF-κB is a master regulator of osteoclastogenesis (Iotsova et al. 1997). It seems that these findings are inconsistent. It appears that the decrease in osteoclastogenesis caused by saliva in the present work occurred irrespective of stimulated NF-κB signaling. The difference in the effects of saliva on osteoclastogenesis and NF-κB signaling reflects the pleiotropic function of the transcription factor, which also plays a pivotal role in the formation of antigen-presenting cells. For example, dendritic cell development and survival require the function of various NF-κB subunits (Ouaaz et al. 2002), and NF-κB signaling is crucial for IL-4-induced macrophage fusion (Yu et al. 2011). The present data suggest that saliva-induced NF-κB signaling plays a role in macrophage differentiation and inhibition of osteoclast formation. This seems plausible considering the complexity of differential activation of the canonical and the noncanonical NF-κB signaling pathway, involving activation of RelA/p50 and RelB/p52 transcription factors, respectively. Thus, the underlying mechanisms responsible for why saliva activates NF-κB target genes but blocks osteoclastogenesis remain to be investigated.

The question may then be asked if cortisol in saliva accounts for the shift of osteoclastogenesis toward

phagocytes. This is rather unlikely because dexamethasone, a synthetic glucocorticoid analog, supports osteoclast-like cell formation in mouse bone marrow cultures (Menaa et al. 2000; Shuto et al. 1994), particularly in cocultures with mesenchymal cells involved in the suppression of GM-CSF (Atanga et al. 2011). Moreover, dexamethasone reduced surface expression of CD40 and CD86 by dendritic cells, which was not the case when bone marrow cells were exposed to saliva (Pan et al. 2001). The published data are in line with our findings that the inhibitory effect of saliva on osteoclastogenesis also occurs in the presence of 1μM dexamethasone. Salivary cortisol level in the present bone marrow culture was less than 2 nM. These data suggest that the impact of salivary cortisol is negligible in the present in vitro model.

One of the main limitations of the study is that we have not yet elucidated the molecular mechanisms underlying the observations. The questions remain: First, what components in saliva mediate the observed effects? Second, what is the mechanism in the target cells that leads to the suppression of the machinery of osteoclastogenesis? In addition, our observation that resorption pits produced by mature osteoclasts were smaller than those in the respective controls requires further analysis. Also, the mechanisms of the formation of phagocytic cells and their characterization need to be clarified. Finally, it is not possible to clearly distinguish phagocytes from osteoclasts through an ingestion test with latex beads. Osteoclasts are also phagocytes capable of ingesting latex beads (Sakai et al. 2001). However, cells developing in the presence of saliva are more active in terms of phagocytosis compared to the osteoclast-like cells in vitro. These findings, with the morphology and the gene expression profile, provide a first insight into the characteristics of bone marrow cells developing in the presence of saliva.

Future studies should evaluate which components of saliva cause the suppression of osteoclastogenesis in favor of the development of phagocytic cells. Samples of saliva, as used in the present study, contain molecules that potentially mimic the effects of whole saliva: for example, whole saliva contains the protease inhibitor cystatin C, a molecule that can suppress bone resorption and that was reported to decrease RANK expression (Stralberg et al. 2013). The present findings, with the cystatin C neutralizing antibody showing no reverse of the salivary effect, speak against this option. Saliva also contains molecules that directly control osteoclastogenesis, but, for example, RANKL exceeds the antagonist osteoprotegerin by far (Tobon-Arroyave et al. 2012). Future studies should also characterize the impact of the formation of phagocytizing cells in more detail, as this observation might be even more relevant than the inhibition of osteoclastogenesis. Clearly, this is a pilot study that leaves room for further research.

The clinical relevance of the present findings is still open for debate. Saliva plays an important role in the oral cavity, as in oral wound healing (Bodner et al. 1991). Saliva can make contact with the bone in many situations, especially during surgical interventions. In many situations—as in extracting a tooth, placing a dental implant, performing a guided bone regeneration procedure, or cleaning a deep periodontal pocket—saliva may get access to the bone surface. How saliva can affect dental treatments like bone regeneration is unknown. The in vitro data suggest that the bone exposed to saliva is less resorbed by osteoclasts and at the same time the innate, even the adaptive, immune response is supported. However, whether the in vitro data translate into a clinical response, as in playing a role in Sjögren syndrome or in cases of osteonecrosis of the jaws, has to be determined in future studies. An in vivo study with desalivated rodents would contribute toward determining whether the in vitro effects occur also in vivo.

Taken together, the data suggest that saliva shifts the differentiation of hematopoietic cells toward the phagocyte lineage, overcoming the simulated microenvironment of osteoclastogenesis. It might be possible that the main relevance of the present study is not that saliva inhibits osteoclastogenesis but the possibility that a major function of saliva may be in stimulating the immune system and allowing the formation of phagocytic cells. The present study sets the foundation for these investigations.

Acknowledgments

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Saliva Suppresses Osteoclastogenesis in Murine Bone Marrow Cultures

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Appendix

Materials and Methods

Saliva Sampling and Treatment. Donors who had no oral inflammation provided saliva samples while chewing paraffin wax (Ivoclar Vivadent AG, Schaan, Liechtenstein). Donors gave their written informed consent to participate in this study. Saliva was collected between 9:00 and 11:00 a.m., and donors abstained from eating and drinking 1 h before collection. The local ethical board of the Canton of Bern approved sampling. Saliva was centrifuged at 4,000 g for 5 min, and filter-sterilized samples were immediately used. For indicated experiments, heating for 5 min at 56°C and 96°C was performed in a thermomixer (Eppendorf AG, Hamburg, Germany), and saliva was used without filter sterilizing. Cortisol analysis was performed by an electro-chemiluminescence immunoassay (Modular Analytics E170; Roche Diagnostics, Mannheim, Germany).

In Vitro Osteoclastogenesis in Bone Marrow Cultures. Bone marrow cells were obtained by flushing the femur and tibiae of 4- to 6-wk-old female mice (strain BALB/c) and seeded at 1 million bone marrow cells per cm² in Eagle's Minimum Essential Medium (Alpha Modification [αMEM]) supplemented with 10% fetal calf serum (FCS) and antibiotics. To induce osteoclastogenesis, the media was supplemented with soluble receptor activator of nuclear factor kappa-B ligand (30 ng/ml; RANKL), macrophage colony-stimulating factor (30 ng/ml; M-CSF), and human transforming factor-beta 1 (10 ng/ml; TGF-β1). All supplemented factors were obtained from ProSpec, Ness-Ziona, Israel. If not otherwise indicated, 25% saliva was included in the media. Experiments were also performed in the presence of a human cystatin C affinity-purified polyclonal antibody AF1196 (R&D Systems, Minneapolis, MN, USA), tumor necrosis factor alpha (10 ng/ml; TNFα) obtained from ProSpec, and dexamethasone at 1 μM (Sigma Aldrich, St. Louis, MO, USA), where indicated. After 3 d, histochemical staining for tartrate-resistant acid phosphatase (TRAP, Sigma Aldrich) was performed. Cells were counted positive for TRAP staining when observed with 3 or more nuclei. For the resorption assay, bone marrow cells were cultured for 6 d with RANKL, M-CSF, and TGF-β1, with a change in media after 3 d. Cells were detached using 0.5 mM EDTA and a scraper. Cells were seeded on dentin disks. After 48 h, disks were cleaned with ultrasound and stained with toluidine blue at 1% dilution to show resorption pits.

Expression of Marker Genes in Bone Marrow Cultures. Total RNA was isolated with the High Pure RNA Isolation Kit (Roche Applied Science, Rotkreuz, Switzerland). Reverse transcription was performed with Transcriptor Universal cDNA Master, and polymerase chain reaction was performed with TaqMan universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) or the FastStart Universal Probe Master Rox on a 7500 Real-Time PCR System (Roche). Probes for CTR, TRAP, CathK, OSCAR, TREM2, FcRγ, DAP12, and beta actin were obtained from the TaqMan Gene Expression Assays service (Applied Biosystems). The FastStart Universal SYBR Green Master Rox (Roche) was used. All other primers were designed with the online Universal ProbeLibrary System (Appendix Table 1). The mRNA levels were calculated by normalizing to the housekeeping gene beta actin via the $\Delta\Delta$Ct method.

Cell Viability and Proliferation. For cell viability, live-dead staining was performed (Enzo Life Sciences AG; Lausen, Switzerland). To understand the impact of saliva on

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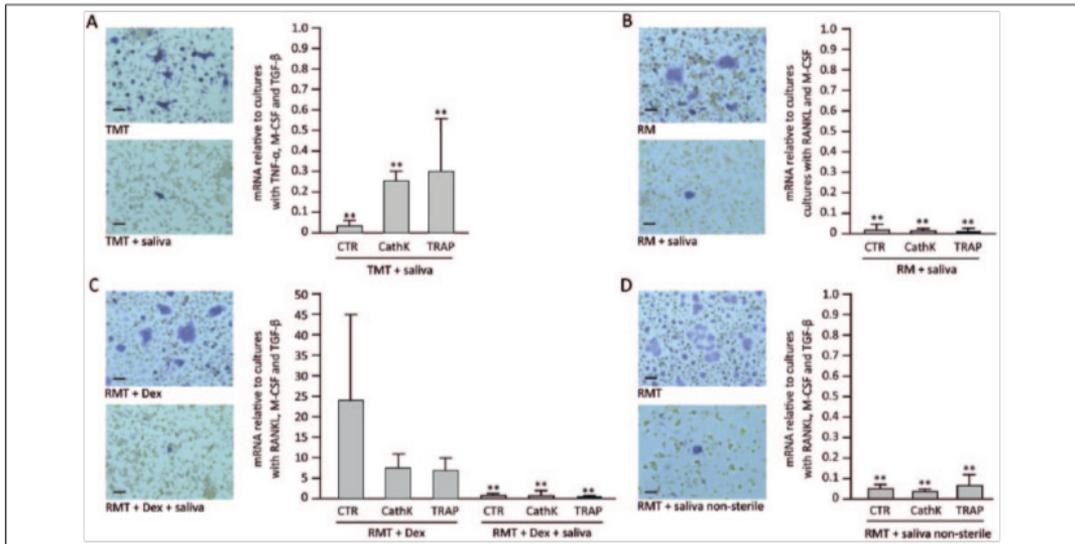[4]Department of Preventive, Restorative and Pediatric Dentistry, School of Dental Medicine, University of Bern, Switzerland
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Appendix Figure. Saliva suppressed osteoclastogenesis in various conditions. (**A**) RANKL was replaced with TNFα, (**B**) TGF-β was left out, and (**C**) dexamethasone was added. (**D**) The addition of nonsterile saliva also led to the blocking of osteoclastogenesis. In TRAP staining pictures, a TRAP$^+$ cell in the saliva groups was taken as a control for the staining. Bars represent 20 μm; **$P < 0.01$.

proliferation, expression analysis of relevant genes were investigated, such as myb-related protein B (MYBL2), polo-like kinase 1 (PLK-1), antigen identified by monoclonal antibody Ki-67 (MKI67), and serine/threonine-protein kinases BUB1 (budding uninhibited by benzimidazoles 1).

Phagocytosis Assay. Phagocytosis was measured with 1-μm Fluoresbrite Dragon Green Carboxylate Microspheres (Polysciences Europe, Eppelheim, Germany). Particles were opsonized with FCS diluted to 50% with Kreb's Ringers PBS. Particles with FCS were incubated at 37°C for 30 min. After the incubation, particles were added to the phagocytosis assay at a concentration equivalent to 5% FCS, and particle density was adjusted to 10^8 particles per mL. Cells were cultured with the beads for 4 h and washed 3 times with cold PBS, fixed with 4% paraformaldehyde, and assembled using mounting medium with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Vectashield, Vector Lab., CA, USA). Slides were visualized with a fluorescence microscope (Olympus Bx-51, Olympus Optical, Tokyo, Japan).

Osteoclast Fusion Assay. Bone marrow cultures were exposed to growth medium supplemented with 30 ng/mL of RANKL, 30 ng/mL of M-CSF, and 10 ng/mL of TGF-β1 over 4 d. After this period, filter-sterilized saliva was added to the cultures for 3 more d. Messenger RNA was isolated, and osteoclast genes were analyzed. TRAP staining of the cultures was also performed.

Statistical Analysis. The data were analyzed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Data represent the mean plus standard deviation of at least 2 independent experiments, each performed in triplicate. Differences in mRNA expression of target genes between cells stimulated with saliva and unstimulated cells were analyzed using the nonparametric Mann–Whitney U test and paired T test. Statistical significance was considered for $P < 0.05$.

Appendix Table 1. Primers for Reverse Transcription Polymerase Chain Reaction.

Gene	Forward Primer	Reverse Primer
m βactin	ctaaggccaaccgtgaaaag	accagaggcatacagggaca
mRANK	gtgctgctcgttccactg	agatgctcataatgcctctcct
m c-fms	gaccatggtgaatggtaggg	ggataacgttgaatcccactg
mTRAF6	ttgcacattcagtgtttttgg	tgcaagtgtcgtgccaag
m c-fos	gcaactttctatgacactgaaacac	tctctctagggctgcattgg
mMITF	gacaccagccataaacgtca	tttccaggtgggtctgc
mPU.1	ggagaagctgatggcttgg	caggcgaatcttttttcttgc
mNFATc-1	ccgttgcttccagaaaataaca	tgtgggatgtgaactcggaa
mDC-Stamp	aagctccttgagaaacgatca	caggactggaaaccagaaatg
mAtp6Od2	aagcctttgtttgacgctgt	gccagcacattcatctgtacc
mCD14	aaagaaactgaagcctttctcg	agcaacaagccaagcacac
mCD86	gaagccgaatcagcctagc	cagcgttactatcccgctct
mCD80	tcgtctttcacaagtgtcttcag	ttgccagtagattcggtcttc
mCD40	gagtcagactaatgtcatctgtggtt	accccgaaaatggtgatg
mCD11c	gagccagaacttcccaactg	tcaggaacacgatgtcttgg
mGM-CSF	gcatgtagaagccatcaaagaa	cgggtctgcacacatgtta
mIL-18	caaaccttccaaatcacttcct	tccttgaagttgacgcaaga
mIL-4	catcggcattttgaacgag	cgagctcactctctgtggtg
mINFγ	atctggaggaactggcaaaa	ttcaagacttcaaagagtctgaggta
mIL10	cagagccacatgctcctaga	tgtccagctggtcctttgtt
mIL33	ggtgaacatgagtcccatca	cgtcacccctttgaagctc
mIFNβ	cacagccctctccatcaacta	catttccgaatgttcgtcct
mMIKI67	gctgtcctcaagacaatcatca	ggcgttatcccaggagact
mPLK1	ttgtagttttggagctctgtcg	cagtgccttcctcctcttgt
mBUB1	tcctgtaagtggccagtcatt	tgaattcatgaacactttgattcac
mCXCL1	gactccagccacactccaac	gactccagccacactccaac
mCXCL2	aaaatcatccaaaagatactgaacaa	ctttggttcttccgttgagg
mCCL2	catccacgtgttggctca	gatcatcttgctggtgaatgagt
mIL6	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa
mPTGS2	gatgctcttccgagctgtg	ggattggaacagcaaggattt

Appendix Table 2. Dose Response Relationship Resulting from the Effect of Saliva on Osteoclastogenesis In Vitro: Number of TRAP[+] MNCs.

Saliva Dilution	w/o	50%	25%	12.5%	6.3%	3.1%
Mean TRAP[+] MNCs	45	0	0	0	9	36
Standard deviation	8	—	—	—	2	5

MNC, multinucleated cell; TRAP, tartrate-resistant acid phosphatase.

Appendix Table 3. Salivary Cortisol Quantification from 8 Donors.

Saliva Donors	1	2	3	4	5	6	7	8	Mean	SD
Salivary cortisol (nM)	8.5	6.5	6.6	8.4	7.4	6.9	8.3	10.6	7.9	1.4

Appendix Table 4. X-fold mRNA Changes with the Addition of Saliva and Cystatin C Neutralizing Antibody in RMT Cultures.

Gene	CTR	CathK	TRAP
Mean	0.1	0.1	0.2
Standard deviation	0.05	0.08	0.1

RMT, RANKL, M-CSF, and TGF-β.

Appendix Table 5. Number of TRAP[+] MNCs per Well in Cultures with RMT and Cultures with RMT + Saliva + Cystatin C Neutralizing Antibody.

	RMT	RMT + Saliva + Cystatin C Neutralizing Antibody
Mean TRAP[+] MNCs	47	0
Standard deviation	6	0

MNC, multinucleated cell; RMT, RANKL, M-CSF, and TGF-β; TRAP, tartrate-resistant acid phosphatase.

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Paper II

Proteomic analysis of porcine bone conditioned medium

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Proteomic Analysis of Porcine Bone-Conditioned Medium

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Purpose: Autografts are considered to support bone regeneration. Paracrine factors released from cortical bone might contribute to the overall process of graft consolidation. The aim of this study was to characterize the paracrine factors by means of proteomic analysis. **Materials and Methods:** Bone-conditioned medium (BCM) was prepared from fresh bone chips of porcine mandibles and subjected to proteomic analysis. Proteins were categorized and clustered using the bioinformatic tools UNIPROT and PANTHER, respectively. **Results:** Proteomic analysis showed that BCM contains more than 150 proteins, of which 43 were categorized into "secreted" and "extracellular matrix." Growth factors that are not only detectable in BCM, but potentially also target cellular processes involved in bone regeneration, eg, pleiotrophin, galectin-1, transforming growth factor beta (TGF-β)-induced gene (TGFBI), lactotransferrin, insulin-like growth factor (IGF)–binding protein 5, latency-associated peptide forming a complex with TGF-β1, and TGF-β2, were discovered. **Conclusion:** The present results demonstrate that cortical bone chips release a large spectrum of proteins with the possibility of modulating cellular aspects of bone regeneration. The data provide the basis for future studies to understand how these paracrine factors may contribute to the complex process of graft consolidation. Int J Oral Maxillofac Implants 2014;29:1208–1215. doi: 10.11607/jomi.3708

Key words: autograft, bone regeneration, conditioned medium, paracrine, proteomics, secretome, supernatant

Autologous bone is extensively used for bone augmentation and reconstruction in oral and maxillofacial surgery.[1,2] The biology of graft consolidation is the key to understand the favorable characteristics of autologous bone.[3] Graft consolidation follows the conserved sequence of intramembranous bone formation, a developmental process where mesenchymal progenitor cells differentiate into bone-forming osteoblasts.[4,5] A fundamental question arises: Is autologous bone merely a space-maintaining tool, or does autologous bone actively support the process of graft consolidation?

Three characteristics of autografts are considered to support the consolidation process.[3,6] First, autologous bone is osteoconductive, providing guidance for the newly formed bone to grow into the defect. Secondly, autologous bone is osteogenic, meaning that it holds mesenchymal cells that can differentiate into osteoblasts.[7] Finally, autologous bone is osteoinductive, as bone morphogenetic proteins entombed in the matrix can initiate the process of endochondral or even intramembranous bone formation.[8] However, it is also likely that other growth factors and proteins released from autografts can support bone regeneration.

The first convincing evidence for a paracrine function of bone is based on the observation that supernatants from murine long bones that were freed from bone marrow as well as periosteal and endosteal cells support myelopoiesis in vitro.[9] In this model, the osteocytes contributed to the production of growth factors that accumulated in the cell culture medium.[9,10] Bone-conditioned medium (BCM) thus contains the complex mixture of secreted proteins from native cortical bone that is commonly termed "secretome."[11,12] In the present study, the authors have characterized the proteins being released from freshly particulated cortical bone chips using a proteomic approach.

Proteomics is a current technology to analyze the entire set of proteins and thus an instrument for

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characterizing tissues, cells, and matrices.[13] Proteomics was recently proposed as a valuable tool in bone biology highlighted by Dufour et al[14] and Lammi et al.[15] The secretome from isolated bone cells was analyzed by proteomics,[11,12,16] and tissue extracts of human alveolar bone obtained from tooth extraction sites[17] and from porcine alveolar bone were subjected to proteomics analysis.[18] However, the proteins being released from the native alveolar process have not been reported so far.

Herein, evidence that cortical bone chips release a broad spectrum of proteins, some that potentially contribute to bone regeneration and others that currently have an unknown role in this process, is presented. The presented data provide the first insight into the paracrine function of autografts.

MATERIALS AND METHODS

Bone-Conditioned Medium (BCM)

Bone was obtained from young pigs within 6 hours postmortem. Bone chips were harvested from the mandibular buccal cortical bone with a "bone scraper" (Hu-Friedy) and placed into sterile plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics and antimycotics (Life Technologies). A ratio of 5 g bone chips per 10 mL culture medium was used. Bone-conditioned medium (BCM) from three independent preparations was collected after 24 hours of incubation, filtered sterile, and kept frozen at -80°C.

Protein Quantification and Identification

Bone-conditioned media from three independent batches were analyzed. Proteins were separated by SDS-PAGE and fractions subjected to the workflow as described for shotgun LC-MS/MS with an LTQ-Orbitrap XL ETD system (Thermo Fisher Scientific) with nonlabeled samples,[19] and MaxQuant software was applied for semiquantitative estimation of protein abundance. Analysis was performed at the Proteomics & Mass Spectrometry Core Facility of the Department of Clinical Research at the University of Bern.

Protein Selection and Study

Proteins were categorized based on the UniProt database (www.uniprot.org). Proteins of the "secreted" and "extracellular matrix" categories in the "cellular component section" were selected for a narrative Medline database search using the keywords "bone," "osteoblast," and "osteoclast." The potential biologic function of the proteins in bone biology is briefly summarized in the Appendix (see online article at www.quintpub.com).

Protein Categorization and Interaction Construction

Selected proteins were analyzed with PANTHER (Protein Analysis Through Evolutionary Relationship; www.pantherdb.org), an online resource to cluster genes and proteins by their functions.[20] In the process, the PANTHER ontology, biologic processes, molecular functions, and pathways were used. The protein-protein interaction map was generated with STRING (Search Tool for the Retrieval of Interacting Genes/Proteins; http://string-db.org). This online tool integrates known and predicted interactions from a variety of sources creating a visual map.[21] The results are visualized as a net of the proteins with their interactions. There are seven different interactions with different colors. A red line indicates the presence of fusion evidence; a green line, neighborhood evidence; a blue line, co-occurrence evidence; a purple line, experimental evidence; a yellow line, text-mining evidence; a light-blue line, database evidence; and a black line, co-expression evidence. This network does not mean that all protein interactions are taking place at the same place and time, but it suggests the possible interactions based on the scientific evidence.

RESULTS

Protein Categorization and Interaction Construction

A total of 175 proteins were detected in BCM with different concentrations and variations among the three batches (Table 1). Out of the 175 proteins, 43 were described as being secreted or present in the extracellular matrix (see Appendix). The PANTHER classification system was used to classify these proteins according to their function or to the biologic process in which they are involved. PANTHER classified the proteins into 13 groups according to their function (Fig 1a) and into 12 groups according to their biologic process (Fig 1b).

The largest group according to the protein function was involved in extracellular matrix processes (15%) and in signaling functions (15%). A significant number of proteins were also involved in enzyme modulation (12%) or transfer carrier (12%). Two major groups of biologic processes, cellular process and metabolic process, were each represented by 16% of the proteins. Other biologic processes are cell communication (13%), transport (10%), developmental process (10%), and cell adhesion (10%). This wide range of categories indicates that proteins present in a conditioned medium of freshly prepared cortical bone chips have diverse cellular functions and they are involved in widespread biologic processes.

Caballé-Serrano et al

Table 1 Summary of All Identified Proteins in BCM According to the Nomenclature of Universal Protein Resource (Uniprot) and Semi-Quantitative Estimation of Protein Abundance

Rank	UniProt ID	Protein quantification	Protein name	Rank	UniProt ID	Protein quantification	Protein name
1	HBB_PIG	5,895.22	Hemoglobin subunit beta	38	SPRC_PIG	129.12	SPARC
2	HBA_PIG	3,914.77	Hemoglobin subunit alpha	39	LDHA_PIG	128.63	L-lactate dehydrogenase A chain (LDH-A)
3	ALBU_PIG	1,618.59	Serum albumin	40	PRDX2_PIG	124.93	Peroxiredoxin-2 (TSA)
4	ACTB_PIG	1,491.38	Actin, cytoplasmic 1, N-terminally processed	41	G3P_PIG	124.54	Glyceraldehyde-3-phosphate dehydrogenase
5	KCRM_PIG	1,018.84	Creatine kinase M-type	42	S10AB_PIG	122.88	Protein S100-A11
6	CYC_PIG	662.74	Cytochrome c	43	TBA1B_PIG	122.68	Tubulin alpha-1B chain
7	GELS_PIG	612.37	Gelsolin (ADF)	44	TTHY_PIG	122.10	Transthyretin [CHAIN 0]
8	PPIA_PIG	484.24	Peptidyl-prolyl cis-trans isomerase A	45	RUXE_PIG	120.58	Small nuclear ribonucleoprotein E (snRNP-E)
9	PTN_PIG	452.86	Pleiotrophin (PTN) (PTF-beta)				
10	FETUA_PIG	419.03	Alpha-2-HS-glycoprotein	46	PGS2_PIG	114.42	Decorin
11	ANXA2_PIG	384.55	Annexin A2 (PAP-IV)	47	CPNS1_PIG	113.29	Calpain small subunit 1 (CSS1)
12	HMGB1_PIG	343.81	High mobility group protein B1 (HMG-1)	48	PGK1_PIG	113.05	Phosphoglycerate kinase 1
13	LEG1_PIG	341.36	Galectin-1 (Gal-1)	49	BGH3_PIG	112.57	Transforming growth factor-beta-induced protein ig-h3 (Beta ig-h3)
14	ACTS_PIG	334.59	Actin, alpha skeletal muscle				
15	COF1_PIG	317.08	Cofilin-1	50	MYL6_PIG	104.67	Myosin light polypeptide 6
16	S10AA_PIG	293.02	Protein S100-A10	51	LDHB_PIG	100.31	L-lactate dehydrogenase B chain (LDH-B)
17	TRYP_PIG	255.77	Trypsin				
18	TRFE_PIG	249.80	Serotransferrin (Transferrin)	52	VINC_PIG	96.40	Vinculin
19	RNAS4_PIG	238.91	Ribonuclease 4 (RNase 4)	53	TPIS_PIG	93.12	Triosephosphate isomerase (TIM)
20	HMGB2_PIG	235.77	High mobility group protein B2 (HMG-2)	54	RSSA_PIG	92.12	40S ribosomal protein SA
21	LYSC2_PIG	234.44	Lysozyme C-2	55	CNN2_PIG	84.51	Calponin-2
22	S10AC_PIG	209.92	Protein S100-A12	56	MIF_PIG	81.58	Macrophage migration inhibitory factor (MIF)
23	ANGI_PIG	201.77	Angiogenin (RNase 5)	57	CAN1_PIG	80.89	Calpain-1 catalytic subunit (CANP 1)
24	FABP4_PIG	190.03	Fatty acid-binding protein, adipocyte (ALBP)				
25	TENA_PIG	186.24	Tenascin (TN) (TN-C) [ISOFORM Major]	58	DHRS4_PIG	79.36	Dehydrogenase/reductase SDR family member 4
				59	COF2_PIG	77.79	Cofilin-2
26	PTBP1_PIG	173.38	Polypyrimidine tract-binding protein 1 (PTB)	60	FA12_PIG	69.53	Coagulation factor XIIa light chain
27	RS12_PIG	163.05	40S ribosomal protein S12	61	MDHM_PIG	69.47	Malate dehydrogenase, mitochondrial
28	TBB5_PIG	154.96	Tubulin beta chain				
29	VIME_PIG	151.30	Vimentin	62	GBLP_PIG	65.03	Guanine nucleotide-binding protein subunit beta-2-like 1 (RACK1)
30	NDKB_PIG	146.78	Nucleoside diphosphate kinase B (NDK B)				
31	ENOB_PIG	138.43	Beta-enolase (MSE)	63	H33_PIG	62.23	Histone H3.3
32	H4_PIG	138.10	Histone H4	64	GSTP1_PIG	61.96	Glutathione S-transferase P
33	PGS1_PIG	133.06	Biglycan	65	MOES_PIG	60.93	Moesin
34	SNRPA_PIG	132.74	U1 small nuclear ribonucleoprotein A	66	UBC_PIG	57.97	Ubiquitin
35	RS15_PIG	132.53	40S ribosomal protein S15	67	RYR1_PIG	55.47	Ryanodine receptor 1 (RYR-1)
36	MYG_PIG	131.89	Myoglobin	68	ANXA1_PIG	53.89	Annexin A1
37	LAC_PIG	129.30	Ig lambda chain C region	69	TRFL_PIG	53.55	Lactotransferrin (Lactoferrin)

Table 1 *continued*   Summary of All Identified Proteins in BCM According to the Nomenclature of Universal Protein Resource (Uniprot) and Semi-Quantitative Estimation of Protein Abundance

Rank	UniProt ID	Protein quantification	Protein name	Rank	UniProt ID	Protein quantification	Protein name
70	CYTB_PIG	51.66	Cystatin-B	100	CAH3_PIG	28.71	Carbonic anhydrase 3 (CA-III)
71	DEST_PIG	50.85	Destrin (ADF)	101	RS21_PIG	28.71	40S ribosomal protein S21
72	ATPO_PIG	49.92	ATP synthase subunit O, mitochondrial (OSCP)	102	RS20_PIG	28.69	40S ribosomal protein S20
73	C1QA_PIG	49.81	Complement C1q subcomponent subunit A	103	RS28_PIG	27.03	40S ribosomal protein S28
74	RL14_PIG	49.04	60S ribosomal protein L14	104	A1AT_PIG	26.79	Alpha-1-antitrypsin
75	H1T_PIG	48.98	Histone H1t	105	THIO_PIG	26.76	Thioredoxin (Trx)
76	IGF2_PIG	48.79	Preptin	106	RS16_PIG	26.49	40S ribosomal protein S16
77	PRDX6_PIG	48.02	Peroxiredoxin-6 (NSGPx)	107	APOA1_PIG	26.32	Truncated apolipoprotein A-I
78	SRSF1_PIG	46.47	Serine/arginine-rich splicing factor 1	108	RL18_PIG	25.40	60S ribosomal protein L18
79	RAB1A_PIG	46.20	Ras-related protein Rab-1A	109	RS3_PIG	25.33	40S ribosomal protein S3
80	ICA_PIG	45.26	Inhibitor of carbonic anhydrase	110	ALDR_PIG	25.32	Aldose reductase (AR)
81	RAB1B_PIG	44.80	Ras-related protein Rab-1B	111	CATD_PIG	25.18	Cathepsin D heavy chain
82	IBP5_PIG	44.20	Insulin-like growth factor-binding protein 5 (IBP-5)	112	SUCA_PIG	25.10	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial
83	TGFB1_PIG	43.73	Latency-associated peptide (LAP)	113	RL6_PIG	25.00	60S ribosomal protein L6
84	ITIH1_PIG	43.73	Inter-alpha-trypsin inhibitor heavy chain H1	114	MDHC_PIG	23.80	Malate dehydrogenase, cytoplasmic
85	CAPZB_PIG	41.91	F-actin-capping protein subunit beta	115	DESM_PIG	23.71	Desmin
86	SRSF2_PIG	41.90	Serine/arginine-rich splicing factor 2 (SC-35)	116	CISY_PIG	23.70	Citrate synthase, mitochondrial
87	B2MG_PIG	41.47	Beta-2-microglobulin	117	RS13_PIG	23.34	40S ribosomal protein S13
88	ICTL_PIG	39.31	Cathelin	118	RPN1_PIG	22.91	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1
89	PMP37_PIG	38.66	Antibacterial peptide PMAP-37				
90	MYO6_PIG	38.60	Unconventional myosin-VI	119	ATP8_PIG	22.76	ATP synthase protein 8
91	RS23_PIG	37.73	40S ribosomal protein S23	120	SODC_PIG	21.79	Superoxide dismutase [Cu-Zn]
92	S10A6_PIG	37.51	Protein S100-A6	121	UGPA_PIG	21.71	UTP--glucose-1-phosphate uridylyltransferase (UDPGP)
93	UB2D2_PIG	33.15	Ubiquitin-conjugating enzyme E2 D2	122	A4_PIG	21.47	C31
94	HS71B_PIG	31.18	Heat shock 70 kDa protein 1B (HSP70.2)	123	CYB5_PIG	20.84	Cytochrome b5 [ISOFORM 2]
95	NFM_PIG	31.08	Neurofilament medium polypeptide (NF-M)	124	G6PI_PIG	20.44	Glucose-6-phosphate isomerase (GPI) (AMF) (NLK) (PGI) (PHI)
96	SPP24_PIG	30.62	Secreted phosphoprotein 24 (Spp-24)	125	PPAC_PIG	20.26	Low molecular weight phosphotyrosine protein phosphatase (LMW-PTP)
97	LMNA_PIG	30.09	Lamin-A/C [ISOFORM C]				
98	GDIB_PIG	29.27	Rab GDP dissociation inhibitor beta (Rab GDI beta) (GDI-2)	126	SEP15_PIG	19.97	15 kDa selenoprotein
				127	C1S_PIG	19.58	Complement C1s subcomponent light chain
99	TGFB2_PIG	29.23	Transforming growth factor beta-2 (TGF- β2)	128	CAZA2_PIG	19.46	F-actin-capping protein subunit alpha-2
				129	RINI_PIG	18.30	Ribonuclease inhibitor
				130	RL29_PIG	18.15	60S ribosomal protein L29

Table 1 *continued* **Summary of All Identified Proteins in BCM According to the Nomenclature of Universal Protein Resource (Uniprot) and Semi-Quantitative Estimation of Protein Abundance**

| Rank	UniProt ID	Protein quantification	Protein name	Rank	UniProt ID	Protein quantification	Protein name
131	KCRB_PIG	17.69	Creatine kinase B-type	153	RL11_PIG	12.65	60S ribosomal protein L11
132	APOC3_PIG	16.82	Apolipoprotein C-III (Apo-CIII)	154	HSP76_PIG	12.58	Heat shock 70 kDa protein 6
133	FUT8_PIG	16.74	Alpha-(1,6)-fucosyltransferase (alpha1-6FucT)	155	ATPK_PIG	12.42	ATP synthase subunit f, mitochondrial
134	SCG1_PIG	16.70	CCB peptide	156	KAD1_PIG	12.26	Adenylate kinase isoenzyme 1 (AK 1)
135	FRIL_PIG	16.69	Ferritin light chain (Ferritin L subunit)	157	HPLN1_PIG	11.90	Hyaluronan and proteoglycan link protein 1
136	CO3_PIG	16.25	Complement C3a anaphylatoxin	158	AK1A1_PIG	11.72	Alcohol dehydrogenase [NADP(+)]
137	MYP2_PIG	16.23	Myelin P2 protein	159	THRB_PIG	11.72	Thrombin heavy chain
138	PIMT_PIG	16.05	Protein-L-isoaspartate (D-aspartate) O-methyltransferase (PIMT)	160	PSME1_PIG	11.69	Proteasome activator complex subunit 1 (PA28a)
139	LEG4_PIG	15.91	Galectin-4 (Gal-4)	161	COX5B_PIG	11.60	Cytochrome c oxidase subunit 5B, mitochondrial
140	FABPH_PIG	15.85	Fatty acid-binding protein, heart (H-FABP) [	162	SCOT1_PIG	11.30	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial
141	CFAD_PIG	15.68	Complement factor D	163	APOE_PIG	10.82	Apolipoprotein E (Apo-E)
142	SMD2_PIG	15.28	Small nuclear ribonucleoprotein Sm D2 (Sm-D2)	164	ACBP_PIG	10.36	DBI(32-86)
143	HEMO_PIG	14.54	Hemopexin	165	SPAST_PIG	10.15	Spastin
144	SAMP_PIG	14.52	Serum amyloid P-component (SAP)	166	HSP7X_PIG	9.60	Heat shock 70 kDa protein
145	FUMH_PIG	14.44	Fumarate hydratase, mitochondrial (Fumarase)	167	RAB14_PIG	9.58	Ras-related protein Rab-14
146	ODO2_PIG	14.06	Dihydrolipoyllysine-residue succinyltransferase (OGDC-E2)	168	LDHC_PIG	9.56	L-lactate dehydrogenase C chain (LDH-C)
147	ITIH4_PIG	14.00	Inter-alpha-trypsin inhibitor heavy chain H4 (ITI heavy chain H4)	169	TFAM_PIG	9.39	Transcription factor A, mitochondrial (mtTFA)
148	ATM_PIG	13.50	Serine-protein kinase ATM (A-T mutated homolog)	170	SAHH_PIG	8.94	Adenosylhomocysteinase (AdoHcyase)
149	ATPA2_PIG	13.12	ATP synthase subunit alpha liver isoform, mitochondrial	171	PLF4_PIG	8.88	Platelet factor 4 (PF-4)
150	SLPI_PIG	12.99	Antileukoproteinase (ALP)	172	CRYL1_PIG	8.87	Lambda-crystallin homolog (Gul3DH)
151	HPRT_PIG	12.80	Hypoxanthine-guanine phosphoribosyltransferase (HGPRT)	173	PPA5_PIG	8.70	Tartrate-resistant acid phosphatase type 5 (TR-AP)
152	EF1G_PIG	12.68	Elongation factor 1-gamma (EF-1-gamma)	174	PK3C3_PIG	8.54	Phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3-kinase type 3)
				175	PROC_PIG	8.22	Activation peptide
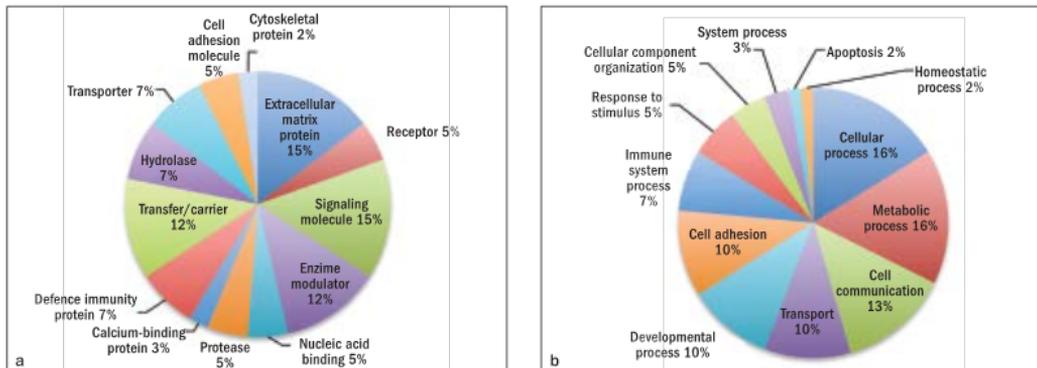
Fig 1 Bioinformatic analysis of identified proteins that are secreted or present in the extracellular matrix of cells. Pie chart representations of the distribution according to the *(a)* molecular function and *(b)* biologic processes of BCM proteins. Categorizations made according to the information provided by the PANTHER classification system.

Protein-Protein Interaction Analysis

To understand the interactions among the 43 proteins, a protein-protein interaction analysis was carried out (Fig 2). Seventy-seven percent (33 out of 43) of the proteins could be networked. Six out of the seven possible interactions were described; interactions with neighborhood evidence were not described. The most interactive protein was decorin (DCN). Decorin was shown to have fusion evidence, co-occurrence evidence, experimental evidence, text-mining evidence, database evidence, and co-expression evidence interactions with biglycan. Moreover, DCN also had two types of interactions with complement C1q subcomponent subunit A (C1QA). These two interactions were fusion evidence and co-expression evidence. Other strong interactions are, for example, those between decorin and transforming growth factor beta-1 (TGF-β1), TGF-β1 and transforming growth factor beta-2 (TGF-β2), and secreted protein acidic and rich in cysteine (SPARC) with TGF-β1. The interaction with text-mining evidence, which are the interactions described in the scientific literature, was the most common among the proteins.

DISCUSSION

Bone autografts are considered to be the gold standard, as they support graft consolidation by being osteoconductive, osteogenic, and osteoinductive.[3] In addition, paracrine factors released from cortical bone might contribute to the overall process of graft consolidation.[9] Based on this assumption, the authors took advantage of proteomics technology to characterize the paracrine factors released from porcine bone chips into the conditioned medium (BCM). The main finding was that BCM contains more than 170 proteins, including 43 growth factors and proteins present in the
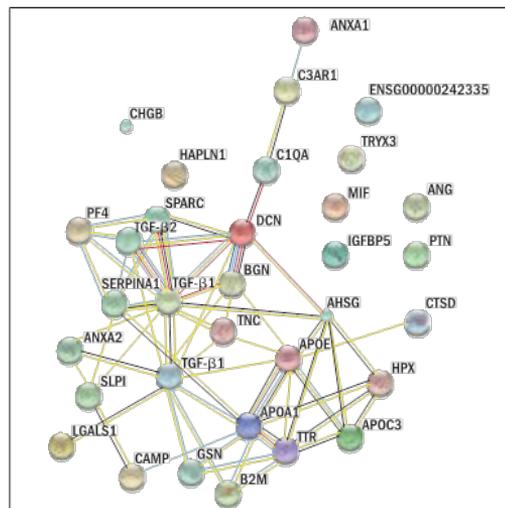
Fig 2 Pathway mapping and interaction of BCM proteins using the STRING online tool. A red line indicates the presence of fusion evidence; a green line, neighborhood evidence; a blue line, co-occurrence evidence; a purple line, experimental evidence; a yellow line, text-mining evidence; a light-blue line, database evidence; and a black line, co-expression evidence. The interaction map shows high interconnection of decorin, biglycan, TGF-β1, and TGF-β2, meaning that these proteins are more likely to have a biologic interaction with other proteins present in BCM.

extracellular matrix. The data present initial evidence that cortical bone is a source of proteins that can be released into the healing blastema. Through this mechanism, cortical bone chips can target cells in the defect site and possibly affect graft consolidation.

Proteomic analysis of porcine and human alveolar bone has recently been reported. However, in contrast to the previous approach where the whole bone was

either demineralized[18] or grinded to powder,[17,22] in the present study, entire bone chips were incubated with culture medium. Here, a clinically inspired approach where bone chips were left in culture medium, allowing passive release of proteins in a physiologic environment, was chosen. Even though the concept differed from previous studies, some proteins are overlapping in the analysis, such as gelsolin, annexin A2, galectin-1, trypsin, serotransferrin, angiogenin, tenascin-c, biglycan, SPARC, DCN, macrophage migration inhibitory factor, annexin A1, lactotransferrin, C1QA, insulin-like growth factor-bindinc protein 5, TGF-β1, TGF-β2, hemopexin, and platelet factor 4.[11,17,18] Thus, the data of the present study add to the current knowledge on proteins related to bone matrix and bone cells.

The present study describes new, presumably unexpected proteins that are highly abundant in BCM and that could participate in the control of bone regeneration. For example, transforming growth factor beta-induced protein, 68kDa, also known as TGFBI, positively regulates bone size and strength.[23] TGF-β2 is also present in BCM and is considered as a biomarker for the assessment of bone turnover and for monitoring antiresorptive therapy in postmenopausal osteoporosis.[24] Moreover, TGF-β2 is involved in endothelial-to-mesenchymal transition and acquisition of a stem cell–like phenotype by endothelial cells[25] and acts as protection from unloading-induced apoptosis in vivo.[14] Application of recombinant hTGF-β2 can increase bone volume in a preclinical model.[26] However, whether TGF-β2 in BCM reaches the threshold level to support bone regeneration remains unknown.

Another example of a protein that is highly abundant in BCM is pleiotrophin, also termed osteoblast-stimulating factor-1 (OSF-1). OSF-1 can promote in vitro adhesion, migration, expansion, and differentiation of human osteoprogenitor cells.[27] Mice overexpressing pleiotrophin display increased bone density.[28] Surprisingly, overexpression of pleiotrophin impairs fracture healing.[29] Pleiotrophin is a secreted component of the bone marrow vascular niche that regulates hematopoietic stem cell self-renewal and retention in vivo.[30] In vivo, pleiotrophin serum levels increased during fracture healing.[31] Thus, pleiotrophin in BCM might have a paracrine function at defect sites.

Galectin-1 (Gal-1) is another protein present in BCM that plays a role in mesenchymal and bone cells. Gal-1 is a widely expressed β-galactoside-binding protein, which exerts pleiotropic biologic functions and is expressed by osteoblasts[32] and bone marrow mesenchymal cells.[33] Mesenchymal cells can undergo myoblastic differentiation in response to Gal-1.[34] Gal-1 exhibits proangiogenic functions during early stages of pregnancy[35] and triggers platelet activation in vitro.[36] Previous work has implicated Gal-1 with a pleiotropic

biologic function that might also be involved in the process of graft consolidation.

The present study has limitations. First, it cannot be defined if this protein mixture exerts bioactivity in vivo. Many proteins are cell components or parts of metabolism pathways; thus, they do not have a receptor. However, in vitro bioassays indicate that BCM modulates osteogenic and adipogenic differentiation of mesenchymal cells.[23,37] Second, bone chips were obtained from animals in a growing phase. Since the number of osteocytes in bone changes with age and diseases,[38,39] the paracrine function of bone probably also changes accordingly. Third, the origin of the proteins that include the bone matrix and the cells is unclear. Fourth, BCM does not cover the proteins that are actively released from the bone matrix upon osteoclast resorption in vivo.[40] Finally, bone was isolated from porcine mandibular cortical bone immediately postmortem; however, potential changes in the composition of the BCM over time remain to be determined. Many questions remain to be answered; yet, this study is considered to be a primer for ongoing research.

Future studies should focus on the biologic activity of BCM and probably identify proteins that are of particular relevance for graft consolidation, also keeping in mind that based on this knowledge, cell-free bone substitutes can be supplemented with those proteins that are naturally released from bone grafts in a "bionic" approach. Furthermore, questions on the source of the respective proteins, particularly the role of osteocytes as a source of paracrine mediators and their role in graft consolidation, would be worthwhile to study, for example, with a genome-wide microarray. In support of this suggestion, preclinical studies testing the capacity of BCM to modulate bone regeneration in vivo would be of great value. Following this trend, the data shown here can provide the scientific basis for future studies and offer a point of departure for more in-depth research.

CONCLUSIONS

The present findings suggest that cortical bone chips release proteins into the culture medium that are partially described to have an impact on bone regeneration. It is currently unclear if this descriptive in vitro analysis somehow reflects the biologic environment and explains the beneficial clinical properties of autografts. Nevertheless, the present study might inspire further research to reveal if proteins in BCM actively contribute to graft consolidation and eventually serve as a master plan for the development of bionic bone substitutes. The study presents pioneer data on the paracrine mechanisms of bone chips, clearly indicating

the presence of growth factors with the potential to modulate the process of graft consolidation.

ACKNOWLEDGMENTS

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Caballé-Serrano et al

Appendix   Proteins Secreted or Present in the Extracellular Matrix

Protein name	Gene name	Function on osteoblasts	Function on osteoclasts
Gelsolin	GSN	Not known	Important for podosome formation and function in osteoclasts[1]
Pleiotrophin	PTN	Involved in osteoblast proliferation and differentiation, response to mechanical stimuli and cross-talk with Wnt signaling [2,3]	Regulates the expansion and regeneration of osteoclast precursors[4]
Alpha-2-HS-glycoprotein	AHSG	Antagonize BMP. Physiologic calcification inhibitor[5,6]	Antagonize TGF[6]
Annexin A2	ANXA2	Involved in osteoblastic mineralization. Expressed 2X in SAOS[7,8]	Heightens osteoclast formation and bone resorption[8,9,10]
Galectin-1	LGALS1	Modulated osteoblastic proliferation and differentiation[11]	Not known
Trypsin	TRZX3	Not known	Not known
Serotransferrin (Transferrin)	TF	Lactoferrin may not play a significant role in modulating osteoblast functions.[12]	May be involved during osteoclast development and function[13]
Lysozyme C-2	LZY2	Not known	Not known
Protein S100-A12	S100B	Not known	Not known
Angiogenin	ANG	Not known	Resorption inhibitory factor[14]
Tenascin	TNC	Influence osteoblast adhesion and differentiation[15]	Not known
Biglycan	BGN	Promotes osteoblast differentiation and matrix mineralization[16]	Biglican deficiency increases osteoclast activity and differentiation. May modulate both formation and resorption.[17,18]
Ig lambda chain C region	IGLC	Not known	Not known
SPARC	SPARC	Involved in bone mineralization[19,20]	Limit osteoclast maturation and function[19,20]
Transthyretin	TTR	KO mice for Transthyretin have increased bone mineral density and trabecular volumen.[21,22]	May play a role on osteoclasts. However, not clear scientific evidence.[21,22]
Decorin	DCN	Produced and expressed on osteoblasts. May play a role in osteogenesis. Plays a role in maintaining an appropriate number of mature osteoblasts by modulating the proliferation and survival of bone marrow stromal cells.[23,24,25]	Antagonize TGF (it is a leucine-rich proteoglycan). Inhibition of osteoclastogenesis.[26,27]
Transforming growth factor-beta-induced protein ig-h3	BIGH3	May play an important role in beta-ig-h3-mediated inhibition of osteoblast differentiation[28]	Not known
Macrophage migration inhibitory factor	MIF	Increase expression of RANKL.[29]	Induction of RANKL. Enhance osteoclastogenesis and osteoclast funtion. [29,30,31]
Annexin A1	ANXA1	May play a role in the regulation of osteoblast differentiation. Can be released by apoptotic cells[32,33]	Not known
Lactotransferrin (Lactoferrin)	LTF	Lactoferrin stimulates the proliferation, differentiation, and survival of osteoblasts,[34]	Potently inhibiting osteoclastogenesis in bone marrow cultures[35]

Appendix *continued*   Proteins Secreted or Present in the Extracellular Matrix

Protein name	Gene name	Function on osteoblasts	Function on osteoclasts
Complement C1q subcomponent subunit A	C1QA	Not known	Enhance osteoclast development[36]
Preptin	IGF2	Is anabolic to bone mediated by ERK/CTGF and may contribute to the preservation of bone mass[37,38]	Induction of osteoclast differentiation and bone resorption[39]
Inhibitor of carbonic anhydrase	ICA	Not known	Inhibits osteoclast differentiation and bone resorption.[40,41]
Insulin-like growth factor-binding protein 5	IGFBP5	Inhibits osteoblast differentiation[42,43,44]	Enhance osteoclast formation and osteoclastic bone-resorbing activity[43,45]
Latency-associated peptide	TGFB1	Not known	Latent TGF that can be activated by enzymes (metalloproteinases) in vivo[46,47]
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	Not known	Not known
Beta-2-microglobulin	B2M	May play a role in osteoblasts[49,50]	Stimulates osteoclast formation[48,49,50]
Cathelin	CAMP	Not known	Not known
Antibacterial peptide	PMAP37	Not known	Not known
Secreted phosphoprotein 24	SPP24	Antagonizes BMPs[52,53,54]	Binds to TGF. May affect osteoclasts[51]
Transforming growth factor beta-2	TGFB2	May protect osteoblasts from apoptosis[55]	Enhances osteoclastogenesis[54]
Alpha-1-antitrypsin	SERPINA1	Can be secreted by osteoblasts. Not clear if it plays a role on osteoblasts[57,58]	May inhibit osteoclast activity[57]
Truncated apolipoprotein A-I	APOA1	Not known	Not known
Cathepsin D heavy chain	CTSD	May play a negative role in mineralization[59]	Not known
Apolipoprotein C-III	APOC3	Not known	Not known
CCB peptide	CHGB	Not known	Not known
Complement C3a anaphylatoxin	C3AR1	May enhance the inflammatory response of osteoblasts[60]	Modulate osteoclast formation, particularly in pro-inflammatory environment[60]
Complement factor D	CFD	Not known	Not known
Hemopexin	HPX	Not known	Not known
Serum amyloid P-component	APCS	Expressed in bone tissue and is associated with the changes of bone mineral density[61]	Expressed in bone tissue and is associated with the changes of bone mineral density[61]
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	Not known	Not known
Antileukoproteinase	SLPI	Not known	Not known
Hyaluronan and proteoglycan link protein 1	HAPLN1	Not known	Not known
Thrombin heavy chain	F2	Not known	Not known
Apolipoprotein E	Apo-E	Has a physiologic role as a regulator of osteoblast function[63]	Inhibits osteoclast differentiation[62]
Platelet factor 4	PF4	Inhibitor of osteoblast-like cells[64]	May regulate osteoclastic bone resorption in vitro[65]

Literature review of proteins present in BCM that are secreted or present in the extracellular matrix. It can be distinguished between the function that the protein does on osteoblasts and on osteoclasts, according to the existing scientific literature.

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Paper III

Conditioned Medium from Fresh and Demineralized Bone Enhances Osteoclastogenesis in Murine Bone Marrow Cultures

Clinical Oral Implants Research, 2015

CLINICAL ORAL IMPLANTS RESEARCH

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Conditioned medium from fresh and demineralized bone enhances osteoclastogenesis in murine bone marrow cultures

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Abstract
Objectives: Osteoclasts rapidly form on the surface of bone chips at augmentation sites. The underlying molecular mechanism, however, is unclear. Soluble factors released from bone chips in vitro have a robust impact on mesenchymal cell differentiation. Whether these soluble factors change the differentiation of hematopoietic cells into osteoclasts remains unknown.
Methods: Osteoclastogenesis, the formation of tartrate-resistant acid phosphatase-positive multinucleated cells, was studied with murine bone marrow cultures exposed to RANKL and M-CSF, and conditioned medium from fresh (BCM) and demineralized bone matrix (DCM). Histochemical staining, gene and protein expression, as well as viability assays were performed.
Results: This study shows that BCM had no impact on osteoclastogenesis. However, when BCM was heated to 85°C (BCMh), the number of tartrate-resistant acid phosphatase-positive multinucleated cells that developed in the presence of RANKL and M-CSF approximately doubled. In line with the histochemical observations, there was a trend that BCMh increased expression of osteoclast marker genes, in particular the transcription factor c-fos. The expression of c-fos was significantly reduced by the TGF-b receptor I antagonist SB431542. DCM significantly stimulated osteoclastogenesis, independent of thermal processing.
Conclusions: These data demonstrate that activated BCM by heat and DBM are able to stimulate osteoclastogenesis in vitro. These in vitro results support the notion that the resorption of autografts may be supported by as yet less defined paracrine mechanisms.

Bone augmentation is a common procedure in oral, maxillofacial, and orthopedic surgery (Chiapasco et al. 2009; Myeroff & Archdeacon 2011; Buser et al. 2013). Malformation, trauma, and dental implant surgery can make bone grafts necessary for reconstruction surgery (Chiapasco et al. 2009). Autografts have unique characteristics, which make them a favorable material for bone regeneration; they contain living cells that can contribute to bone formation (Bohr et al. 1968), serve as a scaffold (Saulacic et al. 2014), and contain bioactive molecules that are released from the bone matrix upon resorption (Crane & Cao 2014). This trinity of properties is known as osteogenicity, osteoconductivity, and osteoinductivity (Khan et al. 2005; Grabowski & Cornett 2013). It does not rule out that there may be further properties, as yet poorly defined, which add to the existing knowledge of how autografts modulate bone regeneration.

The paracrine function, or at least the passive release of soluble factors of harvested bone, can be considered the fourth property of autografts and should be distinguished from the endocrine function of orthotropic bone in whole-organism physiology (Karsenty & Ferron 2012). Proteomic analysis detected numerous molecules, including growth factors, in bone-conditioned medium (BCM) (Caballé-Serrano et al. 2014) and has therefore extended existing knowledge of the composition of whole bone (Kupcova Skalnikova 2013; Romanello et al. 2013). Osteocyte-derived molecules are also found in BCM (Brolese et al. 2014), and BCM potently affects the differentiation of mesenchymal cells in vitro (Peng et al. 2014). Thus, there is increasing evidence that freshly prepared

Caballé-Serrano et al·Bone-conditioned medium increases osteoclastogenesis

bone chips release bioactive molecules, thereby supporting the hypothesis that autografts have a transient paracrine-like function.

Resorption of autografts is a clinical challenge and occurs early during graft consolidation. Multiple osteoclasts rapidly appear when bone chips are placed into bone defects (Saulacic et al. 2014), likely explaining why no residual autografts were detectable 2 years after contour augmentation (Jensen et al. 2014). Resorption occurs with bone harvested from different anatomical regions and placed into different augmentation sites, for example, bone harvested from the iliac crest or ramus and used for sinus floor elevation or ridge augmentation (Spin-Neto et al. 2013; Schmitt et al. 2014). Bone graft resorption is thus not only a clinical principle in regenerative medicine, but also an evolutionary principle removing dead bone after injury or disease. The molecular mechanisms controlling osteoclastogenesis on the surface of bone grafts are unknown. It is possible that molecules released by the bone grafts initiate or at least support the process of osteoclastogenesis.

Osteoclasts originate from hematopoietic stem cells when the key factor – receptor activator of nuclear factor kappa-B ligand (RANKL) – is present (Suda et al. 1999). Osteoclastogenesis also requires the M-CSF receptor (c-fms) (Hofstetter et al. 1992). Osteoclasts are characterized by their multinucleated morphology and the expression of tartrate-resistant acid phosphatase (TRAP), cathepsin k (CatK), and the calcitonin receptor (CTR). Osteoclasts express costimulatory molecules activating the immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway (Koga et al. 2004). Osteoclast-associated receptor (OSCAR) and triggering receptor expressed in myeloid cells 2 (TREM2) are receptors that are associated with the respective adaptor molecules Fc receptor common gamma chain (FcRγ) and DNAX-activating protein 12 kDa (DAP12), respectively. Downstream signaling pathways culminate in the increased expression of transcription factors c-fos and nuclear factor of activated T cells c1 (NFATc1), both master regulators of osteoclastogenesis. Moreover, microphthalmia-associated transcription factor (MITF) and PU.1 can modulate osteoclastogenesis (Sharma et al. 2007). Also relevant are genes that regulate cell fusion, that is, dendritic cell-specific transmembrane protein (DC-STAMP) and the ATPase, H⁺ transporting, lysosomal 38 kDa, and V0 subunit d2 (Atp6v0d2) (Lee et al. 2006; Miyamoto et al.

2012). This study determined the expression levels of the respective genes to understand the role of BCM during osteoclastogenesis *in vitro*.

TGF-β is a heat-stable molecule present in BCM (Caballé-Serrano et al. 2014; Peng et al. 2014) and in conditioned medium from demineralized bone (DCM) (Schuldt Filho et al. 2015) that can considerably increase osteoclastogenesis in bone marrow cultures (Fuller et al. 2000). Thus, current evidence supports the idea that BCM can enhance osteoclastogenesis via a TGF-β-dependent mechanism but not involving c-fos (Fox et al. 2008). Proteomic analysis of BCM (Caballé-Serrano et al. 2014) further revealed other molecules that can stimulate osteoclastogenesis, such as annexin A2 (Menaa et al. 1999) – but maybe more interestingly, also molecules that inhibit osteoclastogenesis, such as apolipoprotein E (Kim et al. 2013), lactotransferrin (Lorget et al. 2002), macrophage migration inhibitory factor (Jacquin et al. 2009), and angiogenin (Morita et al. 2008). Thus, it remains unclear whether the pro-osteoclastogenic or the anti-osteoclastogenic molecules dominate in BCM. The bioassay presented integrates the overall impact of BCM and DCM on the complex process of osteoclastogenesis *in vitro*.

Material and methods

Bone-conditioned medium

Bone-conditioned medium (BCM) was prepared from porcine fresh cortical bone chips as described recently (Caballé-Serrano et al. 2014; Kuchler et al. 2014; Peng et al. 2014). In brief, bone was obtained from adult pigs within 6 h postmortem (Metzger Meinen, Bern, Switzerland). Cortical bone chips were harvested from the mandible and placed into sterile plastic dishes containing DMEM supplemented with antibiotics and antimycotics at 50% (wet weight/volume) (all Life Technologies, Grand Island, NY, USA). BCM was collected after 24 h, filtered sterile, and kept frozen at −20°C. The stocks were thawed immediately before the experiments. BCM was also heated at 85°C for 10 min. If not otherwise indicated, BCM was fourfold. Variations in BCM activity can occur due to various factors, such as techniques used for harvesting, individual characteristics of the animals, and handling of the samples.

Demineralized bone matrix-conditioned medium

Demineralized bone matrix-conditioned medium (DCM) was prepared from porcine cortical bone blocks. Bone blocks were cut from

the buccal side of the porcine mandibles and trimmed to obtain pieces measuring 3 mm in thickness and 12 mm in length. Bone blocks were subjected to defatting and demineralization with 0.5 M HCL at 4°C for 24 h. Defatting was performed twice with acetone and methanol (1 : 1). Residual acid was neutralized by repeatedly washing the bone blocks with demineralized water for 30 min. Following elimination of residual acid, samples were freeze-dried and ground. After 24 h of incubating the demineralized bone matrix with serum-free DMEM containing antibiotics and antimycotics, conditioned medium was filtered sterile and kept frozen at −20°C. Variations on the DCM activity can occur due to various factors, such as processing of the bone.

Osteoclastogenesis in murine bone marrow cultures

Bone marrow cells were prepared by flushing the femur and tibiae of 4- to 6-week-old female mice (strain Balb/c) and seeded at one million bone marrow cells per cm² in Eagle's minimum essential medium-alpha modification (αMEM) supplemented with 10% fetal calf serum (FCS), antibiotics, and macrophage colony-stimulating factor (M-CSF) at 30 ng/ml for 48 h. For osteoclastogenesis, medium was supplemented with M-CSF at 30 ng/ml and soluble receptor activator of nuclear factor kappa-B ligand (RANKL) at 30 ng/ml for 3 days. BCM was used at a fourfold dilution as recently reported (Peng et al. 2014). Recombinant proteins were purchased from Prospec (Ness-Ziona, Israel). SB431542 was used at 10 μM (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After 4 days, histochemical staining for TRAP (Sigma Aldrich, St. Louis, MO, USA) was performed. Cells were considered osteoclasts when positive for TRAP and having three or more nuclei as observed by light microscopy. For the resorption assay, bone marrow cultures were performed on dentin disks, which were cleaned with ultrasound and stained with toluidine blue (1%) to show resorption pits.

Expression of marker genes in murine bone marrow cultures

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science, Rotkreuz, Switzerland). Reverse transcription (RT) was performed with Transcriptor Universal cDNA Master, and PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) or the FastStart Universal Probe Master Rox on a 7500 Real-Time PCR System (Roche).

Table 1. Primers for RT-PCR

Gene	Forward primer	Reverse primer
m c-fms	gaccatggtgaatggtaggg	ggataacgttgaatcccactg
m c-fos	gcaactttctatgacactgaaacac	tctctctagggctgcattgg
m ACTA2	ctctcttccagccatctttcat	tataggtggtttcgtggatgc
m Atp6Od2	aagcctttgtttgacgctgt	gccagcacattcatctgtacc
m BUB1	tcctgtaagtggccagtcatt	tgaattcatgaacactttgattcac
m CTGF	tgacctggaggaaaacattaaga	agccctgtatgtcttcacactg
m DC-Stamp	aagctccttgagaaacgatca	caggactggaaaccagaaatg
m IL11	ctgtgggacatgaactgtg	aggggcaacgactctatctg
m MIKI67	gctgtcctcaagacaatcatca	ggcgttatcccaggagact
m MITF	gacaccagccataaacgtca	ttttccaggtgggtctgc
m MYBL2	gcccataaagtcctgggtaac	cacagcattgtccgtcctc
m NFATc-1	ccgttgcttccagaaaataaca	tgtgggatgtgaactcggaa
m PLK1	ttgtagttttggagctctgtcg	cagtgccttcctcctcttgt
m PU.1	ggagaagctgatggcttgg	caggcgaatcttttttcttgc
m RANK	gtgctgctgttccactg	agatgctcataatgcctctcct
m TRAF6	ttgcacattcagtgtttttgg	tgcaagtgtcgtgccaag
m β-actin	ctaaggccaaccgtgaaaag	accagaggcatacagggaca

Probes for CTR, TRAP, CatK, OSCAR, TREM2, FcRγ, DAP12 and β-actin were obtained from TaqMan Gene Expression Assays service (Applied Biosystems). All other primers were designed with the online Universal Probe Library System (Table 1). The mRNA levels were calculated by normalizing to the housekeeping gene beta-actin using the ΔCt method.

Western blot analysis

For Western blot analysis, RAW 264.7 cells, kindly provided by Jürg Gertsch (Institute of Biochemistry and Molecular Medicine, University of Bern), were serum-starved overnight and stimulated with fourfold BCM for 30 min. Cells were lysed in SDS buffer containing protease and phosphatase inhibitors (Roche). Cell extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking, binding of the first antibody raised against phospho Smad3, phospho JNK, phospho p38 MAPK (all Cell Signaling Technology, Danvers, MA, USA), phospho ERK, and β-actin (both Santa Cruz Biotechnology, SCBT) was detected with the appropriate secondary antibody (LI-COR Biosciences; Lincoln, NE, USA), directly labeled with near-infrared dyes and visualized with the appropriate imaging system (LI-COR).

BCM incubation with blood fresh-frozen plasma

In indicated experiments, BCM was incubated with human fresh-frozen plasma (Österreichisches Rotes Kreuz, Austria) at 1 : 1. Coagulation was activated by adding 5 U/ml thrombin from bovine plasma (Sigma, St Louis, MO, USA). After 12 h of incubation at 37°C, samples were centrifuged and supernatant was immediately used in bone marrow cultures at a twofold dilution.

Cell viability and proliferation

For cell viability, bone marrow cells were exposed to BCMh for 24 h and staining was performed (Live-Dead cell staining kit; Enzo Life Sciences AG, Lausen, Switzerland) where green cells are alive and red cells are dead. To further understand the impact of BCMh

on proliferation, expression analysis of relevant genes such as myb-related protein B (MYBL2), serine/threonine protein kinases BUB1 (budding uninhibited by benzimidazoles 1), polo-like kinase 1 (PLK-1), and antigen identified by monoclonal antibody Ki-67 (MKI67) was investigated.

Statistical analysis

Data are reported using dot plots showing all data points and the median. Experiments were repeated at least four times in duplicates. Paired t-test was used to analyze RT-PCR data of the controls vs the stimulated groups. T-test was calculated with EXEL and P-values indicated in the figures. Figure 1 was based on post-ANOVA comparisons with the Tukey's HSD test (SPSS Software, IBM, NY, USA). In Supplement figures, no statistical analysis was used due to a small sample size.

Results

BCMh supports RANKL-induced osteoclastogenesis *in vitro*

To investigate the impact of BCM on osteoclastogenesis, the number of TRAP⁺ MNCs in the presence of RANKL and M-CSF was deter-
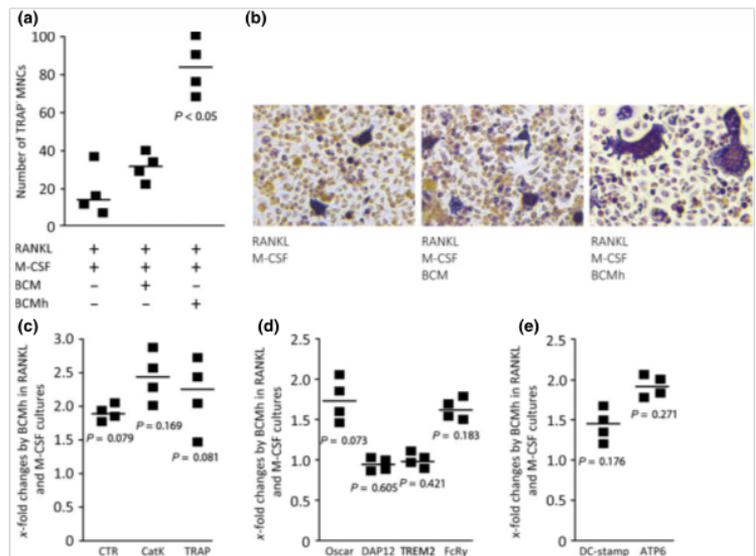
Fig. 1. BCMh supports RANKL-induced osteoclastogenesis *in vitro*. Murine bone marrow cultures were grown in the presence of RANKL and M-CSF supplemented with BCM and BCMh at a fourfold dilution. (a, b) The number and size of TRAP⁺ MNCs increased significantly in the presence of BCMh. Cells counted positive had three or more nuclei. (c) Expression of osteoclast differentiation markers CTR, CatK, and TRAP approximately doubled in the presence of BCMh. (d) BCMh also increased expression of osteoclast costimulatory molecules OSCAR and FcRγ, with the costimulatory molecules DAP12 and TREM2 remaining unchanged. (e) Expression of osteoclast fusion gene DC-Stamp was increased. $P < 0.05$ compared to the two other groups.

mined. As shown in Fig. 1a,b, BCM failed to cause any visible changes in osteoclastogenesis. As TGF-β can enhance osteoclastogenesis (Fuller et al. 2000), TGF-β is found in BCM (Peng et al. 2014), and TGF-β can be activated by heat (Brown et al. 1990), BCM was exposed to 85°C for 10 min. The presence of BCMh increased the number of TRAP⁺ MNCs in the presence of RANKL and M-CSF (Figs 1a,b and S1), but not in the presence of fresh-frozen plasma (Fig. S2). There was also a trend that BCMh increased the expression of osteoclast markers and fusion genes (Fig. 1c,d). A resorption assay performed on dentin disks confirmed the activity of osteoclasts, but no quantification was performed because of the large variations within and between the experiments (Fig. S3). It is noteworthy to mention that most but not all preparations of BCMh supported osteoclastogenesis (data not shown). Together, these findings show that BCMh moderately enhances osteoclastogenesis *in vitro*.

BCMh increases osteoclast signaling molecule expression

To determine whether the effect of BCMh involves increased responsiveness of cells to RANKL or M-CSF, the expression levels of the respective receptors RANK and c-fms were determined. BCMh increased expression of RANK by around twofold but did not increase expression of c-fms (Fig. 2a). There was also a trend that BCMh increased expression of TRAF6 and the respective downstream master regulators of osteoclastogenesis, NFATc1, and c-fos (Fig. 2b). Expression of PU.1 and MITF was not changed considerably (Fig. 2b). Taken together, BCMh moderately enhanced the expression of some but not all osteoclast signaling molecules.

BCMh-induced c-fos expression requires TGF-β signaling

In line with a potential role of TGF-β in mediating the effect of BCMh on osteoclastogenesis, blocking of TGF-βR1 kinase with SB431542 decreased c-fos expression (Fig. 3a). TRAP staining showed the suppression of osteoclastogenesis by SB431542 (Fig. 3b). Further support for a potential role of TGF-β signaling in the bone marrow macrophages comes from observations that BCMh increased the respective target genes IL11, ACTA2, and CTGF in an SB431542-dependent manner (Fig. 4a) and that BCMh slightly stimulated phosphorylation of SMAD3 and p38 in a macrophage cell line (Fig. 4b). Together, these data support the assumption that BCMh mediates at least part of its activity via TGF-β signaling.

DCM supports RANKL-induced osteoclastogenesis *in vitro*

To determine whether DCM, previously prepared by acid demineralization, which possibly activates TGF-β (Schuldt Filho et al. 2015), had an effect on osteoclastogenesis, the number of nuclei per TRAP⁺ MNCs in the presence of RANKL and M-CSF was determined (Fig. 5a,b). DCM increased expression of osteoclast differentiation markers CTR, CatK, and TRAP (Fig. 5c). Expression levels of cell fusion markers DC-Stamp and Atp6v0d2 were also significantly enhanced (Fig. 5d). These findings are in line with the basic concept that acid-activated TGF-β supports osteoclastogenesis in the presence of RANKL and M-CSF.

BCMh does not affect cell viability and proliferation

To assess whether BCM could affect cell viability, live–dead staining of cultures supplemented with BCM and genetic expression analysis of cell-cycle-related genes was executed. Live–dead staining, where green indicates viable cells and red indicates dead cells, showed no differences between the groups with and without BCMh (Fig. S4A). To determine whether BCMh affected proliferation of bone marrow cultures, expression analysis of the cell-cycle-related genes MYBL2, BUB1, PLK1, and MIKI67 was performed. Expression of cell-cycle-related genes was not affected by BCM (Fig. S4B). Therefore, cells remain viable but are not forced to expand when exposed to BCMh.
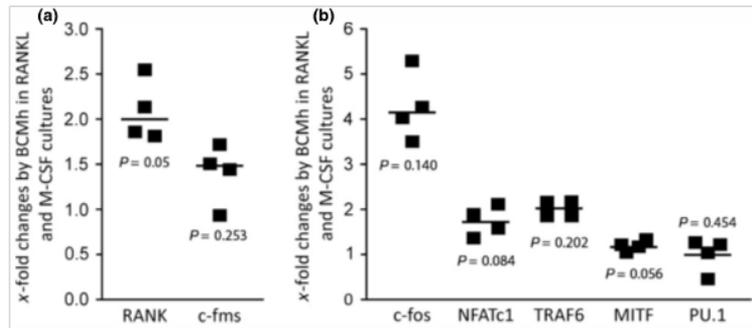
Fig. 2. BCMh increased expression of RANK, signaling molecules, and activity of osteoclasts. To analyze whether the effect of BCMh on bone marrow macrophages was due to increased responsiveness to RANKL or M-CSF, expression of RANK and c-fms receptors was determined. (a) Expression of the RANK receptor was notably increased in the presence of BCMh compared to cultures with RANKL and M-CSF (RM) alone. (b) Expression of TRAF6 and the respective downstream master regulators of osteoclastogenesis, c-fos and NFATc1, was enhanced in the presence of BCMh. Expression of RANK regulators PU.1 and MITF was unchanged.

Fig. 3. SB431542 partially blocks the impact of BCMh on c-fos expression. To understand how BCMh exerts its effect on osteoclastogenesis, the TGF-βR1 antagonist (SB431542) was used. (a) SB431542 reduced but did not block the expression of c-fos. (b) Addition of SB431542 to cultures with RM and BCMh abolished the formation of TRAP⁺ MNCs. TRAP staining is representative for two experiments with two replicates.

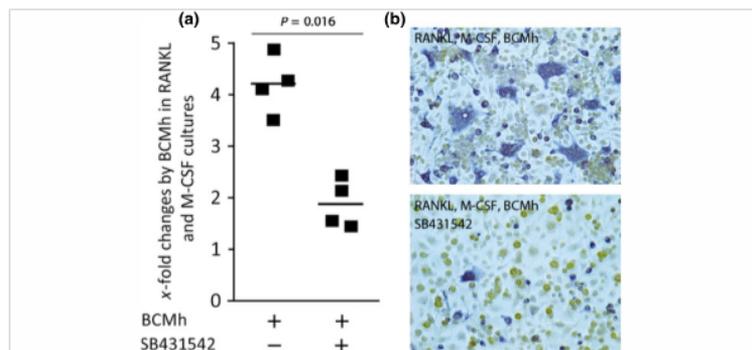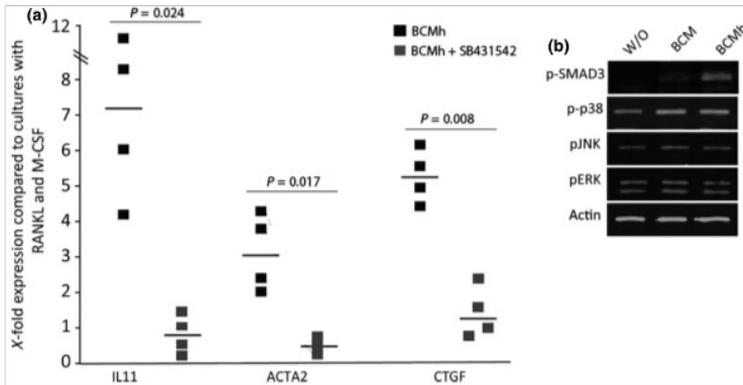Fig. 4. BCMh does not affect cell viability and proliferation. (a) BCMh increased the expression of respective target genes IL11, ACTA2, and CTGF in bone marrow cultures, which were blocked by the inhibitor for TGF-βR1 kinase, SB431542. (b) BCMh stimulated phosphorylation of SMAD3 and p38 in the macrophage cell line RAW 264.7. Western blot data are representative for three experiments with one replicate.
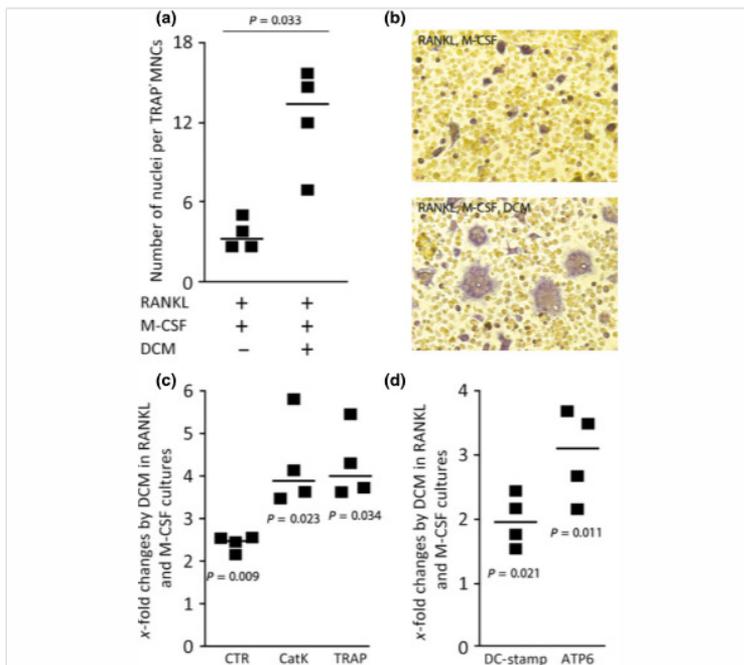

Fig. 5. DCM supports RANKL-induced osteoclastogenesis in vitro. Murine bone marrow cultures were grown in the presence of RANKL and M-CSF supplemented with DCM at a fourfold dilution. (a, b) The number of nuclei per TRAP⁺ MNCs was increased significantly in the presence of DCM. (c, d) Expression of osteoclast differentiation markers CTR, CatK, and TRAP was increased up to fivefold in the presence of DCM. DCM also increased expression of fusion genes DC-Stamp and Atp6v0d2.

Discussion

Resorption of autografts and allografts remains a major challenge in implant den-tistry and other fields dealing with bone reconstruction. Osteoclasts have been detected early, within days, on the surface of transplanted bone (Saulacic et al. 2014). The

underlying molecular mechanism, however, remains unknown. There is reason to suggest that soluble factors released from trans-planted bone support osteoclastogenesis, par-ticularly because BCM and DCM contain TGF-β (Peng et al. 2014; Schuldt Filho et al. 2015), and TGF-β supports in vitro osteoclas-togenesis (Fuller et al. 2000). The results of the present work generally support this notion, but with one premise: BCM had to be exposed to 85°C to enhance osteoclastogene-sis. On the basis of these findings, it can be concluded that in vitro, soluble factors released from bone chips can enhance osteo-clastogenesis.

If we relate our findings to those of other authors, BCM greatly increased IL11 expres-sion in mesenchymal cells, which was inhib-ited by blocking TGF-β signaling (Peng et al. 2014). Also in the present study, BCMh increased IL11 and other TGF-β target genes in bone marrow cultures, again requiring TGF-β signaling. Increased phosphorylation of the TGF-β signaling molecules Smad3 and p38 was also observed in the present study (Cicek et al. 2011; Lou et al. 2013; Peng et al. 2014). Further support for TGF-β signaling comes from observations that SB431542 reduced BCMh-induced c-fos expression (Wy-grecka et al. 2012). Also consistent with the present hypothesis, TGF-β is secreted in a latent form and needs to be activated (Miyaz-ono et al. 1988; Barcellos-Hoff 1996; Koli et al. 2001; Hyytiainen et al. 2004), for exam-ple by heat or low pH (Brown et al. 1990). What was surprising, however, was that TGF-β signaling in mesenchymal cells was achieved without heating BCM (Peng et al. 2014). Also, the effects of recombinant TGF-β are usually stronger than those observed with BCMh (Fuller et al. 2000). Another explana-tion for the enhanced osteoclastogenesis could be the increased responsiveness to RANKL, as BCMh considerably increased expression of RANK. Thus, the effect of BCM on osteoclastogenesis is likely more complex than simply blaming it on TGF-β.

The clinical relevance of the present find-ings can be related to the long-recognized need to understand the mechanisms of bone graft resorption. This information can be used to advance current clinical concepts to predict the outcome of bone augmentation. Early resorption is not restricted to bone grafts, however (Saulacic et al. 2014). Early expression of RANKL (Kon et al. 2001) and inflammatory cytokines such as TNF-α (Ger-stenfeld et al. 2003), both involved in osteocl-astogenesis, is fundamental for fracture healing in mice. Osteoclastogenesis is also a

Caballé-Serrano et al·Bone-conditioned medium increases osteoclastogenesis

hallmark of the early phase following tooth extraction (Cardaropoli et al. 2003) and implant insertion (Vasak et al. 2013). Overall, osteoclastogenesis and bone resorption precede osteoclastogenesis and bone formation – which is a general biological principle in bone regeneration. Whether the function of particulated bone, exemplified by the rather weak *in vitro* impact of BCMh and DCM on osteoclastogenesis, explains the rather strong *in vivo* osteoclastogenesis remains to be determined.

Many questions remain to be answered. First, it is unclear whether the stimulation of osteoclastogenesis by BCMh is due to heat-labile inhibitors or heat-activated stimulators of osteoclastogenesis, or a mixture of both. Second, we cannot simulate *in vitro*, if and how BCM will be activated *in vivo*, considering that even if unheated, BCM has potent effects on mesenchymal cells (Peng et al. 2014). Our attempt to use fresh-frozen plasma to simulate the proteases of blood clots, and thereby activate BCM, failed because of the strong inhibition of osteoclastogenesis (Agis et al. 2013). Third, it remains unclear whether BCMh and DCM use the same mechanisms to stimulate osteoclastogenesis. Fourth, it is still uncertain whether the changes in c-fos caused by BCMh, the partial implication of TGF-β signaling, and

the phosphorylation of Smad3 and other kinases have implications for osteoclastogenesis. Fifth, it is surprising that BCM changes the expression of osteoclast genes to almost the same extent that BCMh does, but only the latter preparation visibly supports osteoclastogenesis after histochemical staining of TRAP. Finally, BCM mainly contains proteins released because of mechanical deterioration of the bone matrix and from the remaining viable cells, including osteocytes (Brolese et al. 2014; Caballé-Serrano et al. 2014) – but not selectively those being actively released by osteoclasts during bone resorption (Crane & Cao 2014). Many questions remain unanswered. Nonetheless, the present data provide a scientific basis for more in-depth research in the field of bone regeneration – and specifically, in graft resorption.

Future studies should determine the role of osteocytes as a source of paracrine factors and their role in osteoclastogenesis during bone regeneration in general and in particular the resorption of autologous bone grafts. Osteocytes are important because their apoptosis initiates osteoclastogenesis in mouse models (Tatsumi et al. 2007) and because they also control this process during normal bone remodeling, even being responsible for the adaptation of bone to loading (Nakashima

et al. 2011; Xiong et al. 2011). Nevertheless, we have no direct evidence that osteocytes contribute to the activity of BCMh in osteoclastogenesis; exploring this remains a central aim of future studies. Furthermore, a genome-wide microarray of hematopoietic cells subjected to BCM would possibly provide valuable clues to a deeper understanding of the paracrine function of particulated autologous or demineralized and freeze-dried allogenic bone at regeneration sites.

In conclusion, this pioneer study presents preliminary data on how soluble factors released from bone grafts affect osteoclastogenesis. This information likely serves as one piece of a puzzle allowing us to understand the complex process of graft resorption within the overall sequential process of graft consolidation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Independent preparations of BCMh, with equally enhanced osteoclastogenesis shown by purple TRAP staining, compared to cultures with RANKL and M-CSF alone.

Figure S2. BCM incubated with fresh frozen plasma (FFP) failed to reverse negative effects of blood plasma on osteoclastogenesis.

Figure S3. Activity of osteoclasts was assessed by resorption of dentin discs, indicating that also isolated osteoclasts exposed to BCMh were capable of resorbing bone. No attempts were made to quantify dentine resorption.

Figure S4. BCMh does not affect cell viability and proliferation.

Paper IV

Conditioned Medium from Fresh and Demineralized Bone Enhances Osteoclastogenesis in Murine Bone Marrow Cultures

Journal of Visualized Experiments, 2015

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Bone Conditioned Medium: Preparation and Bioassay

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Abstract

Autologous bone grafts are widely used in oral and maxillofacial surgery, orthopedics, and traumatology. Autologous bone grafts not only replace missing bone, they also support the complex process of bone regeneration. This favorable behavior of autografts is attributed to the three characteristics: osteoconductivity, osteogenicity, and osteoinductivity. However, there is another aspect: Bone grafts release a myriad of molecules, including growth factors, which can target mesenchymal cells involved in bone regeneration. The paracrine properties of bone grafts can be studied *in vitro* by the use of bone-conditioned medium (BCM). Here we present a protocol on how to prepare bone-conditioned medium from native pig cortical bone, and bone that underwent thermal processing or demineralization. Cells can be directly exposed to BCM or seeded onto biomaterials, such as collagen membranes, previously soaked with BCM. We give examples for *in vitro* bioassays with mesenchymal cells on the expression of TGF-β regulated genes. The presented protocols should encourage to further reveal the paracrine effects of bone grafts during bone regeneration and open a path for translational research in the broad field of reconstructive surgery.

Video Link

The video component of this article can be found at http://www.jove.com/video/52707/

Introduction

Autologous bone is widely used to bridge defects that occurred as a consequence of malformation, resective surgery, reconstructive trauma surgery, and prior to implant placement [1,2]. Understanding the biological principles of how bone grafts support the process of graft consolidation is not only key to understand why autografts are considered to be the gold standard in reconstructive surgery, it is also bionic to the improved design of bone substitutes [3]. Still, graft consolidation is faster with autologous bone compared to bone substitutes [4,5]. Thus, it is imperative to reveal the molecular and cellular mechanisms that make autologous bone so effective to support bone regeneration.

There are three textbook characteristics of autografts that are considered to support the consolidation process [6,7]. First, autologous bone is osteoconductive, providing guidance for the newly formed bone to grow into the defect. Secondly, autologous bone is osteogenic, meaning that it contains mesenchymal cells that can differentiate into osteoblasts [8]. Third, autologous bone is osteoinductive as growth factors like bone morphogenetic proteins entombed in the matrix can initiate the process of endochondral or even intramembranous bone formation [9]. There is another aspect: freshly prepared bone chips hold a paracrine function based on the *in vitro* observations with "bone-conditioned medium" [10-15]. Also the impact of myelopoiesis should be mentioned [16]. A similar term "demineralized bone matrix-conditioned medium" was already coined in 1996 and supports the overall concept of a paracrine function of bone, even when processed by demineralization [17]. For our purposes, BCM can be prepared from fresh pig mandibles [10,11]. Proteomic analyses of BCM revealed the complex composition, including growth factors and constituents of the extracellular matrix [10], also extending existing knowledge on the proteasome of whole bone [18,19]. Thus, BCM should reflect the released activity of various modifications of bone grafts *in vitro*.

What happens when mesenchymal cells, for example those isolated from bone chips or from oral soft tissue, are exposed to BCM? *In vitro*, BCM reduces osteogenic and adipogenic differentiation, and provokes a strong increase of IL11 expression [11]. Genome wide microarray revealed more genes to be differentially expressed in mesenchymal cells in response to BCM. Among these genes are adrenomedullin (ADM), IL11, IL33, NADPH oxidase 4 (NOX4), proteoglycan 4 (PRG4, or lubricin) and pentraxin 3 (PTX3) [15]. BCM obtained from autoclaved bone chips failed to change the expression of the respective genes [14]. BCM from bone chips that underwent pasteurization and freezing was able to change gene expression [14]. Also conditioned medium of demineralized bone matrix (DBM-CM) changes the expression of TGF-β-regulated genes [20]. Interestingly, collagen barrier membranes used to shield the bone chips from the surrounding soft tissue [21,22], adsorbed those parts of BCM that are responsible for the changes in gene expression [23]. BCM research can be extended to other cell types involved in bone regeneration such as

bone-resorbing osteoclasts and endothelial cells, to name a few. Overall, the accumulating *in vitro* data provide the scientific basis for the design of a preclinical study.

The present protocol is two-fold: First, it shows how to prepare BCM. Secondly, it shows how to test its biological activity based on mesenchymal cells *in vitro*.

Protocol

1. BCM Preparation

1. Obtain pig mandibles from the local butcher as fresh as possible. Place the mandibles onto a firm surface and release a full thickness flap paying special attention not to leave any soft tissue or periosteum attached to the bone. Work in a clean environment without the need to work under the flow hood.
2. Once a full thickness flap is released, use a bone scraper to harvest the bone chips from the buccal side. Please note that the bone scraper has to be sharp. Handle firmly the bone scraper and with long movements collect the bone. Discard bone chips smaller that 1mm.
   1. To maintain native bone chips, place directly the bone chips in plastic dishes of 10cm diameter with Dulbecco's Modified Eagle's medium (DMEM) supplemented with 1% antibiotics and antimycotics not letting them to dry out.
   2. To evaluate the impact of thermal processing, subject bone chips to pasteurization for 30 min at 80 °C or autoclave for 20 min at 121 °C.
   3. To evaluate the impact of demineralization, shake bone chips in 1 M HCL for 4-6 hr at 4 °C and wash repeatedly with culture medium until the pH is neutral.
3. Place a total of 5 g of bone chips per 10 ml fresh DMEM supplemented with 1% antibiotics and antimycotics into a new plastic dish.
4. Place the plastic dishes in a humidified atmosphere at 37 °C for 24 hr. Then, harvest BCM. Centrifuge the BCM at 200 x g for 10 min to remove debris, filter it sterile (0.2 nm), and keep aliquots frozen at -80 °C.
5. Thaw the BCM stock immediately before use and avoid repeated cycles of freezing and thawing.
6. For indicated experiments, soak collagen membranes with BCM or serum-free medium for 1 hr at room temperature (RT). Wash vigorously the membranes with PBS and place them into 96 well plates. Wet membranes are seeded with cells.
7. BCM preparation process is summarized in **Figure 1**.

2. Bioassays Based on Mesenchymal Cells

1. Seed human mesenchymal cells (for example bone cells, gingival and periodontal ligament fibroblasts) into a 12-well plate with a concentration of 30,000 cells/cm^2. To seed the cells use growth medium consisting of DMEM, 10% fetal calf serum and antibiotics. Let the cells attach to the plate over night.
2. Discard the culture medium and wash the cells with pre-warmed PBS at 37 °C. Stimulate the cells by adding pre-warmed serum-free culture medium with and without 20% BCM. Place the cells in a humidified atmosphere at 37 °C for 24 hr.
3. Discard the culture medium, rinse the cells with pre-warmed PBS and extract the RNA according to your preferred protocol.
4. Adjust the concentration of RNA in order to have the same amount of RNA in each sample. Prepare cDNAs and perform a qRT-PCR to analyse the selected genes using the primers shown in **Table 1**.
   NOTE: These are the dilutions of every component: 2x SYBR Green, 20x primer forward, 20x primer reverse, 5x sterile DD water, 5x cDNA. The qRT-PCR is performed in 40 cycles of 95 °C 15 sec and 60 °C 1 min.
5. Calculate the relative expression levels by normalizing to the housekeeping gene GAPDH using the Δ(ΔCt) method where ΔCT is CT target - CT GAPDH and Δ(ΔCT) is ΔCT stimulated - ΔCT control.
6. After this quality control, add BCM to culture medium to stimulate all types of cells including mesenchymal cells, hematopoietic cells or endothelial cells.

Representative Results

Bone Conditioned Medium is prepared from fresh porcine bone chips. General overview of the process to prepare BCM and to use biomaterials in combination with BCM is shown in **Figure 1** and **Figure 2** respectively. During the BCM preparation, it is important to obtain large bone chips with long movements as short movements or very small bone chips can affect the quality of the final BCM. Quality of BCM can be controlled by analyzing the gene expression of BCM target genes: ADM, PTX3, IL11, IL33, NOX4 and PRG4 (**Figure 3**). ADM and PTX3 are down-regulated down to 0.4-fold and IL11, IL33, NOX4 and PRG4 can be up-regulated to 200-fold. If oral fibroblasts do not express BCM target genes at the level shown, check the health of the cells or prepare new BCM from new mandibles. **Figure 4** displays typical results from the expression of BCM target genes in oral fibroblasts seeded onto a collagen barrier membrane. Oral fibroblasts stimulated with 20% of conditioned medium from pasteurized bone chips and conditioned medium from demineralized bone chips, showed similar gene expression to cells stimulated with BCM (**Table 2** and **Table 3**). However, gene expression of oral fibroblasts exposed to conditioned medium from sterilized (121 °C) bone chips, was comparable to un-stimulated controls.
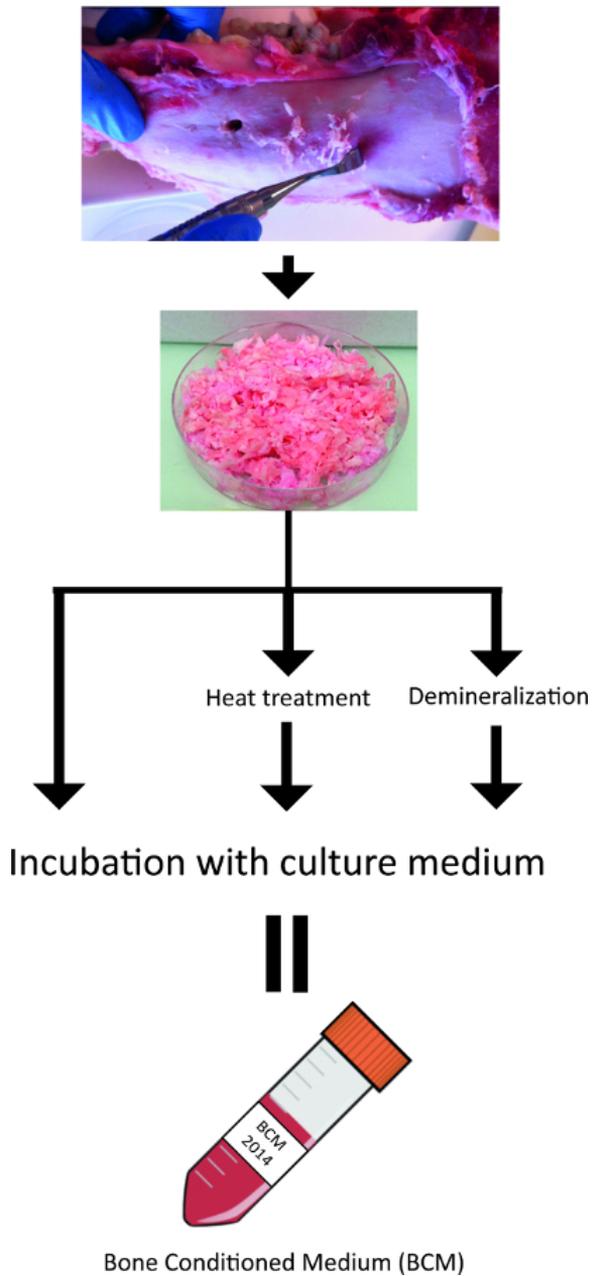
Figure 1: Summary of the process used to prepare bone-conditioned medium from fresh pig mandibles.
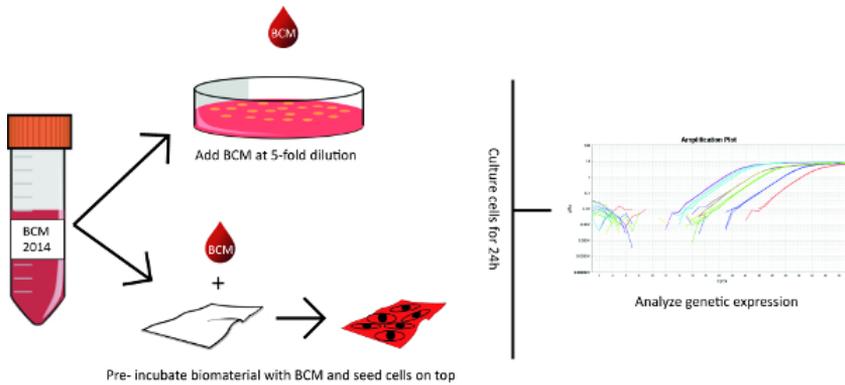
Figure 2: Summary of the bioassays based on mesenchymal cells with BCM.

Figure 3: Gene expression of bone-conditioned medium target genes in oral fibroblasts. Typical results of six genes used to control the quality of BCM. Genes ADM and PTX3 are downregulated (**A**) and IL11, IL33, NOX4, PRG4 are upregulated (**B**).
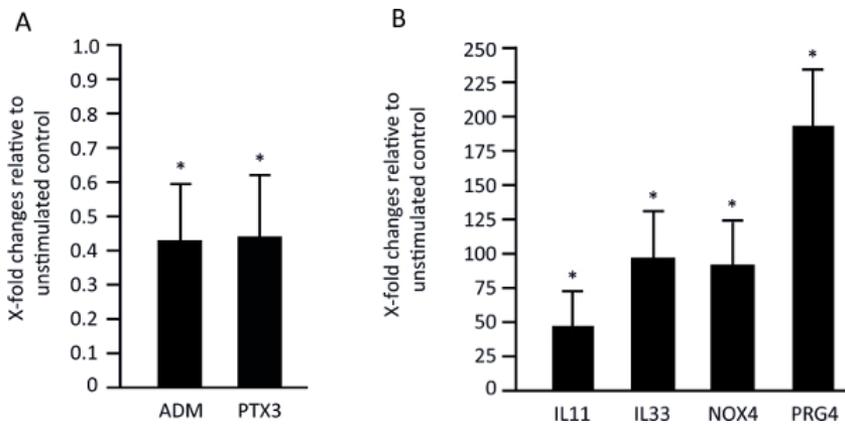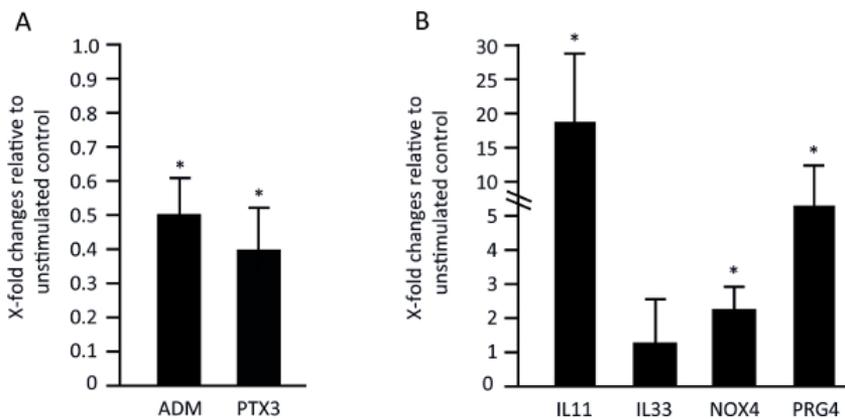

Figure 4: Gene expression of bone-conditioned medium target genes in oral fibroblasts seeded onto a collagen barrier membrane.

Abbreviation	Primer forward	Primer reverse
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
ADM	GGACATGAAGGGTGCCTCTC	TGTTCATGCTCTGGCGGTAG
IL11	TGCACCTGACACTTGACTGG	AGTCTTCAGCAGCAGCAGTC
IL33	TCAGGTGACGGTGTTGATGG	GGAGCTCCACAGAGTGTTCC
NOX4	TCTTGGCTTACCTCCGAGGA	CTCCTGGTTCTCCTGCTTGG
PRG4	CGACGCCCAATGTAAGAAGT	GGTGATGTGGGATTATGCACT
PTX3	TGTATGTGAATTTGGACAACGAA	CATTCCGAGTGCTCCTGAC

Table 1: Primer sequence of the 6 genes used.

Genes	80 °C Mean ± SD	121 °C Mean ± SD
ADM	0.2 ± 0.1	1.1 ± 0.2
PTX3	0.1 ± 0.1	0.9 ± 0.2
IL11	20 ± 10	1.5 ± 1
IL33	15 ± 5	1.2 ± 4
NOX4	35 ± 15	2 ± 1
PRG4	40 ± 10	1.8 ± 1

Table 2: Typical gene expression of ADM, PTX3, IL11, IL33, NOX4 and PRG4 in oral fibroblasts stimulated with 20% of conditioned medium from heat-treated bone chips.

Genes	Mean ± SD
ADM	0.1 ± 0.1
PTX3	0.1 ± 0.1
IL11	15 ± 5
IL33	20 ± 10
NOX4	60 ± 15
PRG4	50 ± 20

Table 3: Typical gene expression of ADM, PTX3, IL11, IL33, NOX4 and PRG4 in oral fibroblasts stimulated with 20% of conditioned medium from demineralized bone chips.

Discussion

Bone-conditioned medium reflects the released activity of bone grafts during the early stages of bone regeneration. The protocol described here can be adapted to study the response of different types of cells involved in bone regeneration. Furthermore, the protocol can be used to prepare conditioned medium from processed bone or bone fillers. The methods are easy to perform and rely on a simple concept: the factors released from various native and processed bone. Understanding how BCM affects mesenchymal cells can help to learn more about graft consolidation and properties of bone autografts. Based on this concept we have accumulated knowledge on the impact of BCM obtained from native [11,15] and processed bone [14,20] on gene expression of mesenchymal cells, but also on proliferation, migration, and differentiation into the three main lineages; osteoblasts, adipocytes and chondrocytes [11]. BCM was also examined for its capacity to target hematopoietic cells, for example with respect to the modulation of osteoclastogenesis [13]. Many potential target cells are waiting to respond to BCM *in vitro*, the protocols presented here, can serve as a primer for this research.

The presented protocols should also animate to further reveal the molecular mechanisms of how BCM activates particularly TGF-β-regulated genes in mesenchymal cells. For example, the TGF-β receptor I antagonist SB431542 blocked the effect of BCM on the expression of the gene panel ADM, IL-11, NOX4, PRG4, and PTX3 [11,15]. Interestingly, alkaline phosphatase and IL33 were not reversed by SB431542 [11,15] suggesting that other as yet unknown pathways are regulated by BCM. Another open question is what are the molecules in BCM being responsible for the cellular response? BCM contains TGF-β but is does not explain the complex cellular reactions [10,11]. Besides the in vitro cellular aspects, the overall question remains: to which extend does the released activity of bone grafts as reflected by BCM, have an impact on the in vivo process of bone regeneration? The protocols and data from bioassays should introduce research in this direction.

This protocol has limitations. BCM cannot be fully standardized because of variations between donors and harvesting techniques. Moreover, how enzymes present in vivo can affect the composition or activity of BCM remains unknown. Future studies should, for example, focus on how harvesting techniques affect the "biological activity" of BCM. The role of osteocytes on the composition of BCM should also be studied in detail. BCM contains sclerostin, a molecule released almost exclusively by osteocytes [12]. Limitations, however, provide the inspiration for the next steps in research. Even though the clinical relevance of research with BCM remains hypothetical, our protocols support the long-standing concept that

bone grafts, either native or after processing, release a "biological activity". Understanding how BCM affect cells in vitro can presumably help to understand how bone autografts will work *in vivo*.

Disclosures

Acknowledgements

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Annex I - Summary of the PhD thesis in Catalan

Resum

La present tesis doctoral és un compendi de quatre publicacions ampliant el coneixement de l'osteoclastogènesis en les regeneracions òssies, més especialment sobre els efectes de la saliva i el medi condicionat ossi en l'osteoclastogènesis. La reabsorció dels empelts ossis i de l'os de l'hoste, pot ser un repte especialment quan un defecte ossi ha de ser regenerat en condicions desfavorables o grans atròfies com per exemple després de traumatismes, diverses patologies, edat avançada o extraccions múltiples. En la cavitat oral, la saliva pot entrar en contacte amb superfícies mineralitzades, tot i això la relació entre saliva i reabsorció òssia és encara desconeguda. En la present tesis hem examinat si la saliva afecta el procés de l'osteoclastogènesis in vitro, possiblement afectant a la regeneració i cicatrització òssia. La regeneració òssia és un procés comú en traumatologia, periodòncia, cirurgia oral i maxil·lofacial que involucra l'ús de substituts ossis. Els empelts d'os autòleg són considerats l'estàndard d'or dels substituts ossis degut a la seva trinitat de propietats: osteoconductivitat, osteoinducció i osteogènesis. Els factors paracrins alliberats pels empelts d'os autòleg podrien contribuir en el conjunt de processos que donen com a resultat la consolidació del empelts, tanmateix els mecanismes que regeixen aquest processos no són coneguts. En el present treball hem pogut caracteritzar un conjunt de proteïnes alliberades per partícules d'os cortical porcí en el medi condicionat ossi (BCM) per imitar l'ambient paracrí dels empelts d'os cortical. Alguns dels factors alliberats pels empelts d'os autòleg podrien influenciar la reabsorció òssia explicant per què els osteoclasts es formen ràpidament a la superfície de les partícules d'os autòleg en els llocs regenerats. Tot i això els mecanismes moleculars que regeixen aquest procés, encara son desconeguts. Factors solubles alliberats pels empelts d'os autòleg in vitro tenen un impacte robust a la diferenciació de cèl·lules mesenquimals. En la present tesis doctoral, hem determinat si aquests factors solubles son capaços de canviar la diferenciació de cèl·lules mare hematopoètiques a osteoclasts, desconegut abans de realitzar els estudis aquí presentats.

Basant-nos en els resultats in vitro aquí presentats, es pot observar que la saliva suprimeix l'osteoclastogènesis i promociona el desenvolupament de cèl·lules amb un fenotip fagocític, afectant a la funció dels osteoclasts, les cèl·lules encarregades de reabsorbir l'os. La reabsorció dels empelts d'os autòleg es pot atribuir a l'efecte d'algunes de les proteïnes detecta-

des en les secrecions dels auto-empelts, anomenant aquestes secrecions Medi Condicionat d'Os (BCM). Un estudi proteòmic del BCM va mostrar que aquest medi condicionat conté més de 150 proteïnes, de les quals 43 es van caracteritzar com "secretades" i presents en la matriu extracel·lular. Vàrem descobrir que alguns dels factors continguts en el BCM com per exemple pleiotropina, galectina-1 o TGF-β1 poden afectar processos cel·lulars involucrats en la regeneració òssia. El resultats presentats en aquesta tesis sobre l'influencia del BCM en l'osteoclastogènesis demostra que el BCM termo-activat és capaç d'estimular l'osteoclastogènesis in vitro. Aquests resultats in vitro suporten la noció que la reabsorció dels auto-empelts ossis pot ser que estigui estimulada per mecanismes reguladors encara no definits. En aquesta línia, els protocols presentats sobre l'ús del BCM haurien d'animar a revelar els efectes paracrins dels empelts d'os autòleg durant el procés de regeneració òssia i obrir nous camins a investigacions translacionals en l'ampli camp de la cirurgia reconstructora.

Resumint-ho tot, podem concloure que la saliva afecta la reabsorció òssia promocionant el desenvolupament de cèl·lules amb un fenotip fagocític, i que no només la saliva pot afectar a la reabsorció òssia, sinó que també les secrecions dels injerts d'os autòleg. En aquest punt, hi ha suficient evidencia per concloure que els auto-empelts d'os no només tenen tres propietats, sinó una més: la propietat reguladora, la quarta dimensió dels empelts d'os autòleg.

Objectius

Objectius generals
Estudiar el comportament dels osteoclasts i les seves cèl·lules progenitores sotmeses a diferents condicions presents en la cavitat oral in vitro.

Objectius secundaris
1; Comparar l'activitat dels osteoclasts sotmesos a diferents factors de creixement.

2; Analitzar l'expressió proteica i genètica utilitzant diverses tècniques de biologia molecular.

3; Dur a terme estudis de fagocitació amb diversos tipus de cèl·lules sotmeses a condicions diverses.

4; Analitzar la composició de les substàncies estimulants utilitzades, especialment del medi condicionat d'os i la saliva.

5; Provar substàncies estimulants en fibroblasts orals com a proba de principis.

Objectius dels articles

Article I
L'objectiu de l'estudi va ser examinar si la saliva afectava al procés de l'osteoclastogènesis i a la formació de cèl·lules amb capacitat fagocítica in vitro.

Article II
L'objectiu de l'estudi va ser caracteritzar els factors paracrins del medi condicionat d'os mitjançant un estudi proteòmic.

Article III
L'objectiu de l'estudi va ser analitzar l'impacte de medi condicionat d'os fresc I desmineralitzat en la formació d'osteoclasts.

Article IV
L'objectiu de l'estudi va ser presentar com preparar medi condicionat d'os i provar la seva activitat in vitro.

Conclusions

- La saliva desvia la diferenciació de les cèl·lules hematopoètiques cap a un fenotip fagocític superant el microambient pro-osteoclastògènic creat in vitro. (Article I)

- Les partícules d'os cortical alliberen proteïnes en el medi de cultiu, algunes d'elles amb efectes en la regeneració òssia, incloent quaranta-tres factors de creixement diferents. (Article II)

- Els factors solubles alliberats per partícules d'os cortical afecten a l'osteoclastogènesis, incrementant l'expressió de marcadors osteoclàstics i la reabsorció de discs de dentina. (Article III)

- La capacitat d'alliberar molècules d'empelts d'os frescs i desmineralitzats pot ser avaluada mitjançant l'ús de medis condicionats. (Paper IV)

Perspectives de futur

L'esperança de vida a tot el mon està incrementant com també l'edat dels nostres pacients. És comú veure pacients de la tercera edat que han perdut les dents i necessites una rehabilitació oral, incloent procediments quirúrgics per incrementar la quantitat d'os. Aquests processos de regeneració òssia i la conseqüent consolidació dels empelts, a vegades és complex i requereix més d'una cirurgia. En els casos exitosos, al cap de 4 o 6 mesos un teixit similar a l'os està preparat per rebre un implant dental. Tot i això, a vegades, la regeneració òssia no té l'èxit esperat degut a grans atròfies de l'os del pacient o degut a un fallo en la consolidació de l'empelt ossi.

Investigacions i estudis futurs en el camp de la regeneració òssia haurien d'adreçar aquestes falles en la regeneració òssia. Estratègies futures s'han de desenvolupar per millorar aquests procediments i materials per recolzar la regeneració òssia d'una manera més ràpida i segura sense la necessitat de recollir os autòleg, de moment encara l'estàndard d'or. En els diferents estudis aquí presentats, es va dur a terme un intent per caracteritzar en profunditat els empelts d'os autòleg i explorar la seva activitat un cop col•locats en el lloc de la regeneració. Aquests resultats pioners, han de ser els iniciadors de futurs estudis sobre la caracterització dels auto-empelts ossis, sabent que un millor coneixement dels auto-empelts pot ajudar a dissenyar futurs materials de regeneració. Entenent cóm els factors de creixement continguts en el BCM juguen un paper in vivo, hauria de ser també un aspecte a tenir en compte. Estudis in vivo col•locant BCM, per exemple liofilitzat, amb un material de transport i col•locat en un defecte ossi podria ajudar a entendre com els factors de creixement alliberats pels empelts d'os autòleg funcionen. També seria interessant col•locar BCM subcutàniament en ratolins nus per determinar com el BCM afecta als teixits tous. Podria ser que els 43 factors de creixement continguts en el BCM, també juguin un paper en la supervivència i proliferació de les cèl•lules del teixit tou. Un altre aspecte que seria interessant investigar es la contribució dels osteòcits a la composició del BCM. El BCM conté esclerostina, una molècula que és nomes segregada pels cementoblasts i els osteòcits. Què contindria un BCM provinent d'un os sense osteòcits? Seria el seu efecte comparable al BCM d'os normal?

Els resultats aquí presentats sobre la saliva i els osteoclasts son intrigants. La saliva esta present a la cavitat oral de quasi tot els humans i dels mamífers en general. Tanmateix, no es coneix massa el seu efecte sobre l'os. Equips multidisciplinaris incloent immunòlegs, cirurgians orals i científics haurien d'aprofundir en els efectes aquí descrits de la saliva sobre

l'osteoclastogènesis i la funció osteoclàstica. La saliva no només inhibeix l'osteoclastogènesis, possiblement afectant a la regeneració òssia i a la consolidació dels empelts ossis, sinó que també inicia el desenvolupament de cèl•lules del sistema immunitari. Aquesta troballa tant interessant pot ser utilitzada per dissenyar noves estratègies en l'estudi de la xerostomia i el Síndrome d'Sjögren com també l'influencia de la saliva en la curació de ferides al os i la regeneració òssia. Estudis amb models desalivats – ratolins, rates o gossos- podria servir per investigar la curació i regeneració òssia després d'una extracció dental o una exposició òssia a la cavitat oral. Així mateix, què passaria si saliva for col•locada en un defecte ossi, per exemple concentrada o liofilitzada? Estudis clínics randomitzats podrien ser dissenyats per estudiar l'èxit de les regeneracions òssies en pacients amb un flux baix de saliva.

En conjunt, més recerca és necessària en ambdós tòpics: regeneració òssia i la saliva. En el futur llunyà, nous biomaterials amb les quatre propietats dels empelts d'os autòleg podrien ser desenvolupats. També en el futur llunyà, les proteïnes de saliva responsables dels efectes en la reabsorció òssia podrien ser isolades i aplicar-les terapèuticament per evitar la reabsorció òssia i incrementar les respostes del sistema immunitari in situ.

Annex II - Scholarship from the Foundation of Dental Research and Education

Klinik für Oralchirurgie und Stomatologie, Freiburgstrasse 7, CH-3010 Bern

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**UNIVERSITÄT
BERN**

Medizinische Fakultät

Zahnmedizinische Kliniken

**Klinik für Oralchirurgie und
Stomatologie**

Einsatzbestätigung

Personalien	Herr Dr. Caballé Serrano Jordi Geb. 21.10.1988 Spanier Pass A4614938500
Funktion	Gastwissenschaftler / Zahnarzt
Beschäftigungsgrad	100% (42 Std./Woche)
Anstellungsdauer	1 Jahr vom 1.10.2013 bis 30.9.2014
Finanzierung	FDR Foundation for Dental Research and Education c/o centerVision AG, 4052 Basel CHF 30'000.--- für ein Jahr (s. Beilage)
Adresse in der Schweiz	Bahnstrasse 98 3008 Bern

Bern, 18. September 2013

Prof. Daniel Buser
Klinikdirektor

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Annex III - Acceptance of the PhD protocol & co-director

Universitat Internacional de Catalunya | Escola de Doctorat

Barcelona, 25 de noviembre de 2013

Sr. Jordi Caballé Serrano
Mandri, 1 4º 1º.
08022, Barcelona.

Estimado Sr.

Por la presente, le comunico que la Comisión Académica del Doctorado en Ciencias de la Salud, en la su sesión del 12 de noviembre de 2013, y una vez estudiada su solicitud ha acordado:

Se acuerda admitir al Sr. Jordi Caballé Serrano al Periodo de Investigación del Doctorado en Odontología.

Se acuerda aprobar el Proyecto de Tesis titulado "In-Vitro study of osteoclastogénesis under simulated bone augmentation", y nombrar al Dr. Jordi Gargallo Albiol como Director y al Dr. Xavier Rodríguez Ciurana como Codirector de la Tesis.

Se le informa que deberá ajustar el proyecto a la realidad de la información.

Adicionalmente, se le informa que la normativa de la UIC establece que debe obtener una evaluación favorable del Comité de Ética en la Investigación, antes de la puesta en marcha de la investigación. Deberá aportar este informe cuando lo obtenga.

Aprovecho la oportunidad para saludarlo cordialmente,

Jaime Oliver Serrano
Secretario Comisión Académica
Doctorado en Ciencias de la Salud

Universitat Internacional de Catalunya
VICERECTORAT DE RECERCA

Universitat Internacional de Catalunya
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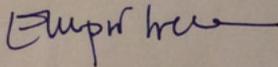Universitat Internacional de Catalunya | Escola de Doctorat

JORDI CABALLÉ SERRANO
MANDRI,1,4° 1ª
08022 BARCELONA

Benvolgut Sr. Caballé

Amb la present, li comunico que la Comissió Acadèmica del Doctorat en Ciències de la Salut, en la seva sessió del passat 8 d'abril, i una vegada estudiada la seva sol·licitud va acordar aprovar la modificació del seu projecte de tesi "In Vitro study of osteoclastogenesis under simulated bone augmentation" i nomenar el Dr. Reinhard Gruber codirector.

Per qualsevol qüestió que vulguin comentar no dubtin en posar-se en contacte amb nosaltres.

Atentament,

Universitat Internacional de Catalunya | Escola de Doctorat

Empar Lorda
Secretària de la Comissió Acadèmica Doctorat en Salut
Escola de Doctorat
Universitat Internacional de Catalunya

Barcelona, 11 d'abril de 2014

Annex IV - Acceptance of the ethical committee

CER Comitè d'Ètica de Recerca | **Universitat Internacional de Catalunya**

CARTA DE CONFORMITAT DEL CER PER A PROJECTES AVALUATS I APROVATS PER UN CEIC

Codi de l'estudi:CIR-ELB-2013-02
Versió del protocol:1.0
Data de la versió:26/11/13
Títol:"In-Vitro study of osteoclastogenesis under simulated bone augmentation"

Sant Cugat del Vallès,07 de gener de 2014

Investigador: Jordi Caballé Serrano

Títol de l'estudi: "In-Vitro study of osteoclastogenesis under simulated bone augmentation"

Benvolgut (da),

Valorat el projecte presentat, el CER de la Universitat Internacional de Catalunya, considera que, des del punt de vista ètic, reuneix els criteris exigits per aquesta institució i, per tant, ratifica l'aprovació dels CEICs aportada, d'acord amb el reglament vigent.

Em permeto recordar-li que si en el procés d'execució es produís algun canvi significatiu en els seus plantejaments, hauria de ser sotmès novament a la revisió i aprovació del CER.

Quedo a disposició per a qualsevol dubte o aclaració al respecte.

Atentament,

Dr. Josep Argemí
President CER-UIC

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Annex V - Acceptance of the tutor abroad and approval of the European PhD application

Universitat Internacional de Catalunya | **Escola de Doctorat**

Barcelona, 28 de febrer de 2014

Sr. Jordi Caballé Serrano
C/ Mandri,1, 4º 1ª
08022 Barcelona

Benvolgut Sr. Caballé

Amb la present, li comunico que la Comissió Acadèmica del Doctorat en Ciències de la Salut, en la seva sessió del passat 11 de febrer, va acordar aprovar la seva sol·licitud d'optar al doctorat amb Menció Europea del Sr. Jordi Caballé i s'anomena com a tutor de la seva estada de Recerca a la universitat de Berna al Dr. Reinhard Gruber, cap del laboratori de Biologia Cel·lular, del Departament de Cirugia Oral de la Facultat d'Odontologia de la Universitat de Berna.

Per qualsevol qüestió que vulguin comentar no dubtin en posar-se en contacte amb nosaltres.

Atentament,
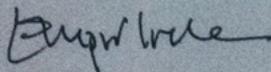
Empar Lorda
Secretaria Comissió Acadèmica Doctorat en Ciències de la Salut
Escola de Doctorat
Universitat Internacional de Catalunya

Annex VI - Acceptance of PhD manuscript CAD

uic
barcelona

JORDI CABALLÉ SERRANO

Benvolgut Jordi,

Amb la present et comunico que la Comissió Acadèmica de Doctoral en Salut, en la passada edició del 9 de juny de 2015, va aprovar la teva tesi "In vitro study of osteoclastogenesis under simulates bone augmentation. The effects of bone-conditioned medium and saliva on osteoclastogenesis".

Atentament,

Esther Belvis
Secretaria Tècnica de l'Escola de Doctorat
Escala de Doctoral

Barcelona, 15 de juny de 2015