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**Periosteum derived stem cells for regenerative medicine proposals: boosting current knowledge**

Ferretti C *et al*. Periosteum-derived stem cells and regenerative medicine

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**Abstract**

Periosteum is a thin fibrous layer that covers most bones. It resides in a dynamic mechanically loaded environment and provides a niche for pluripotent cells and a source for molecular factors that modulate cell behaviour. Elucidating periosteum regenerative potential has become a hot topic in orthopaedics. This review discusses the state of the art of osteochondral tissue engineering rested on periosteum derived progenitor cells (PDPCs) and suggests upcoming research directions. Periosteal cells isolation, characterization and migration in the site of injury, as well as their differentiation, are analysed. Moreover, the role of cell mechanosensing and its contribution to matrix organization, bone microarchitecture and bone stenght is examined. In this regard the role of periostin and its upregulation under mechanical stress in order to preserve PDPC survival and bone tissue integrity is contemplated. The review also summarized the role of the periosteum in the field of dentistry and maxillofacial reconstruction. The involvement of microRNAs in osteoblast differentiation and in endogenous tissue repair is explored as well. Finally the novel concept of a guided bone regeneration based on the use of periosteum itself as a smart material and the realization of constructs able to mimic the extracellular matrix features is talked out. Additionally, since periosteum can differentiate into insulin producing cells it could be a suitable source in allogenic transplantations. That innovative applications would take advantage from investigations aimed to assess PDPC immune privilege.

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**Key words**: Periosteum; Mesenchymal stem cells; MicroRNA; Bone tissue engineering; Bone turn-over

**Core tip:** Periosteum provides a niche for pluripotent cells. Elucidating periosteum regenerative potential is a hot topic in orthopaedics. This review discusses the state of the art of osteochondral tissue engineering rested on periosteum derived cells and suggests upcoming research directions aimed to the development of new standards of care for the maintenance of bone mass both in post-trauma healing process and in physiological turn-over.

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**INTRODUCTION**

The field of Tissue Engineering and Regenerative Medicine (TERM) has burgeoned in the last decade. The term "Regenerative Medicine" was first found in a 1992 Leland Kaiser’s paper[1] as “a new branch of medicine that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems”.

Products for regenerative medicine can consist in proteins, able to stimulate endogenous repair, living cells or even organs. Advances in regenerative medicine applications have been useful to develop new standards of care for the treatment of several diseases such as neurological, cardiovascular, metabolic (*e.g.,* diabetes), oncologic and orthopaedic disorders.

The idea of using cells to restore damaged tissue is intuitively based on their native role in tissue development and homeostasis. Cells could be delivered to the patient alone or combined with a natural or synthetic biomaterial. The interactive ‘‘diamond’’ concept of TERM suggests that in addition to cell type, 3D dimensional structure/architecture, mechanical/physical signals, and bioactive factors in the environment are critical and act in concert to direct tissue repair and regeneration[2]. Each of those areas is currently under dynamic investigation. In this review we will focus on cell-based therapeutic applications in skeletal tissue repair.

Mesenchymal stem cells (MSCs) represent the leading cell type for regenerative medicine purposes. They are multipotent stromal cells capable of both self-renewal and differentiation into lineages of mesenchymal tissue, including cartilage, bone, adipose tissue and skeletal muscle[3]. MSCs were originally identified in the bone marrow stroma, where they regulate key stages of haematopoiesis. Ever since, they have been isolated from other anatomical sites, such as amniotic fluid[4], Wharton’s jelly[5],umbilical cord blood[6], adipose tissue[7], skin[8],synovial membrane[9], articular cartilage[10] and compact bone[11].

The main challenge in osteochondral tissue repair is the healing of critical-size defects that don’t bridge on their own. They result from pathological events (*e.g.,* tumour, trauma, inflammation or congenital malformation) and can be lead to a delayed union or non-union fracture[12]. Surgical procedures employed for bone gaps treatment may be time-consuming, expensive and exposing patients to high risk of complications and discomfort[13]. To overcome these issues regenerative medicine is working to restore structure and function of damaged tissues by TERM approaches.

Since bone marrow contains osteogenic progenitors, its use was proposed to lead efficient bone regeneration and, effectively, preclinical and clinical investigations corroborated this speculation[14]. Periosteum has been identified as an intriguing niche for cells of the osteoblastic lineage as well.

Periosteum is a specialized highly vascularized connective tissue that envelopes bone surfaces (Figure 1). It is composed of an external fibrous layer containing elastic fibres and microvessels and an inner cambium layer where reside periosteum derived progenitor cells (PDPCs) that act as major players in bone development and fracture healing[13,15].

**Regenerative potential of periosteum**

The paramount importance of the periosteum in bone healing process was suggested since 1800s when de Mourgues[16] discovered that transplanted periosteal tissue induced new bone growth. In 1932, Fell[17] was the first to successfully culture periosteum and in 1990s Nakahara *et al*[18] explored the osteogenic potential of PDPCs in bone tissue engineering. At the same time O’Driscoll *et al*[19] underlined the possibility to regenerate cartilage in damaged joints by periosteum transplantation.

The use of autologous periosteum graft has long been known in orthopaedic surgery. However, it’s only after recent progresses that the contribution of the different sources of MSCs in bone repair, as well as their response to growth factors favouring specific differentiation processes has been examined in depth.

Periosteum as a whole have been used in thousands of orthopaedic surgeries as covering layer in autologous chondrocyte transplantation (ACT)[20], in the treatment of non-union fractures[21], as a graft for reconstruction of the patellar articulation[22], or as tissue engineered bone transplant for maxillary sinus floor augmentation[23].

However, only in 2009 Colnot[24] provided direct evidence that periosteum, endosteum, and bone marrow are the major sources of skeletal stem/progenitors cells and that they differently contribute to osteogenesis and chondrogenesis. In bone healing, periosteum and endosteum both give rise to osteoblasts, whereas periosteum is the only source of chondrocytes. The distinct cellular contributions of periosteum, endosteum, and bone marrow suggested the presence of both intrinsic dissimilarities within these residing stem cell populations and differences in the tissue environment. The correct identification of *in vivo* adult skeletal progenitor sources as well as their response to nutrients, metabolites and growth factors will therefore have profound implications in cell-based therapies for the treatment of recalcitrant fractures or bone and cartilage diseases. Exploring and optimising the governing factors that controls PDPCs osteogenesis and chondrogenesis will be a considerable benefit. It is worth noting that periosteum meets the three primary requirements for tissue engineering: cell font, scaffold for cell retaining and delivery, as well as source of local growth factors. These peculiar features endorse its use as a whole, in autologous grafts. The injection of cell suspensions and the transplantation of cells within scaffolds have been largely employed as well[20,23,25].

**Periosteum as cell source**

PDPCs hold promise in osteochondral repair applications due to their ease of isolation and expansion potential. Several studies reveal periosteum as a better cell source for bone regeneration than either bone marrow or other mesenchymal cell origins. This is due to the fact that PDPCs display multipotency at single cell level[3] and a higher proliferation rate while retaining their ability to differentiate *in vitro*[26]. Furthermore, PDPCs from elderly show performances comparable to that of cells from younger subjects[3,27,28]. This may be related to telomeres stability, since *in vitro* analysis showed that after 24 population doublings telomere lengths and telomerase activity are similar to those of the parental population[3].

Harvest site, donor conditions and technical factors could affect periosteum regenerative potential: load-bearing bones have a more osteogenic periosteum than flat bones, and also inter-individual differences influence periosteum biology[29,30]. Moreover, resection methods and cell isolation procedure could affect periosteum regenerative properties as well. To this end, the use of instruments (like forceps) that can disrupt the inner cambium layer should be avoided[13]. After dissection, cells are typically obtained by egression or enzymatic digestion. Despite of isolation method, culture expanded cells retain their osteochondral potential[31,32].Even though both techniques are commonly used, cell egression from their native environment may maintains their physiological state, without artefacts[33]. The choice of basal medium is equally important to preserve MSC characteristics and multipotent properties, even after prolonged culture *in vitro*.

Despite there is still a lack of consensus on the ideal method of culturing MSCs, it has been demonstrated that the use of DMEM-F12 preserves MSC stemness andability to differentiate for more than 25 sub-culture passages[34]**.**

A long-debated issue is the obtainment of a pure PDPC population, since no exhaustive markers to identify MSC populations are established. PDPCs were commonly characterized by the classic MSC antigenic profile in agreement with the minimal criteria of the International Society for Cellular Therapy (Table 1)[35]. Yet, additional efforts are required to circumvent the isolation of contaminant cells, such as fibroblasts. The use of two additive surface markers, CD166 and CD9 and the comparison of their expression levels on MSCs and fibroblasts, could address this item (Table 1). The expression of CD166 is generally higher on MSCs than on fibroblasts, while CD9 expression has the opposite pattern[36]. Moreover, MSCs with a “fibroblast-like” expression pattern (*i.e.,* low CD166 and high CD9) display a poor osteogenic differentiation[36].

Further markers enable to identify periosteum mesenchymal progenitors (Table 1) could be STRO-1, stage-specific embryonic antigen-4 (SSEA4), ScaI and CD146, also known as melanoma cell adhesion molecule (MCAM)[37,38].

In addition, it could be helpful to evaluate the gene expression profile of transcription factors, such as SRY (sex determining region Y)-box 2, also known as Sox2, octamer-binding 4 (Oct4) and Homeobox protein Nanog, associate to pluripotency and stemness[39].

Population enrich,ment for a cell-type specific surface markers by cell-sorting is recommended, too. At last, novel isolation and characterization strategies, from a heterogeneous population, are currently developing. One example is an innovative droplet-based microfluidic device as a platform for the identification and quantification of distinct cell phenotypes[30].

**Molecular pathways in periosteum**

The potential use of mesenchymal cells for *in situ* repair of osteochondral defects is related to their migration and homing. Understanding how MSCs migrate into tissue injured sites is therefore useful to augment cell transplantation efficiency by enhancing cell targeting.

PDPCs show a dose-dependent migratory effect under chemokine receptor ligands stimulation[40]. Interestingly, PDPCs express chemochine (C-X-C motif) receptor 4 and chemochine (C-X-C motif) receptor 5 that respectively respond to the stromal cell-derived factor 1 (SDF-1) and B cell-attractive chemokine 1 (BCA1). Osteoblasts derived from post-traumatic or osteoarthritis patients express SDF1 and BCA1 in the bone remodelling area, indicating the potential role of these chemokines not only as chemo-attractant but also as a signaling molecule for *in situ* bone regeneration. Additional studies showed that the expression of SDF-1 is up-regulated in periosteal cells at the sites of injury and it serves as a potent chemo-attractant to recruit circulating or residing CXCR4 expressing MSCs[41], to promote their proliferation (Figure 2). Apparently, the involvement in PDPCs of the SDF-1/CXCR4 axis during bone repair has not been fully elucidated. However, SDF-1 or CXCR4 blocking clearly inhibits BMP2-induced osteogenic differentiation, probably interfering with Smads and MAP-kinase activation[40].

Bone graft integration depends on the orchestrated activation of growth factors and cytokines in both host and graft. Activation, expansion and differentiation of periosteal progenitor cells act as an essential step for successful bone remodelling. Understanding the molecular events that initiate these actions (*e.g.,* BPM2 signaling) provides insights into endogenous regeneration of periosteum and offers information for optimizing tissue engineering constructs[42].

BMP2 is a bone morphogenic protein that belongs to the transforming growth factor-beta (TGFβ) superfamily. TGFβ/BMPs signaling have widely recognized role in bone formation during mammalian development. Signaling TGFβ/BMPs transduction is performed by both canonical Smad-dependent and non-canonical Smad-indipendent (*e.g.,* p38 mitogen-activated protein kinase pathway, MAPK) pathways. Smad and p38 MAPK pathways converge to runx2 gene and control mesenchymal precursor cells differentiation[43].

BMP2 is at the apex of the signaling cascade that starts periosteal progenitor proliferation and differentiation during repair and regeneration. *In vivo* studies highlight that in the absence of BMP2, periosteal progenitors remain quiescent and healing does not initiate[44]. In addition, the expression of Sox9, a chondrogenic marker is reduced as well. Thus, BMP2 is essential for the activation of periosteal progenitor cells and their subsequent differentiation along the osteo-chondrogenic lineage[44]. The relevance of BMP2 in triggering osteochondral tissue remodelling is related to its involvement in all crucial osteogenic pathways: Wnt/ß-catenin cascade, Fibroblast growth factor-2 (FGF2) and Hedgehog (Hh) signaling[43]. Multiple Wnt proteins and their modulators are expressed in periosteum. Their cross-talk with Hh intermediates enhances fracture healing[42]. The role of Hh pathway in the promotion of osteogenic and chondrogenic differentiation of PDPCs in adult bone repair has been recently confirmed by *in vivo* investigations[45]. FGF2 signaling has a critical function at the early stage of fracture repair it improves new bone volume and mineral content and it also takes part in angiogenesis[45].

BMP2 also functions as focal point for the interaction of Smad and Notch signaling during osteoblast differentiation. The latter enhances BMP-induced Alkaline Phosphatase (ALP) activity and formation of calcified nodules *in vitro*[43,44].

In-depth knowledge on BMP2 and its related signaling-pathways, hence, would provide interesting targets to promote osteochondral repair.

It is also emerging that cartilage and bone regenerative techniques are related to NF-kß/p65 signaling, which determines the early expression of Sox9 and facilitates the subsequent chondrogenic differentiation[46,47].

**Mechanosensing in periosteum**

It is now well accepted that MSC differentiation and phenotypic expression can be influenced by cues from surrounding environment, both soluble (*e.g.,* cytokines and growth factors) and insoluble (*e.g.,* ECM density and stiffness). Due to its external localization on bone, periosteum is particularly sensitive to mechanical stimuli and, even in absence of other stimulations, mechanical load induces new bone formation from periosteum[48], suggesting that this is a highly specialized mechanosensitive tissue[13].

Several studies show that substrate stiffness affects cell shape thus controlling MSCs fate, including self-renewal and lineage commitment[13]. The native environment of PDPCs is mechanically regulated by a combination of tension and shear. PDPCs ability to carry intracellular tension through their microfilament network controls a signaling cascade that, in turn, is responsible for the expression of soluble factors that modulate bone and cartilage growth[13].

In critical size defects, applying tensions in periosteum after surgery leads to rapid *de novo* bone healing. Therefore, mechanical signaling at the tissue level may be responsible for the start of bone regeneration at cell level[13].

Periosteum mechanobiology is probably related to its local microstructure and collagen content[13]. Some studies evidence the emerging role of periostin in the correct collagen fibrillogenesis. Periostin belongs to the matricellular proteins family and regulate cell functions and cell-matrix interaction. Periostin is expressed at high level in the periosteum during embryogenesis and it is re-expressed after mechanical stress and fracture[48]. It is also present in connective tissues subjected to mechanical stress, such as periodontal ligament, heart valves and tendons. Periostin preferential expression in collagen-rich tissues submitted to mechanical stresses (*i.e.,* periosteum) suggests it may play an essential role in bone maintenance and regeneration[48].

As matter of fact, the regulation of the periostin expression occurs by Wnt pathways; BMP2, TGFβ and retinoic acid stimulate periostin expression as well[49-51].

Through interaction with several integrins, periostin recruits and attaches osteoblasts to bone matrix and activates pro-survival signaling, by caspases inactivation, resulting in increasing bone formation[48]. In addition, periostin interacts with BMP1 to augment its deposition in the fibronectin matrix, in close proximity of lysyl oxydase (LOX), an enzyme that catalyses the collagen cross-linking[48].At last, periostin has a binding site for glycoproteins, glycosaminoglycans and proteoglycans, suggesting a role of this protein in supporting mechanical strength in periosteum[48]. Taken together these data suggest that periostin, contributing to matrix organization, bone microarchitecture and bone strength[48], may acts as a support, thus playing a clear role in the intrinsic mechanobiology of periosteal tissue.

These insights in understanding and harnessing the innate mechanosensing of both periosteum and its cells provide a unique opportunity to induce differentiation without perturbing the biochemical environment[14].

**microRNAs and periosteum**

MicroRNAs (miRs) are small noncoding RNAs that have emerged as crucial post-transcriptional regulators of gene expression by either inhibiting mRNA translation or inducing mRNA degradation[52,53]. miRs can be transcribed individually or in clusters and are encoded by introns or intergenic regions. After being transcribed, primary miRs are processed by protein complexes containing the endonuclease Drosha into the precursor miR (pre-miR), which is approximately 70 nucleotides. Pre-miR is subsequently exported to the cytoplasm[52,53]. Next, the endonuclease Dicer further cleaves the pre-miR, resulting in the generation of the approximately 22-bp miR duplexes, which are incorporated in the RNA-induced silencing complex. One strand is then retained in the complex and becomes the mature miR, which binds to the 3′ untranslated region of the target mRNA.

Hundreds of miRs have been described and currently approximately 1500 miRs are considered to be expressed in humans. Each miR binds up to several hundred complementary mRNAs, thereby modulating gene expression patterns rather than single genes. In the past decade, miRs were extensively investigated and were shown to act as key players in various critical cellular processes such as proliferation, cell cycle progression, apoptosis and differentiation.

As far as stem and progenitor cells are concerned, distinct miRs regulate their functions, modulating cell survival and homing or controlling differentiation and maturation. Additionally, experimental studies shown that miRs regulate endogenous tissue repair and might potentially be useful to enhance bone regeneration[54].

The switch between self-renewal and differentiation requires rapid widespread changes in gene expression. Since miRs can repress the translation of many mRNA targets, they are good candidates to regulate cell fate[55]. Throughout recent years extensive molecular studies have unraveled genetic and epigenetic mechanisms involved in osteoblasts differentiation and functions[54].

As mentioned above, differentiation of MSCs into the osteogenic lineage is tightly regulated by local growth factors (*e.g.,* BMPs, FGFs) that activate specific intracellular pathways, thus triggering the expression of crucial transcription factors such as Runx2 and Osterix (Osx)[54]. miRs regulate each differentiation step by targeting multiple proteins and various signaling pathways, exerting a positive or a negative effect on osteogenesis.

miR-29b, miR148b, miR196a, miR-210, miR-2861 and miR-3960 have been reported to cause down-regulation of various inhibitors of osteoblasts differentiation, thus exerting stimulatory effects. For instance, miR-29a potentiates osteoblastogenesis by modulating Wnt signaling through a positive feedback loop[56].

On the contrary, miR138, miR-133 and miR-204 are associated with a low bone mineral density. Particularly, miR138 was shown to attenuate the ERK-dependent pathway, phosphorylation of Runx2, and Osx expression, being able to inhibit osteoblasts differentiation and bone formation by human MSCs both *in vitro* and *in vivo*[57].

Elucidating the molecular mechanisms that regulate MSC differentiation is important not only for the treatment for orthopaedic trauma, but also for regenerative medicine purposes in case of the loss of functions that naturally occurs with age. Bone homeostasis is in fact strictly related to the balance between bone deposition and resorption as well as to the correct response to mechanical forces.

miRs act as key regulators of both bone formation and remodelling and degeneration, as well. Deregulation of miRs-mediated mechanisms is pathologically linked to bone-related diseases, such as osteoporosis[58]. Indeed, since miRs control differentiation of osteoblast from stem cells and differentiation of osteoclasts from hematopoietic precursors[58], deregulation at these levels could affects osteoclast-related bone remodelling[58].

At present, no data are available on miRNA expression in periosteum. Therefore, profiling of miRs in PDPCs could be useful in elucidating crucial mechanisms governing pre-osteoblasts differentiation during bone development and remodelling. Moreover, advances in miR expression knowledge could also provide information on bone tissue metabolism during lifespan, with particular attention to changes related to inflammation and/or ageing.

**Periosteum and cartilage regeneration**

The chondrogenic potential of periosteum is well documented both *in vitro* and *in vivo*[19,59], in fact free autogenous periosteal grafts restore cartilage defects[60]

Immediately following cortical bone injury, periosteum undergoes a series of changes to initiate bone formation at the fracture site. Cells at the periphery of the cortex adopt an osteogenic fate whereas cells near the cortical bone junction differentiate into chondroprogenitors[42]. Chondrocytes within the fracture callus are primarily derived from the periosteum inner cambium-layer as indicates the presence of Sox-9 expressing chondroprogenitor cells in the periosteum adjacent to the fracture site[61].

The development and maturation of neochondrocytes involves several growth factors, encompassing Insulin Growth Factor 1 (IGF-1), TGFβ1, TGFβ3, Growth Differentiation Factor 5 (GDF-5) and BMP2[62]. In addition the expression of adhesion molecules, such as N-cadherin, play a role in the regulation of chondrocytic phenotype[63]. At last, for resurfacing arthoplasty in humans, periosteum has been used alone or in combination with continue passive motion to stimulate joint neochondrogenesis[62].

With aging the chondrogenic potential of periosteum decreases, as the number of chondrocytes precursors decline in the cambium layer[62]. However sub-periosteal injection of both TGFβ1[64,65] and TGFβ3 has been shown to stimulate the proliferation of PDPCs and to induce their chondrogenic differentiation[63]. Yet, a recent study showed that a subperiosteal injection of a chondroinductive growth factor mixture do not stimulate tissue differentiation of an autologous osteoperiosteal graft[66]. This suggests that the repair of cartilage defects could benefit from an *in vitro* pre-treatment of micromass PDPCs cultures with TGFβ3, which improves periosteum ability to undergo chondrogenesis and produce hyaline cartilage[66]. Quality of tissue harvest, choice and amount of appropriate stimulating molecule, time of exposure, as well as intervals between injections, may influence healing. Mechanical stimulations could affect the clinical outcome as well.

Tissue engineering approaches in cartilage tissue regeneration could be also useful to potentiate the *in vivo* outcomes. Recently, Casper *et al*[67] showed the potential of PDPCs to infiltrate poly-epsilon caprolactone (PCL) nanofiber scaffolds in a rabbit model and the possibility to produce engineered cartilage *in vitro*. The same group has also demonstrated that the application of a directional fluid flow to periosteal explants seeded onto PCL scaffolds enhances cell proliferation, chondrogenic differentiation and organization, thus modifying the biomechanical properties of the engineered cartilage[68].

In order to generate 3D artificial cartilage resembling native articular one, a recirculating flow-perfusion bioreactor, which simultaneously offer shear stress and hydrodynamic pressure, was also developed and, in presence of periosteum/PCL constructs, good ECM composition, cell distribution and mechanical properties were obtained[59].

**Periosteum and bone healing**

In fracture healing, periosteum is the major responsible for bridging the callus formation and participating to endochondral and intramembranous ossifications.

Steps of fracture bone repair have been well summarize by Shapiro[69]. After fracture, cells from the inner cambium layer of periosteum proliferate and differentiate: at the periphery of the fracture the inner layer arranges a collar of bone by intramembranous ossification; nearer to the fracture site the cambium layer produces a mass of cartilage around the fracture location that, subsequently, undergoes to endochondral ossification[69]. Osteoblastic potential of periosteum differs not just with age but also by location: calvaria periosteum showed less osteogenic potential than tibia ones[29,70].

Even though the use of periosteal autografts for the treatment of bone fractures is a well-established procedure[21,51], only recently it was demonstrated that autologous periosteal precursor cells cultured on a 3D matrix are responsible to promote the healing of a distal femur atrophic non-union[71]. Unfortunately, autografts are not always feasible, also due to donor-site morbidity, and alternatives have to be sought. Indeed, the use of allografts for the treatment of critical sized bone defects remains a challenge. Allografts avoid donor site pain and morbidity and fill the need for large volumes of graft materials[72]. Yet, clinical evidences showed that where periosteum orchestrates bone remodelling, allograft healing ability is lower if compared to autograft[73]: allografts exhibit minimal engraftment and a 60% failure rate 10-years-post-transplantation[74,75].

Alternatives to the use of native periosteum for critical size defects healing could be hence hypothesized. For instance, when periosteum contains too few PDPCs or has been damaged, it is possible to create a tissue engineered periosteum (TEP)[13]. At present, few studies have well characterized TEP mechanical properties. Therefore, this approach is currently intended only for use in oral applications, where TEP would experience less mechanical strain than in a dynamically loaded environment (*i.e.,* femur)[13].

It is been a long time since the need to realize constructs that reproduce the intrinsic properties of autogenous bone, by culturing PDPCs *ex-vivo* and subsequently seeding into a natural or synthetic scaffold, has emerged[33]. The success of this approach is strictly related to the use of an appropriate material able to improve PDPC differentiation, with a corrected structure/topography and able to provide adequate support for nutrients and growth factors[2] (Figure 3).

For the development of an engineered tissue, elucidating the steps that can enhance PDPC osteogenic differentiation is advantageous as well[76].In mesenchymal stromal cells this involves the following processes: cell proliferation, cell migration–aggregation and cell differentiation with the dynamic expression of osteogenic transcription and growth factors[77]. Moreover, early MSC osteogenic differentiation is characterized firstly by a proliferative burst, including the formation of nodule-like structures, accompanied by the expression of ALP.

To replicate this differentiation profile, PDPC culture conditions reproducing these key events are required. It has been widely demonstrated that under osteogenic conditions, PDPCs express mRNAs for bone markers (*e.g.,* collagen type I, osteopontin and osteocalcin), whilst in a chondrogenic environment they display chondrogenic markers such as collagen type II and aggrecan[76]. Moreover, the addition of foetal bovine serum (FBS) and dexamethasone (Dex) to the culture media has a positive effects on osteocalcin and ALP expression, in the early differentiation stages[78]. For the expression of the main transcription factors governing osteogenesis and hence differentiation towards a mature osteoblast, the subsequent combination of trans-retinoic acid (atRA), FBS, Dex and BMP2 is required[78]. At last, also Vascular Endothelial Growth Factor (VEGF) plays a role in osteogenesis and it is express in human normal periosteum as well as in periosteum after fracture healing: the addition of VEGF to a basal culture medium enhance PDPC osteoblastic differentiation. That was corroborated by our results as well[79].

In bone tissue engineering approach, scaffolds are generally used as temporary substitutes of the original tissue after injury. As well-known, 3D scaffolds should be tolerated by the body, provide cell attachment, migration and proliferation, allow for biochemical signaling and possess a bone-like stiffness and degradation rate commensurate to bone healing[2,80]. Canonical classification includes natural and synthetic scaffolds. Natural scaffolds such as chitosan, collagen, gelatine, fibrin glue and hyaluronic acid show several advantages, such as an ECM-like chemistry and structure, the presence of cell-adhesive sequences and a resorbability driven by enzymes, with the production of non-toxic easily excreted molecules. Natural materials are also often used as drug carries for their aptitude to retain growth factors that encourage cellular migration and proliferation[81]. Drawbacks in their use include limited availability, low mechanical resistance and potential immunogenicity[80]. In this respect synthetic scaffolds display many advantages, encompassing easy modulation of chemical and mechanical properties, biodegradability and avoidance of infections or immunogenicity.

Hydroxyapatite or its analogues (including natural bone matrix) are the most popular inorganic components for bone replacement, due to their chemical similarity to the mineral component of mammalian bone[80]. Collagen/demineralised bone powder scaffold combined with PDPCs has been proposed as a potential tool for bone tissue engineering[82]. In our experience scaffolds with an increased amount of inorganic phase were able to modulate stem cells behaviour[11] as well as periosteal-derived stem cells osteogenic properties[83]. The rationale for the use of Calcium Phosphate (CaP) biomaterials and the evaluation of their bone forming capacity in the presence of PDPCs has been recently summarized by Roberts *et al*[84].

Modern bone regenerative medicine strategies aim to “take lesson from Nature” in scaffold development. To this respect a chitosan-heparin coating acting as a synthetic periosteum was recently proposed for the improvement of bone allografts outcomes[85]. Several biomaterials, such as naturally derived acellular matrices, commercially available collagen-based sponges and synthetic polymers[86-88] have also been investigated as periosteum mimicking. These materials improve cell localization but show an inadequate cell survival[86-88]. Instead, the use of hydrogels, which emulate mechanical properties and hydration of the native periosteum ECM, seems a promising approach. Hydrogels may be properly tailored for correct degradation, inclusion of biomolecules and cell-adhesion ligands in order to elicit a specific cell functions[85]. It has been shown that hydrogel-based tissue engineered periosteum enhance osteoblast progenitor cells infiltration, bone callus formation and allograft biomechanical stability[72,73].

Besides, peculiar surgical techniques have been used as a tool for mimicking periosteum. Since 1986 Masquelet[89] developed a simple method to reconstruct long bone defects based on the insertion of a cement spacer that maintains the space for bone reconstruction and promotes the formation of a synovium-like membrane. This induced membrane (IM) prevents the graft resorption and favours its re-vascularization. Moreover, the membrane acts as an *in situ* growth factors delivery system, which is capable of enhancing bone graft healing[89].

Recently, Cuthbert*et al*[90] investigated the morphology, molecular properties and gene expression pattern of IMs from patients undergoing large bone defects surgery, showing that IMs share strong architectural similarities, vascular features and growth factor expression of periosteum[90]. Moreover, cells expanded from IMs revealed a mRNA profile similar to PDPCs[90]. Cuthbert *et al*[90] thus, provided evidences that the IM technique generates a dynamic periosteum-like structure, offering important insights into new bone regeneration approaches. Nevertheless, further studies are required to establish if this surgical technique could be suitable for all bone regeneration applications despite of the nature of disease, the lesion site and the patient-related features.

**PDPCs in oral and maxillofacial tissue engineering**

Periosteum has found great use in enhancing bone formation in the field of dentistry and maxillofacial reconstruction[91,92]. Even though human jaw periosteal cells (JPC) are a promising source for the engineering of cell-based osseoinductive grafts in oral surgery[93], their harvesting and subsequent characterization is not particularly easy. Specific surface markers can facilitate the isolation of a cell pure population, while an accurate analysis of the gene expression profile can allow a detailed comprehension of the JPCs.

In the last years, several markers have been suggested to enrich the osteogenic progenitor cell fraction from the entire JPCs population. Among these, particular attention has received mesenchymal stem cell antigen-1(MSCA-1) and CD 166. MSCA-1+ enriched JPCs have an higher osteogenic potential compared with MSCA-1, as well as CD166+ respect to CD166--[93].Magnetic-Activated Cell Sorting (MACS) isolation technology was also recommended for increasing recovery and purity of rare MSCA-1+cells from jaw periosteum[93].

The high osteogenic potential of MSCA-1+ cell fraction is strictly related to the expression of specific markers, such as lipoprotein receptor-related protein 6 (LRP-6), a key component of the WNT receptor complex. MSCA-1+/LRP-6+ also induce an high expression of stanniocalcin 1 (STC-1) and of tissue inhibitor of metalloproteinases-4 (TIMP-4)[93]. STC-1 is involved in endochondral and intramembranous bone formation while TIMP-4 is tangled in ECM remodelling during JPCs osteogenesis[93].

In spite of PDPCs derived from periosteum, other sources of stem cells such as dental pulp[94,95] and periodontal ligament[95] have been proposed for dentistry applications. Harvest morbidity and patient acceptance should affect the final choice of the appropriate cell source for regenerative medicine purposes.

***Cutting-edge applications***

The great plasticity of mesenchymal stromal cells, due to their ability to differentiate into multiple lineages, makes them good candidates for *in vivo* regeneration innovative procedures. The use of allogeneic MSCs in regenerative medicine is also encouraged by their immunosuppressive and immunomodulatory features.

MSCs derived from different sources have been studied for the generation of Insulin-Producing Cells (IPCs) in the treatment of type 1 diabetes. Kim *et al*[95] examined the differentiation in IPCs of MSCs isolated from different sources: bone marrow, adipose tissue, Wharton’s jelly and periosteum. Even though cultured under similar conditions, only IPCs derived from PDPCs showed a significant increase in insulin secretion under glucose stimulation[96].

These results indicate the periosteum as a suitable source of multipotent progenitor cells that could be employed in allogenic transplantations.

However, even if MSC immune privilege is well known for cells derived from bone marrow, umbilical cord blood and adipose tissue, no studies confirm that PDPCs have similar properties. Therefore, this aspect needs to be further investigated in order to accomplish PDPCs innovative applications[96].

**Conclusion**

Small bone defects can be bridged with conventional grafting[97], whilst bone regeneration in large bone defects is challenging and several factors (*i.e.,* defect site and patient related factors) may affect treatment outcomes. The healing of large size defects, hence, looks into tissue engineering strategies, including the use of exogenous stem cells, growth factors and bioactive scaffolds[2,98]. Recently relevant breakthroughs in designing and creating bone substitutes have been achieved. After the sophisticated approaches combining biomaterials/stem cell constructs the concept of a guided bone regeneration has received attention: the use of smart, bioactive-induced membranes started gaining momentum.

Scientific word is therefore going on with investigations understanding molecular basis of cell/tissue endogenous repair, as well as, improving scaffold design. To this end, periosteum will offer new intriguing cues for further investigation.

Periosteum plays a key role in ECM architecture and cell cytoskeletal reorganization under mechanical stress, by the activation of the mechanosensing signaling. The comprehension of cell molecular mechanisms associated with mechanosensing and cell intrinsic repair abilities has underlined a critical role of periostin. Its expression is up-regulated in the presence of mechanical stress in order to preserve bone tissue integrity and function. Periostin up-regulation leads to the activation of specific pathways that support cell survival. It also ensures a correct collagen fibrillogenesis and matrix organization, opening intriguing perspective in designing future strategies for bone tissue regeneration.

In addition, a further characterization of cellular epigenetic mechanisms miRs related is encouraged: directing the mRNAs expression, miRs affect pivotal differentiation pathways and could therefore represent important targets in promoting osteochondral regeneration.

Finally, considering periosteum dynamic response to environmental and mechanical stimuli, two strategies have been pursued: the use of periosteum itself as a “smart material” (*i.e.,* TEP) and the realization of constructs (*e.g.,* chitosan-heparin coating and PEG-hydrogels) able to mimic the ECM features of this tissue. At present TEP constructs are not tuned for the repair of a dynamically loaded environment such as long bones.

Taken together, the data highlight periosteum involvement in bone anabolic pathways and suggest novel TERM approaches in osteochondral tissue repair. Moreover, a deeper understanding of the molecular basis of cell mechanosensing, as well as of microRNA involvement in PDPC differentiation responses, could be useful for the development of new procedures for the maintenance of bone mass both in post-trauma healing process and in physiological turn-over (therefore preventing osteoporosis).

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**Table 1 surface markers of periosteum-derived cells**

|  |  |  |
| --- | --- | --- |
|  |  | **Ref.** |
| **Minimal criteria for MSCs** |  |  |
| CD73 | **+** | 13,35,79,94 |
| CD90 | **+** | 13,35,79,94 |
| CD105 | **+** | 13,35,79,94 |
| CD45 | **-** | 13,35,79,94 |
| HLA-DR | **-** | 13,35,79,94 |
| CD14 | **-** | 13,35,79,94 |
| CD34 | **-** | 13,35,79,94 |
|  |  |  |
| **Integrins** |  |  |
| CD29 | **+** | 13,94 |
| CD49e | **+** | 13,94 |
|  |  |  |
| **Adhesion molecules** |  |  |
| CD31 | **-** | 13,94 |
| CD44 | **+** | 13,94 |
| CD166 | **+** | 13,36,94 |
| CD54 | **+** | 13,94 |
| CD146 | **+** | 37,38 |
|  |  |  |
| **MHC class** |  |  |
| HLA-ABC | **+** | 13,94 |
|  |  |  |
| **Hematopoietic markers** |  |  |
| CD14 | **-** | 13,94 |
| CD33 | **-** | 13,94 |
| CD34 | **-** | 13,94 |
| CD45 | **-** | 13,94 |
| CD133 | **-** | 13,94 |
|  |  |  |
| **Additional markers** |  |  |
| MSCA-1 | **+** | 93 |
| CD9 | **+/-** | 13,36,94 |
| CD13 | **+** | 37,38 |
| STRO-1 | **+** | 37,38 |
| SSEA-4 | **+** | 37,38 |
| ScaI | **+** | 37,38 |
| Sox2 | **+** | 39 |
| Oct4 | **+** | 39 |
| Nanog | **+** | 39 |

CD: Cluster of differentiation; HLA: Human leucocyte antigen; MSCA-1: Mesenchymal stem cell antigen 1; STRO-1: Stromal cell antigen -1; SSEA-4: Stage specific embryonic antigens 4; ScaI: Stem cell antigen I; Sox2: Sex determining region Y box; Oct4: Octamer-binding 4.

**Figure 1 Schematic representation of periosteum as well as the distribution of cell populations and extracellular matrix that contribute to its biological and mechanical properties.** PDPCs: periosteum-derived precursor cells.

**Figure 2 stromal cell-derived factor 1/chemochine receptor 4 can recruit mesenchymal stem cells to induce fracture repair in skeletal healing.** stromal cell-derived factor 1 (SDF-1) is expresses on the periosteum of the bone graft and recruited chemochine (C-X-C motif) receptor 4 (CXCR4) expressing mesenchymal stem cells (MSCs) in the acute phase of bone repair.

**Figure 3 interactive ‘‘diamond’’ concept of Tissue Engineering and Regenerative Medicine suggests that in addition to cell type, 3D dimensional structure/architecture, mechanical/physical signals, and bioactive factors in the environment are critical and act in concert to direct tissue repair and regeneration. Cell activity is dynamically regulated by the other key cornerstones of the diamond.**