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OVERCOMING PHYSICAL CONSTRAINTS IN BONE ENGINEERING: 'THE IMPORTANCE OF BEING VASCULARIZED'.

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Abstract

Bone plays several physiological functions and is the second most commonly transplanted tissue after blood. Since the treatment of large bone defects is still unsatisfactory, researchers have endeavoured to obtain scaffolds able to release growth and differentiation factors for mesenchimal stem cells, osteoblasts and endothelial cells in order to obtain faster mineralization and prompt a reliable vascularization.

Nowadays, the application of osteoblastic cultures spans from cell physiology and pharmacology to cytocompatibility measurement and osteogenic potential evaluation of novel biomaterials. To overcome the simple traditional monocultures *in vitro*, co-cultures of osteogenic and vasculogenic precursors were introduced with very interesting results. Increasingly complex culture systems have been developed, where cells are seeded on proper scaffolds and stimulated so as to mimic the physiological conditions more accurately. These bioreactors aim at enabling bone regeneration by incorporating different cells types into bio-inspired materials within a surveilled habitat.

This review is focused on the most recent developments in the organomimetic cultures of osteoblasts and vascular endothelial cells for bone tissue engineering.

Introduction

The multifunctional skeletal system usually displays a good regenerative capability, but the natural healing does not always occur properly. This is the case of bone non-unions, delayed bone formation after fracture, bone lacunae after demolitive surgery, inborn malformations, and alveolar resorption. Unsurprisingly, treatment of bone defects conveys major interest in the biomedical field, as bone is the second most commonly transplanted tissue after blood, with over 2.2 million bone grafting procedures performed annually worldwide (Giannoudis *et al.*, 2005). For an effective bone regeneration to occur, three key elements must coexist: a scaffold, growth and differentiation factors, and living functional cells capable of synthesizing extracellular matrix (Olender, Brubaker, Wojtowicz, & Kaminski, 2014). Notably, in the last years, a great effort has been provided to obtain scaffolds that release growth and differentiation factors, so as to enhance the recruitment and differentiation of mesenchimal stem cells, osteoblasts or/and endothelial cells, to promote vascularization and mineralization (Nath, Abueva, Kim, & Lee, 2015; Perets et al., 2003; Suliman et al., 2015; Yufeng Zhang et al., 2015; J. Zhao et al., 2013; Shichang Zhao et al., 2014).

The presence of cells is mandatory to allow osteogenesis. Since the first *in vitro* culture of human osteoblasts (Bard, Dickens, Smith, & Zarek, 1972), several cell lines, mainly of tumoral origin or otherwise immortalized, along with primary cells and pluripotent stem cells, have been developed as models for bone studies. Osteoblastic cultures are currently used for cell physiology and pharmacology, as well as for cytocompatibility testing and osteogenic potential evaluation of novel biomaterials. However, the experimental settings should take into account the advantages and limitations of the cell types employed, especially if aimed at regenerative approaches. While cell lines are easy to obtain and handle, primary cells are less available and display higher variability. On the other hand, being immortalized or tumor derived, cell lines may not represent the best model to reproduce the physiological behavior. Pautke et al. pointed out that normal human osteoblasts differ significantly from osteosarcoma cell lines (SaOs-2, MG-63 and U-2 OS) as for immunocytochemical markers and matrix produced (Pautke et al.,

2004). Another relevant issue deals with interspecific differences, since human cells likely provide more relevant information in the study of human osteogenesis. Recently, factors influencing the phenotype of osteoblasts derived from different sources, along with the advantages and limitations of these cell types, have been thoroughly reviewed (Czekanska, 2012).

The conventional monocultures *in vitro* are hindered by over-simplification and unable to reproduce the complex features of the processes investigated. To address this pitfall, co-cultures of osteogenic and vasculogenic precursors were introduced with very interesting results (Dohle et al., 2014; Leszczynska, Zyzynska-Granica, Koziak, Ruminski, & Lewandowska-Szumiel, 2013a; Pedersen et al., 2013a; Santos, Unger, Sousa, Reis, & Kirkpatrick, 2009; Shi, Andrukhov, Berner, Schedle, & Rausch-Fan, 2014; Tao, Sun, Wang, & Liu, 2009a). In the last few years, research has been prompted to develop increasingly complex 3D culture systems in bioreactors, where cells are seeded on proper scaffolds and stimulated so as to mimic the physiological conditions more accurately. These bioreactors aim at enabling bone regeneration by incorporating different cells types, such as osteoblasts and endothelial cells (ECs), into bio-inspired materials within a surveilled habitat (Brennan, Davaine, & Layrolle, 2013; Deb, Mandegaran, & Di Silvio, 2010; Dorst, Oberringer, Grässer, Pohlemann, & Metzger, 2014; Gao et al., 2014; Hofmann et al., 2008; Santos et al., 2009; Thein-Han & Xu, 2013).

This review is focused on the most recent developments in the organomimetic cultures, as regards the role played by osteoblasts and vascular endothelium.

Complexity of bone physiology

Autogenous grafting has been widely used for repair and reconstruction of bony skeletal defects and stimulation of bone formation (Hubble, 2002), along with allografts (i.e. coming from the same species), xenografts (coming from other species) and a broad spectrum of synthetic biomaterials. Nevertheless, as the treatment of large bone defects is still an unmet challenge, tissue engineering techniques (Langer & Vacanti, 1993) have been proposed, in the last few years, generating a huge deal of research. As non-functionalized scaffolds largely failed in enhancing bone formation in vivo (Szpalski, Sagebin, Barbaro, & Warren, 2013), researchers recur to functionalizing scaffolds with a large array of growth factors, biomimetic surface coating, adhesion proteins, and signaling molecules (Cushnie, Khan, & Laurencin, 2010; Sahoo, Ang, Goh, & Toh, 2010; Yanoso-Scholl et al., 2010). Indeed, the cell types present within bone tissue are numerous including: 1) the osteogenic line spanning from the mesenchymal stem cells (MSCs) to the committed osteocytes, through pre-osteoblasts, osteoblasts and bone lining cells; 2) osteoclasts belonging to monocyte-macrophage system; 3) endothelial cells (ECs), 4) neurons (ANS). This high complexity of bone also owes a great deal to the extracellular matrix (Paiva & Granjeiro, 2014) and the environmental factors alike (Szpalski et al., 2013).

In addition to the chemical composition of the matrix, also mechanical and electrical stimuli are critical for the healthy maintenance of bone. Bone cells *in vivo* are exposed to compressive, tensile, and torsional stresses due to bone loading and to shear stress from interstitial flow (Burr, Robling, & Turner, 2002), which influence, via osteocytic guidance of osteoclast and osteoblast activity, bone mass and microstructure (Zaidi, 2007).

In particular, cells are affected by the nature of the loading applied (frequency and strain rate) [De R, Zemel A, Safran SA. Theoretical concepts and models of cellular mechanosensing. Methods Cell Biol. 2010;98:143-75. doi:10.1016/S0091-679X(10)98007-2.]. The strain rate of specific waveforms may be adjusted to achieve desired results (Riehl, Park, Kwon, & Lim,

2012). Stretch frequency has been shown to orchestrate cell orientation through the remodeling of focal adhesions and stress fibers in endothelial cells (Hsu, Lee, & Kaunas, 2009). These cells are physiologically subjected to unique mechanical stimuli consisting of flow-induced shear stress and tensile strain due to distension and stretching of the tissues. Under cyclic stretch, vascular ECs increase stress filament area in response to shear stress and regulate autocrine and paracrine signaling for angiogenesis and endothelial remodeling (Chien, 2007; Haghighipour, Tafazzoli-Shadpour, Shokrgozar, & Amini, 2010; Yung, Chae, Buehler, Hunter, & Mooney, 2009). Cyclic stretch, shear stress, and pressure on mesenchymal stem cells (MSCs) produce significant differences in cell morphology and lineage specification (Maul, Chew, Nieponice, & Vorp, 2011). Cell differentiation may also be tuned by strain magnitude. Finally, the role of strain in keeping the bone healthy is achieved through the osteocytes, which are subject to a remarkable mechanical stimulus at the membrane level (You, Cowin, Schaffler, & Weinbaum, 2001; Zaidi, 2007). Being numerous and capable to form an extensive network through long intercellular processes, osteocytes fulfil a complex "mechano-responsive" task (Hoey, Kelly, & Jacobs, 2011; Klein-Nulend, Bakker, Bacabac, Vatsa, & Weinbaum, 2013; Nguyen & Jacobs, 2013; Xiao et al., 2011). Indeed osteocytes not only detect, but also respond to mechanical stimuli regulating the activities of osteoblasts as well as of other cells (Litzenberger, Kim, Tummala, & Jacobs, 2010; Nakashima et al., 2011; Robling et al., 2008; Tu et al., 2012; Xiong et al., 2011; Yue Zhang et al., 2011).

In addition to mechanical stimulation, electrical properties of bone are suggested to play a feedback role on its remodeling and development (Fukada & Yasuda, 1957; Guzelsu & Demiray, 1979; Rubinacci, Black, Brighton, & Friedenberg, 1988). Bone bio-potentials may be either strain-related or non-strain-related (Gittens, Olivares-Navarrete, Tannenbaum, Boyan, & Schwartz, 2011). When strain-related, bio-potentials are due to the piezoelectric behavior (*i.e.*, electric potential in response to applied forces) of bone, which is related to the structure and dipolar charge of collagen, as well as to the streaming potentials associated with the flow of fluid and ions through porous bone. Non-strain-related bio-potential result from contribution of

biological processes such as osteoblast membrane potential, extracellular matrix acidification and ion release caused by osteoclast bone resorption, and cell junctions of osteocytes. *In vivo*, these electrical signals work in concert to provide the correct environment for normal bone growth and development, but can be disrupted or altered by injury after trauma and during healing.

Reproducing tissue complexity: what has been done so far

The need for osteogenesis: osteoblast and osteocyte cultures

For sake of clarity, a brief outline of the main osteoblastic and osteocytic models is here reported. Of great interest in the study of human osteogenesis, primary human cells are a heterogeneous cell population. As a consequence, they exhibit relevant phenotypic differences depending on a series of variables among which the anatomical district of origin and the donor features are worth remembering (C. Li et al., 2015).

In the last 30 years, several methods to isolate primary human osteoblasts have been developed. The main advantages in using these cells are their potential clinical applicability (when autologous) and the reduced interspecies differences (Kasperk et al., 1995) along with the possibility to better portray osteoblast to osteocyte transition reducing the current gap between in vitro data and in vivo results [Czekanska EM1, Stoddart MJ, Ralphs JR, Richards RG, Hayes JS. A phenotypic comparison of osteoblast cell lines versus human primary osteoblasts for biomaterials testing. J Biomed Mater Res A. 2014 Aug;102(8):2636-43.]. More recently, primary murine osteoblasts have been employed to elucidate differentiation mechanisms such as a specific role of mTORC1 in the transition from preosteoblasts to mature osteoblasts [Chen J, Long F. mTORC1 Signaling Promotes Osteoblast Differentiation from Preosteoblasts.PLoS One. 2015 Jun 19;10(6):e0130627.] and the effect of Histone Demethylase Utx on the expression of Runx2 and Osterix [Yang D, Okamura H, Teramachi J, Haneji T. Histone Demethylase Utx Regulates Differentiation and Mineralization in Osteoblasts. J Cell Biochem.

2015 Apr 28.doi: 10.1002/jcb.25210.]. With the same intent of attaining the most physiological setting possible, primary human osteoblasts were used to test the differentiation occurring on different titanium implant surfaces pre-treated with blood. [Kopf BS, Schipanski A, Rottmar M, Berner S, Maniura-Weber K. Enhanced differentiation of human osteoblasts on Ti surfaces pre-treated with human whole blood.Acta Biomater. 2015 Jun;19:180-90. doi: 10.1016/j.actbio.2015.03.022.]

Primary mesenchymal stem cells mainly deriving from the bone marrow are also very important when dealing with bone regenerative purposes. The first isolation method relied on the ability these cells have to adhere to tissue culture plastic (Friedenstein, Chailakhjan, & Lalykina, 1970; Friedenstein, Petrakova, Kurolesova, & Frolova, 1968; Friedenstein, Piatetzky-Shapiro, & Petrakova, 1966). To reduce the high heterogeneity of whole bone marrow cultures, Haynesworth et al. introduced the separation of the MSCs through gradient centrifugation (Haynesworth, Goshima, Goldberg, & Caplan, 1992). In later studies, these cells were able to properly differentiate into bone cells (Pittenger et al., 1999). More recently, adipose tissue was proposed as an alternative source of mesenchymal cells, i.e. adipose derived stem cells (ASCs), which have been employed in osteogenic models with contrasting results (Chou, Zuk, Chang, Benhaim, & Wu, 2011; Lin et al., 2013; P. A. Zuk et al., 2001; P. Zuk, Chou, Mussano, Benhaim, & Wu, 2011).

Among the osteoblast cell lines, MC3T3-E1 cells are endowed with a pre-osteoblastic phenotype. Notably, several sub-clones of these widely diffused murine cells have been established. Some of them undergo mineralization with the addition of ascorbic acid and inorganic phosphate. (Quarles, Yohay, Lever, Caton, & Wenstrup, 1992; Wang et al., 1999). Interestingly, in sub-clone 4, specific temporal changes from proliferation to nodule formation and mineralization have been described, resembling the intramembranous osteogenesis in vivo. (Sudo, Kodama, Amagai, Yamamoto, & Kasai, 1983). MC3T3-E1 cells represent a reliable alternative to primary human osteoblasts as in vitro cell model for various research areas (Czekanska, 2012).

The most used human osteoblast cell line, SaOs-2cells were originally isolated from an 11-year old Caucasian female in 1975. SaOs-2 cells display a mature osteoblast phenotype and tend to form a calcified matrix typical of woven bone (Rodan et al., 1987). SaOs-2 cells share with primary human osteoblasts a similar expression profile of cytokines, growth factors and receptors for parathyroid hormone (Bilbe, Roberts, Birch, & Evans, 1996). The structure of the collagen synthesized by SaOs-2 turned out to be similar to that of collagen produced by primary human osteoblast cells. (Fernandes, Harkey, Weis, Askew, & Eyre, 2007).

MG-63 cell line derives from a juxtacortical osteosarcoma diagnosed in the distal diaphysis of the left femur of a 14-year-old male. (Billiau et al., 1977) MG-63 cells represent an immature osteoblast phenotype. Although inconsistent data are available in literature as regards their mineralization capabilities (Czekanska, 2012), MG-63 cells have been used in spite of their limitations in long-term studies concerning cell behavior on biomaterials (Lincks et al., 1998).

As osteocytes are terminally differentiated cells embedded within a mineralized matrix, they are quite difficult to obtain in culture for in vitro study (Kartsogiannis & Ng, 2004). So far, research has been limited to chick, rat, and mouse (Gu, Nars, Hentunen, Metsikkö, & Väänänen, 2006; Halleux, Kramer, Allard, & Kneissel, 2012; Nijweide, van der Plas, Alblas, & Klein-Nulend, 2003; Semeins, Bakker, & Klein-Nulend, 2012). Among others, Bonewald's group deserves to be mentioned for developing immortalized cell lines such as: MLO-Y4, MLO-A5 and IDG-SW3. Kato established the clonal line MLO-Y4 (murine long bone osteocyte Y4) with osteocyte-like characteristics (Kartsogiannis & Ng, 2004; Kato, Windle, Koop, Mundy, & Bonewald, 1997). These cells are endowed with extensive, complex dendritic processes, produce large amounts of osteocalcin and are positive for osteopontin and connexin 43. The MLO-Y4 cells also support osteoclast formation and activation (S Zhao, Zhang, Harris, Ahuja, & Bonewald, 2002). The mouse derived MLO-A5 cell line representing late osteoblast/early osteocytes was shown to mineralize spontaneously in culture even in the absence of beta-glycerophosphate and ascorbic acid (Kato et al., 2001). Both MLO-Y4 and MLO-A5 are usually cultured in monolayer on collagen-coated plastic. The clonal cell line named IDG-SW3

was generated from mice carrying a Dmp1 promoter driving GFP crossed with a thermolabile SV40 large T antigen regulated by interferon γ (IFN-γ). As a result these cells can be expanded at 33 °C supplementing IFN-γ and then let differentiate at 37 °C, in the absence of IFN-γ. Thus, IDG-SW3 cells are Dmp1-GFP(-) and T antigen(+) under immortalizing conditions, whilst become Dmp1-GFP(+) and T antigen(-) under osteogenic conditions. IDG-SW3 cells have been cultured both in 2D and 3D settings. The authors claim that this cell line may prove useful for studying osteoblast-to-osteocyte transition, mechanisms for biomineralization and osteocyte function (Woo, Rosser, Dusevich, Kalajzic, & Bonewald, 2011).

The need for vascularization: endothelial cells

Tissue engineering *in vitro* is severely limited by a number of biological drawbacks related to the huge simplification of the complex architecture of native organs (Hegen et al., 2011). Nonetheless, a deeper and universal constraint is chemico-physical and owes to the coupling between the surface/volume ratio and the dissipative nature of living beings (open thermodinamic state). As open systems, biological tissues need to manage fluxes of mass and energy with the surrounding environment: this exchange occurs through their surfaces (boundary). On the other hand, diffusion of biomolecules is a volumetric process. During tissue growth, the ratio between surface and volume decreases (in the absence of change in form), so that simple diffusional processes do not result adequate to support such a critical balance. Consequently, the cells located in the deeper regions are poorly oxygenated and accumulate toxic catabolites leading to tissue necrosis.

In most animals, this diffusional limitation is overcome by the mass transport of internal fluids (blood) flowing through a vasculature under a pressure generated by one or more pumps (hearts). Is has been calculated that the average distance from cells to vessels is about 100-200 μm in mammals (Lovett, Ph, Lee, Edwards, & Kaplan, 2009). A great novelty occurring during vertebrate evolution was the appearance of endothelial cells that line vessels. Indeed, ECs not

only divide blood from tissue matrix physically, but also manage the biochemical crosstalk between the two environments acting as a selective and flexible barrier (Aird, 2012; Monahan-Earley, Dvorak, & Aird, 2013).

ECs play a crucial role in tissue neovascularization during physiological processes (i.e. development, tissue growth and repair) as well as in several human diseases (Aird, 2008; Carmeliet, 2003). Appropriate pore size and interconnections between macropores are essential to promote scaffold vascularization and were thoroughly reviewed elsewhere. However, no consensus exists as for the optimal porous structure needed to support blood vessel ingrowth. Recently, to evaluate how the interconnection size influence biomaterial vascularization, human umbilical vein endothelial cells were used on a series of beta-tricalcium phosphate samples with the same pore sizes and variable interconnections scaffolds. Scaffolds with a 150 μM interconnection size ameliorated endothelial cell function in vitro and significantly improved neovascularization upon implantation in rabbits. Also, this study shed a light on the role played by the PI3K/Akt/eNOS pathway in supporting implant vascularization.

The main challenge in bone engineering is how to exploit the great potentiality of endothelium as a mandatory vehicle to drive prevascularization of scaffolds as well as the angiogenesis of an implanted bone (Brennan et al., 2013; Chung & Shum-Tim, 2012a; Hegen et al., 2011; Rouwkema, Rivron, & van Blitterswijk, 2008; Sakaguchi et al., 2013). Indeed, vascularization of engineered bone tissues is still the primary factor limiting their suitability for clinical use.

Integrating the system: 2D and 3D co-cultures

Two-dimensional co-culture approaches (by the use of two chambers-based systems or conditioned media) are providing increasing mechanistic details on the paracrine cross-talk between ECs and osteoblast cell lines (Dohle et al., 2014; Leszczynska et al., 2013; Pedersen et al., 2013a; Santos et al., 2009; Shi et al., 2014; Tao et al., 2009). Outgrowth of endothelial cells and primary osteoblasts *in vitro* enhances both angiogenesis and osteogenesis (Herzog, Dohle, Bischoff, & Kirkpatrick, 2014). A number of growth factors critically contribute to these

processes, including VEGF, PDGF, angiopoietins, BMPs, and IGFs. The addition of soluble factors has been recently explored in 2D monocultures of MSCs with innovative approaches (Honda et al., 2013). Beside the paracrine diffusible factors, also physical cell-cell contacts such as connexins are involved (Herzog et al., 2014).

Establishing tridimensional co-cultures embedded in a variety of scaffolds may improve our knowledge of bone remodeling and possibly enable future clinical application (Brennan et al., 2013; Deb et al., 2010; Dorst et al., 2014; Gao et al., 2014; Hofmann et al., 2008; Santos et al., 2009; Thein-Han & Xu, 2013). In particular, co-cultures within scaffolds under proper environmental stimuli have been proposed to explore the interaction of ECs with other cell types already seeded in the scaffold or attracted by the surrounding implanted (hosting) tissue. To be efficient, three-dimensional co-culture systems ought to rely on the most suitable scaffold technology currently available. The key contribution of cell milieu is supported by a great deal of research, part of which related to the sophisticated mimicking of structural and functional details of the extracellular matrix with artificial of semi-synthetic materials. (Cao et al., 2014; Fu et al., 2014; Shim, Ankeny, Kim, Nerem, & Khang, 2014; Tanase et al., 2013; Yeatts et al., 2014; Q. Zhao et al., 2010).

The feasibility of co-culture or co-implantation strategies has been so far tested in several studies, focusing on the quality of the engineered bone substitute (Dariima, Jin, Lee, Wall, & Kim, 2013; Fu et al., 2014; Kokemüller et al., 2014; Leszczynska et al., 2013; R P Pirraco et al., 2013; Rogério P Pirraco et al., 2014). Interestingly, mineralization can be achieved without osteogenic media or additional growth factors through co-cultures of normal human primary osteoblats and Human umbilical vein ECs (HUVECs) mainly by recurring to the manipulation of the coculture ratio (Shah, Wenke, & Agrawal, 2014). Following the same route, Ma and coworkers (J. Ma et al., 2011) determined optimal cell culture medium and cell ratio for cocultures of human marrow stromal cells (HMSCs) and HUVECs, in 2D and 3D conditions. To replicate, even partially, the complexity of a living tissue *ex-vivo* is a hard task that entails

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reproducing, at least in a simplified way, also the functional and environmental features proper of the tissue itself (Desmoulin, Enns-Bray, Hewitt, & Hunter, 2013).

The need for function: mechano-electrical stress

Mechanical strain and stress affect the healthy maintenance of bone (Howard, Grill, & Bois, 2011): thus, establishing biomimicking stretch conditions is expected to improve regenerative approaches. Indeed, the mechanical stimulation of cocultures in a biaxial bioreactor promotes greater mineralization than the static systems (Liu et al., 2013). To investigate the effect of loading on osteocyte–osteoblast interactions, Vazquez et al. developed a 3D in vitro co-culture system, in which MLO-Y4 cells were embedded in type I collagen gels and MC3T3-E1 or MG63 cells layered on top. In addition, a possible link between mechanical stimulation and nutrition supply was demonstrated by the increased oxygen transport observed in MSCs embedded fibrin matrix stressed by moderate cyclic mechanical loading (Witt, Duda, Bergmann, & Petersen, 2014). Consistently, dynamic compression enhances the vasculogenic activities of circulating endothelial precursors (EPCs) seeded in the demineralized bone matrix scaffolds (Kong et al., 2012). The relationship between mechanical inputs and resulting biological tissue structure, composition, and metabolism is reported and discussed in several other papers and reviews (David et al., 2008; Desmoulin et al., 2013; Gardel, Serra, Reis, & Gomes, 2014; Gaspar, Gomide, & Monteiro; S.-T. Li et al., 2014; van Griensven et al., 2009).

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Moreover, to confer suitable piezoelectric properties (see previous discussion on bone complexity), novel materials have been proposed and are currently under development. A porous composite scaffold fabricated by freeze casting hydroxyapatite/barium titanate (HA/BT) suspensions (Yan Zhang, Chen, Zeng, Zhou, & Zhang, 2014) combined biocompatibility with the ability to generate electrical activity, when mechanically deformed. Polyvinylidine fluoride (PVDF) (Damaraju, Wu, Jaffe, & Arinzeh, 2013) prepared by electrospinning at different voltages (12 and 25 kV) was compared to tissue culture polystyrene.

MSCs cultured on PVDF-25 kV scaffolds had the greatest alkaline phosphatase activity and early mineralization by day 10 as compared to the other conditions, supporting the potential for the use of PVDF scaffolds for bone tissue engineering applications.

Critical concerns

The current detailed knowledge on EC functional properties, along with a critical and careful assessment of the related pitfalls, is theoretically sufficient to drive a successful bone vascularization. However, defining 'a priori' pros and cons of each endothelial feature is not an easy task. Particular cell behaviors may result advantageous or unwanted depending on the conditions. Such is the case of the great plasticity displayed by ECs in tumor angiogenesis (Bussolati, Grange, & Camussi, 2011). Although this characteristic makes the clinical approaches less predictable, it could be potentially very advantageous to induce the formation of new blood vessels in avascular scaffolds.

Great attention should be focused on the remarkable heterogeneity of ECs, an evolutionary conserved feature, detectable in morphology, gene expression and function (Aird, 2012; Chi et al., 2003; Langenkamp & Molema, 2009; Ramcharan, Lip, Stonelake, & Blann, 2011; Regan & Aird, 2012; Yano et al., 2007). The endothelium of arteries and veins, as well as from large and small vessels, display different behaviors that should be taken into account for a suitable strategy of EC subtype selection to vascularize a scaffold. Endothelial permeability and oxygen sensitivity can be highly variable and are expected to have a relevant impact on the overall process (Aird, 2012; Langenkamp & Molema, 2009; Yano et al., 2007). Indeed, microvascular endothelium, that line capillary or small arteriolar districts, reacts to tissutal hypoxia with an 'activation' pathway: this multistepped event involves proangiogenic factors leading to an increase of vessel leakage, regarded as the first step towards neovascularization.

One of the factors causally involved in endothelial heterogeneity is the complex local environment surrounding ECs, which includes tissue-specific diffusible factors, interaction with other cell types and extracellular matrix. This high plasticity strengthens the idea that

epigenetic factors are likely to concur significantly to the actual endothelial features, an issue of great relevance in tissue engineering.

Another component concurring to vascular variability is related to the origin of ECs: new blood vessels can arise either from mature ECs or through the differentiation of tissue-resident or circulating endothelial precursors (EPCs) (Carmeliet, 2005). Recently, EPCs (also in co-culture with osteoblast precursors) were introduced to obtain vascularized bone scaffolds (Papadimitropoulos et al., 2011; Tao et al., 2009).

The 'endothelialization' of bone scaffolds for regenerative medicine requires ECs to play a number of functions, not necessarily all at the same time. As a basic precondition to serve as a suitable replicate of the extracellular matrix (ECM), the scaffold has to reveal a good biocompatibility that depends on its chemical composition (polymers, glasses, glass–ceramics and composites) as well as on the geometry of its surface (Chung & Shum-Tim, 2012b; Lovett, Lee, Edwards, & Kaplan, 2009). Nonetheless, it needs to be permissive for a correct EC attachment. Detailed information is available on the ability of ECs to attach, spread and proliferate on different organic and inorganic substrates in 2D culture monolayers, while our knowledge on 3D conditions is still relatively poor. This is a very relevant matter, since it is now widely accepted that properties of ECs change dramatically when cultured on 2D or 3D substrates. Endothelium grown in 3D conditions by the use of matrigel or hydrogel substrates displays the typical ability to form interconnected tubules and its gene expression and cell signaling are clearly different from the monolayer cultures (Blobel, 2010; Engelse, Laurens, Verloop, Koolwijk, & van Hinsbergh, 2008; Hofmann et al., 2008; X. Ma et al., 2013; Nishiguchi, Matsusaki, Asano, Shimoda, & Akashi, 2014; Pedersen et al., 2013b).

Conclusions and perspectives

Cell therapy based on autologous osteoblasts or progenitors such as MSCs implanted into bone defects has been depicted as a viable option (Olender et al., 2014; Shah, Cornejo, et al., 2014; Tohma et al., 2008) along with traditional grafting. Clinical treatment of large bone defects,

however, entails the presence of a solid structure able to support cells colonization and ensure mechanical resistance. This makes the scaffolds unavoidable, whenever clinical protocols are involved, and urges to optimize the homing of osteoblasts and osteoclasts and, foremost, the onset of a vascular network. As ECs not only generate a vascular network but also increase bone formation (Goerke, Obermeyer, Plaha, Stark, & Finkenzeller, 2015; R. Zhang et al., 2012), they are the key factor to achieve a successful bone engineering. Based on this observation, it is surprising that the implementation of complex 3D systems capable to promote the interaction of vascular and strictly defined osteogenic cells is still in its infancy, although bioreactors have been widely used both to culture scaffolds and to keep the explanted bone vital, under the control of some parameters (temperature, pH, oxygen concentration, growth factors, and mechanical stimuli, among others).

The complexity of the bony environment has been reproduced only rudimentary by current technology. The exploitation of ECs' plasticity and regenerative potential, within a sophisticated biomimetic milieu mimicking exogenous stimuli, will hopefully lead to more satisfactory and powerful solutions.

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