UNIVERSITA' DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO IN SCIENZE FARMACOLOGICHE

Dipartimento di Scienze Farmacologiche e Biomolecolari

Corso di Dottorato in Scienze Farmacologiche

Settore Scientifico Disciplinare BIO/14

Ciclo XXVI

FROM *IN VITRO* STUDIES TO A LARGE ANIMAL MODEL: A MULTISTEP DISSECTION ON THE FUTURE ROLE OF ADIPOSE-DERIVED STEM CELLS FOR MUSCULOSKELETAL TISSUE ENGINEERING

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Anno Accademico 2012/2013

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ABSTRACT

Tissue engineering is an emerging interdisciplinary field, born with the purpose to provide an alternative solution for the regeneration of lesioned or lost tissues, combining cells, biocompatible scaffolds and bioactive factors. The cells for this approach should be non-immunoreactive and non-tumorigenic. Moreover, they should be available in large amount and possess, or be able to acquire, a specific protein expression pattern similar to that of the damaged tissue and/or act as a pool of trophic factors for resident cells. All these reasons, make mesenchymal stem cells (MSCs) good candidates for applications in regenerative medicine. Although bone marrow is still the most common source of MSCs, these cells could be harvested from all vascularised tissues, and, interestingly, from tissues that are normally discarded, such as fat, placenta or umbilical cord. One of the most convenient source of MSCs, is unequivocally, the adipose tissue due to the easily accessible anatomical location and the abundance of subcutaneous adipose tissue. Adipose-derived stem cells (ASCs) are similar to MSCs isolated from bone marrow in morphology, immunophenotype, and differentiation ability, and own interesting features such as immunoregolatory and anti-inflammatory properties.

In the recent years, many strategies for the cure of musculoskeletal tissues critical lesions, mainly in orthopaedic, oral and maxillo-facial surgery, have been under investigations. In this contest, the regeneration of structures including different tissues, such as the periodontium and the osteochondral unit, are particularly challenging.

Periodontal regeneration is especially demanding, as it requires regeneration of three quite diverse and unique tissues such as the alveolar bone, the periodontal ligament and the cementum, that have to interface with each other to restore their complex structure. Since the promising results obtained with ASCs in preclinical studies of periodontal diseases arouse the curiosity of maxillofacial and dental surgeons, we decided to identify a novel source of ASCs, i.e, the buccal fat pad, convenient for these specialists. For this purpose, we studied human adipose derived-stem cells from buccal fat pad (BFP-ASCs), comparing them with cells from the subcutaneous adipose tissue (SC-ASCs) of the same donor (n=2). In parallel, considering the need for preclinical studies in which the effect of allogenic cells should be tested, and swine as an accepted animal model in tissue engineering applications, we also characterized porcine cells (n=6). With preclinical and clinical application prospective, we

also investigated ASC interactions with oral tissues, natural and synthetic scaffolds and Amelogenin, an oral bioactive molecule. First of all, we showed that it is feasible to isolate ASCs even starting from very limited amounts of tissue (0,5 ml) and that the cellular yield is influenced by species, but not by the site of harvesting ($1.1x10^5 \pm 1.4x10^4$ human BFP-ASCs/ml and $1.15x10^5 \pm 7.1x10^3$ human SC-ASCs/ml; $3.0x10^4 \pm 9.3x10^3$ porcine BFP-ASCs/ml and $5.5x10^4 \pm 3.3x10^4$ porcine SC-ASCs/ml). Despite the lower yield, the pASCs great proliferation rate allows to obtain high number of cells (potentially, 10^8 - 10^9) after few (3, 4) passages in culture. After the isolation, a great amount of cells deriving from all the tissues, adhered to cell culture plates showing the MSC fibroblast like morphology, with only mild shape differences constituted by the higher elongation and dimension of human SC-ASCs. Moreover, all the cells are easily expandable and showed good clonogenic ability at early passages. Cells of the same species, from both the harvesting site, displayed the same surface markers profile, that, in particular for human ASCs, was the typical one of hMSC (CD90+, CD105+, CD73+, CD14-, CD31-, and CD34-). Human and porcine BFP-ASCs, as SC-ASCs, are multipotent; indeed, when induced towards osteogenic and adipogenic lineages, they up-regulated significantly ALP activity, collagen and calcified extracellular matrix deposition and lipid vacuoles productions, respectively, already after 14 days of differentiation *in vitro*.

Next, since cell/scaffold interaction is fundamental for the outcome of a tissue engineering approach, in sight of a preclinical study, we combined porcine BFP and SC-ASCs to both clinical grade (titanium) and innovative [silicon carbide–plasma-enhanced chemical vapor deposition (SIC-PECVD)] biomaterials, and studied cell adhesion and their differentiation ability. All the cells nicely grew on both scaffolds and, when osteoinduced, significantly increased the amount of calcified ECM compared to control cells; interestingly, titanium is osteoinductive even per se on pASCs (+284% and +91 for BFP- and SC-ASCs). Considering the importance of cell interaction with tissue of the lesion site, and with materials commonly used during surgical practices, we studied human BFP- and SC-ASC adherence to several supports. SEM analysis confirmed that both cell type nicely stick on alveolar bone, periodontal ligament, collagen membrane and polyglycolic acid filaments. Finally, we found that amelogenin, the most abundant enamel matrix protein seems to be an early osteoinductive factor for BFP-hASCs, whereas this effect is not manifested for SC-hASCs.

For future cellular therapy, and since the use of FBS pose the risk of xenogenic contaminations leading to immunological complications during transplant, we tested cells growth in the presence of autologous supplements. Interestingly, both hASCs adapted rapidly to human serum, increasing their proliferation rates compared to standard culture condition, while porcine autologous or heterologous sera, did not improve pASC growth.

In conclusion, we identified a cell population derived from a tissue easily available to dentists and maxillofacial surgeons, whose multipotent features and interaction with clinical grade scaffolds make proper candidate for future uses in tissue engineering approaches of periodontal diseases.

In parallel, part of my PhD project was focused on the study of a critical osteochondral defect regeneration performed in a large animal preclinical model.

The main obstacles for clinicians in treating this defect arises from the disparity concerning anatomy, composition and, most importantly, rate of healing of the articular cartilage (AC) and the subchondral bone. The key points of our study are the use of an innovative hydrogel of oligo(polyethylene glycol)fumarate (OPF) to fill the osteochondral defect, and of either porcine, or human ASCs, to create bioconstructs to be implanted in non-immuno-compromised minipigs. In particular, four critical osteochondral defects (diameter 9mm, depth 8mm) were created in the peripheral part of the trochlea of seven animals (defect n=28), and then treated with the different pre-made constructs. Untreated defects and defects filled by just scaffold were included as controls.

No side-effects have been observed during the six-moths follow-up. At the end of this period, animals were sacrificed and knees explanted. Gross appearance analyses showed quite satisfactory filling of all the lesions, with the exception of one animal, whose joint appeared infected and not healed.

MRI analyses revealed that in all the scaffold treated groups an overall improvement of the tissue quality at the osteochondral lesion site, was induced. More accurate evaluations (histological and immunohistochemistry analyses) revealed that some important tissue features were significantly improved by the association of OPF and ASCs. Indeed, regarding the subchondral bone, in all the OPF+ASCs groups, a mature bone appeared, with higher deposition of collagen type I compared to untreated or unseeded OPF groups. Moreover, the use of ASCs associated to scaffolds induced an improvement in newly formed cartilage features such as collagen type II deposition, and histological scores associated to these

samples indicated a significant increase in matrix staining, tissue morphology and formation of tidemark, together with a reduction in vascularisation (a positive aspect in cartilage) compared to unseeded scaffolds.

However, the histology indicated that in all the samples cartilage regeneration was still immature, most likely due to the limited time of follow up and/or the insufficient stimuli for cartilage complete regeneration. Despite this, biomechanical tests revealed that the neo-cartilage found in the cell-loaded scaffold groups possessed poroelastic behaviour, as well as indentation modulus and creep curves comparable to native cartilage. This important result suggest that the ASC presence at the lesion site, is able to enhance newly formed cartilage functionality.

In conclusion, this *in vivo* study provides the evidence that both porcine and human adipose-derived stem cells associated to OPF hydrogel improve osteochondral defect regeneration, even though, at the moment, we are not able to define if the implanted ASCs are responsible per se of the new tissue formation or if they help spontaneous regeneration process by paracrine actions.

INTRODUCTION

Tissue engineering

Tissue engineering has been defined by Langer and Vacanti as ”an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (Langer and Vacanti, 1993).

Tissue engineering aim to provide an alternative solution for the regeneration of lesioned or lost tissue/organ, avoiding the disadvantages associated to traditional therapy, combining and integrating physics, chemistry, engineering and material, cell and medical sciences (Langer and Vacanti, 1993). The field relies extensively on the use of porous 3D scaffolds to provide the appropriate environment for the regeneration of tissues and organs. These scaffolds essentially act as a template for tissue formation and are typically seeded with cells and occasionally growth factors, or subjected to biophysical stimuli by the use of bioreactors. These cell-seeded scaffolds are either cultured *in vitro* to synthesize tissues which can then be implanted into an injured site, or are directly implanted into the injured site, using the body’s own systems, where regeneration of tissues or organs is induced *in vivo*.

Cell source

Regardless the tissue to be repaired, the cells to be used in tissue engineering approaches should have some important features.

First of all they should be non-immunoreactive (unable to induce rejection and graft-versus-host disease, GvDH), and non tumorigenic. These populations should be available in large amount, expandable *in vitro* for many generations, and they should possess or be able to acquire a specific protein expression patterns similar to that of the tissue to be regenerated, and finally, have to adequately integrate within the surrounding tissues. Recently, also the ability to act as trophic factor pool for resident cells has been proposed to be an important characteristic.

All these reasons, make mesenchymal stem cells (MSCs) good candidates for applications in regenerative medicine: there are no limitations to their practical use related to ethical or religious considerations, and techniques of isolation and culture are simple to implement.

Moreover, their phenotypic stability, multipotentiality and low immunogenicity give these cells a high therapeutic potential (Gimble, 2003).

The presence of non-hematopoietic stem cells in bone marrow was observed for the first time by Cohnheim almost 150 years ago (Cohneim et al., 1867) and has been confirmed only in the 1960s by McCulloch and Till (Becker et al., 1963) and in the 1970s by Friedenstein et al. who named these cells the stromal cells colony forming unit-fibroblasts (Friedenstein et al., 1974). Caplan successively (1991) named them mesenchymal stem cells because of their capacity of self-renewal and differentiation (Caplan, 1991), and, three years ago, focusing on their secretive paracrine activities, he proposed that MSCs should be an acronym for "*medicinal signaling cells*" (Caplan, 2010).

MSCs can be isolated simply through a series of passages in culture. *In vitro* they adhere to plastic, showing a typical fibroblast-like morphology and forming colonies. MSCs should also express a minimum specific set of surface antigens - CD105 (endoglin, SH2), CD73 (ecto-5'-nucleotidase) and CD90 (Thy1) - and should not express the hematopoietic markers - CD45, CD19 or CD79, CD14 or CD11b, and HLA-DR. Moreover they should own multipotent capacity being able to differentiate into osteoblasts, adipocytes or chondroblasts under standard *in vitro* differentiating conditions (Dominici et al., 2006). Although bone marrow is still the most common source of MSCs, in the last two decades there has been a continuous effort to identify alternative sources of MSCs, mainly driven by a constant quest for a source with lower donor site morbidity. Therefore, it is particularly interesting that MSCs have been found in tissues that are normally discarded, such as fat from liposuction, or placenta and umbilical cord. Although it could appear funny that cells deriving from very different tissue are so similar, a possible explanation have been proposed. Indeed, Caplan et al suggested that MSCs are pericytes, (Caplan, 2008) basing this theory on recent evidences. First of all, for almost every blood vessel in the body, mesenchymal cells are observed in perivascular locations (on both arterial and venous vessels) (Crisan et al., 2008). Moreover, isolated pericytes exhibit a panel of cell surface markers that are identical to those expressed by isolated MSCs (Crisan et al., 2008). This intriguing hypothesis, could also explain the great variety of clinical applications of MSCs, including Parkinson's and Alzheimer's disease, spinal cord injury, stroke, burns, arthritis, heart disease, diabetes, osteoarthritis and rheumatoid arthritis (ClinicalTrials.gov). Almost all these trials, utilize MSCs in a therapeutic manner that

is quite distinct from the differentiative capacity but based on two therapeutic activities: immunomodulation and trophic activities.

One of the most convenient source of MSCs, is unequivocally, the adipose tissue due to the easily accessible anatomical location and the abundant existence of subcutaneous adipose tissue. Since leptin identification in 1994, this tissue was no longer considered as a fat store only, but as a true secretory tissue (Zhang et al., 1994). About ten years later, adipose tissue was further dignified, thanks to the discovery of an abundant number of mesenchymal stem cells (ASCs, adipose derived- stromal cells) (Zuk et al., 2002). The concentration of multipotent stem cells in adipose tissue is around 2% of nucleated cells (Zuk et al., 2002), which is approximately 100–300 times higher than that of MSCs in bone marrow. These cells own all the MSCs features and have been used successfully in many pre-clinical studies not only for musculoskeletal regeneration (de Girolamo et al., 2011) but also for experimental models of, for example, multiple sclerosis, lupus erythematosus (Choi et al., 2011), rheumatoid arthritis (Gonzalez-Rey et al., 2010), colitis model (Gonzalez-Rey et al., 2009). Furthermore, about hundred clinical trials are investigating the potential of ASC in the treatment of fistulas, vascular disease, joint disease, inflammatory bowel disease, osteoarthritis and many other diseases (ClinicalTrials.gov).

Other cell types that can be appealing for tissue regeneration could be the direct progenitors of, or even terminally differentiated cells of the tissue to be replaced or diametrically opposed, pluripotent stem cells. Both approaches are interesting but present important drawbacks.

Considering the physiological role of progenitors for the maintenance of tissue homeostasis, replacing terminally differentiated cells, and differentiated cells as the proper cells to populate and modify tissue from which they derive, it appears obvious their use in regenerative medicine. A fascinating example is that of autologous stem cells of the limbus (the narrow zone between the cornea and the bulbar conjunctiva) cultivated on fibrin glue and then used with a resulting permanent restoration of a transparent, renewing corneal epithelium (Rama et al., 2010). Nevertheless, must be considered that in general, progenitors are not present in abundant quantity, or easy available with mild donor discomfort.

Finally, huge interest has developed in the scientific and clinical communities around pluripotent stem cells (both Embryonic Stem Cells or induced Pluripotent Stem Cells)

therapeutic potential, due to these cell ability to be programmed into new mature differentiated cells of all lineages. Particular attention has been focused on their potential use in cell-based therapy for diseases that are refractory to conventional treatments, such as neurodegenerative diseases and immunodeficiency. The list of pathologies that in theory can be treated using stem cells includes Alzheimer's, Parkinson's and Huntington's disease, stroke, diabetes, age-related disorders, haematological disorders, cardiovascular disease and bone and muscle regeneration.

Nevertheless, even overlooking ethical or religious considerations related to ESCs use, the huge differentiative potential of pluripotent stem cells make them not so easy to be handled. Furthermore, their use as therapy is clouded by their potential tumorigenicity (Romeo et al., 2012).

Biomaterials

According to the European Society for Biomaterials (ESB) a biomaterial is a "material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body".

General features going beyond the specific tissue application have to be considered for proper scaffold choice.

First of all, the scaffolds have to be biocompatible; cells must adhere, function, and migrate onto the surface and eventually through the scaffold pores or channels before laying down new matrix. After implantation, the scaffold or tissue engineered construct must elicit a negligible immune reaction in order to prevent severe inflammatory response that might reduce healing or cause rejection by the body.

Moreover, scaffolds and constructs, are not intended as permanent implants. The scaffold must therefore be biodegradable and the products of degradation should also be non-toxic and able to be eliminated by the body without interfering with other organs. Ideally, the scaffold should also posses mechanical properties consistent with the anatomical site into which has to be implanted and, from a practical perspective, it must be stiff enough to allow surgical handling during implantation.

Another key aspect of scaffold to be used for tissue engineering is its architecture. Scaffolds should have interconnected pores to ensure cellular infiltration and adequate diffusion of nutrients to cells within the construct, and of waste products out of the scaffold (Ko et al.,

2007; Phelps and Garcia, 2009). Concerning the size of the pores, they should be large enough to allow cells to migrate into the structure, but small enough to allow efficient binding of a critical number of cells to the scaffold (O'Brien et al., 2005). Finally, the development of manufacturing processes to good manufacturing practice (GMP) is critically important in ensuring successful translation of tissue engineering strategies to the clinic (Hollister, 2009).

Typically, four groups of biomaterials are used in the production of scaffolds for tissue engineering: natural polymers, synthetic polymers, ceramics and metals.

Biological materials such as collagen, alginate-based substrates, fibrin and chitosan present similarities to the ECM, typically good biological characteristics and inherent cellular interactions. Unlike synthetic polymer-based scaffolds, natural polymers are biologically active and able to promote cell adhesion and growth. Furthermore, their biodegradability, bioresorbability and versatility, facilitate their use in cell delivery applications. However, these scaffolds generally have poor mechanical properties, which limits their use in load-bearing orthopaedic applications and when harvested from animal or human sources they requires purification procedures.

Among synthetic polymers, the most used in tissue engineering approaches are polylactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers (e.g., poly(lactic-co-glycolic) acid (PLGA). These materials can be produced with specific architecture and degradation characteristics (Lu et al., 2000; Rowlands et al., 2007); however, they have also drawbacks including the risk of rejection due to reduced bioactivity. In addition, the degradation process of PLA and PGA by hydrolysis, produce carbon dioxide that induces a local decrease of the pH resulting tissues necrosis (Liu et al., 2006).

Ceramic scaffolds, such as hydroxyapatite (HA), tricalcium phosphate (TCP), CaPs, and calcium sulphates have been widespread use in bone regeneration applications because of their high mechanical stiffness. Furthermore, they exhibit excellent biocompatibility due to their chemical and structural similarity to the mineral phase of native bone. The interactions of osteogenic cells with ceramics are important for bone regeneration (Ambrosio et al., 2001). Various ceramics have been used in dental and orthopaedic surgery to fill bone defects and to coat metallic implant surfaces to improve their integration with the host bone. However, their clinical applications for tissue engineering has been limited because of their brittleness, difficulty of shaping for implantation and the fact that new bone formed in

a porous HA network cannot sustain the mechanical loading needed for remodeling (Wang, 2003). In addition, although HA is a primary constituent of bone and might seem ideal as a bone graft substitute, some problems exist to control its degradation rate.

Metallic materials are also particularly suitable for the replacement of hard tissues such as bones and teeth and for the production of structures able to support loads. The main shortcoming is linked to the possible release of metal ions *in situ* and their accumulation in other body districts, following the corrosion operated by biological fluids. The metals used as biomaterials for the manufacture of prostheses are iron, cobalt, nickel, titanium, and tungsten. In particular, due to its biocompatibility, corrosion resistance and excellent mechanical properties, titanium is suggested in oral, maxillofacial and orthopaedic surgery.

Growth factors

Growth Factors are cytokines secreted by various cell types, that act as signal molecules and are essential for tissue formation. Since the binding of the growth factors and their receptors is responsible for the modulation of cellular adhesion, proliferation, migration and differentiation related pathway, these proteins play a fundamental role in tissue engineering. The growth factors investigated for these applications are over all bFGF (basic Fibroblast Growth Factor o FGF-2), IGF-I (Insulin-like Growth Factor), VEGF (Vascular Endothelial Growth Factor), PDGF (Platelet Derived Growth Factor) (Jadlowiec et al., 2003) and the TGF- ß superfamily (Transforming Growth Factor Beta).

FGF-2 is a growth factor involved in the endothelial cell proliferation and in the bone remodelling. It also promote angiogenesis.

IGF-I is one of the main activator of Akt pathway, and therefore act stimulating cell proliferation and inhibiting apoptosis. Concerning bone, IGF-I stimulate collagen I synthesis and matrix deposition during fractures.

PDGF is produced by platelets, monocytes and macrophages and it is implicated in growth and differentiation. Autologous PDGF from the platelet-rich-plasma (PRP) is already used in clinical practice in maxillofacial and vascular surgery and in orthopaedic practices for musculotendinous pathologies, pseudoarthrosis and prosthesis replacement (Filardo et al., 2013; Lubkowska et al., 2012; Volpi et al., 2010).

TGF- ß superfamily proteins are involved in various functions such as tissue regeneration, cell differentiation, embryonic development and immune system modulation. In particular,

they regulate MSC proliferation, osteoblast, fibroblast and endothelial cell mobility, collagen production and secretion and the mitogenic effects of the other growth factors (Everts et al., 2006). Bone morphogenetic proteins (BMPs) are transforming growth factor-β (TGF-β) superfamily members, known to be potent inducers of bone formation . More than 30 BMP-related proteins have been identified. They are synthesized by skeletal cells, and play a crucial role in early embryogenesis, skeletogenesis and in the maintenance of bone mass also during adulthood. They are involved in the differentiation of bone marrow stem cells toward osteoblasts , chondrocytes and adipocytes. Moreover, they are involved in the development and in diseases of a variety of tissues, in particular vascular and neural ones (Biver et al., 2013). Also VEGF could be potentially useful in tissue engineering for its angiogenic activity. This protein is usually released at the injured site and regulate new vascularization through the recruiting of endothelial cells.

Dynamic Culture systems

Bioreactors can be defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions such as pressure, temperature, nutrient supply, and waste removal.

The *in vitro* cultivation of 3D-constructs in bioreactors allow the media perfusion through a porous scaffold, providing homogenous nutrient and oxygen concentrations to cells. As nutrient deprivation and hypoxia often occur in static culture, this ability of bioreactor systems makes them a key part of an *in vitro* culture strategy (Yeatts AB 2013).

Moreover, another interesting skill is the possibility to apply mechanical stimulation to direct cellular activity, differentiation and function, that is important for the development of functional grafts (Chen HC 2006). Bioreactors, extensively used in the culture of MSCs, include simple systems such as spinner flask and rotating wall bioreactors and more complicated systems including perfusion and dynamic loading bioreactors. In particular, perfusion and dynamic loading systems have been demonstrated to be very effective in MSC culture enhancing their proliferation and also chondrogenesis and osteogenesis.

Clinical needs and current strategies

In the recent years, many strategies for the cure of musculoskeletal tissues critical lesions, mainly in orthopaedic, oral and maxillofacial surgery, have been under investigations.

A great challenge is represented by the repair of defects that include more kinds of tissue, as occurred in periodontal and osteochondral diseases.

Periodontal regeneration is especially demanding, as it requires regeneration of three quite diverse and unique tissues such as the alveolar bone, the periodontal ligament and the cementum, that have to interface with each other to restore their complex structure (Chen et al., 2010; Young et al., 2005).

In parallel, one of the main obstacles for osteochondral defect regeneration arises from the disparity concerning anatomy, composition and, most importantly, rate of healing of the articular cartilage (AC) and the subchondral bone. Nevertheless, despite their heterogeneity, these two tissues together constitute a unique unit which requires the interconnected activity of both components to carry out the physiological function of absorbing mechanical stress and attenuate the loads through the joints (Brown and Vrahas, 1984; Hoshino and Wallace, 1987; Imhof et al., 2000; Radin et al., 1970; Radin and Rose, 1986) and in which the modifications of each tissue induce a remodelling of the other (Mahjoub et al., 2012).

Periodontal disease

Periodontal diseases are inherited or acquired disorders of the tissues surrounding and supporting the teeth (periodontium) including alveolar bone, the periodontal ligament (PDL) and root cementum (figure 1). These diseases are highly prevalent and can affect up to 90% of the worldwide population. Periodontitis results in loss of connective tissue and bone support which could lead to tooth loss. These diseases are mainly caused by pathogenic microorganisms in the biofilm; however, also genetic and environmental factors contribute to origin the pathology. Periodontal manifestations could also derive from dermatological, haematological, granulomatous, immunosuppressive, and neoplastic disorders. A part from tooth loss, common forms of periodontal disease have been associated with adverse pregnancy outcomes, cardiovascular disease, stroke, pulmonary disease, and diabetes, even though the causal relations have not been established. Prevention and treatment are aimed at controlling the bacterial biofilm and other risk factors, arresting progressive disease (tooth cleaning/scaling, root planning and periodontal debridement) and restoring lost tooth

support (Pihlstrom et al., 2005). Several procedures have been attempted to achieve periodontal regeneration, including bone graft placement, guided tissue/bone regeneration and the use of various growth factors and/ or host modulating agents such as Emdogain and parathyroid hormone. However, these techniques did not achieve successful results in restoring the interconnected structure formed by alveolar bone, PDL and cementum that is necessary for proper functioning of the periodontium (Chen et al., 2012). Each of the periodontal components has its very specialized structure and this directly define its function.

Cementum is the hard, avascular connective tissue that coats the roots of teeth and that serves primarily to invest and attach the principal periodontal ligament fibers.

The periodontal ligament is a soft, specialized connective tissue situated between the cementum covering the root of the tooth and the bone forming the socket wall (alveolo-dental ligament). It ranges in width from 0.15 to 0.38 mm, with its thinnest portion around the middle third of the root, showing a progressive decrease in thickness with age. It is a connective tissue particularly well adapted to its principal function, supporting the teeth in their sockets and at the same time permitting them to withstand the considerable forces of mastication. In addition, the periodontal ligament has the capacity to act as a sensory receptor necessary for the proper positioning of the jaws during mastication and, very importantly, it is a cell reservoir for tissue homeostasis and repair⁄regeneration.

The alveolar process is that bone of the jaws containing the sockets (alveoli) for the teeth. It consists of outer cortical plates (buccal, lingual, and palatal) of compact bone, a central spongiosa, and bone lining the alveolus (alveolar bone). The cortical plate and bone lining the alveolus meet at the alveolar crest. The bone lining the socket is specifically referred to as bundle bone because it provides attachment for the periodontal ligament fiber bundles (Nanci and Bosshardt, 2006).

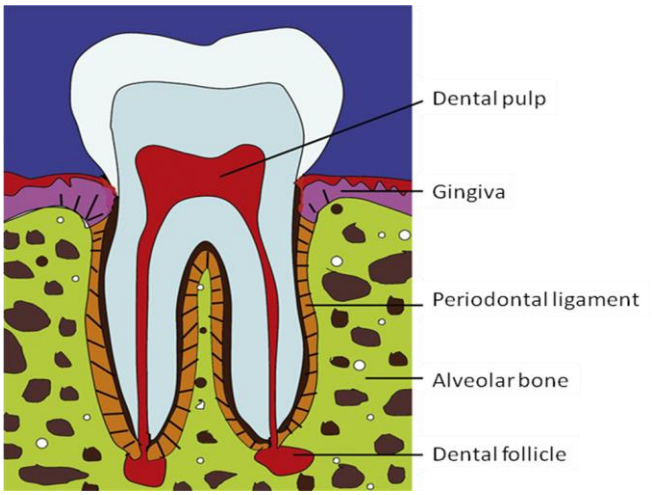

Figure 1. Modified by Chen FM 2012 (Chen et al., 2012)

Since the importance of the periodontium complex integrity, and the progresses in cell-based therapeutics, different tissue-engineering approaches for a robust periodontium regeneration with efficacy and predictability are currently under investigation. Concerning cell type, a possible choice consist in Dental Stem Cells, which include Dental Follicle Cells, Dental Pulp Stem Cells and Dental Apical Papilla Stem Cells and PDL-derived MSCs (PDLSCs) (Chen et al., 2012).

Among them, PDLSCs seem to be the most proper candidate. Since their first isolation by Seo et al. (Seo et al., 2004), their ability to regenerate PDL tissues have been verified in rodent and swine models of periodontal defects (Ding et al., 2010; Liu et al., 2008; Seo et al., 2004) and recently, a three-patient experiments confirm their ability to improve periodontal disease also in humans (Feng et al., 2010).

However, the difficulty in the generation of enough PDLSCs from one donor source due to the variation of stem cell potential between donors and the disease state of each patient represent a problem for possible autologous uses. This disadvantage, is shared by all Dental Stem Cells. Therefore, a valid alternative is represented by mesenchymal stem cells both from bone marrow (BMSCs) and from adipose tissue (ASCs). BMSCs can efficiently regenerate not only bone tissue but also the periodontal tissue in various animal models. BMSCs autologous transplantation in dogs induced periodontal (cementum, PDL and alveolar bone) regeneration in experimental class III furcation defects (Kawaguchi et al., 2004). These results are consistent with that obtained in a rat model in which GFP-labelled BMSCs were detectable in the repaired tissue four weeks after transplantation. In particular,

cementoblasts, osteoblasts, and fibroblasts of the repaired tissue were positive for GFP, suggesting that implanted BMSCs could survive and differentiate into periodontal tissue cells (Hasegawa et al., 2005).

Nonetheless, bone marrow harvest is an expensive and quite complex procedure.

Differently, adipose tissue is easy to obtain and normally present in larger quantity. Recently, the ASC ability to promote periodontal regeneration have been investigated *in vivo* both in a rat and in a dog model. In particular, after 8 weeks from implantation, ASCs mixed with PRP induced the formation of PDL-like and alveolar bone-like structures into rat periodontal defects (Tobita et al., 2008) and bone formation in a canine model of dental root bifurcation defects (Tobita and Mizuno, 2010).

Concerning the delivery of cells for periodontal regeneration, both scaffold mediated or scaffold-free approaches have been tested. The first choice is guided by the fact that biomaterials can maximise the beneficial effects of cellular therapies improving persistence and controlling cell delivery (Chen FM 2012). The scaffolds used for this purpose include natural biomaterials such as collagen (Grimm et al., 2011), gelatin (Kuo et al., 2008) or fibrin (Soffer et al., 2003). These biomaterials shown the ability to guide cells in the lesion site accelerating periodontal defects healing, even though the requirement of extensive purification protocols and potential pathogen contamination when harvested from animal or human sources must carefully considered before application (Chen et al., 2012). For these reasons, a valid alternative are synthetic polymers as poly(lactic-co-glycolic acid (Shang et al., 2010), or ceramics (Jiang et al., 2010) that have been recently applied with promising results in preclinical models of periodontal defects (Jiang et al., 2010; Shang et al., 2010). Finally, also metals, such as titanium (a material largely used in orthopaedic practises), have been shown to support and guide autologous PDLSCs to organize periodontal tissues in a rat preclinical model, suggesting a possible clinically relevant methods for autologous PDL regeneration on titanium implants in humans (Lin et al., 2011). Scaffold-free approaches include cell sheets (Flores et al., 2008a; Flores et al., 2008b; Iwata et al., 2009) or cell pellet transplantation (Yang et al., 2009). Using both the approaches it was induced a good periodontal regeneration, with restoration of the cementum/periodontal-ligament complex. However, some doubt around their use include the delicate structure difficult to handle of the cell sheets and, the perplexity about the non-tumorigenicity of the cell pellet microtissues (Demirbag et al., 2011).

Osteochondral defects

Osteochondral defects derive from traumatic injuries, osteochondritis dissecans and chondromalacia and are associated with the risk of developing degenerative joint diseases. The characteristic of this type of lesion is that two very different tissues such as the cartilage and the bone are affected. Articular cartilage is an aneural, avascular tissue, that consists of water, collagen, proteoglycans, and only a cell type, the chondrocytes. Each component of cartilage holds a specific role in maintaining its supportive nature. Glycosaminoglycans (GAGs), attached to the proteoglycans found in cartilage extracellular matrix, have a high density of negative charges that attracts osmotically active cations in water. The following excess of water causes the turgor which enables the resistance to high compressive forces. GAGs (mainly chondroitin and keratin sulphates) and proteoglycans (mainly aggrecan) constitute a network interlaced through the structured collagen (mainly of type II), which fibers allow structural and elastic strength of the cartilage (Nukavarapu and Dorcemus, 2013). According to the different concentrations of each component, articular cartilage can be divided into four zones (figure 2): the superficial or tangential zone (10–20% of articular cartilage), the middle zone (subsequent 40–60%), the deep zone and the calcified cartilage (Pearle et al., 2005). The superficial zone present densely packed collagen fibers and flattened cells parallel to the cartilage surface, and a small amount of proteoglycans. This zone has low permeability but is the main responsible for the wear and frictional properties of the tissue. Differently, the middle zone, contains abundant proteoglycans, that confer to it high compressive modulus, but has a low number of cells, and obliquely oriented collagen fibers. In the deep zone, both the cells and the collagen fibrils are oriented perpendicularly to the articular cartilage's surface, and the fibrils are anchored in the underlying subchondral bone. This zone also has a high compressive modulus, even though presenting less proteoglycans than the middle zone. Between this zone and calcified cartilage there is a thin line called “tidemark” that present few cells and the collagen fibers extending from the upper to the lower zone. The calcified zone has a fundamental role since it constitutes the transition between the pliable cartilage and the rigid subchondral bone (Nukavarapu and Dorcemus, 2013) (figure 2).

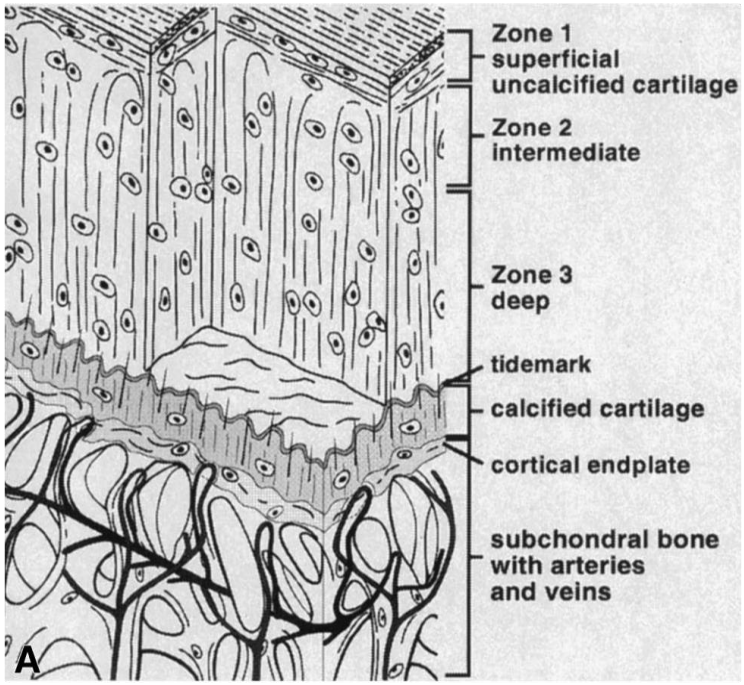

Figure 2. Schematic drawing of different layers of hyaline cartilage and subchondral bone with vessels (Imhof et al.,)

The area underlying the calcified cartilage and composed of the bony lamella and the trabeculae is defined subchondral bone (Madry et al., 2011). The bony lamella (or subchondral bone plate) is a solid mass of bone with a varying thicknesses ranging from 0.2–0.4 mm in humans. Differently from cartilage, the trabecula of the subchondral bone is highly vascularized and contains nutrients for both itself and the adjacent articular cartilage, and also contains unmyelinated free nerve endings. The main functions of subchondral bone are the absorption of compressive stress and the maintainance of the joint shape (Kawcak et al., 2001).

It appears clear that these tissues have interconnected functions and are both essentials for the joint health. For these reason, therapies for their contemporaneous repair in the case of osteochondral lesions, have been developed.

Current procedures include mosaicplasty or autograft osteochondral transplant (Bobic, 1996; Matsusue et al., 1993) and osteochondral allograft placement (McDermott et al., 1985). Mosaicplasty involves taking osteochondral plugs from a non-load-bearing area of the patient's own joint (autografts) and transplanting these plugs into the disease site. Although it represents a promising approach to restore the biological and mechanical functionality of the joint the clinical use of autologous osteochondral grafts suffers from several limitations such as the low amount of material available, the donor site morbidity and the difficulty to

match the topology of the autografts with the injured site (Martin et al., 2013). Allograft transplantation allow to bypass these shortcomings but it presents the possibility of disease transmission, rejection of allograft tissue, graft versus host disease, and potential need for immunosuppression (Elisseeff et al., 2005).

For these reason, a broad variety of tissue engineering approaches for osteochondral regeneration are developing. Three-dimensional (3D) tissue grafts of pre-defined size and shape can be engineered by combining cells with 3D porous biomaterials, which provide the template for tissue development (Martin et al., 2013).

As clearly reported in a recent review by Martin et al. (Martin et al., 2013) different approaches could be classified on the basis of different cell and scaffold types (figure 3). According to this classification, cells could be I) of a single source and have chondrogenic capacity, (II) of two cell sources and have either chondrogenic or osteogenic capacities, (III) of a single cell source and have both chondrogenic and osteogenic differentiation capacity, (IV) or could also be absent (cell-free approach). Moreover, the construct can be generated using: (A) a scaffold for the bone component (ceramics, poly (lactic-co-glycolic acid) or polylactic acid scaffolds, or devitalized bone) but a scaffold-free approach for the cartilage component, (B) different scaffolds for the bone (as above) and the cartilage (polylactic acid scaffolds, hyaluronic acid sponges or poly (glycolic acid) meshes) components combined at the time of implantation, (C) a single but heterogeneous composite scaffold, or (D) a single homogenous scaffold for both components such as poly (ethylen-glycol) hydrogels.

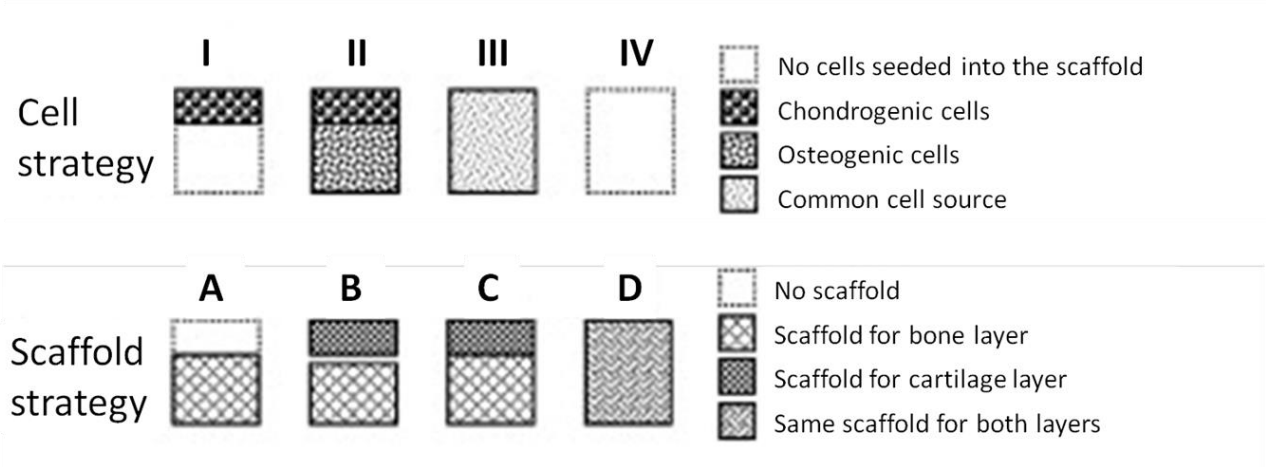

Figure 3. Modified from Martin et al, 2013 (Martin et al., 2013).

Regarding the cell type to be used for the constructs generation great attention have been paid on chondrocytes and mesenchymal stem cells (either differentiated or not). Taking into account the greater healing potential of bone compared to cartilage (an avascular, aneural tissue) and the presence of osteoprogenitors in the blood deriving from subchondral bone fractures, an obvious strategy could be to favour cartilage regeneration using differentiated chondrocytes (Figure 3 I, II). Despite this premise, the use of chondrocytes is not always so effective (Kon et al., 2009). Moreover, the use of autologous chondrocytes present some complications; first of all, for clinical purposes, chondrocytes should be culture expanded before their use. This, has been proved to lead to a de-differentiation of these cells, with changes in morphology and down-regulation of specific genes. Although this problem seem to be resolvable culturing chondrocytes with specific growth factor such as FGF-2 or TGF-β1 (Barbero et al., 2003) this technique imply a further manipulation of the cells. To avoid the limitations of de-differentiated chondrocytes some groups are investigating the potential effect of non-digested finely minced cartilage fragments in the repair of experimental cartilage defect (Frisbie, 2005; Lu et al., 2005). However, in all these approaches, the donor site morbidity represent an important drawback. All these reasons, pushed several groups to investigate the potential of MSCs for both bone and cartilage repair. Recent studies report the use of BMSC for osteochondral defect repair in different animal models (Gao et al., 2001; Oshima et al., 2004; Uematsu et al., 2005) and in a few clinical cases (Wakitani et al., 2004). Nonetheless, whether the regenerated cartilage does not remodel into bone in the long-term, still has to be validated. This concern arouses from the fact that when chondrodifferentiated BMSCs are exposed to osteogenic stimuli, they express hypertrophic chondrocytes markers (Mackay et al., 1998; Winter et al., 2003) and mineralize the deposited matrix (Mackay et al., 1998; Muraglia et al., 1998). Among MSC, also adipose-derived stem cells have been used in preclinical model of osteochondral defects (Nathan et al., 2003). Although the promising results, whether these cells are able to regenerate the tissue or to provide support to resident cells, still have to be established. Moreover, to validate these cell efficacy for osteochondral defect repair, a larger animal study, is required.
Concerning the choice of the scaffold a possible approach is to use a biomaterial supporting bone repair and to seed on its top cells for cartilage regeneration (Figure 3 A). Good results were obtained both *in vitro* (Tuli et al., 2004; Wang et al., 2004) and *in vivo* (Kandel et al., 2006) seeding chondrocytes (Kandel et al., 2006; Wang et al., 2004) or MSCs “committed”

with chondrogenic stimuli (Tuli et al., 2004), on ceramic materials. Alternative techniques consider that different supporting structure are required for both bone and cartilage regeneration (3 B, C). One approach consists in the creation of two different bioconstruct *in vitro* and then in the suture of the two part before the *in vivo* implantation. Two procedures, both successful, included cells terminally differentiated (chondrocytes and periosteal-derived cells) (Schaefer et al., 2000) or progenitors (MSCs) cultured before the seeding with either chondrogenic or osteogenic supplements (Gao et al., 2001; Schaefer et al., 2002). Another approach, consists in heterogeneous scaffolds composed of two distinct but integrated layers for the cartilage and bone regions. Various groups choose this option, combining the multilayer scaffolds with different kind of cells (Duan et al., 2013; Frenkel et al., 2005; Kim et al., 2013; Kon et al., 2011; Kon et al., 2010a; Martin et al., 2007; Niederauer et al., 2000; Schek et al., 2004).

Finally, different procedures have been applied to an homogeneous scaffold approach. The use of two cell types having chondrogenic (rib chondrocytes) and osteogenic (MSCs) capacity was adopted by Xue et al. to a polycaprolactone (PCL) scaffolds (Xue et al., 2013). Another strategy consist in the use of cells from a single source, but previously *in vitro* differentiated. In the Alhadlaq et al study (Alhadlaq et al 2004) rat bone marrow-derived MPC were expanded, induced separately to chondrogenic or osteogenic differentiation and then loaded on the scaffold.

Also undifferentiated MSCs have been used in association with homogenous scaffold in a porcine model (Lim et al., 2013). Cell-free approaches in which the scaffold was either impregnated (Fukuda et al., 2005) or not (Hui et al., 2013) with fibroblast growth factor-2 (FGF-2) were also described.

In order to improve cell differentiation and tissue development many groups have developed bioreactors to apply mechanical stimuli to cell-seeded scaffolds, in order to simulate the specific physiological forces acting during joint loading. Indeed physical stimuli can modulate chondrocytes and osteoblasts metabolism, and upregulate the production of extracellular matrix components.

A number of studies have shown that dynamic compression applied with a specific magnitude and frequency stimulated chondrocyte metabolism and enhanced cartilage ECM production (Buschmann et al., 1995; Davisson et al., 2002; Kisiday et al., 2004; Lee and Bader, 1997). Similarly, physiological strain magnitudes applied with a four-point bending

bioreactor enhanced osteogenic differentiation and mineralized matrix deposition of human BMSC, pre-cultured within partially demineralized bone matrix (Mauney et al., 2004).

Swine as animal models

In vivo animal studies are essential to test biocompatibility, tissue response and mechanical function of a cell/scaffold construct, in order to close the gap between *in vitro* experiments and human clinical trials.

A part from the application, desirable characteristics of an animal model include similarity with humans, both in terms of physiological and pathological aspects, together with the possibility to observe numerous subjects over a relatively short time frame (Egermann et al., 2005; Liebschner, 2004). Other features to be considered are the costs for the animal purchase and housing, availability, acceptability to society, tolerance to captivity and ease of housing. In addition to this, the lifespan of the species chosen should be suitable for the duration of the study. More specifically, for studies investigating implant interactions, an understanding of the species specific tissue characteristics, such as microstructure and composition, are important for the results extrapolation to the human situation. Small animal models such as mice, rats and rabbits offer advantages for mechanistic study, evaluation of allogenic and xenogenic strategies, and initial feasibility studies. However considering their use for orthopaedic tissue engineering studies, these models present important drawbacks such as the gross differences in the bone anatomy between these animals and the human one. Moreover, the joints of small animals do not adequately mimic those of humans, being much smaller and having thinner cartilage, and consequently they do not allow the creation of defects comparable in dimension with human ones. For these reasons, large animal models (canine, caprine, swine, and equine models) could replicate the human clinical scenario in a better way. Among them, swine are reported as the subjects of choice in a wide variety of the dental, maxillofacial and orthopaedic tissue engineering studies (Ciocca et al., 2009; Wang et al., 2007). These animal bone share several features with the human one, such as rate of healing, morphology, anatomy (Thorwarth et al., 2005), mineral density and composition (Aerssens et al., 1998). Furthermore, the oral maxillo-facial region of these animals is similar in anatomy, development, physiology, patho-physiology, and disease occurrence to the human one (Wang et al., 2007). Therefore these animals might be considered appropriate models of oral disease and in oro- facial research, indeed

they have been recently used in preclinical models of dental implantology (Gahlert et al., 2007; Nkenke et al., 2003; Terheyden et al., 1999) and maxillo-facial surgery (Henkel et al., 2005; Wilson et al., 2012; Wiltfang et al., 2004). Concerning the study of osteochondral defects treatment, swine are an appropriate model as their joint size, weight-bearing requirements, and cartilage thickness more closely imitate the human condition than canine and smaller animal models. Despite pig models have been used to study osteochondral defect treatment (Chang et al., 2011), these animals are generally considered undesirable for orthopaedic research due to their large growth rates and excessive final body weight. However, the development of minipigs has partially overcome this problem.

Minipig have docile and gentle behaviour, and also present similarity with adult humans in terms of weight (70-80 kg). Different studies have shown that, in these animal models the process of bone remodeling, bone structure, and cartilage thickness are quite similar to humans (Frisbie et al., 2006; Zelle et al., 2007). Different studies of chondral and osteochondral defect healing have been performed using this animal model (Harman et al., 2006), showing promising results in the field of tissue engineering.

MATERIALS AND METHODS

Patient and swine enrolment and tissues harvesting

For the *in vitro* study, human adipose tissues were harvested from patients that underwent tooth extraction and were selected by using the clinical criteria reported in Table 2. The enrolled patients (table 1) were in good health without any systemic complication. The informed consent and experimental protocols for this study were reviewed and approved after written consent by the Ethics Committee and Institutional Review Board of University of Milan (authorization #17/12 of April 23, 2012).

The surgery was performed by a maxillofacial surgeon. Local anaesthesia was performed (carbocaine 2% and adrenaline 1:100,000) either in the oral cavity or in the subcutaneous fat tissue; the surgical access for liposuction was performed at the navel using the lipofilling technique: fat was harvested with a sterile 10-mL BD Luer Lock syringe performing a constant manual negative pressure (plunger positioned between 1 and 2 mL) (Ciuci and Obagi, 2008; Coleman and Saboeiro, 2007) The tooth was extracted and different tissues were collected: alveolar bone, as a result of modelling the residual extraction socket; periodontal ligament; and tooth portions, coronal and root. Finally, BFP was approached, cutting the mucosa in proximity to the second molar teeth, 0.5 cm from the fornix. Blunt dissection was performed to prevent damage, to close the anatomical structures, and to allow the identification and exteriorization of the fat pad. BFP was stretched into the wound to close the residual defect and extra tissue was cut (Fig. 1). This technique is widely used either for aesthetic or reconstructive reasons (Amin et al., 2005; Pelo et al., 2008). At the end of the procedure, Ethilon 5.0 sutures were used to close the surgical access.

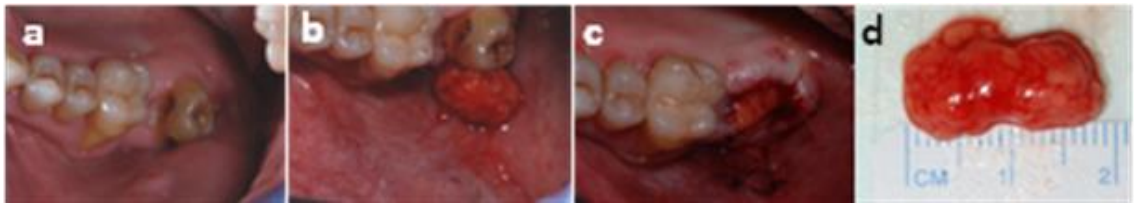

Figure 1. Extraction of Buccal/Bichat's fat pad (BFP). (a) Preoperative conditions. Poor oral hygiene; 26 compromised by periodontal disease, absence of 27, and caries infiltrating 28. (b) Exposure of BFP. (c) Tunnelling and positioning of the BFP to repair defect. (d) Waste material of the BFP at the end of surgery.

Porcine fat tissues and blood samples were collected from 6 swine (3 pigs and 3 minipigs, table 1), at the end of preclinical studies approved by the Italian Ministry of Health and were

performed at the CRABBC (Biotech Research Centre for Cardiothoracic Applications) (Rivolta d'Adda - CR). All the procedures were carried out in conformity with institutional guidelines in compliance with national (Law 116/92, Authorization n.169/94-A issued December 19, 1994, by the Italian Ministry of Health) and international laws and policies (EEC Council Directive 86/609, OJ L 358. 1, December 12, 1987).

			Gender	Age	n	Raw adipose tissue (ml)
ASCs	human	BFP	1 ♂, 1 ♀	35,5± 2,7 years	2	0,8± 0,2
		SC	1 ♂, 1 ♀	35,5± 2,7 years	2	32,5±22,9
	porcine	BFP	3 ♂, 3 ♀	≥5 months	6	5,7 ± 1,5
		SC	3 ♂, 3 ♀	≥5 months	6	12,3 ± 3,6

Table 1. Gender and age of human donors and animals used for the *in vitro* study. Amount of harvested fat from buccal fat pad (BFP) and subcutaneous adipose tissue (SC) is also reported (data are expressed as mean±SEM).

Clinical inclusion criteria	• Patients classified as ASA I - II. • Males and females, between 18 and 70 years of age, with compromised wisdom teeth and thin bone at the maxillary sinus. • Possibility of withdrawing subcutaneous adipose tissue from abdominal region at the same time.
Clinical exclusion criteria	• Disorders of the parotid glands. • Disorders affecting fat and carbohydrate metabolism • Presence of adequate bone support under remaining teeth. • No risk of oro-sinusal comunication after tooth extraction. • Medical contraindications to elective surgery.

Table 2. Enrolment Criteria for Patients

For the *in vivo* study, seven adult (12 months old) male Yucatan minipigs, with an average weight of 73.5±2.2 kg (range 71-77 Kg), were included. Animal care and surgery were approved by the Ethical and Technical Committee of the Italian Ministry of Health (CRABCC-22-2011); all the animal experiments were performed at CRABCC, Italy, in accordance with

both policies and principles of laboratory animal care and with the European Union guidelines (86/609/EEC) approved by the Italian Ministry of Health (Law 116/92).

During the first surgical procedure, upon shaving and disinfection, adipose tissue (~ 8 g) was harvested from the interscapular region of all the animals. General anaesthesia was induced by intramuscular injection of a combination of ketamine (10mg/kg) and midazolam (0.5mg/kg) and maintained via inhalation of a mixture of isoflurane 4% in oxygen. The small incision was then sutured and the animals were administered once with tramadol (1mg/kg) and meloxicam (0.4 mg/kg) to control pain.

Human ASCs to be used in the *in vivo* study were isolated from waste adipose tissue from aesthetic liposuction of a female donor (age 61, BMI<30 kg/m^2, no metabolic disease) after informed consent and Institutional Review Board (IRB) approval of Galeazzi Orthopaedic Institute, Milano, Italy, as previously described (de Girolamo et al., 2009).

All the tissue harvesting procedures were carried out by maxillofacial, plastic or veterinary surgeons.

Characterization of Adipose-derived Stem Cells (ASCs)

Isolation

Adipose-derived stem cells (ASCs) were isolated as previously described (de Girolamo, Arrigoni). Briefly, human and porcine tissues were enzymatically digested at 37°C with 0.075 and 0.1 % type I collagenase (225 U/mg; Worthington, Lakewood, NJ) for 30 and 60 minutes, respectively. The stromal vascular fraction (SVF) was centrifuged, filtered and 10^5cells/cm^2 were plated in DMEM (Sigma-Aldrich, Milan, Italy) supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine (Sigma-Aldrich) (control medium, CTRL). Cells were maintained at 37°C in a humidified atmosphere with 5% CO2. When human and porcine ASCs reached 70-80% confluence, they were detached with 0.5% trypsin/ 0.2% EDTA (Sigma-Aldrich) and plated at a density of 10^4 and 5 x10^3 cells/cm^2, respectively.

Proliferation

Both cell types were maintained in culture for 21 days and counted every week. Doubling time was calculated as (ln [N/N_0])/(ln 2), where N is the number of the counted cells and N_0 represents the number of plated cells.

MTT cell viability assay

To test the viability of cells, $3x10^4$ human ASCs/cm^2 and $1.5x10^4$ porcine ASCs/cm^2 were plated in 96-well plates, and monitored at day 1 and 7. 100 µL of MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide Sigma-Aldrich) (final concentration 0.5 mg/ml in DMEM) were added and cells were maintained for 4 additional hours at 37°C. Formazan precipitates were solubilized by 100% DMSO (dimethyl-sulphoxide, Sigma-Aldrich) and absorbance was read at 570 nm in a Wallac

Victor II plate reader (Perkin Elmer Western Europe, Monza, Italy).

Fibroblast-colony-forming unit assay (CFU-F)

ASCs, were plated in DMEM supplemented with 20% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine, in 6-well plates by serial dilution starting from 1000 cells/well. After 6 days the medium was replaced and, at day 14 and 10 for human and porcine cells, respectively, ASCs were washed, fixed in 100% methanol and stained with 0.5% crystal violet (Fluka, Buchs, Switzerland). The frequency of the CFU-F was established by counting individual colonies (of at least 25 cells) compared to the number of seeded cells.

Flow cytometry analysis

ASCs (3×10^5) in PBS with 1% FBS/0.1% NaN_3 per sample were incubated for 30 minutes on ice with monoclonal antibodies raised against CD14, CD31 CD34, CD73, CD90, and CD105 (Ancell, Bayport, MN). Specific binding was revealed by either streptavidin-PE– or fluorescein isothiocyanate–conjugated sheep anti-mouse antibody. Samples were acquired by FACS Calibur flow cytometer (BD Biosciences Europe, Erembodegem, Belgium) and data were analyzed using CellQuest software (BD Biosciences Europe).

Osteogenic differentiation

$7{,}75x10^3$ hASCs/cm^2 at 4^{th} and 7^{th} passages and $5x10^3$pASCs/cm^2 at 3^{rd} and 4^{th} passages were maintained for 14 days in either control or osteogenic medium (OSTEO, DMEM, 10% FBS, 10 nM dexamethasone, 10 mM glycerol-2-phosphate, 150 µM L-ascorbic acid-2- phosphate, 10 nM cholecalciferol, Sigma-Aldrich).

To evaluate alkaline phosphatase (ALP) enzymatic activity, both undifferentiated and differentiated ASCs were lysed in 50 µl of 0.1% Triton X-100 and incubated at 37°C with

10mM p-nitrophenylphosphate dissolved in 100 mM diethanolamine and 0.5 mM $MgCl_2$, pH 10.5. Samples were read at 405 nm and ALP activity was standardized respect to the sample protein concentration determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL). To determine collagen production, cells were stained with 0.1% (w/v) Sirius Red F3BA in saturated picric acid (Sigma-Aldrich) for 1 hour at room temperature, and then the samples were extracted with 0.1 M NaOH for 5 minutes and absorbance was read at 550 nm (Tullberg-Reinert and Jundt, 1999). Standard curve of known concentration of calf skin type I collagen (Sigma-Aldrich) was used to determine the concentration of secreted collagen.
Extracellular matrix (ECM) calcification was determined on fixed ASCs stained by 40 mM Alizarin Red-S (AR-S, pH 4.1; Fluka). Mineral deposition was quantified by incubating the stained sample with 10% w/v cetylpyridinium chloride (CPC; Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) for 15 minutes to extract AR-S. Absorbance was read at 550 nm with Wallac Victor II plate reader (Halvorsen et al., 2001).

Adipogenic differentiation

ASCs from both sites of harvesting were induced to differentiate towards the adipogenic lineage as previously described with some modifications (de Girolamo et al., 2009). Briefly $1.5x10^4$ ASCs /cm^2 were plated and cultured in control medium supplemented with 1 µM dexamethasone, 10 µg/ml insulin, 500 µM 3-isobutyl-1-methylxanthine, and 200 µM indomethacin (Sigma-Aldrich). 14 days later, cells were fixed in 10% neutral buffered formalin for 1 hour and stained by fresh Oil Red O solution (20 mg/mL [w/v] Oil Red O in 60% isopropanol) for 15 minutes. Lipid vacuoles were quantified by extraction with 200 µl of 100% isopropanol for 10 minutes and reading the absorbance of 50 µl at 490 nm with Wallac Victor II plate reader.

Chondrongenic differentiation

$5x10^5$ ASCs were cultured in micromasses in chondrogenic medium (DMEM supplemented with 1% FBS, 100 nM dexamethasone, 110 mg/l sodium pyruvate, 150 µM L-ascorbic acid-2-9 phosphate, 1x insulin-transferrin selenium (ITS) and 10 ng/ml TGF-β1) for 21 days. Glycosaminoglycans (GAGs) production was assessed by dimethylmethylene blue (DMMB) assay as previously described (Farndale et al., 1986; Wolf et al., 2008). Briefly, micromasses were digested at 56°C overnight by 100µl of 50 µg/ml proteinase K in 100 mM K2HPO4 (pH

8.0). After 10 minutes at 90°C to inactivate the enzyme, the samples were spun at 14000 g for 10 minutes and each supernatant was collected for GAGs. Samples were then incubated at room temperature in 40 mM glycine/NaCl (pH 3) with 16 mg/ml DMMB and the absorbance was read at 500 nm with the Wallac Victor II plate reader. The amount of GAGs was determined respect to known concentrations of chondroitin sulfate (Sigma-Aldrich).

ASCs and biomaterials

pASC culture and osteogenic differentiation on biomaterials

Titanium is a material widely used in dental surgery, due to its high mechanical and corrosion resistance as well as its biocompatibility. Titanium disks were kindly provided by Permedica S.p.A., Merate, Italy.

Silicon carbide (SIC), with its hardness and wear-resistance, could be an innovative material suitable to coat metallic implants giving an adequate protection to the material and decreasing the wear rate of the inserted devices. SIC used in this study was kindly provided by CETEV - Centro Tecnologico del Vuoto, Carsoli - AQ, Italy).

Both BFP- and SC-pASCs were seeded at $5x10^3/cm^2$ on titanium disk and silicon carbide–plasma-enhanced chemical vapour deposition (SIC) fragments in a 24-wells plate either in CTRL or OSTEO medium. In order to determine cells adhering to the biomaterials, both undifferentiated and differentiated ASCs for 21 days, were lysed in 0.1% Triton X-100 and protein concentration was determined by BCA Protein Assay. Meanwhile, in adjacent wells, calcified ECM deposition was determined, as described above, and compared to the one produced by plastic-adherent (PA) cells.

To visualize the adhesion of ASCs on scaffolds we cultured both ScI- and BFP-ASCs on titanium disks for seven days. Then, cells were stained with Calcein-AM (Fluka) and Hoecst 33258 and examined with a confocal microscope (Leica model TCS SP5; Leica Microsystems CMS GmbH, Mannheim, Germany) using a 20x dry objective (HC PL FLUOTAR 20.0 x 0.50 DRY) and 40x immersion oil objective (HCX PL APO Lambda blue 40,0X 1,25 oil UV).A 488 nm laser line was used to excite calcein while the fluorescent emission was detected from 500 to 540 nm. Hoechst was excited with 405 nm laser line, and its fluorescent emission was detected from 615 to 510 nm). Moreover, using a third laser line (633 nm) in reflection mode, it was possible to determine with high accuracy the titanium disk (starting acquisition point) reflecting surface.

hASCs-scaffold interaction determination by scanning electron microscopy

ASCs (1 x 10^5 and 3 x 10^4) were grown for 4 days on natural supports (alveolar bone and periodontal ligament) and on synthetic scaffolds (collagen membrane; Euroresearch, Milan, Italy and polyglycol acid filaments; Resorb, Sweden and Martina, Padova, Italy), respectively. The constructs were fixed in glutaraldehyde (2% in 0.1M sodium cacodylate buffer) overnight at 4°C and dehydrated with a series of graded ethanol (from 75% to 100%) for 4–5 h at room temperature and subjected to drying using critical point drying (CPD; Baltec C.P.D. 030). Samples were mounted on aluminium stubs, sputter-coated with gold (Balzers MED 010) and analyzed by scanning electron microscopy (SEM; Sem Quanta Feg 250 esem).

hASCs and Amelogenin (AM) treatment

Amelogenin (30mg/ml) was provided in a propylen glycol alginate water solution (Emdogain, Straumann, Basel, Switzerland). Both BFP- and SC-hASCs (1.5 x 10^4/well) were seeded in a 24-well culture plate, with wells previously spread with a drop of AM (~100µl), and cultured in either control or osteogenic medium for seven days. Then ALP activity and collagen deposition were evaluated as previously described. As control, we considered ASCs cultured in the absence of AM.

Proliferation of ASCs in medium supplemented with autologous or heterologous sera

Serum collection

10 ml of blood from each human donor and animal were allowed to clot for 30-45 min at 37°C and then transferred at 4°C for 30 minutes. After centrifugation (1000 g for 10 minutes) sera were collected under sterile conditions (Schwarz et al., 2012) and maintained at -20°C until their use.

Cell maintenance

ASCs (6 x 10^4/well) were cultured in 6-well plates in DMEM supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine and either 10% or 5% autologous serum (AS) or 5% heterologous serum (HS). ASCs were also grown in the presence of 10%

FBS as control. Cells were cultured for four passages; they were detached and replated every 7 days and counted each time.

In vivo experiments

Scaffold preparation

Oligo(polyethylene glycol) fumarate (OPF) was synthesized by MAYO Clinic (Rochester, Minnesota) using polyethylene glycol (PEG) with the initial molecular weight of 10,000 as previously described (Jo et al., 2001). Porous negatively charged hydrogels were made by dissolving 1 g OPF macromer and 0.1 g sodium methacrylate in 2 ml deionized water containing 0.15% (w/w) Irgacure 2959 (Ciba-Specialty Chemicals, Tarrytown, NY, USA) and 300 µl N-vinyl pyrrolidinone. To obtain hydrogels with 75% porosity, the resulting solution was mixed with sodium chloride (particle size, 300 µm) at a ratio of 1:3 (W/W), and polymerized using 365 nm UV light at the intensity of ~8 mW/cm2 (Black-Ray Model 100AP, Upland, CA, USA) for 30 min. Cylindrical hydrogel scaffolds were cut using a cork borer and placed in deionized water for 48h with 4-5 changes.

We received OPF in a not sterile dehydrated form (figure 2A). To prepare the scaffold for its use, restoring its structure, properties and dimension (9 mm in diameter, 8 mm in depth), we hydrated it with PBS1X (figure 2B). Then we sterilized it trough immersion in 70% ethanol for one hour. After three consecutive washing in PBS1x (30 minutes/each) the scaffolds were put in colture medium (figure 2C).

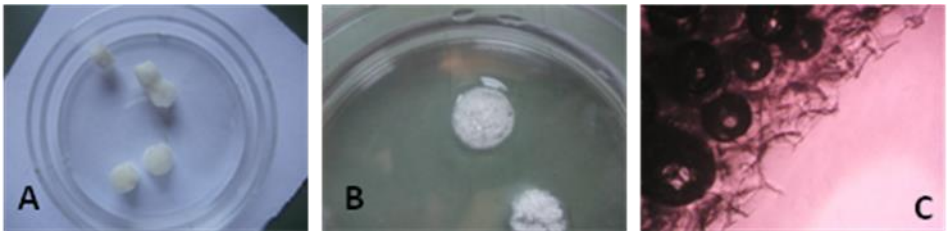

Figure 2. *Dehydrated hydrogel (A); hydrated hydrogel in PBS1X (B); microphotographs of the the hydrogel in colture medium (C, 40x magnification).*

Experimental design and surgical procedure

After the removal of the medium excess from the scaffolds, laying them down on sterile lint for few seconds, undifferentiated porcine or human ASCs, detached at 3rd passage, (3×10^6 cells/300 µl medium) were loaded on OPF. Seeded scaffolds were maintained for 30 minutes

in the incubator in order to allow cells-scaffold interaction. Then 4 ml of control medium were added to the constructs that were kept in the incubator overnight. Cell-free scaffolds followed identical procedure. Next day, the minipigs were surgically treated under general anaesthesia by veterinary surgeons. With the animals in dorsal recumbence, a medial parapatellar incision and arthrotomy were performed on the right hind leg to expose the anterior aspect of the distal femur. Using a standardized core punch, four osteochondral lesions (9 mm in diameter, 8 mm in depth each) per stifle joint were created in the trochlea periphery (Figure 3).

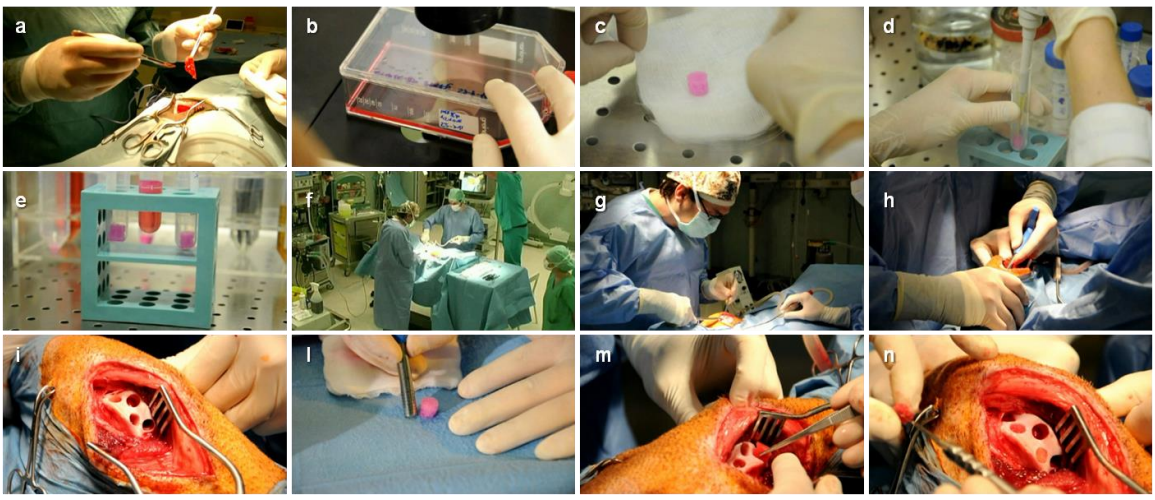

Figure 3. Images of the early phases of the study. Adipose tissue harvesting from the interscapular region (a); ASC expansion *in vitro* (b); OPF scaffold preparation (c); ASC seeding on biomaterial (d); incubation of the construct (e); creation of osteochondral lesions in the trochlea periphery of the knee joint of minipigs (f-g-h); construct preparation and implantation (i-l-m-n).

After implantation, the joint capsule was closed and the wound was sutured in layers with bioabsorbable stitches. After surgery the minipigs were treated at first with enrofloxacin (2 mg/kg IV) and amoxicillin (15mg/kg IM); then, with enrofloxacin (5mg/kg die for 5 days IM) and meloxicam (0,4 mg/kg die for three days) to control pain. The animals were allowed to free movements and carefully monitored until full recovery.

Six months later, all the animals were euthanized by IV injection of pentothal sodium (50 mg/kg) and potassium chloride (20 mg/kg); the right hind legs were explanted and then analysed by magnetic resonance imaging (MRI). Then, the joints were dissected, the treated portions retrieved en bloc and cut into four pieces corresponding to the four osteochondral defects, to allow independent analyses of each defect. Each single defect was prepared for further analyses. The contralateral hind legs were used as controls.

At the same time fat from buccal fat pad and interscapular region was also removed and processed as described above.

Macroscopic analysis

Macroscopic signs of infection, inflammation, hypertrophy of the synovial membrane and tissue adhesions were assessed. The gross chondral surface was evaluated, taking into account the neo-formed tissue and the interface implant-host tissue. Signs of incomplete filling, matrix degradation, fusion between the different osteochondral defects and other possible features were recorded.

MRI analysis

The right hind leg of all animals and one control left leg were imaged by MRI. Examinations were performed using two 1,5 T magnetic field super conducting MR Systems (Avanto, Siemens Medical Solution, Erlangen, Germany, gradient strength 45 mT/m, slew rate 200 T/m/ms; Espree, Siemens Medical Solution, Erlangen, Germany, gradient strength 33 mT/m, slew rate 170 T/m/ms), with a dedicated 8-channel knee coil (Invivo, Gainesville, FL, USA).

The hind limb of minipigs were positioned with the knee extended and with the joint space in the middle of the coil. Lesions were studied with the following sequences:

- PD-TSE FS SPACE sequence on sagittal plane (Repetition Time [TR]/Echo Time [TE]: 1200/35; Field of View [FOV]: 170x170 mm; Matrix: 320x320; Slice Thickness: 1 mm; Voxel Size: 0.5x0.5x1 mm; Number of Slice: 60; scan time: 9 min, 2 sec;
- T2-weighted TSE sequence on sagittal plane (TR/TE: 3300/83; Flip angle: 150°; FOV: 180x180 mm; Matrix: 320x320; Slice Thickness: 3 mm; Voxel Size: 0.6x0.6x3 mm; number of slice: 20; scan time: 4 min, 1 sec;
- T1-weighted Vibe Water Excitation (WE) GE sequence on sagittal plane (TR/TE: 15.6/6.65; Flip angle: 12°; FOV: 200x160 mm; Matrix: 256x256; Slice Thickness: 1 mm; Voxel Size: 0.8x0.8x1 mm; number of slice: 72; scan time: 5 min, 45 sec;
- T1-weighted sequence on sagittal plane (TR/TE: 560/10; Flip angle: 143°; FOV: 160x160 mm; Matrix: 384x310; Slice Thickness: 2.5 mm; Voxel Size: 0.5x0.4x2.5 mm; number of slice: 20; scan time: 5 min, 14 sec.

Post-processing was performed on a dedicated workstation (Leonardo, Siemens Medical Solution, Forchein, Germany). Images evaluation was performed by two experienced senior

musculoskeletal radiologists. MRI data were analysed by a modified 2D MOCART score for the evaluation of *ex vivo* osteochondral samples (Goebel et al., 2012).

Histological and immunohistochemical analysis

The samples were dissected free of soft tissue, fixed in 10% buffered formalin for 24 hours at room temperature, and then decalcified in a formic acid-sodium citrate solution as described elsewhere (de Girolamo et al., 2011). After decalcification procedure, samples were rinsed for 10 min in running water, and processed for paraffin embedding through a graded ethanol series. Four micrometer-thick sections were obtained and stained with Safranin-O following a standard protocol, for the evaluation of the structural details and GAGs deposition.

After rehydration, sections for immunohistochemical analyses were incubated in an aqueous solution of 1% H_2O_2 for 30 min at room temperature, washed 3 times in PBS and then incubated overnight with either mouse anti-collagen type I antibody or anti-collagen type II (both Chondrex Inc, Redmond, WA, USA; 1:500). Antigen retrieval was performed by treating the sections in citrate buffer, pH 6.0, in a microwave oven (2 times for 5 min at 500 W) for anti-collagen type I and with 2% hyaluronidase solution (Sigma-Aldrich) at room temperature for 30 min for anti-collagen type II. Antigen–antibody complexes were detected with a peroxidase-conjugated polymer which carries secondary antibody molecules directed against mouse immunoglobulins (EnVisionTM+, DakoCytomation, Milan, Italy) applied for 60 min at room temperature. Peroxydase activity was detected with diaminobenzidine (DAB, DakoCytomation) as substrate. Finally, the sections were incubated with mouse anti-human nuclei (Millipore, Billerica, MA, USA, 1:50) and treated as described above. For all the immunohistochemical procedures the samples were weakly counterstained with Mayer's hematoxylin, dehydrated, and permanently mounted. The specificity of anti-collagen type I or type II antibody was also assessed. Photomicrographs were taken with an Olympus BX51 microscope (Olympus, Milan, Italy) equipped with a digital camera and final magnifications were calculated. The sections were also analyzed with a polarized light microscope (Leica DM LP microscope, Leica Microsystems, Wetzlar, Germany) in order to investigate the tissue organization, referring to collagen fibers of the newly formed structures.

Histological scoring

Oriented histological specimens for each group, with the subchondral bone, and stained with Safranin-O, were evaluated independently by three experienced blind researchers using the ICRS II scoring system (Mainil-Varlet et al., 2010).

Biomechanical analysis by nanoindentation tests

The indentation tests and the following calculations have been performed by our collaborators at Politecnico di Milano. Unfortunately, due to the limited number of implanted constructs constituted by scaffold combined with allogenic or human cells, no tests on these samples have been conducted.

Experiments

The experiments were performed using a NanoTest Indenter (Micro-Materials Ltd., Wrexham, UK) equipped with a liquid cell to keep samples in a hydrated and fully saturated state. The sample preparation protocol is the following: I) each sample is thawed in a thermal bath at 37 °C for 45 min; II) the sample is glued on a cylindrical aluminum stub equipped with a glass chamber and placed into the nanoindenter; III)the chamber is filled with physiological solution (0.90% w/v of NaCl) and kept at rest before running the tests for the thermal and swelling equilibrium to be achieved.

Nanoindentation tests are carried out following a multiload schedule in load control mode. The maximum load is applied by a series of load steps of increment 0.1 mN with a holding time of 120 sec at the end of each load step. The maximum indentation force is ranging between 0.3 mN and 1 mN according to the sample compliance consistently with the limit of 24 µm set on the maximum indentation depth. The loading and unloading rates are 1 mN/sec and the tests are carried out with two spherical tips having radii R_{25}=25 µm and R_{400} = 400 µm. The indentation modulus is computed according to the Hertz theory for spherical indentation (Johnson et al., 1993) by fitting the force-penetration data achieved at equilibrium. Table 3 reports the details of the indentation tests.

Sample	Tip Radius R [µm]	Total Load Fτ [mN]	Load steps	Indentation sites
Healthy control	25	0.9	0.1	8
Healthy control	400	1	0.1	6
OPF+mpA-ASCs	25	0.7	0.1	3
OPF+mpA-ASCs	400	1	0.1	4
OPF	25	1	0.1	4
OPF	400	1	0.1	4
UNT	25	0.3	0.1	5
UNT	400	0.6	0.1	4

Table 3. Maximum load, load step size and total number of indentation sites for each sample type.

Data Analysis

Parameter P (poroelasticity) proportional to the product of indentation modulus and tissue permeability has been identified by adapting the analytical solution for the vertical displacement of the top surface of poroelastic layer subjected to a load-controlled unconfined test proposed by Biot (Biot et al., 1955) to fit the force-displacement data collected during the spherical indentation: to this purpose, a dimensionless time has been introduced by following Oyen et al. (Oyen et al., 2012). Then, a two-parameters function is used to best fit the creep curves (Taffetani et al., 2013)

Here, the detailed data analysis description is reported.

Consider j the index that counts the load level. The output data at each load level F_L^j of the experimental tests are:

1. The equilibrium (long term) h_{eq}^j and the short term h_s^j indenter displacement or depth. The equilibrium depth represents the penetration depth measured at the end of the j-th creep phase $h_{eq}^j = h^j(t = 120)$. The short term response is that obtained at the end of the loading phase (i.e. at the beginning of the holding phase)
2. The time-domain data $h^j(t)$ which is the displacement history measured for each j-th load level during the holding phase.

Following the theory of the spherical indentation of a purely elastic half space, the load-penetration $(F - h)$ relationship is (Johnson et al., 1993)

$$F = \frac{4}{3} E^* \sqrt[2]{R} h^{3/2} \quad (1)$$

where, E^* is the indentation modulus of the deformable body and R is the radius of the indenter. Eq. (1) can be used to fit the $(F_L - h_{eq})$ and $(F_L - h_s)$ data points to identify equilibrium (M_{eq}) and short term (M_s) indentation moduli. To this purpose the following fitting equations are introduced:

$$F_L = \frac{4}{3} M_{eq} \sqrt[2]{R} h_{eq}^{3/2} \quad (2)$$

$$F_L = \frac{4}{3} M_s \sqrt[2]{R} h_s^{3/2} \quad (3)$$

The indenter displacements measured during the holding phase at each load level $h^j(t)$ is interpolated using the following functions in which the dimensionless time τ^j has been introduced.

$$h^j(\tau) = h_u^j + \overline{P_1^j}\left[g(\tau^j)\right] \quad (4)$$

$$g(\tau) = \sum_{m=0}^{\infty} \frac{8}{(1+2m)^2\pi^2}\left[1 - e^{-(1+2m)^2\pi^2\tau}\right] \quad (5)$$

$$\tau^j = \frac{P_2^j t}{a^2} = \frac{P_2^j t}{R h^j(t)} \quad (6)$$

The parameters P1 and P2 are two fitting parameters: $\overline{P_1^j}(h)[nm]$ is the difference between the equilibrium and the short term displacements; $\overline{P_2^j}(h)[nm^2/s] = k_{ind}^j M_{eq}$ plays the role of a diffusivity parameter having the same physical units of c introduced in Oyen (Oyen et al., 2012) and k_{ind}^j is a permeability parameter for the indentation problem.

From the diffusivity parameter $\overline{P_2^j}(h)$, the indentation permenability is obtained, for each load level j, as

$$k_{ind}^j = \frac{P_2^j}{M_{eq}} \quad (7)$$

To determine a permeability-strain relationship, the information on depth h_{eq}^j is rewritten in terms of equivalent deformation, ϵ_{eq}^j, as proposed by Lin (Lin D 2007)

$$\epsilon_{eq}^j = \frac{\sqrt{2Rh_{eq}^j - h_{eq}^{j\,2}}}{R} \quad (8)$$

where R is equal to R_{400} or R_{25} depending on the test.

A proper normalization for the indenter displacement during the creep phase can be also introduced for each load level j as

$$\overline{h_F^j} = \frac{(h^j(t) - h_{eq}^j)}{(h_s^j - h_{eq}^j)} \quad (9)$$

Statistical analysis

Data are expressed as mean±standard error of the mean (SEM) and statistical analysis was performed using Student's t-test, where not differently indicated. For the histological scoring, analysis of variance stratified for each item was used. In all cases $p<0.05$ was considered statistically significant.

RESULTS 1. Mesenchymal Stem Cells from buccal Fat Pad as a novel population for periodontal regeneration: *in Vitro* Comparison with Adipose-Derived Stem Cells from Subcutaneous Tissue

Characterization of human (h) and porcine (p) buccal fat pad (BFP) and subcutaneous tissue (SC) derived ASCs

Isolation, culture, morphology, clonogenic ability and immunophenotype

We analyzed cell populations derived from two donors enrolled for surgery for upper wisdom tooth extraction with sinus communication, and from 6 swine at the end of preclinical studies (Table 1, materials and methods). MSCs were isolated from both buccal fat pad (BFP-ASCs) and abdominal (for the human donors) or interscapular (for the swine) subcutaneous adipose tissue (SC-ASCs). Cells plated in culture dishes, nicely adhered to plastic assuming a MSC typical fibroblast-like morphology with only mild differences in shape constituted by the more pronounced elongation of human SC-ASCs compared to both human BFP-ASCs and porcine cells (figure 4).

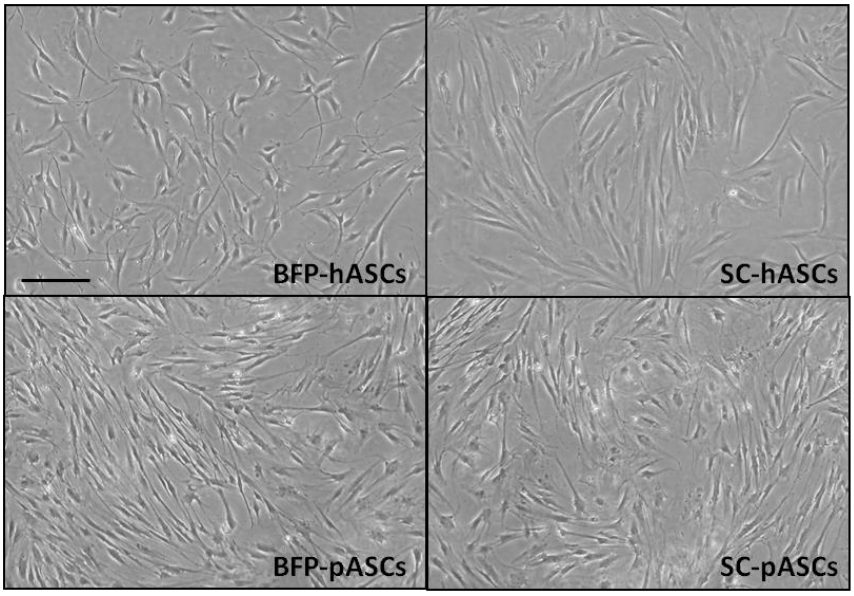

Figure 4. Human (upper panels) and porcine (lower panels) BFP- (left panels) and SC-ASCs (right panels) morphology by optical microscopy (100X magnification, scale bar 50μm).

Despite the different amount of raw tissue harvested from the two sites, we isolated a quite similar amount of cells per millilitre of BFP and SC, while the cellular yield was influenced by the species of origin, being more abundant in human compared to porcine tissues

($1.1x10^5±1.4x10^4$ human BFP-ASCs/ml and $1.15x10^5±7.1x10^3$ human SC-ASCs/ml; $3.0x10^4±9.3x10^3$ porcine BFP-ASCs/ml and $5.5x10^4±3.3x10^4$ porcine SC-ASCs/ml). However, the lower amount of cells from porcine tissues did not hamper subsequent experiments as it was opposed to their high proliferation rate that was constant for both porcine cell populations with a mean doubling time (DT) of about 72.5±8.2 hours for BFP-pASCs and 82.9±11.5 hours for SC-pASCs. This could have allowed us to collect $1.59x10^7±7.4x10^6$ BFP- and $1.00x10^7±4.62x10^6$ SC-pASCs starting from only $6x10^4$ cells, in 21 days (figure 5, right panel). For human cells was observed a slight difference in the proliferation rate related to the tissue source; indeed, while SC-hASCs proliferated as fast as pASCs, the average BFP-hASC doubling time was slightly higher (126.5±33.6 hours compared to 73.5±17.2 hours of SC-hASCs). Anyway, also human cells proliferated efficiently and, after 21 days, starting from $6x10^4$ ASCs, $1.3x10^6±3.0x10^5$ SC-ASCs and $5.9x10^5±2.6x10^5$ BFP-ASCs were collected (figure 5, left panel).

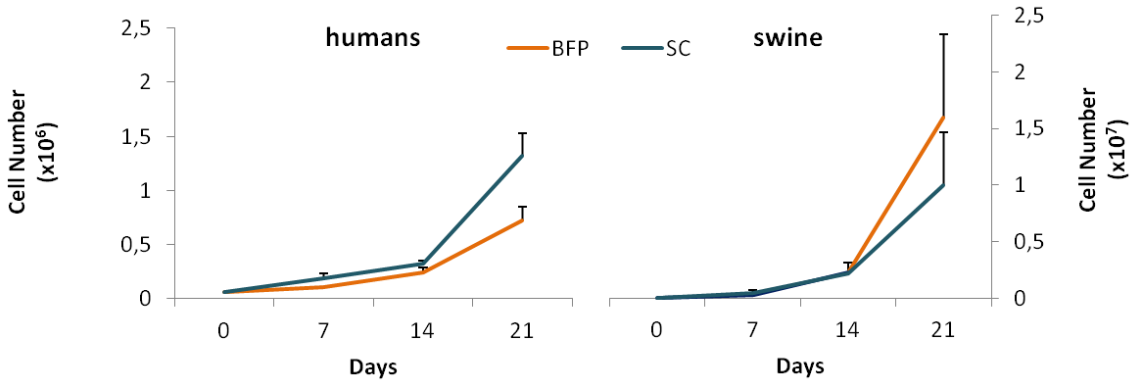

Figure 5. Proliferation trend at early passages of human (left panel, n=2) and porcine (right panel, n=6) BFP- and SC-ASCs assessed by cell counting once a week for 21 days (data are expressed as mean±SEM).

These observations were also confirmed by viability test, indeed, MTT incorporation by SC-hASCs was mildly higher than by BFP-hASCs (figure 6, left panel), while pASCs viability data were superposable (figure 6, right panel).

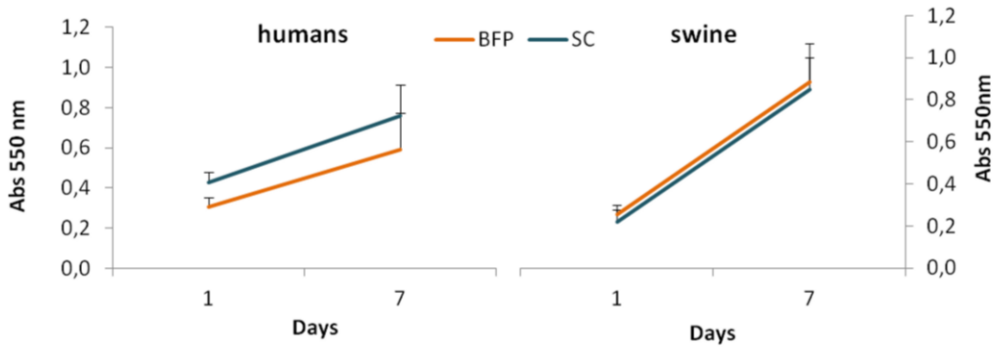

Figure 6. Viability of human (left panel, n=2) and porcine (right panel, n=6) BFP- and SC-ASCs maintained for 1 week in undifferentiated condition (data are expressed as mean±SEM).

All the cell populations, held a good clonogenic ability, at early passages, and only scarce differences were detectable among cells; in particular, while for human cells BFP-ASC CFU-F frequency was slightly superior than SC-hASC one (10.1±2.7% for BFP- and 5.0±0.9% for SC-hASCs, figure 7A), for porcine cells the tendency was inverted (7.8±1.1 for BFP- and 11.9±1.4 for SC-pASCs, figure 7B).

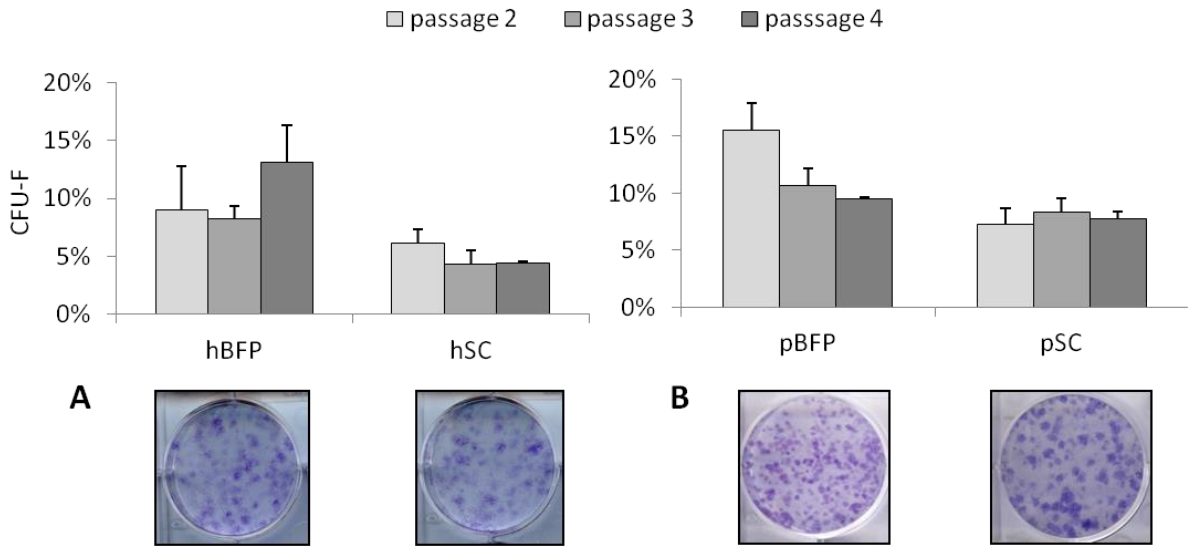

Figure 7. Human (A, upper panel) and porcine (B, upper panel) ASC clonogenicity from passage 2 to 4 expressed as colony forming units (CFU-F) percentage (ratio of number of colonies/number of plated cells x 100) (data are expressed as mean±SEM, n=2 and 6 for human and porcine BFP- and SC-ASCs, respectively). Representative BFP- and SC-ASCs plates stained with crystal violet (lower panel).

The BFP-ASCs were also characterized for their immunophenotype, and a representative cytofluorimetric analysis of BFP-ASCs compared with SC-ASCs is shown in figure 8. Cells from different sources appeared similar even though BFP-hASCs size and granularity were slightly reduced compared to SC-ASCs. Both types of ASCs isolated from human donors expressed

the specific mesenchymal stem cell markers such as CD73, CD90, and CD105, whereas they did not express lymphocyte or leucocyte antigens and hematopoietic markers such as CD14, CD31, and CD34 (table 4). Also porcine cells expressed CD90, whereas no cross-reactivity was found on both SC- and BFP-pASCs for lymphocyte or leucocyte antigens and, unfortunately, for CD73 and CD105.

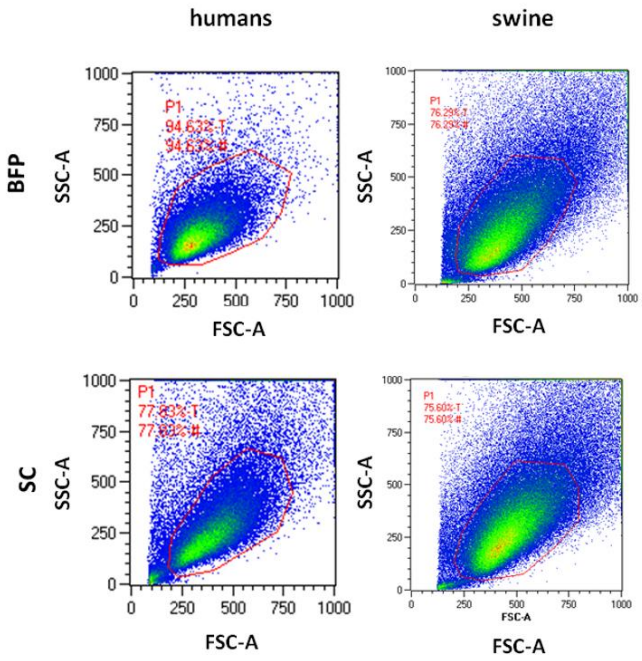

Figure 8. Human (left panels) and porcine (right panels) BFP- (upper panels) and SC-ASCs (lower panels) size and granularity by FACS analysis.

	Human ASCs		Porcine ASCs	
	BFP	SC	BFP	SC
CD90	+	+	+	+
CD73	+	+	ND	ND
CD105	+	+	ND	ND
CD14	-	-	-	-
CD45	-	-	-	-
CD34	-	-	-	-

Table 4. Specific mesenchymal stem cell markers expressed on human and porcine BFP- and SC-ASC populations (+ indicate a number of positive gated cells >98%, n=2).

Multi-differentiative potential

Osteogenic potential

After 14 days of osteogenic induction (OSTEO), both BFP-ASCs and SC-ASCs showed a significant up-regulation of bone specific markers such as ALP activity (figure 9A and B)and collagen (figure 9C and D) and calcified extracellular matrix (ECM) (figure 9E and F) deposition. Basal ALP activity was higher in BFP rather than SC-ASCs from human donors (p=0.058), but the increment during osteogenic induction was almost identical (+435% for BFP- and +456% for SC-hASCs) (figure 9A). In porcine cells we observed an opposite behaviour with increases in ALP activity of about 130% and 363% for BFP and SC-pASCs, respectively (figure 9B). Evaluating the collagen and the calcified extracellular matrix (ECM)

deposition of human cells (figure 9C and E), we observed a contrary tendency respect to the ALP activity data; indeed, SC-hASCs produced more collagen (figure 9C) and calcified ECM (figure 9E) compared to BFP-hASCs, even though this difference is statistically significant only for collagen deposition. Also porcine BFP-ASCs showed a slightly lower basal level of collagen deposition respect to SC-pASCs, but this difference at 14 days was not significant (figure 9D). Regarding calcified matrix deposition, porcine cells were almost the same with an increase in this marker of about 159 and 137% for BFP and SC-pASCs (figure 9F).

No significant differences between human and porcine cells were observed.

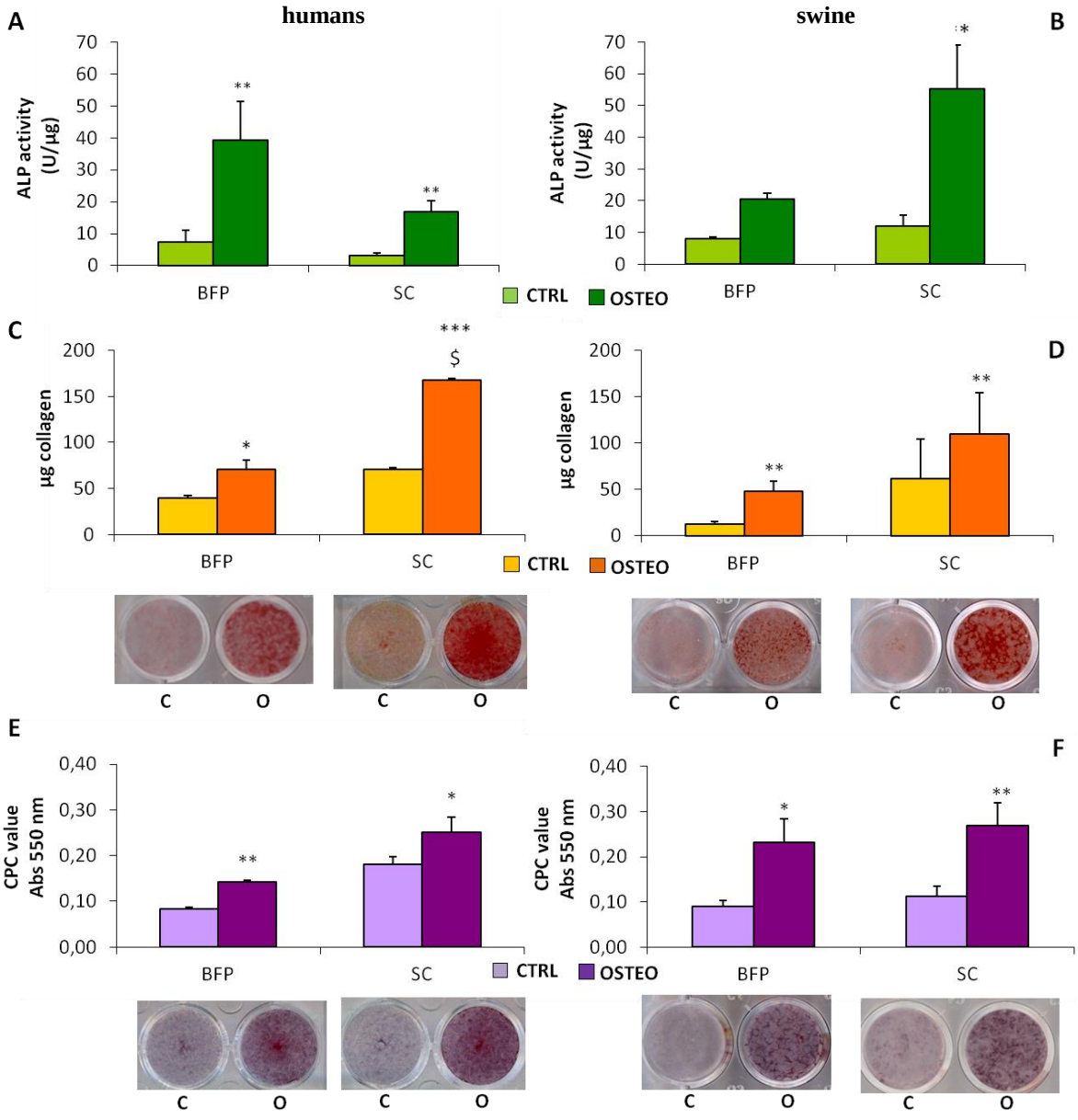

Figure 9. Quantification of alkaline phosphatase activity (A, B), collagen (C, D) and calcified Extracellular Matrix (E, F) deposition, in undifferentiated (CTRL, lighter bars) and osteo-differentiated (OSTEO, darker bars) human

(A, C, E) and porcine (B, D, F) BFP- and SC-ASCs (ALP activity is normalized on protein concentration; data are expressed as mean±SEM, n=4 and 12 for human and porcine cells, respectively). OSTEO vs CTRL *p<.05; **p<.01; ***p<.001. SC- vs BFP-hASCs $<.05. Images of BFP- and SC-ASC wells stained with Sirius Red (C and D, lower panels) and Alizarin Red-S (E and F, lower panels)(C, control; O, osteodifferentiated).

Adipogenic potential

After 2 weeks of adipogenic induction, an important morphological change was induced. The fibroblast-like shape was lost, and all the populations showed intracellular accumulation of lipid vacuoles as shown by Oil Red O (ORO) staining (Figure 10, lower panels). ORO extraction and quantification showed a significant increase in the production of lipid vacuoles in differentiated both human (figure 10A, upper panel) and porcine (figure 10B, upper panel) ASCs, compared to control cells. No difference was observed in the adipogenic potential of ASCs derived from the two anatomical regions.

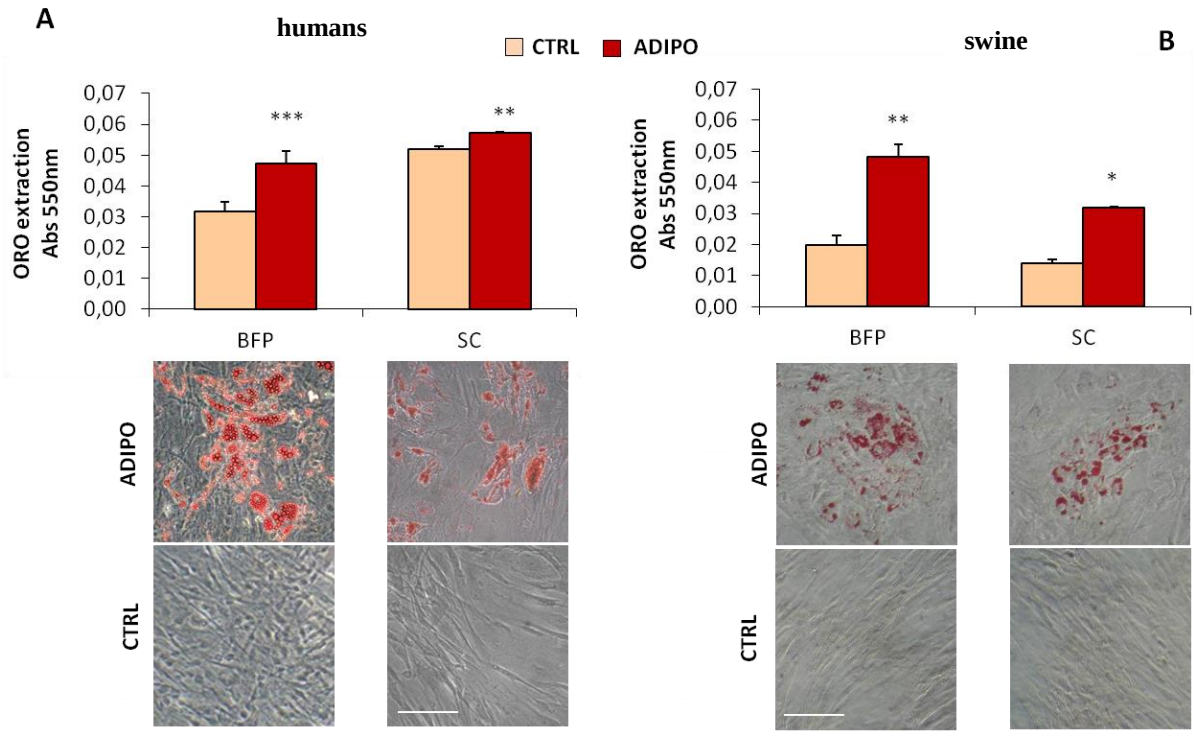

Figure 10. Quantification of lipid vacuoles formation in human (A) and porcine (B) BFP- and SC-ASCs, by Oil Red extraction (upper panels, data are expressed as mean±SEM, n=2). Representative microphotographs of BFP-ASCs and SC-ASCs maintained for 14 days in control (CTRL) and adipogenic medium (ADIPO), after lipid vacuoles staining by Oil Red O (lower panels)(optical microscopy, 200x magnification, scale bar 50µm).

BFP- and SC-ASCs interactions with scaffolds

BFP- and SC-pASC on titanium disks (TIT) and silicon carbide–plasma-enhanced chemical vapour deposition (SIC) fragments

The capability of pASCs to grow and differentiate on a widely used biomaterial in dental practice (titanium) and on a promising candidate for the implant coatings (SIC) was assessed. pASCs cultured for 21 days on both biomaterials, either in the presence or in the absence of osteogenic stimuli, efficiently adhered to them; indeed, there were not significant differences between the protein concentration of either plastic adherent cells or scaffold associated ones (figure 12B). This observation was also confirmed by a preliminary experiment in which BFP- and SC-pASCs cultured for 7 days on the titanium disks appeared alive and tightly layed on them when observed by confocal microscopy (figure 11).

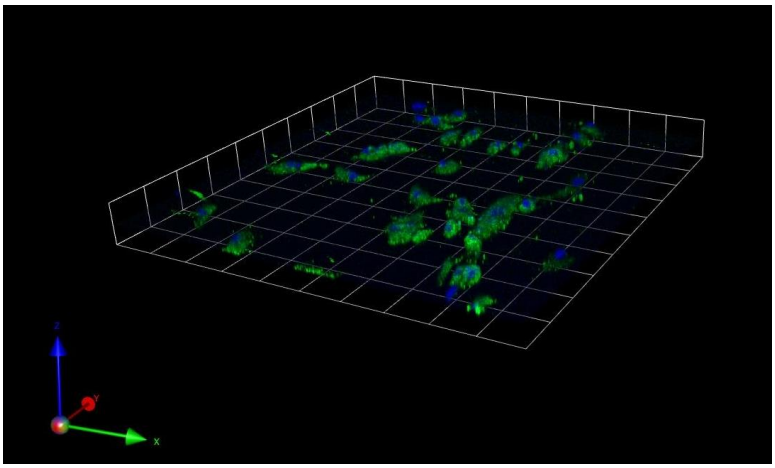

Figure 11. Representative image of pASCs adhering to titanium (SC-pASCs; due to reflection problems were not possible to visualize the titanium level; 20X objective)

Both pASC populations cultured on biomaterials, differentiated towards cells of the osteogenic lineage. Indeed, osteo-differentiated ASCs seeded on TIT, increased the amount of calcified ECM, compared to CTRL cells, of about 37% and 46% for BFP - and SC-pASCs, respectively; similarly, BFP- and SC-pASCs on SIC, increased ECM deposition of 200% and 90%, respectively, compared to CTRL cells (figure 12A).

Interestingly, TIT appears to be osteoinductive per se for pASCs, *in vitro*; indeed, increases of calcified ECM of about 234% in CTRL BFP-pASCs, and of about 91% in CTRL SC-pASCs, compared to plastic-adherent cells, were quantified (figure 12A).

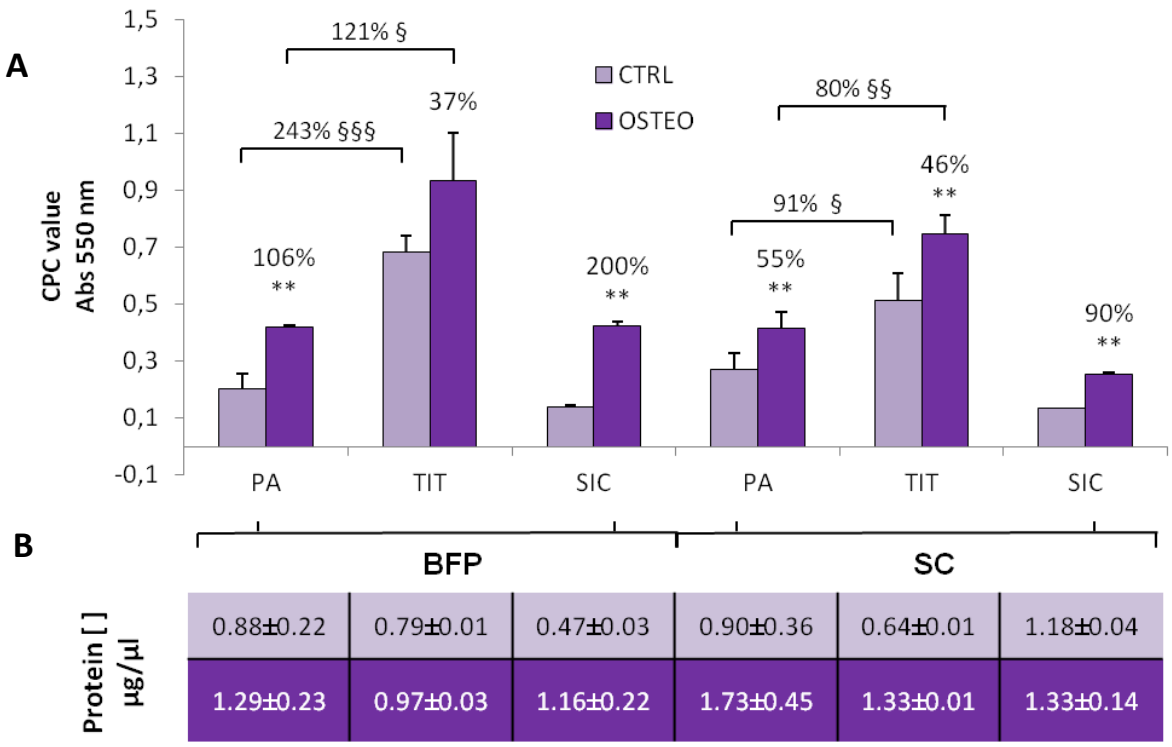

Figure 12. Calcified ECM deposition in undifferentiated (CTRL, lighter bars) and osteogenic-differentiated (OSTEO, darker bars) BFP- and SC-pASCs, cultured for 21 days on monolayer (plastic adherent-PA), or seeded on titanium disks (TIT) or on silicon carbide–plasma-enhanced chemical vapor deposition (SIC) fragments (panel A). Protein concentrations of pASC samples are also shown(B). Data are expressed as mean±SEM (*n*= 3). OSTEO vs CTRL **p<.01; TIT vs PA §p<.05; §§p<.01; §§§p<.001

BFP- and SC-hASCs on natural and synthetic scaffolds

ASCs are able to nicely adhere to and grow on several tissues and types of scaffolds routinely used in periodontal and oral bone regeneration; indeed, undifferentiated BFP- and SC-hASCs cultured on alveolar bone, periodontal ligament, collagen membrane, and polyglycolic acid filaments for 4 days, were found nicely stick on all the scaffolds. The only slight difference was observed when cells were kept on suture filaments of polyglycolic acid where SC-ASCs seemed to adhere more tightly to it, compared to BFP-hASCs (figure 13).

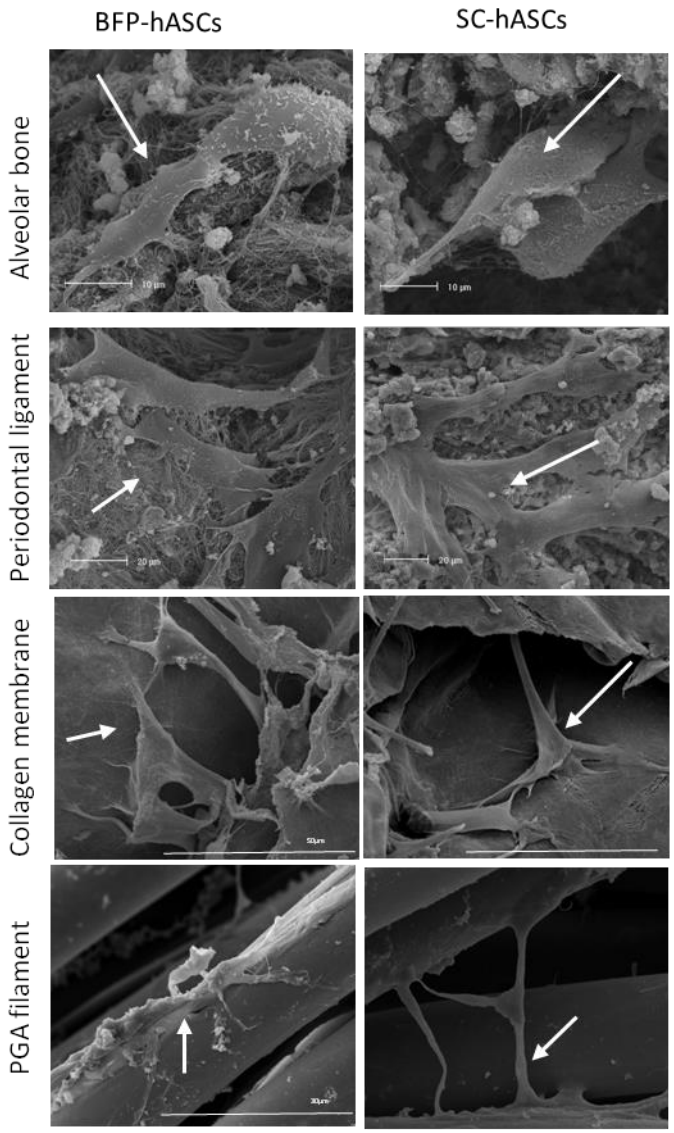

Figure 13. Undifferentiated BFP- (left) and SC-hASCs (right) maintained on natural and synthetic scaffolds for 4 days. Alveolar bone fragment (scale bar, 10µm), periodontal ligament (scale bar, 20 µm), collagen membrane (scale bar, 50 µm), and polyglycol acid (PGA) filament (scale bar, 30 µm.)

Amelogenin-treated hASCs

In a preliminary experiment, perfomed only on cells derived from one donor, BFP- and SC-hASCs were cultured for 7 days in the presence of 3% Amelogenin (AM), an enamel matrix protein applied by dentists to promote periodontal regeneration. Its presence *in vitro* was able to induce ALP activity per se, both in undifferentiated and OSTEO-differentiated BFP-hASCs. Amelogenin effect was primarily observed with BFP-hASCs, suggesting that cells derived from the oral cavity area are more sensitive to it. In particular, ALP activity was increased by AM of 220% in undifferentiated cells (-/ +), and further up-regulated when BFP-ASCs were OSTEO-differentiated (+ / +), showing a specific synergic effect (+ 950.4%; figure 14 A).

Collagen deposition was also up-regulated about 32.5% for both OSTEO-differentiated ASCs (figure 14 C, D).

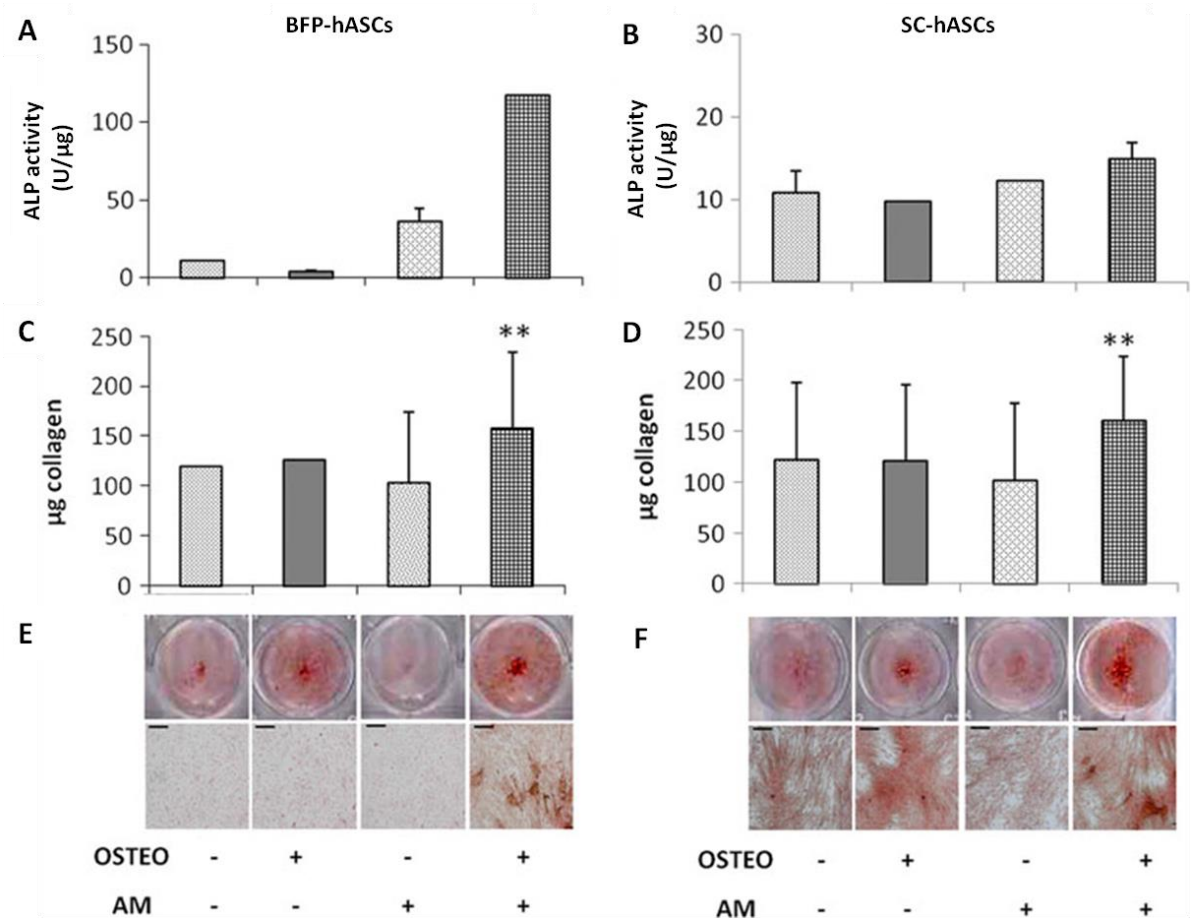

Figure 14. ALP activity of undifferentiated and 7-day-osteodifferentiated BFP and SC-hASCs either in the presence (+/- and +/+) or absence (-/- and +/-) of amelogenin (panel A and B). Quantification of collagen deposition is shown in C and D. Pictures and respective microphotographs of Sirius Red stained BFP- and SC-hASCs are in panel E and F top and bottom, respectively (magnification 40 X , scale bar 300 µm). + / + vs. -/-: **p < 0.01.

ASC culture with autologous and heterologous sera

Considering a possible future clinical application of hASCs and the requirement of pre-clinical studies, we evaluated the growth *in vitro* of both ASCs in medium supplemented with either autologous or heterologous serum (AS and HS, respectively). Human and porcine cells were cultured in the presence of 10% or 5% AS or 5% HS, and their proliferation rates were compared for 21 days to cells maintained in standard conditions (10%FBS).

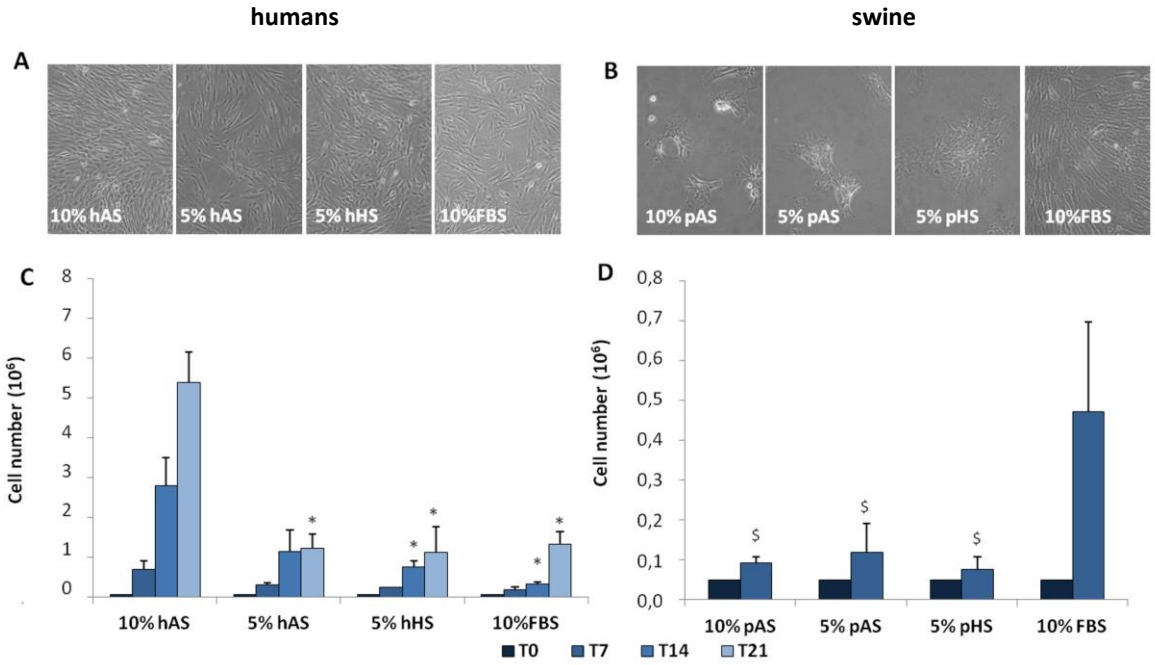

Figure 15. Microphotographs of human (A) and porcine (B) ASCs cultured for 21 days in the presence of different sera (optical microscopy, 100x magnification). Cell count of human (C, n=4) and porcine (D, n=4) ASCs cultured in different serum condition, assessed once a week (data are expressed as mean±SEM). AS, autologous serum; HS, heterologous serum; FBS, fetal bovine serum.

In all the culture conditions, the hASC maintained their fibroblast-like shape (figure 15 A), and the presence of 10% hAS induced a prompt increase in cell number (already within 7 days) compared to other sera (figure 15C, table 5), and after 3 passages, starting from $6x10^4$, we have harvested $5.9x10^6$ ASCs, while, in standard condition, we have collected just $1.5x10^6$ cells. Human ASCs cultured in the presence of either 5% hAS and 5% hHS or 10%FBS have a similar growth trend at day 7 (figure 15C), whereas, after 14 days, human sera increased the cell number of 257 and 131%, respectively, compared to FBS (table 5 -standard condition set as 100%). In contrast, porcine cells did not show any improvement by FBS substitution. Indeed, already after few days, pASCs, grown in the presence of either autologous or heterologous serum, aggregated in small clusters and became smaller and rounder, compared to cells cultured in 10% FBS (figure 15B). The cells proliferated slower then cells maintained in standard condition (figure 15D) and, after three weeks, the number of pASCs collected was about 2.27±1.1% compared to cells grown in standard condition and set as 100% (table 5).

		% cells harvested compared to cell grown in 10%FBS		
		T7	T14	T21
hASCs	10% hAS	369	874	406
	5% hAS	163	357	92
	5% hHS	129	231	85
pASCs	10% pAS	20	4	0,4
	5% pAS	25	6	4,1
	5% pHS	16	6	2,3

Table 5. ASCs cultured in the presence of autologous and heterologous serum for 21 days. Data are expressed as the ratio of number of cells grown with AS or HS/number of cells cultured with FBS x 100. AS, autologous serum; HS, heterologous serum; FBS, fetal bovine serum.

RESULTS 2. Osteochondral regeneration of a critical size defect in a minipig model using Adipose-derived Stem Cells in association with an hydrogel of oligo(polyethylene glycol) fumarate

ASCs *in vitro* expansion and analyses

mpASCs were isolated from seven minipigs. The average cellular yield was $5.6\times10^4\pm1.4\times10^4$ cells/ml of subcutaneous interscapular adipose tissue, quite comparable to the one of the human subcutaneous adipose tissue of this study ($8.5x10^4$ cells/ml) (table 6). As described above (Results 1 section), ASCs isolated from swine appeared smaller and with a rounder shape compared to human ASCs. All the cellular features are summarized in table 6; briefly they showed good proliferative and clonogenic abilities with a mild better behaviour of porcine cells: (doubling time: 60.3±3.68 and 101.9±22.8 hours for mp and hASCs, respectively; clonogenic ability: 16.1±2.7% and 10.1 ±1.4% for mp and hASCs, respectively).

	mpASCs-1	mpASCs-2	mpASCs-3	mpASCs-4	mpASCs-5	mpASCs-6	mpASCs-7	hASCs
ASCs/ml	$3,7X10^4$	$1,8X10^4$	$5,4X10^3$	$9,1X10^4$	$6,3X10^4$	$4,2X10^4$	$1,4X10^4$	$8,5X10^4$
Doubling time (hours)	63,68	70,16	52,91	46,94	57,90	112,28	57,41	101,96
Clonogenicity (%)	11,6±3,1	17,5±8,4	12,4±2,3	30,5±6,7	13,8±2,4	9,96 ±0,5	15,1±0,2	10,1±1,4

Table 6. Cellular yield (upper row) for each animal and the human donor. Proliferation rate expressed as doubling time (DT) (central row), and clonogenic ability expressed as the number of colonies/number of plated cells x100 (lower row) of mp- and h-ASCs at three passages (P2-P4).

To test their multiple differentiative potential *in vitro,* mp- and hASCs were induced by osteogenic and chondrogenic stimuli and in suitable culture conditions for 2 and 3 weeks, respectively. *In vitro* osteo-differentiated ASCs, compared to control cells, significantly increased alkaline phosphatase enzymatic activity (figure 16A), collagen production (figure 16B) and calcified extracellular matrix deposition (figure 16C) of about 440%, 151%, and 110%, respectively, for mpASCs, and of about 104%, 152% and 401% respectively for human cells. In addition, mpASCs and hASCs both chondrodifferentiated in pellet culture for 21

days, deposed a significantly increased amount of GAGs compared to undifferentiated cells (+92 and 28%, respectively) (figure 16D).

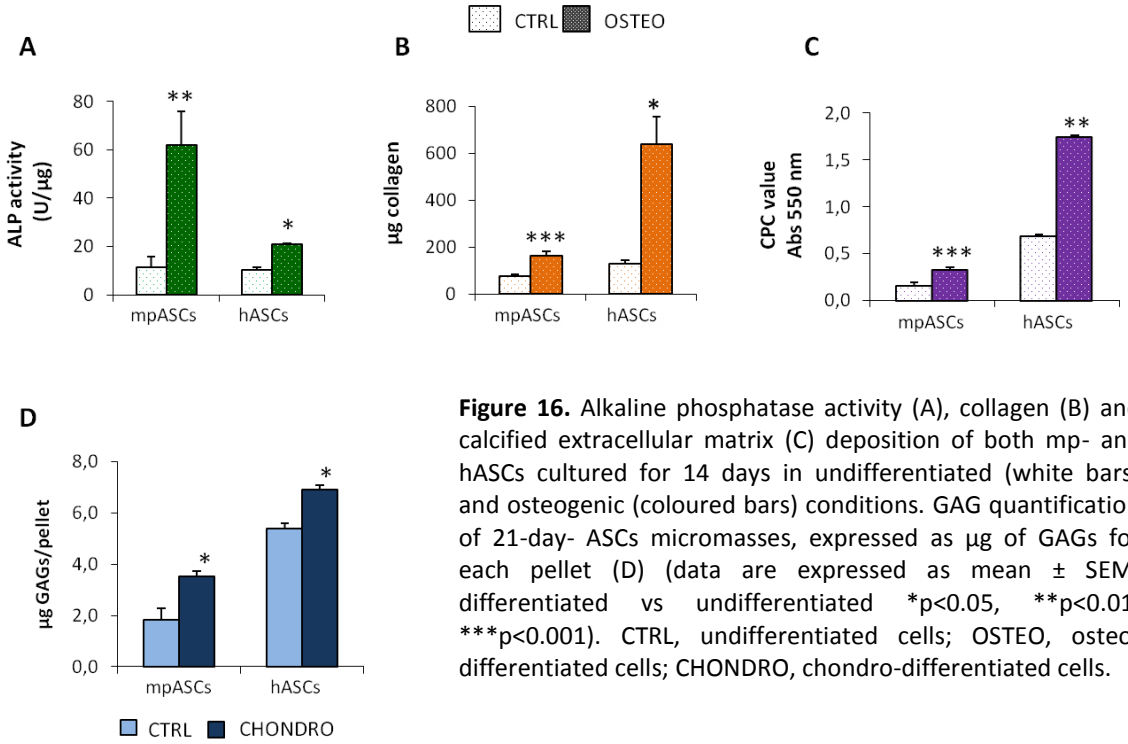

Figure 16. Alkaline phosphatase activity (A), collagen (B) and calcified extracellular matrix (C) deposition of both mp- and hASCs cultured for 14 days in undifferentiated (white bars) and osteogenic (coloured bars) conditions. GAG quantification of 21-day- ASCs micromasses, expressed as µg of GAGs for each pellet (D) (data are expressed as mean ± SEM, differentiated vs undifferentiated *p<0.05, **p<0.01, ***p<0.001). CTRL, undifferentiated cells; OSTEO, osteo-differentiated cells; CHONDRO, chondro-differentiated cells.

Surgical procedure for the osteochondral defect and its treatment

$3x10^6$ undifferentiated ASCs, collected at passage four, from minipigs or human donor, were used for construct production. For the scaffold+ASCs groups, ASCs were loaded on rehydrated oligo(polyethylene glycol) fumarate (OPF) hydrogel, and maintained in a humidified incubator overnight (about 16 hours). Next day, under general anaesthesia, four osteochondral lesions (9 mm in diameter, 8 mm in depth each) were created in the trochlea periphery of each right knee for all the minipigs. The 28 performed defects are summarized in table 7. Untreated defects were the negative controls (UNT group, n=7), and each scaffold was inserted into the lesion by press-fit technique.

Treatment	n
Untreated (UNT)	7
Unseeded scaffold (OPF)	7
OPF + Autologous ASCs (OPF+mpA-ASCs)	7
OPF +Heterologous ASCs (OPF-mpHe-ASCs)	3
OPF + Human ASCs (OPF-hASCs)	4

Table 7. Groups of treatment for the 28 critical osteochondral defects in the minipigs' right knees.

All the animals well tolerated the surgical treatments. After a short period of limping, lasting maximum 14 days from surgery, all the minipigs recovered a normal gait, without any functional limitations. Knee swelling was present post-operatively in all the animals for about 3 weeks.

Radiological and macroscopical analyses

Six months later, all the animals were sacrificed and magnetic resonance of all the treated joints was performed immediately after the explant of the limbs, before the arthrotomy. MRI data were analysed by a modified 2D MOCART scale for the evaluation of *ex vivo* osteochondral samples. The considered parameters for the analysis are reported in table 8. The scale ranges between 0 and 100, (0 the worst and 100 the best scoring).

Parameter	Item	Score
Defect fill	Subchondral bone exposed	0
	Incomplete < 50%	5
	Incomplete > 50%	10
	Complete	20
	Hypertrophy	15
Cartilage	Complete	15
	Demarcating border visible	10
	Defect visible < 50%	5
	Defect visible > 50%	0
Surface	Intact	10
	Damaged < 50% of depth	5
	Damaged > 50% of depth	0
Adhesion	Yes	5
	No	0
Structure	Homogeneous	5
	Inhomogeneous or cleft formation	0
Signal intensity	Normal	30
	Nearly normal	10
	Abnormal	0
Subchondral lamina	Intact	5
	Non intact	0
Subchondral bone	Intact	5
	Granulation tissue, cyst, sclerosis	0
Effusion	No	5
	Yes	0

Table 8. 2D MOCART scale developed by Marlovits et al. and modified by Goebel et al. 2012

According to 2D MOCART scale, all the treated defects showed significantly improved scores in comparison to untreated ones (figure 17); however, no differences among the OPF and OPF+cell groups were observed.

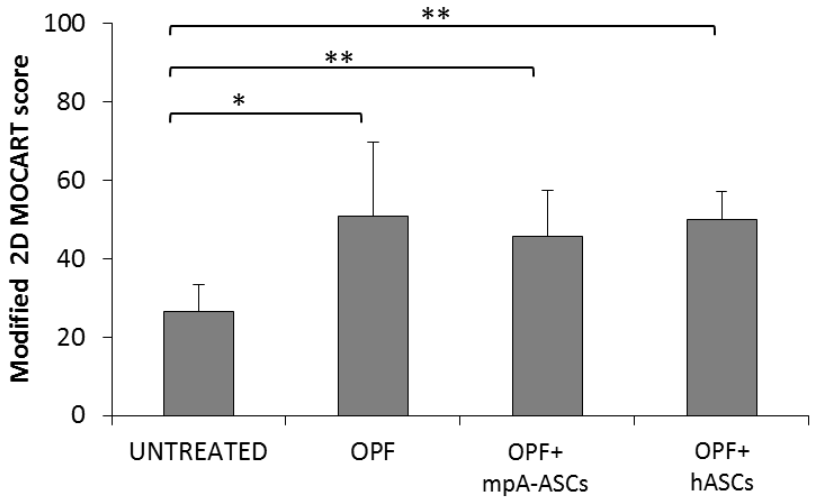

Figure 17. Scoring associated to MRI data analysed by a modified 2D MOCART (data are expressed as mean ± SEM, 100 corresponding to healthy joint). mpA-ASCs, minipig autologous ASCs.

Then, joints were dissected, the treated portions retrieved en bloc and then cut into four pieces corresponding to the four osteochondral defects, to allow independent analyses of each defect. Following arthrotomy, signs of local infection were observed in pig #6, and after joint inspection it was excluded from the study. The loss of this animal, treated with heterologous cells, provoked the uselessness of the data about this cell source and just qualitative data will be mentioned. With the exception of this animal, the joint inspection did not revealed any sign of inflammation, as well as adhesion, hypertrophy or fibrosis of synovial membrane. The surface of all the defects showed small cylindrical lacks of substance, sometimes combined with signs of hemorrhages (figure 18).

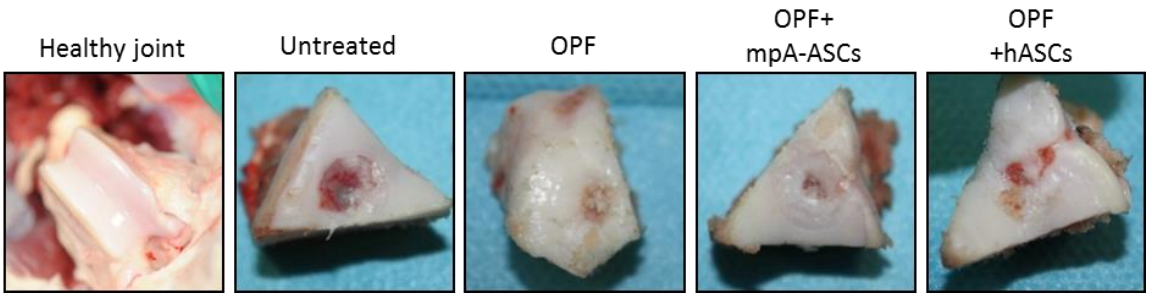

Figure 18. Representative images of some explanted defects after 6 months of follow-up. mpA-ASCs, minipig autologous ASCs.

Histological and immunohistochemical analyses

In both unseeded and mpA-ASC or hASC seeded scaffolds most of the tissue filling the superficial part of the defects, was fibrocartilage, mainly located in the central part of the lesions, while, in the untreated defects, the tissue was mainly fibrous tissue, with blood vessels and a disorganized extracellular matrix. All the samples were Safranin-O less intensely positive respect to native articular cartilage (figure 19, A), suggesting a lower content of glycosaminoglycans in the neo-formed tissues. However, in the scaffold-treated groups the tissue was stained slightly more intensely respect to the untreated groups, and the cartilage appeared at the edge of the defects (Figure 19, A2-A5). In terms of subchondral bone repair, in OPF+mpA-ASC and +hASCs groups, the bone formation process is pronounced, and osteoblasts lined the surfaces of the construct (Figure 19, B3-B5). Interestingly, more mature bone, characterized by bone lamellas, was observed in OPF+hASC treated defects. In contrast, the restoration of the subchondral bone was insufficient and very immature in untreated samples, as it started with endochondral ossification at the borders of the defect (Figure19 B2).

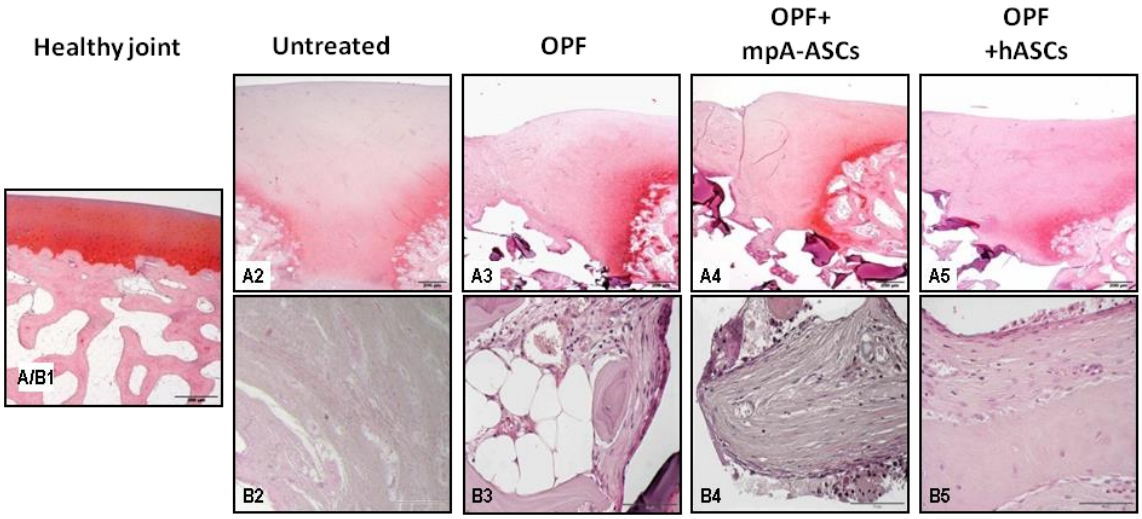

Figure 19. Histological analyses of the superficial (A2-5) and deeper (B2- B5) layers of the defects stained by safranin O; healthy joint is shown as control (A/B1) (scale bar -200µm for healthy joint and cartilage layer, 50µm for subchondral bone layer).

More accurately, analysis by polarized light microscopy allowed to evaluate the presence of the collagen components of primary and secondary bone, the differentiation between mature and developing osteons, and the bone lamellas and their orientation in the healthy joint sample (figure 20.1). In the untreated group, collagen fibers were not organized in

lamellar form (figure 20.2), whereas in both seeded and unseeded OPF defects, a more organized distribution of collagen fibers was detected. Indeed, the deposition of lamellar bone occurred within the pores of the scaffolds, without any relevant difference among unseeded and mpA-ASC or h-ASC seeded scaffolds (figure 20).

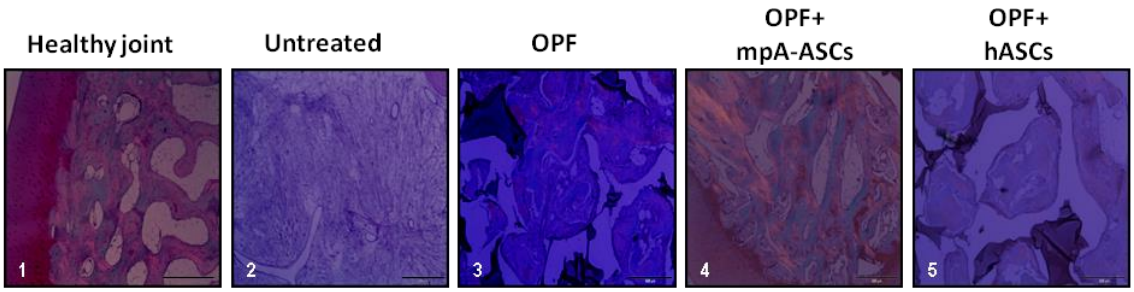

Figure 20. Histological sections observed by polarized light
(scale bars 200µm for healthy joint, 100µm for other samples)

Selected cartilage and bone tissue proteins, such as collagen type II and collagen type I, respectively, were also evaluated by immunohistochemical analyses. In both OPF+mpA-ASCs and OPF+hASCs treated defects collagen type II was nicely expressed (figure 21, A4-A5), and it was comparable to the one of the healthy cartilage (figure 21, A1). In contrast, a poor collagen II immunoreactivity was evident in both untreated and unseeded OPF groups (figure 20, A2-A3). Unfortunately, the inner part of the neo-cartilages contained scarce collagen II immunopositive reparative tissue independently of the groups, (data not shown).

Similarly, the neo-formed bone was more positive for collagen type I in OPF+mpA-ASCs and OPF+hASCs groups (figure 21 B4-B5) in comparison to unseeded OPF and untreated groups (figure 21, B2-B3).

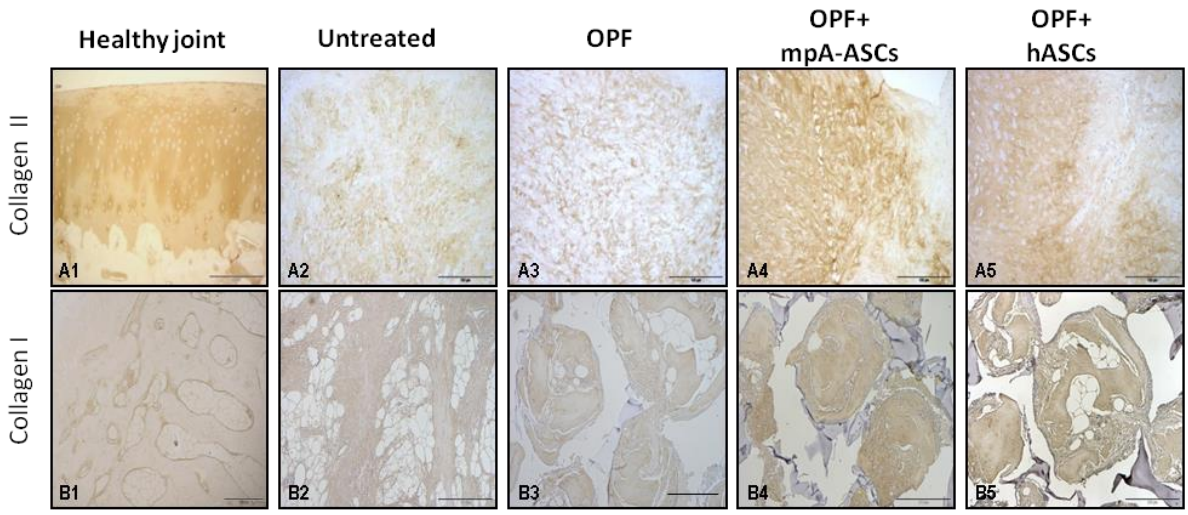

Figure 21. Expression of collagen type II (upper panels) and collagen type I (lower panels) in sections of the superficial and deeper layers of the joint , respectively (scale bar 100μm).

Histological scoring

In order to associate a score to histological observations, the ICRS II scoring system was chosen. It contains 14 parameters: an overall assessment and 13 parameters relating to both chondrocyte and tissue features (table 9, left columns). Each parameter was scored using a 100 mm VAS (Visual Analog Scale), where 0 was considered as poor quality and 100 as very good quality. According to ICRS II scores, significant differences were found between OPF and OPF+mpA-ASCs for some parameters. In more details, tissue morphology, matrix staining, cell morphology, formation of tidemark and vascularization scored higher in the OPF+mpA-ASCs treated groups. On the other hand, surface assessment score was lower in OPF+mpA-ASCs compared to OPF (table 9, right columns). Unfortunately, these scores were quite distant to the ones of s healthy cartilage (used as standard reference and scoring 100 for each parameter). For all the other features no differences were found between the two groups (table 9, right columns).

Score	Histological parameters	OPF	OPF+ mpA-ASCs
0: full thickness collagen fibers 100: normal cartilage birefringence	**Tissue morphology (polarized light)**	22.9±9.1	25.9±11.0*
0: no staining 100: full metachromasia	**Matrix staining (metachromasia)**	25.0±5.4	31.3±10.4**
0: no round/oval cells 100: mostly round/oval cells	**Cell morphology**	20.4±10.1	25.0±3.9**
0: no calcification front 100: tidemark	**Formation of tidemark**	22.9±22.0	28.3±18.5**
0: present 100: absent	**Vascularization (within the repaired tissue)**	38.8±28.1	51.7±13.6***
0: total loss or complete destruction 100: resembles intact AC	**Surface/superficial assessment**	27.5±9.7	19.3±11.9*
0: present 100: absent	**Chondrocyte clustering (4 or more grouped cells)**	99.2±1.7	100±0
0: delamination or major irregularity 100: smooth surface	**Surface architecture**	51.7±11.3	53.7±19.7
0: no integration 100: complete integration	**Basal integration**	52.5±21.8	60.0±17.2
0: abnormal 100: normal marrow	**Subchondral bone abnormalities**	54.2±27.3	51.0±19.0
0: present 100: absent	**Inflammation**	100±0	100±0
0: present 100: absent	**Abnormal calcification/ossification**	100±0	97.0±5.1
0: fibrous tissue 100: normal hyaline cartilage	**Mid/deep zone assessment**	24.6±12.6	26.3±12.6
0: bad (fibrous tissue) 100: good (hyaline cartilage)	**Overall assessment**	21.3±12.0	23.3±7.3

Table 9. ICRS II scoring system parameters OPF+mpA-ASCs vs OPF *p<0.05, **p<0.01, ***p<0.001

Biomechanical testing

An important feature of healthy cartilage is its ability to conteract mechanical stimuli; for this reason we decided to investigate the biomechanical features of the restored tissue trough nanoindentation tests.

The indentation modulus at equilibrium M_eq (computed by fitting the force-penetration data achieved at equilibrium), identified for healthy control, OPF-A, OPF and untreated samples are reported in figure 22 for both the R_25 and R_400 tips. R^2 fitting parameter (referred to Eq. 2, materials and methods) was found to range between 0.85 and 0.9, the lower values where found for untreated samples. In all cases, indentation moduli found using the small tip radius is larger than those found with the large tip radius. Healthy cartilage exhibits an indentation modulus (603±199 kPa) similar to that found for OPF+mpA-ASCs samples (545±96 kPa) with R25 tip. A similar consideration holds for the large tip radius: healthy cartilage and OPF-A samples exhibited an indentation modulus of 238 kPa±68 kPa and 272 ±29 kPa, respectively. Both OPF and untreated samples showed an indentation modulus at equilibrium significantly smaller than that found for healthy cartilage and OPF-A samples. In particular, M_eq for OPF sample was 268±102 kPa and 110±10 kPa for R_25 and R_400, respectively (both $p<0.05$ with respect to native cartilage); M_eq for untreated sample was 292±47 kPa and 81±25 kPa for R_25 and R_400, respectively (both $p<0.05$ with respect to native cartilage). No appreciable difference was found between OPF and untreated samples (figure 22).

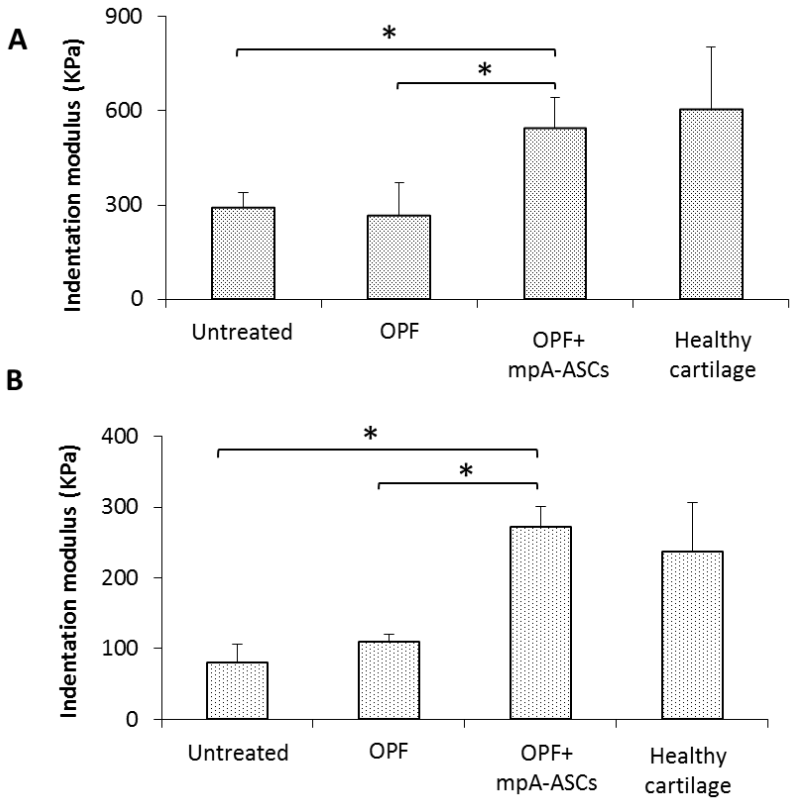

Figure 22. Indentation modulus at equilibrium M_eq, for the R_25 (A) and R_400 (B) tips (data are expressed as mean±SD)

The tissue permeability k_ind is identified as the ratio between the poroelasticity (parameter P) and the equilibrium indentation modulus M_eq for both indenter tips and for each load level (Eq. 7, materials and methods). The k_ind parameter was calculated for healthy and OPF+mpA-ASCs samples, since only for these, the creep response was fitted with R^2 values greater than 0.7. The OPF and untreated samples exhibited creep curves which were fitted with R^2 values lower than 0.7, therefore the poroelastic fitting model was considered unsuited.

Averaged values of the tissue permeability over all the load levels are: $2.61\pm1.10\text{x}10^{-16}$ for R_400 and $1.39\pm0.68\text{x}10^{-17}$ for R_25 in case of healthy cartilage sample; $1.83\pm0.45\text{x}10^{-16}$ for R_400 and $1.87\pm1.11\text{x}10^{-17}$ for R_25 in case of OPF+mpA-ASCs sample. In figure 23 the decreasing trend of permeability is instead presented for both the samples (mean values are shown) with respect to the equivalent deformation, computed as the ratio between the current contact length and the indenter radius. Engineered construct appeared slightly less permeable at smaller deformation, compared to native cartilage (figure 23).

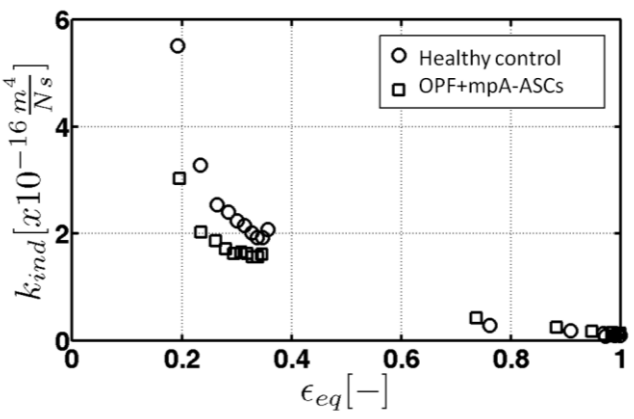

Figure 23. Mean permeability values with respect to the equivalent deformation.

In Figure 24 creep curves are plotted in function of dimensionless time (Eq. 6, materials and methods) when, for each of them, also the depth is normalized as the ratio of the difference between current and instantaneous depths and the jump between equilibrium and instantaneous depths (Eq 9 materials and methods); only selected curves are presented for the healthy cartilage (figure 24A), OPF+mpA-ASCs (figure 24B) and OPF unseeded (figure 24C) samples for both the two tips. In each graph, the selected curves are shown with respect to natural time (t) and dimensionless time (t normalized). As expected, a complete overlap between creep curves collected at different load and different tip radii is found for the control sample. In the case of OPF+mpA-ASCs sample the overlapping is good enough to consider the material governed mainly by poroelasticity, whereas OPF sample showed a more complex behavior. Curves collected at different depths overlap within the same tip when normalized time is used; between the tips, instead, the overlapping does not occur. No data are presented for the untreated sample since no acceptable poroelastic fitting can be found.

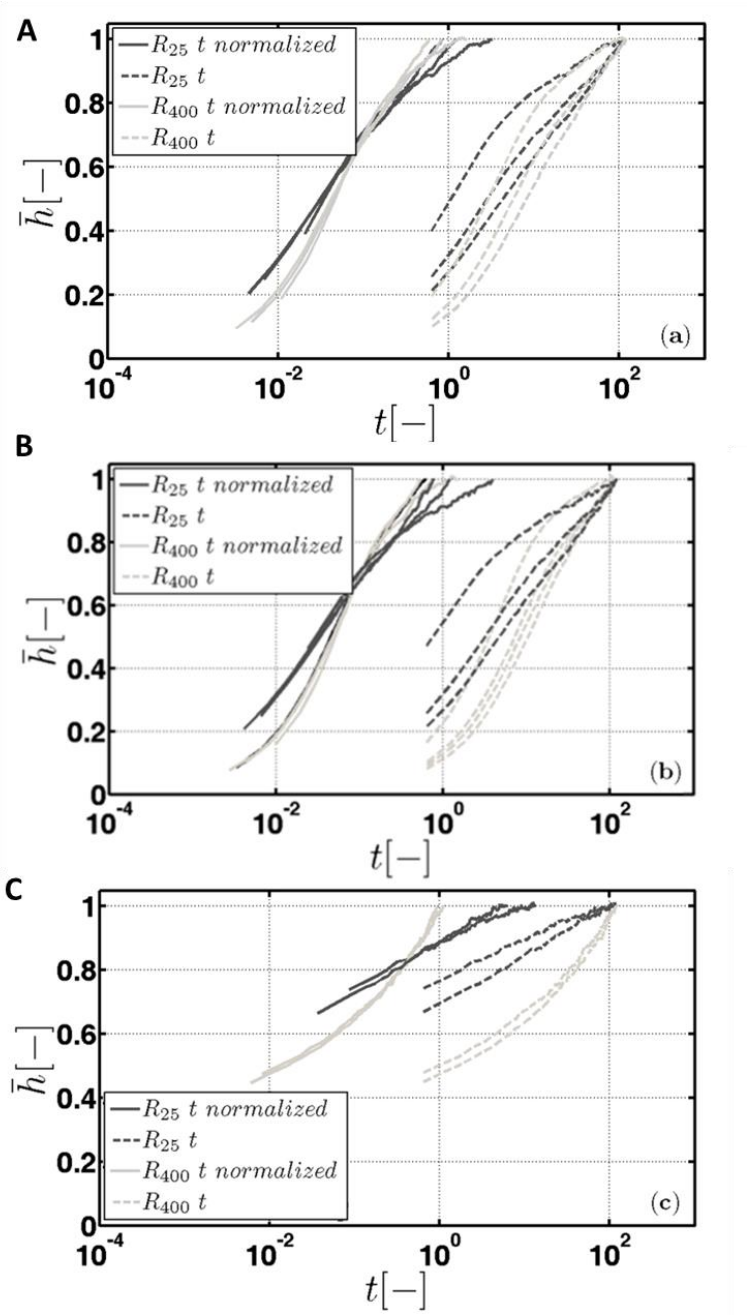

Figure 24. Creep curves presented for the healthy cartilage (A), OPF-A (B) and OPF (C) samples for both R_25 (dark lines) and R_400 (light lines) tips. In each graph, the selected curves are shown with respect to natural time t (dotted lines) and dimensionless time t (continuous lines).

DISCUSSION

Part of my work was dedicated to the investigation of a possible novel source of mesenchymal stem cells, the Buccal Fat Pad (BFP).

Human adipose-derived stem cells from Buccal fat pad (BFP-ASCs) might be quite easily applied in oral tissue engineering, since this tissue is rapidly accessible by dentists and maxillofacial surgeons (Hwang et al., 2005). However, before moving to clinic, it is mandatory to perform approved pre-clinical studies to validate the safety and efficacy of cellular therapies. The most used large size animal models for the human oral bone defects repair are swine (Schwarz et al., 2012; Wilson et al., 2012) [Wilson SM, 2012; Schwarz C, 2012], since these animals present a healing potential comparable to the human one. Nowadays, several studies have been conducted using stem cells in oral diseases and oro-facial research: Wilson et al. have investigated bone regeneration in the pig mandible ramus by either local or systemic ASC injection, concluding that both treatments accelerate the healing process, without any significant difference between the two routes of administration (Wilson et al., 2012). In another study, similar results were obtained combining decidua stem cells with a β-TCP scaffold in a minipig model (Zheng et al., 2009).

For these reason, we decided to compare the BFP-ASCs with the well characterized ASCs from subcutaneous tissue (Arrigoni et al., 2009; De Girolamo et al., 2008; Zuk et al., 2001) both from human donors and from swine, and to evaluate their behavior *in vitro*, also in association with different scaffolds, to identify a convenient source for future pre-clinical studies. Here, we showed that it is feasible to isolate a proper quantity of stem cells even by starting from a small amount of raw adipose tissue (0.5-1 ml for human samples) such as BFP, and that the cellular yield is not influenced by the site of harvesting. The number of isolated cells per ml of withdrawn human adipose tissue, was higher than the porcine one; anyway, thanks to the great proliferation rate of pASCs, after 30 days in culture, we could have obtained a homogeneous populations of about 10^8-10^9 porcine cells with still a pronounced clonogenic ability. All the cells nicely adhered to the tissue culture plates when maintained in standard culture conditions and showed fibroblast-like morphology, even though both porcine ASC populations and BFP-hASCs were slightly smaller and less elongated than SC-hASCs.

Human ASCs derived from both site of harvesting expressed the mesenchymal stem cell markers CD73, CD90, and CD105, whereas they did not express lymphocyte or leucocyte antigens and hematopoietic markers such as CD14, CD31, and CD34; these results are consistent with the minimal criteria for defining multipotent mesenchymal stromal cells (Dominici et al., 2006) and adipose tissue-derived stromal/stem cells (Bourin et al., 2013), and with data previously obtained in our lab with human SC-ASCs (de Girolamo et al., 2009). Also porcine cells expressed CD90, as already described on pMSCs from different tissues , whereas, no cross-reactivity was found on both SC- and BFP-pASCs for lymphocyte or leucocyte antigens. Unfortunately, the antibodies raised against human CD73 and CD105 in our hands did not crossreact with porcine antigens. Although incomplete, our data are consistent with the ones published by Noort et al. on porcine BMSCs (Noort et al., 2012).

Moreover, BFP-ASC stemness has been confirmed by the good clonogenic ability of all the populations tested.

Human and porcine BFP-ASCs are multipotent, indeed they can be induced to differentiate, up-regulating ALP activity and collagen and calcified extracellular matrix deposition as well as lipid vacuoles productions already after 14 days of differentiation *in vitro*. This data are consistent with that described by Farre´-Guasch for human BFP-ASCs (Farre-Guasch et al., 2010) and by us and other groups for SC-hASCs (De Girolamo et al., 2008; Zuk et al., 2001) and -pASCs (Arrigoni et al., 2009; Qu et al., 2007; Tang et al., 2012). In our experimental conditions, osteogenic differentiation was detected in the absence of bone morphogenic proteins, which seemed to be necessary in the study of Shiraishi et al. (Shiraishi et al., 2012). This could be due either to the different applied osteogenic stimuli (particularly the absence of vitamin D and the substitution of ascorbic acid-2- phosphate with ascorbic acid in their culture medium) or to the possible variability among cells derived from healthy donors of our study and patients with jaw deformity of their experimentation.

Since in Tissue Engineering technology the fundamental players are cells, growth factors and supports that have to be colonized, and since our final future aim is to use ASCs in the clinic, I have decided to test their interaction with appropriate supports. We evaluated the ability of both pASCs to grow and differentiate onto two synthetic scaffolds: the former, a widely used biomaterial in dental surgery (titanium), and the latter, a promising candidate for the coating of some portions of implant (SiC-PECVD). Like human ASCs (Lopa et al., 2011), porcine pASCs adhere and differentiate on both scaffolds. Moreover, the osteoinductive

properties of titanium on hASCs (Lopa et al., 2011) , was maintained on both porcine progenitor cells, whereas SIC-PECVD did not affect their osteogenic differentiation.

In addition, we investigated the behavior of BFP-hASCs in association either with natural supports, such as alveolar bone and periodontal ligament, or biocompatible materials that might be in contact with these cells in a clinical setting. It is known that SC-hASCs can be successfully seeded on demineralized and decellularized bone (Frohlich et al., 2010; Kim et al., 2012; Shi et al., 2012). Here, we also showed a stable interaction of SC-hASCs and BFP-hASCs with healthy tissues such as autologous alveolar bone and periodontal ligament. Moreover, all the cells also efficiently adhered to collagen membrane and polyglycolic acid filaments, two materials chosen for their resorbable property and the widespread use in surgery. The observed adaptability of ASCs, to survive and grow on all the supports assayed and used in oral surgery, represents a prominent quality for a future regenerative medicine approach, not just of hard tissue, but also of soft ones. Interestingly, we also observed that osteo-differentiation of BFP-hASCs is specifically induced and up-regulated by Amelogenin (AM), as also previously reported for BMSCs (Izumikawa et al., 2012; Jingchao et al., 2011; Tanimoto et al., 2012). AM is the most abundant enamel matrix protein and dentists favour it for the repair of periodontal defects. The success of this treatment depends on which cell type fills the defect; in particular, it is important to promote selectively growth of periodontal ligament cells and reduce that of gingival and epithelial cells to allow a correct tissue formation next to the dental root. Indeed, it is known that AM enhances proliferation of periodontal MSCs that secrete cytokines and cause the synthesis of new cementum and periodontal fibers. Subsequently, the regeneration of the periodontium creates the precondition for new formation of functional attachment and for bone regeneration (Bosshardt et al., 2005). A review by Bosshardt et al. (Bosshardt, 2008) also sheds light on other biological mechanisms of this molecule that seems to affect a great variety of cells types included MSCs, pre-osteoblasts, and osteoblasts, influencing their proliferation, expression of transcription factors and cytokines, and their differentiation, showing the direct influence of AM also on bone regeneration. We showed a synergic effect of AM *in vitro* on human ASC osteogenic differentiation with other osteo-inductive factors. We may assume that this synergy, observed *in vitro*, could be maintained *in vivo*, where several growth and differentiation factors are released during the healing process (Dimitriou et al., 2005). Moreover, this effect is more pronounced on BFP-hASCs than on SC-hASCs,

suggesting that ASC source could influence these cells making them more responsive to stimuli naturally secreted in the same region. With prospective clinical applications, we have also cultured ASCs, without bovine serum, in media supplemented with homologous sera in self and non-self-conditions. We noticed that the presence of human serum improved the proliferation rate of both BFP- and SC-hASCs, despite the interdonor variability. This effect has not been previously observed on ASCs, since in other studies the growth was not influenced by autologous serum (Im et al., 2011). However, the heterogeneity in the response to autologous supplements has been also described for bone marrow stem cells (Kuznetsov et al., 2000; Mizuno et al., 2006; Shahdadfar et al., 2005; Yamamoto et al., 2003) and could be explained by the differences of sera and ASCs from different donors. Growing human ASCs in the presence of autologous serum, avoiding the concerns about animal proteins used during cell expansion, could be a safe procedure and a must, thinking their future clinical application. In contrast, pASCs cultured in the presence of porcine serum, dramatically reduced their growth. In addition, cells aggregated in clusters and their morphology is deeply affected. Our results agree with previous data by Schwarz et al., where equine ASCs cultured in the presence of autologous serum proliferate less compared with cells maintained in medium supplemented with FBS (Schwarz et al., 2012). Although we have observed that both pASCs behaved similarly, this issue requires further investigations.

The originality of our study, compared to the already published ones (Farre-Guasch et al., 2010; Shiraishi et al., 2012), consists in the comparison of ASCs derived from different body areas but from identical donors, and the parallel characterization of human and porcine cells. Furthermore, experiments performed on natural and synthetic supports, such as clinical grade and prototype scaffolds, allow us to suggest the use of these bioconstructs in preclinical studies of regenerative medicine. We think that comparing cells from the same donor is quite relevant since differences in the features of mesenchymal stem cells harvested from patient differing for gender, age or pathological conditions, such as obesity, have been highlighted by various groups (Buschmann et al., 2013; de Girolamo et al., 2009; de Girolamo et al., 2013; De Girolamo et al., 2008; Fossett et al., 2012; Mojallal et al., 2011; Shu et al., 2012). Moreover, all the ASCs, harvested from diverse patients, show some slight intrinsic physiological variation *in vitro* regarding proliferation rate and clonogenic or differentiative ability, that is unrelated to gender, age or weight of the patient. Our choice avoid all these inter-donor variability.

In our opinion, also the study of animal cells is important, since, in pre-clinical studies it is often required to prove the efficacy of a cell therapy using autologous, at least allogenic, cells.

In conclusion, we identified a cell population derived from a tissue easily available to dentists and maxillofacial surgeons, whose multipotent features and good adhesion to clinical grade scaffolds make proper candidate for future uses in tissue engineering approaches.

In addition, the comparison of human BFP-ASCs with porcine mesenchymal stem cells derived from the same body sites will allow their allo- or xenogenic use in preclinical models of oral bone reconstruction and periodontal disease.

In parallel, part of my PhD project was focused on the study of a critical osteochondral defect regeneration performed in a minipig preclinical model. This large animal study which last, almost a year, involved several specialists (biologists, surgeons, vets, medical doctors, bioengineers, statisticians), whose contribution was required to plan and perform it. In our opinion, the more innovative parameters of this study have been the use of an oligopolyetylenglycol fumarate (OPF) hydrogel to fill the osteochondral defect, and the use of either autologous or heterologous pASCs, or also human ASCs, to create the bioconstructs to be implanted in non immunocompromised minipigs. In addition, to reproduce the complete cartilage formation and the absence of spontaneous reparative process occurring in adult patients, the use of adult animals (one year old at the beginning of the study) has been a forced choice. The results of our preclinical study show that, at six months from implantation, expanded undifferentiated ASCs (autologous, heterologous or human) seeded on OPF hydrogel are able to promote a good subchondral bone healing and to improve the quality of the newly formed cartilage.

Radiological analyses revealed that the scaffold by itself was able to induce an overall improvement of the tissue quality at the osteochondral lesion site, most likely due the resident cells which are recruited in the damaged area and then adhered to the hydrogel. In contrast, the untreated lesions, were mainly filled by fibrous and unorganized tissue. Interestingly, more accurate evaluations revealed that some important tissue features were significantly improved by the association of OPF and ASCs. Indeed, regarding the subchondral bone, in all the OPF+ASCs groups a mature bone appeared, with higher expression of collagen type I compared to untreated or unseeded OPF groups. Moreover,

although histological observations indicated that, for all the samples, cartilage regeneration was still immature at the end of the follow up, the use of ASCs associated to scaffolds induced an improvement in collagen type II expression, matrix staining, tissue morphology and formation of tidemark, compared to unseeded scaffolds.

Quite unexpectedly, since ASCs have been described for their pro-angiogenic potential both *in vivo* and *in vitro* (Rubina et al., 2009; Suga et al., 2013), mainly due to their secretion of growth factors such as VEGF, HGF and TGF-β (Rehman et al., 2004), the presence of ASCs seemed to inhibit the vascularization of the newly formed cartilage. Although this have to be considered a positive result, we still do not have a clear answer to this phenomenon. We believe that ASCs could be led to release, or not, angiogenic factors, following the cross-talk between them and the resident cells, at the site of injury (Dimarino et al., 2013).

However, this interesting issue requires further investigations.

Overall, biomechanical tests shown that ASCs induced a significant increase in the cartilage properties compared to untreated or OPF unseeded samples; in particular, one of the most important results of this study is that only the neo-cartilage found in the cell-loaded scaffold groups possessed poroelastic behavior, as well as indentation modulus and creep curves comparable to native cartilage. However, these findings do not strictly correlate to the histological results, since in these samples, although a collagen type II expression comparable to that of healthy cartilage was observed, the amount of GAGs was just about 25% if compared to healthy cartilage. Even though this discrepancy, these data are quite promising since the functionality of the repaired tissue was our main goal.

Recently, a wide variety of approaches have been under investigation for the engineering of osteochondral grafts, such as the use of multilayer scaffolds to provide a proper support to the different tissue to be repaired (Duan et al., 2013; Frenkel et al., 2005; Kim et al., 2013; Kon et al., 2011; Kon et al., 2010a; Martin et al., 2007; Niederauer et al., 2000; Schek et al., 2004), the use of progenitor cells or terminally differentiated chondrocytes (Kon et al., 2010b), and even an *in vitro* maturation stage of engineered cartilage (Moretti et al., 2005; Rotter et al., 2002). Although these methods have shown promising results, they still have to be validated, and the development of an engineered graft prior to implantation to support an optimal repair, still have to be studied. One obstacle in understanding the most suitable approach to be used is the great heterogeneity among the various studies, depending for example, on the choice of different animal models with completely different body weight

and joint characteristics [rat (Gao et al., 2001), rabbit (Schaefer et al., 2002), sheep (Kandel et al., 2006; Kon et al., 2010b), pigs (Schek et al., 2004)]. Another factor to be considered is the size of the lesions, since the osteochondral defect size greatly vary among studies, both in term of area and depth, and thus, consequently, they cannot always be considered as critical (Hui et al., 2013; Lim et al., 2013).

In this study we selected key experimental parameters, such as the use of undifferentiated progenitor cells and of a monophasic not-associated-to-growth-factors hydrogel.

Our choice of the "simplest" type of graft to be implanted have been taken on the basis of the following rationale. We chose undifferentiated ASCs not only because their manipulation makes their use more difficult for future clinical application, but also basing this decision on the recent evidences on the mechanism of action of mesenchymal stem cells. Indeed, while at the beginning the attention was mainly focused on the ability of MSCs to differentiate in specific lineage cells, the recent opinion is that MSC therapeutic benefits are largely dependent on their capacity to act as a trophic factor pools (Caplan and Dennis, 2006; Xu et al., 2007) and as modulators of immune response (Gonzalez et al., 2009; Yanez et al., 2006). From this point of view, the main action of mesenchymal stem cells could be to support the resident cells which are thought to be the major effectors of tissue replacement. Moreover, the soluble mediators generated by MSCs, able to promote angiogenesis, tissue regeneration and remodeling, immune cell activation or suppression, and cellular recruitment, are released according to the requirements of the environment (Dimarino et al., 2013). Following this criteria we decided to implant a construct in which cells were undifferentiated and "free" to respond to the most proper stimuli, i.e. the ones naturally present at the lesion site. This issue also influenced the choice of a monophasic scaffold without loading it with growth factors. The use of OPF hydrogel associated to progenitor cells (Lim et al., 2013) or not (Hui et al., 2013) has been shown to be helpful for cartilage regeneration in other studies of osteochondral regeneration performed in swine models (Hui et al., 2013; Lim et al., 2013).

In our experiments, in which the dimension of the defects (9 mm in diameter, 8 mm in depth) were largely higher than in previous studies (Hui et al., 2013; Lim et al., 2013) 6 mm in diameter, 1 mm in depth), and the follow up was of six months, the chosen approach was sufficient to regenerate a proper subchondral bone, while the cartilage was still immature. This could depend on the limited time of the follow-up and/or the insufficient stimuli for

cartilage regeneration deriving from the microenvironment. In this view we should perform experiments with a longer follow up and/or loading the OPF hydrogel with chondro-inductive molecules such as TGF-β1 or -β3. Indeed, one interesting feature of this biomaterial is the potentiality to absorb molecules that can be subsequently, gradually released (Dadsetan et al., 2010).

Another key experimental parameter of this study was the use of human ASCs in a construct to be implanted in minipigs. The use of xenogenic ASCs in preclinical models is largely diffused, as it showed therapeutic benefits with no side effects (immune reactions raised against xenogenic cells) (Sacerdote et al., 2013; Zeppieri et al., 2013; Zhu et al., 2013a). This approach highlight the non-immunogenicity of these cells, an interesting features also for possible allogenic clinical use, and investigate directly the *in vivo* effects of human cells. Indeed, the slight differences between human and animal-derived ASCs could represent an obstacle for the translation of preclinical data into clinical application. On the other side, the most important criticism on this methodology is that cells could act in a different way while responding to xenostimuli (cytokines, growth factors, etc) and/or, at the same time, they could respond to injury signals differently from autologous implanted ones. In other words, the cross-talk between implanted and resident cells might be distorted.

Nonetheless, studies comparing autologous and xenogenic effect in preclinical models have not yet been carried out.

In our study we decided to use both approaches. Although the limited number of samples using hASCs did not allow any quantitative extrapolation, we can state that hASCs could be used safely in porcine preclinical models, and that OPF+hASCs constructs, as well as OPF+mpA-ASCs, are able to improve the regeneration of subchondral bone and the newly formed cartilage properties if compared to unseeded scaffold. Interestingly, the more mature bone, with the presence of bone lamellas, was observed in OPF+hASC treated defects. A limitation of our study concerns the cell seeding technique on OPF scaffolds; indeed, although a large amount of both human and porcine ASCs seeded on the OPF scaffolds were able to colonize and form clusters within the pores of the scaffold, as also previously observed by Dadsetan (Dadsetan et al., 2010) and Lim (Lim et al., 2013) for BMSCs, some cells were not able to adhere. For this reason, since this problem could be due to the big dimension of the scaffold pores, to ameliorate the cell seeding efficiency we could use OPF with smaller pores.

In our work we were unable to trace whether the new tissues were derived from the implanted or the resident cells. We cannot exclude that the construct served only as a supporting, trophic template that was later resorbed and replaced by host cells. This could sustain the theory described above about MSCs as supporting element at the injury site. From this perspective, it will be interesting to determine which are the last effectors of these cells mechanism of action in order to reputedly maintain the cell effect in a cell-free approach.

Recently, great attention has been paid on microvesicles, that, containing specific proteins, mRNA and miRNA, and spreading from MSCs (Ratajczak 2006) are considered important effectors of their communication with other cells. Various *in vivo* models confirm their effect on several pathologies (Biancone et al., 2012; Bruno et al., 2012; Zhu et al., 2013b), and one of our future interest is to investigate their action in a preclinical model of osteochondral defect.

In conclusion, this study provide the evidence that both porcine and human adipose-derived stem cells associated to OPF hydrogel improve osteochondral defect regeneration. Various parameters, including scaffold pore dimensions, possible association with growth factors, number of cells, biodynamic construct formation or also new approaches, such as the use of ASC-derived microvesicles combined to scaffold instead of cells, need to be considered before moving to phase I clinical trials.

ACKNOWLEDGMENTS

I would like to thank all the people I have directly collaborated with:

Dr. E Broccaioli, Dr. F. Baruffaldi Preis, Dr. A. Addis, and Dr. G Rasperini, for their precious clinical work.

All the laboratory of Prof. C Domeneghini, Veterinary Medical School of University of Milan and the engineers of the Prof P Vena group at Politecnico di Milano .

All the people of the I.R.C.C.S. Galeazzi Orthopedic Institute involved in the projects (particularly Prof. G. Banfi, Dr L. de Girolamo and Dr D. Stanco) and also Dr. S Fenu (Fondazione INGM, Istituto Nazionale di Genetica Molecolare, Milano) for his valuable help with confocal microscopy.

My PhD program was financially supported by I.R.C.C.S. Galeazzi, and this studies were partially supported by the Italian Ministry of Health (2007-656853), University grant FIRST-2008 and RC-2044 IRCCS Istituto Ortopedico Galeazzi (Milano).

Foremost, I would like to express my sincere gratitude to my tutor Prof. Anna Teresa Brini for the continuous support during my Ph.D study and research, for her great motivation and enthusiasm.

I thank all my fellow labmates, present and past; a particular thought goes to Dr. Elena Arrigoni and to Dr. Lorena Maria Josè Ferreira for their help and support.

Last but not least I would like to thank my family and all my dear ones for their encouragement and support.

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PAPERS ENCLOSED

Since their first isolation in 2002 by Zuk et al, great interest has developed around adipose-derived stem cells. The intriguing point, that also pushed me to become fond of these cells, is that a tissue normally discarded (e.g. after liposuction or aesthetic surgery) could be the source for the repair of tissues that cannot spontaneously or completely regenerate and could provide therapeutic tools for the cure of a large variety of pathologies.

During these years, we have investigated various aspects of these cells, also in collaboration with other Research groups.

In this last part of my thesis I take the liberty to enclose the title-page of the papers about studies in which planning and performing I was involved during my PhD period.

Stem Cell Research & Therapy

Porcine adipose-derived stem cells from buccal fat pad and subcutaneous adipose tissue for future preclinical studies in oral surgery

Stem Cell Research & Therapy 2013, **4**:148 doi:10.1186/scrt359

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ISSN 1757-6512

Article type Research

Submission date 9 May 2013

Acceptance date 26 November 2013

Publication date 11 December 2013

Article URL http://stemcellres.com/content/4/6/148

BioResearch Open Access
Volume 0, Number 0, Month 2012
© Mary Ann Liebert, Inc.
DOI: 10.1089/biores.2012.0291

ORIGINAL ARTICLE

Mesenchymal Stem Cells from Bichat's Fat Pad: *In Vitro* Comparison with Adipose-Derived Stem Cells from Subcutaneous Tissue

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Abstract

Adipose-derived stem/stromal cells (ASCs) are progenitor cells used in bone tissue engineering and regenerative medicine. Since Bichat's fat pad is easily accessible for dentists and maxillo-facial surgeons, we compared the features of ASCs from Bichat's fat pad (BFP-ASCs) with human ASCs from subcutaneous adipose tissue (SC-ASCs). BFP-ASCs isolated from a small amount of tissue were characterized for their stemness and multidifferentiative ability. They showed an important clonogenic ability and the typical mesenchymal stem cell immunophenotype. Moreover, when properly induced, osteogenic and adipogenic differentiation markers, such as alkaline phosphatase activity, collagen deposition and lipid vacuoles formation, were promptly observed. Growth of both BFP-ASCs and SC-ASCs in the presence of human serum and their adhesion to natural and synthetic scaffolds were also assessed. Both types of ASCs adapted rapidly to human autologous or heterologous sera, increasing their proliferation rate compared to standard culture condition, and all the cells adhered finely to bone, periodontal ligament, collagen membrane, and polyglycol acid filaments that are present in the oral cavity or are commonly used in oral surgery. At last, we showed that amelogenin seems to be an early osteoinductive factor for BFP-ASCs, but not SC-ASCs, *in vitro*. We conclude that Bichat's fat pad contains BFP-ASCs with stemness features that are able to differentiate and adhere to biological supports and synthetic materials. They are also able to proliferate in the presence of human serum. For all these reasons we propose BFP-ASCs for future therapies of periodontal defects and bone regeneration.

Key words: amelogenin; biomaterials; buccal fat pad; mesenchymal stem/stromal cells; oral bone regeneration

Introduction

Mesenchymal stem/stromal cells (MSCs) represent important suitable candidates in regenerative medicine applications for the treatment of tissues damaged by trauma or pathological diseases. They have been isolated from bone marrow, adipose tissue, tendon, periodontal ligament, synovial membranes, trabecular bone, skin, periostium, and muscle.[1] Even though bone marrow represents the more used source of stem cells (BMSCs) in the clinical field, adipose tissue is a valid alternative source of MSCs. It is easily accessible in large quantities with a minimal invasive harvesting procedure and allow a high number of adipose-derived mesenchymal stem/stromal cells (ASCs) to be obtained.[2,3] ASCs show a multilineage differentiation capacity similar to that of BMSCs.[4–6] The growth factor secretome of MSCs was characterized by Wang et al.,[7] and the secretory activity of MSCs favors a regenerative microenvironment at sites of tissue injury.[8]

Adipose tissue withdrawn during plastic surgery is a discarded tissue, and the usual anatomical regions from which this tissue is collected are the abdomen, breast, buttock, knee, and thigh. In this study, we have characterized human ASCs isolated from the buccal fat pad, usually called Bichat's fat pad (BFP), one of the encapsulated fat masses in the cheek. It is a deep fat pad located on either side of the face between the buccinator muscle and several more superficial muscles, including the masseter, the zygomaticus major,

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JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med (2013)
Published online in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/term.1772

SHORT COMMUNICATION

Chondrogenic potential of human mesenchymal stem cells and expression of Slug transcription factor

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Abstract

The scientific literature rarely reports experimental failures or inconsistent outcomes in the induction of cell differentiation; however, researchers commonly experience poor or unsuccessful responses to differentiating agents when culturing stem cells. One way of investigating the underlying reasons for such responses is to look at the basal expression levels of specific genes in multipotent stem cells before the induction of differentiation. In addition to shedding light on the complex properties of stem cells and the molecular modulation of differentiation pathways, this strategy can also lead to the development of important time- and money-saving tools that aid the efficient selection of cellular specimens – in this case, stem cells that are more prone to differentiate towards specific lineages and are therefore more suitable for cell-based therapeutic protocols in regenerative medicine. To address this latter aspect, this study focused on understanding the reasons why some human mesenchymal stem cell (hMSC) samples are less efficient at differentiating towards chondrogenesis. This study shows that analysis of the basal expression levels of Slug, a negative regulator of chondrogenesis in hMSC, provides a rapid and simple tool for distinguishing stem cell samples with the potential to form a cartilage-like matrix, and that are therefore suitable for cartilage tissue engineering. It is shown that high basal levels of Slug prevent the chondrogenic differentiation of hMSCs, even in the presence of transforming growth factor-β and elevated levels of Sox9. Copyright © 2013 John Wiley & Sons, Ltd.

Received 30 November 2012; Revised 11 April 2013; Accepted 16 April 2013

Keywords chondrogenic differentiation; gene expression; human mesenchymal stem cells (hMSCs); Slug transcription factor

Despite the availability of established protocols, researchers working with stem cells are often faced with difficulties in stimulating cells to differentiate towards a certain lineage. The failure to achieve successful differentiation is usually attributed to the heterogeneity of cell populations or to slight variations in culture conditions. Thus, most investigations directed at improving success in differentiation are focused on identifying the best sources of progenitor cells and the ideal 'cocktail' of differentiation inducers.

Much evidence now exists showing differences in the gene expression profile in differentiated stem cells, but the evaluation of basal levels of specific genes expressed in pre-differentiation conditions is often lacking. In this context, the major challenge is to recognize informative differences in the basal expression levels of a particular gene between stem cell samples and to correlate them with differences in the differentiation potential of the cells. Such analyses could provide clues as to why '*in vitro*' cell differentiation induction fails in some cells, while providing a tool to help select the stem cell specimens more likely to differentiate towards specific lineages.

Pre-differentiation gene expression analysis is not only able to help elucidate the complex properties of stem cells and the molecular mechanisms that regulate specific

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Biochemical and Biophysical Research Communications xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Chemical and genetic blockade of HDACs enhances osteogenic differentiation of human adipose tissue-derived stem cells by oppositely affecting osteogenic and adipogenic transcription factors

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ARTICLE INFO

Article history:
Received 4 October 2012
Available online xxxx

Keywords:
hASCs
HDACs
Runx2
PPARγ
Osteo-differentiation

ABSTRACT

The human adipose-tissue derived stem/stromal cells (hASCs) are an interesting source for bone-tissue engineering applications. Our aim was to clarify in hASCs the role of acetylation in the control of Runt-related transcription factor 2 (Runx2) and Peroxisome proliferator activated receptor (PPAR) γ. These key osteogenic and adipogenic transcription factors are oppositely involved in osteo-differentiation. The hASCs, committed or not towards bone lineage with osteoinductive medium, were exposed to HDACs chemical blockade with Trichostatin A (TSA) or were genetically silenced for HDACs. Alkaline phosphatase (ALP) and collagen/calcium deposition, considered as early and late osteogenic markers, were evaluated concomitantly as index of osteo-differentiation. TSA pretreatment, useful experimental protocol to analyse pan-HDAC-chemical inhibition, and switch to osteogenic medium induced early-osteoblast maturation gene Runx2, while transiently decreased PPARγ and scarcely affected late-differentiation markers. Time-dependent effects were observed after knocking-down of HDAC1 and 3: Runx2 and ALP underwent early activation, followed by late-osteogenic markers increase and by PPARγ/ALP activity diminutions mostly after HDAC3 silencing. HDAC1 and 3 genetic blockade increased and decreased Runx2 and PPARγ target genes, respectively. Noteworthy, HDACs knocking-down favoured the commitment effect of osteogenic medium. Our results reveal a role for HDACs in orchestrating osteo-differentiation of hASCs at transcriptional level, and might provide new insights into the modulation of hASCs-based regenerative therapy.

1. Introduction

The human adipose-tissue derived stem/stromal cells (hASCs) differentiate towards various lineages including bone, dependent on the stimuli (osteogenic supplements, collagen scaffolds) and growth factors [1,2]. Based on the regenerative properties and the scarcely invasive procedure for collection of adipose tissue, hASCs are important tools for therapy of bone diseases [3]. No significant differences between hASCs and bone-marrow derived mesenchymal stem cells from the same patient are observed with regard to the yield of adherent cells, their growth kinetic, cell senescence, differentiation capacity, and gene transduction efficiency [3]. Numerous preclinical applications for hASCs in musculoskeletal tissue engineering are reported [4–7].

Notwithstanding the growing interest in the differentiation plasticity and therapeutic potential of hASCs, molecular mechanisms favouring engagement towards osteogenic lineage have been scarcely investigated. There is few evidence on the epigenetic mechanisms involved in the control of transcription-factor network driving osteo-differentiation, through the expression of osteoblast-maturation genes and extracellular matrix mineralization.

Our funding hypothesis is that osteogenesis is a complex process associated with dramatic changes of gene expression, and the epigenetic control via histone acetylation might be a key molecular mechanism for osteo-differentiation of hASCs. Histone deacetylases (HDACs)/acetylases (HATs) spatio-temporal interplay is critical for gene expression changes [8]. HDACs influence histone acetylation and chromatin condensation, but they act also as co-repressors or co-activators of transcription factors [9–11]. In the present paper we add insights on the role of acetylation in hASCs differentiation towards bone lineage by regulating transcription factors.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.10.044

Please cite this article in press as: P. Maroni et al., Chemical and genetic blockade of HDACs enhances osteogenic differentiation of human adipose tissue-derived stem cells by oppositely affecting osteogenic and adipogenic transcription factors, Biochem. Biophys. Res. Commun. (2012), http://dx.doi.org/10.1016/j.bbrc.2012.10.044

STEM CELLS AND DEVELOPMENT
Volume 00, Number 00, 2013
© Mary Ann Liebert, Inc.
DOI: 10.1089/scd.2012.0398

ORIGINAL RESEARCH REPORT

Systemic Administration of Human Adipose-Derived Stem Cells Reverts Nociceptive Hypersensitivity in an Experimental Model of Neuropathy

Paola Sacerdote,[1] Stefania Niada,[2,3] Silvia Franchi,[1] Elena Arrigoni,[2] Alice Rossi,[1] Vijay Yenagi,[2] Laura de Girolamo,[3] Alberto Emilio Panerai,[1] and Anna Teresa Brini[2,3]

Over the last decade, it has been proved that mesenchymal stem cells (MSCs) elicit anti-inflammatory effects. MSCs from adipose tissue (hASCs) differentiate into cells of the mesodermal lineage and transdifferentiate into ectodermal-origin cells. Although there are various etiologies to chronic pain, one common feature is that painful states are associated with increased inflammation. We believe in hASCs as a therapeutic tool also in pathologies involving neuroinflammation and neuronal tissue damage. We have investigated the effect of hASCs injected in a model of neuropathic pain [(mouse sciatic nerve chronic constriction injury (CCI)]. hASCs from 5 donors were characterized, and no major differences were depicted. hASCs were cryopreserved and grown on demand. About 1×10^6, 3×10^6, and 6×10^6 hASCs were intravenously injected into normal immunocompetent mice. No mouse died, and no macroscopic toxicity or behavioral changes were observed, confirming the safety of hASCs. hASCs, intravenously (i.v.) injected into C57BL/6 mice when the neuropathic pain was already established, induced a significant reduction in mechanical allodynia and a complete reversion of thermal hyperalgesia in a dose–response fashion, already 1 day after administration. Moreover, the hASCs effect can be boosted by repeated administrations, allowing a prolonged therapeutic effect. Treatment decreased the level of the CCI-induced proinflammatory cytokine interleukin (IL)-1β and activated the anti-inflammatory cytokine IL-10 in the lesioned nerve. hASCs treatment also restored normal inducible nitric oxide synthase expression in the spinal cord of CCI animals. Our data suggest that hASCs are worthy of further studies as an anti-inflammatory therapy in the treatment of neuropathic pain or chronic inflammatory diseases.

Introduction

Mesenchymal stem cells (MSCs) are a heterogeneous population that can be isolated from several tissues, expanded in vitro, and purified by plastic adherence.

Adipose tissue is an attractive abundant source of MSCs (ASCs, adipose-derived stromal/stem cells), which are able to differentiate into cells of the mesodermal lineage such as osteoblasts, adipocytes, chondrocytes, and myocytes [1–3], and also to transdifferentiate into cells of the ectodermal lineage [4–6]. In the last 10 years, the knowledge about their features and their application potential in life science have increased rapidly, and the interest in cell-based therapies involving ASCs is also exponentially growing. Until recently, ASCs have been applied in the regenerative medicine field, since they are able to promote the regeneration of damaged soft and hard tissues. We previously showed that ASCs are not significantly affected by the donor's age [7], and that the stromal vascular fraction (SVF) from subcutaneous fat tissue contains NGF^+ ASCs, precursor/progenitor cells that efficiently differentiate toward osteogenic- and chondrogenic-like cells [8], confirming this tissue as a source of agents prone to regenerate various tissues.

Furthermore, ASCs, as other MSCs, are known to be immunomodulatory through the regulation of immune cells [9] by mechanisms that include both direct cellular contact and release of soluble factors such as trasforming growth factor-β, interleukin (IL)-10, leukemia inhibitory factor (LIF), and others [10]. It has been shown that ASCs reduce allogeneic lymphocyte response by displaying potent immunosuppressive effects that could be mediated by LIF [11], and that MSCs suppressed both effector T-cell and inflammatory responses. Gonzalez-Rey et al. [12] showed that human ASCs produced IL-10 interacting with monocytes, and this cell–cell contact also committed monocytes to produce high levels of IL-10, suggesting that human ASCs (hASCs) indirectly suppress T-cell activation by increasing the production of IL-10 by antigen-presenting cells. Confirming their features, ASCs

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INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY AND PHARMACOLOGY Vol. 26, no. 1 (S), 51-59 (2013)

TWO BONE SUBSTITUTES ANALYZED *IN VITRO* BY PORCINE AND HUMAN ADIPOSE-DERIVED STROMAL CELLS

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Nowadays, the repair of large bone defects is an important goal in orthopaedic and dental fields. Tissue engineering, applied to increase the bone regeneration process, combines suitable scaffolds with either terminally differentiated cells or Mesenchymal Stromal Cells. In vitro studies with Adipose-derived Stromal Cells (ASCs) may identify new bioactive supports, to be tested in preclinical model. In this study, we evaluated the biocompatibility and the osteoinductive properties of two bone substitutes, RegenOSS™ (RO-1) and a new generation scaffold (RO-2), on both porcine and human ASCs. Porcine ASCs need a prolonged initial phase to adapt to both substitutes; indeed, their growth was initially reduced respect to cells cultured in their absence. In contrast, human ASCs were not negatively affected. However, no toxicity of RO-1 and -2 was observed on both ASC populations which are able to stick to both biomaterials. RO-1 and -2 supported osteogenic differentiation of porcine and human ASCs in a different manner: the presence of RO-1 up-regulated both alkaline phosphatase (ALP) activity and collagen production of human ASCs, whereas in porcine ASCs, RO-2 seemed to up-regulate ALP activity, while the production of collagen is mainly stimulated by the presence of RO-1. We suggest to use not just human ASCs, but also animal ones to select suitable scaffolds to generate bio-constructs in vitro, which then need to be tested in animal model before reaching the market.

Loss of bone tissue and treatment of severe bone defects remain two most important issues in orthopaedic and dental surgery. Surgical techniques should provide stability to reach osseous integration and remodelling of bone grafts or substitute materials. None of the currently available synthetic materials provides osteoconduction and osteoinduction comparable to human autograft and allograft. Nowadays, an innovative approach to overcome this aspect includes tissue engineering techniques in which the use of autologous or heterologous progenitor cells in association with bio-degradable or bio-absorbable scaffolds is required. Adult Mesenchymal Stromal Cells (MSCs) have the potential to generate several cell types *in vitro,* and, *in vivo,* they are sensitive to biochemical and mechanical stimuli either alone or in combination with supports [1]. Even if bone marrow is, at the moment, the most common source of MSCs, they have also been found in tissues that are usually discarded, such as adipose tissue, deciduous teeth, placenta and umbilical cord [2]. Adipose tissue is a highly complex system which consists of mature adipocytes, preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident macrophages and lymphocytes, and it contains also a fraction of Adipose-derived Stromal Cells (ASCs) [3-6]. The use of MSCs, derived from several sources, in association to selected scaffolds, allows to develop new biocontructs, in particular in orthopaedic and dentistry fields. Biomaterials provide three-dimensional supports, where structural/physico-chemical/mechanical parameters, administration form and reorganization, reabsorption and degradation rate have to be tuned according to the clinical applications [7,8]. Scaffolds able to improve bone regeneration should mimic bone morphology, structure, and function in order to optimize integration into the surrounding

Key words: Adipose-derived Stromal Cells; Bone substitute; Osteoinduction; Orthopaedics; Dentistry; Bone regeneration.

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0394-6320 (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY AND PHARMACOLOGY Vol. 26, no. 1 (5), 11-21 (2013)

STEMNESS AND OSTEOGENIC AND ADIPOGENIC POTENTIAL ARE DIFFERENTLY IMPAIRED IN SUBCUTANEOUS AND VISCERAL ADIPOSE DERIVED STEM CELLS (ASCs) ISOLATED FROM OBESE DONORS

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Today adipose tissue is not just considered as the primary energy storage organ, but it is also recognized as an important endocrine tissue and an abundant source of mesenchymal stem cells (adipose-derived stem cells, ASCs). During the last decade, several studies have provided preclinical data on the safety and efficacy of ASCs, supporting their use in cell-based therapy for regenerative medicine purposes. Little is known about the effect of obesity on ASCs properties. Since ASCs differentiation and proliferation are determined by their niche, the differences in body fat distribution and the obesity-related co-morbidities may have several consequences. In this study we compared ASCs of subcutaneous adipose tissue from obese (obS-ASCs) and non-obese (nS-ASCs) donors in order to compare their immunophenotype and osteogenic and adipogenic potential. Moreover, in order to evaluate the possible difference between subcutaneous and visceral fat, obS-ASCs were also compared to ASCs derived from visceral adipose tissue of the same obese donors (obV-ASCs). Our results show that subcutaneous and visceral ASCs derived from obese donors have an impaired cell proliferation, clonogenic ability and immunophenotype. Nevertheless, obS-ASCs are able to differentiate toward osteogenic and adipogenic lineages, although to a small extent with respect to non-obese donors, whereas obV-ASCs lose most of their stem cell characteristics, including multi-differentiation potential. Taken together our findings confirm that not all ASCs present the same behavior, most likely due to their biological microenvironment in vivo. The specific stimuli which can play a key role in ASCs impairment, including the effects of the obesity-related inflammation, should be further investigated to have a complete picture of the phenomenon.

Since 1994, when leptin was identified, adipose tissue was no longer just considered as a fat store, but as a true secretory tissue (1). About ten years later, the perception of adiposity changed again, thanks to the discovery of an abundant number of mesenchymal stem cells (ASCs, adipose derived-stem cells), that possess similar properties to bone marrow mesenchymal stem cells (2). During the last decade, several preclinical studies have provided data on the safety and efficacy of ASCs, supporting the use of these cells in future clinical applications in various medical fields such as plastic, orthopaedic, oral and maxillofacial and cardiac surgery (3-5).

Key words: Adipose-derived stem cells; obesity; stem cell properties; osteogenic differentiation; adipogenic differentiation

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0394-6320 (2013)

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