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Review

Impact of developmental origin, niche mechanics and oxygen availability on osteogenic differentiation capacity of mesenchymal stem/stromal cells*

Natalia Bryniarska[1,3#]✉, Andrzej Kubiak[2,3#], Anna Łabędź-Masłowska[3] and Ewa Zuba-Surma[3]✉

[1]Department of Experimental Neuroendocrinology, Maj Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland; [2]Institute of Nuclear Physics, Polish Academy of Sciences, Kraków, Poland; [3]Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

Mesenchymal Stem/Stromal Cells (MSCs) have been widely considered as a promising source of cells for tissue regeneration. Among other stem cells, they are characterized by a high osteogenic potential. Intensive studies in this field had shown that even if basic osteogenic differentiation is relatively simple, its clinical application requires more sophisticated approaches to prepare effective and safe cell therapy products. The aim of this review is to underline biological, physical and chemical factors which play a crucial role in osteogenic differentiation of MSCs. Existence of two distinct mechanisms of ossification (intramembranous and endochondral) indicate that choosing a proper source of MSCs may be critical for successful regeneration of a particular bone type. In this context, Dental Pulp Stem Cells representing a group of MSCs and originating from neural crest (a structure responsible for development of cranial bones) are considered as the most promising for skull bone defect repair. Factors which facilitate osteogenic differentiation of MSCs include changes in forces exerted on cells during development. Thus, culturing of cells in hydrogels or on biocompatible three-dimensional scaffolds improves osteogenic differentiation of MSCs by both, the mechanotransductive and chemical impact on cells. Moreover, atmospheric oxygen concentration routinely used for cell cultures *in vitro* does not correspond to lower oxygen concentration present in stem cell niches. A decrease in oxygen concentration allows to create more physiological cell culture conditions, mimicking the ones in stem cell niches, which promote the MSCs stemness. Altogether, factors discussed in this review provide exciting opportunities to boost MSCs propagation and osteogenic differentiation which is crucial for successful clinical applications.

Key words: mesenchymal stem/ stromal cells, dental pulp stem cells, ossification, neural crest, hypoxia, bone repair

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✉e-mail: ewa.zuba-surma@uj.edu.pl (EZS) natalia.bryniarska@outlook.com (NB)
#These authors contributed equally to this work
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Abbreviations: Adult Stem Cells, ASCs; Alkaline Phosphatase, ALP; Bone Morphogenetic Protein 2, BMP2; Cluster of Differentiation, CD; Contact Inhibition of Locomotion, CIL; Cranial Neural Crest, CNC; Dental Pulp Stem Cells, DPSCs; Extracellular Matrix, ECM; Hypoxia-Inducible Factor 1, HIF-1; Mesenchymal Stem/ Stromal Cells, MSCs; Neural Crest Stem Cells, NCSCs; Pyruvate Dehydrogenase Kinase, PDK; Stem Cells, SC; Stromal Cell-derived Factor-1, SDF-1; Two-dimensional, 2D; Three-dimensional, 3D; Vascular Endothelial Growth Factor, VEGF.

STEM CELL TYPES AND PERSPECTIVES OF THEIR USE IN REGENERATIVE MEDICINE

Nowadays, stem cells (SCs) represent one of the most intensely studied subjects in cell biology. Their basic features include the ability to proliferate and differentiate into more specialized cells (Reya *et al.,* 2001). These properties make SCs an attractive biological material with a wide application potential for its further use in regeneration of injured tissues. Various SC fractions may be distinguished based on their origin and potential for differentiation. SCs of an embryonic origin are the most primitive cells, and therefore they have the widest differentiation potential. Cells arising after zygote divisions (up to the 8-cell stage) are classified as totipotent cells and are able to give rise to all cell types of a developing embryo, along with placental tissues (Zychowicz, 2012). Embryonic stem cells (ESCs) isolated from the blastocyst inner cell mass also represent pluripotent cells capable of differentiation into cells derived from the three germ layers (Murray *et al.,* 2006). Although human ESCs may potentially represent a rich source of somatic cells for transplantation, the research conducted on these cells is associated with an ethical controversy. Therefore, scientists have been looking for other methods of obtaining primary SCs with a wide differentiation potential, alternative to ESCs (Zychowicz, 2012). The second largest group of SCs are adult stem cells (ASCs). Several cell fractions can be distinguished among ASCs, including multipotent cells (able to differentiate into a specific pool of cells derived from a given germ cell) and unipotent or progenitor cells capable of differentiating into a specific type of more specialized cells (Fortier, 2005). Mesenchymal Stem/Stromal Cells (MSCs) of various origin represent one of the most intensely studied populations of ASCs, which have been also widely employed in several clinical trials in humans.

MSCs were described in the 70s of the last century as cells isolated by adhesion to a plastic surface, which are characterized by high *in vitro* proliferation potential and

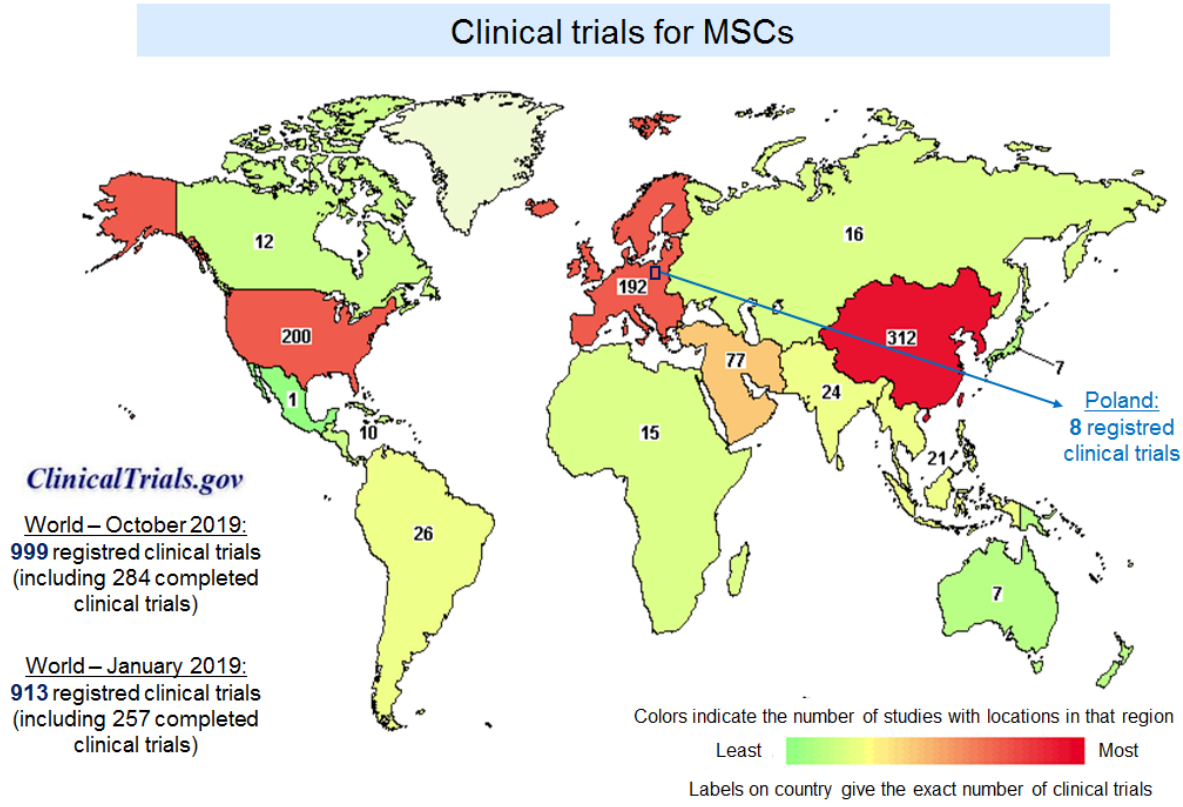

Figure 1. World-wide clinical trials using MSCs. Source: ClinicalTrials.gov – provided by the U.S. National Library of Medicine.

the ability to differentiate into a wide range of tissues - mainly of the mesodermal origin (Bajek *et al.,* 2011).

The main advantages of using MSCs in regenerative therapies include their effective isolation and culture, low immunogenicity and safety of transplantation (Reya *et al.,* 2001; Afzal *et al.,* 2015). These cells exhibit immunomodulatory properties which include secretion of immunomodulatory factors, such as interleukin 6 (IL-6) that may play a dual function in regulating the process of inflammation, hepatocyte growth factor (HGF), prostaglandin E2 and many others (Williams & Hare, 2011). Importantly, it has been shown that MSCs exhibit predominantly anti-inflammatory properties, favoring processes accompanying tissue repair. Moreover, they exhibit low immunogenicity following allogenic transplantations. Therefore, they are an attractive material for allogenic transplantations (Zhang *et al.,* 2015). Nowadays, 999 clinical trials using MSCs in human patients are registered in the worldwide ClinicalTrials.gov database and that number has significantly increased over the last 9 months (Fig. 1).

A large number of studies conducted on MSCs, with sometimes conflicting results, e.g. regarding the potential of MSCs to differentiate into neuronal tissues (Bianco *et al.,* 2013), has led to the need to harmonize guidelines on MSCs characteristics. For human MSCs, the minimal criteria for defining MSCs include:

– ability to adhere to plastic surfaces when maintained under standard culture conditions *in vitro*,

– ability to differentiate into three mesodermal lines *in vitro*: osteoblasts, chondroblasts and adipocytes,

– specific expression profile of surface antigens: expression of CD73, CD90, CD105 and lack of expression of CD34, CD45, CD14 or CD11b, CD79 or CD19, HLA-DR (Dominici *et al.,* 2006) as presented in Fig. 2.

MSCs can be isolated from various tissues, including bone marrow, umbilical cord blood, umbilical cord Wharton's Jelly, adipose tissue or peripheral blood (Kern *et al.,* 2006), as presented in Fig. 2. However, the presence of cells with MSC characteristics has been also demonstrated in many other tissues, such as the heart (Carlson *et al.,* 2011) or lungs (Foronjy & Majka, 2012).

In 2000, Gronthos and others (Gronthos *et al.*, 2000) described a unique population of cells with mesenchymal characteristics (such as ability to adhere to plastic surfaces, fibroblast-like morphology, lack of expression of CD14, CD34 or CD45, potential to differentiate into osteoblast) and of ectomesenchymal origin, which were isolated from dental pulp and called Dental Pulp Stem Cells (DPSCs). DPSCs have been subsequently compared with other MSC populations, including bone marrow-derived cells (BM-MSCs), indicating a similar phenotype of these two cell populations in terms of their antigenic phenotype (Alge *et al.*, 2010). It has been shown that DPSCs express several markers typical for BM-MSCs, such as CD29, CD44, CD73, CD90, CD105, and do not possess CD34, CD45, CD14 or CD19 and HLA-DR surface molecules (Luo *et al.*, 2018). Interestingly, DPSCs have displayed a significantly higher clonogenic potential than BM-MSCs (Alge *et al.*, 2010). Moreover, DPSCs were shown to produce more calcium deposits and alkaline phosphatase (ALP) during *in vitro* osteogenic differentiation, when compared to BM-MSCs, suggesting their greater osteogenic capacity. The authors have also performed a functional comparison by *in vivo* evaluation of bone formation in a porcine critical-size bone defect

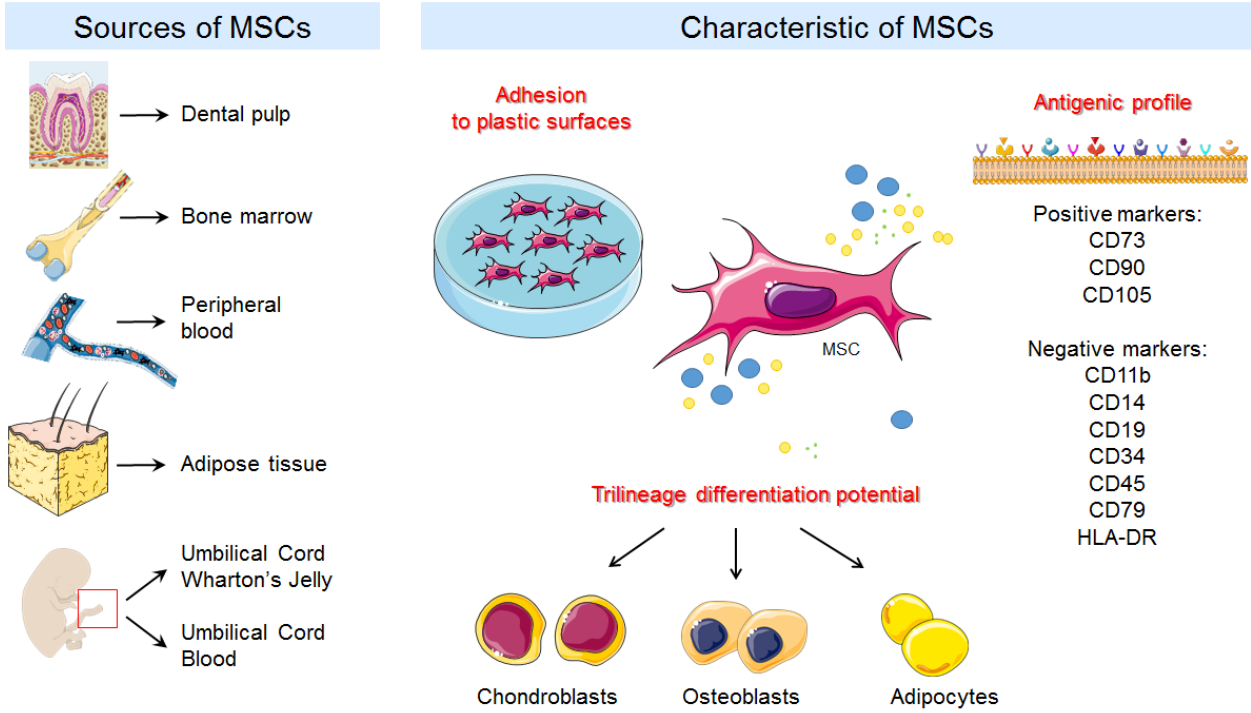

Figure 2. Main sources of MSCs and characteristics of MSCs according to specific criteria provided by the International Society for Cellular Therapy (ISCT).

model, where DPSCs and BM-MSCs were implanted on polycaprolactone – hyaluronic acid – tricalcium phosphate scaffolds. Similarly to the *in vitro* studies, DPSCs generated more bone tissue than BM-MSCs when seeded on the scaffold and transplanted *in vivo* (Jensen *et al.*, 2016). In another study, employing a rabbit calvarial bone defect model *in vivo*, the animals implanted with DPSCs or BM-MSCs seeded on commercially available scaffolds, exhibited a similar bone mineral density and potential for new bone formation, as well as expression of osteogenesis-related proteins, confirming their osteogenic capacity (Lee *et al.*, 2019). Herein, it is important to emphasize that the general characteristics and immunophenotype define DPSCs as a population resembling "mesenchymal stem/stromal cells" (MSCs), as it was also postulated by Ledesma-Martinez *et al.* in 2016. However, some investigators have pointed out that the exact status of DPSCs as a MSC population is still not fully defined and requires further investigations (Lan *et al.*, 2019). However, taking into consideration that stem/ stromal cells isolated from different tissues are today classified as MSCs when they fulfill the "MSC classification criteria" published by ISCT, we may consider DPSCs as a MSC population, but we should still consider their unique properties, including developmental origin.

Going back to the biological potential of DPSCs, when these cells were cultured *ex vivo* and implanted subcutaneously in immunodeficient mice, they showed the ability to form dentin-like structures (Gronthos *et al.,* 2000). An important feature of DPSCs is their ontogenetic origin, strictly related to the dental pulp tissue arising mainly from the ectodermal neural crest cells (Hall, 2009). This feature means that many studies based on DPSCs are focused on their use in regeneration of the nervous system (Kern *et al.,* 2006). This is favored by the fact that mesoderm-derived BM-MSCs are already being used in clinical trials to treat ischemic stroke (Steinberg *et al.,* 2016), amyotrophic lateral sclerosis (Mazzini *et al.,* 2009) or mechanical brain damage (Zhang *et al.,* 2008). Therefore, the use of MSCs derived from the ectomesenchyme, such as DPSCs, may be a very attractive perspective for the treatment of injuries of the nervous system. This has been envisioned by the fact that DPSCs are capable to give rise to neuron-like cells expressing a number of neuronal markers, as well as to some electrophysiological activity as evidenced in both, the mouse (Ellis *et al.,* 2014) and human models (Gervois *et al.,* 2015), while the neural differentiation was more prominent in human DPSCs. Recently, Lan *et al.* reviewed the use of DPSCs in a rodent stroke model. In all publications considered by authors of that review, the use of DPSCs has led to a significant improvement of brain function or a decrease in the infract size after stroke (Lan *et al.*, 2019).

ECTODERMAL ORIGIN OF DPSCS AND ITS IMPACT ON THEIR POTENTIAL – BIOLOGY OF THE NEURAL CREST DEVELOPMENT

The neural crest is formed in the course of embryonic development through interaction between the neural tube and the epidermal ectoderm (Hall, 2009). This structure has exceptional cellular plasticity. Neural crest stem cells (NCSCs) differentiate into a wide variety of cell types – these include the pigmented cells, peripheral neurons, Schwann cells, glial cells (Zhang *et al.,* 2014; Hall, 2009), adrenal medullary cells, cranial chondrocytes and osteocytes, myofibroblasts, and smooth muscle cells of the neck and head (Dupin & Sommer, 2012), arterial pole, endocardial pads (Vincent & Buckingham, 2010) or dental pulp (Young *et al.,* 2013). Particularly interesting is the ability of neural crest cells to differentiate into bone and cartilage-like cells. Cranial neural crest (CNC) cells correspond to a population that, at the relatively early stage of development (in mice between 9 and 10 days of

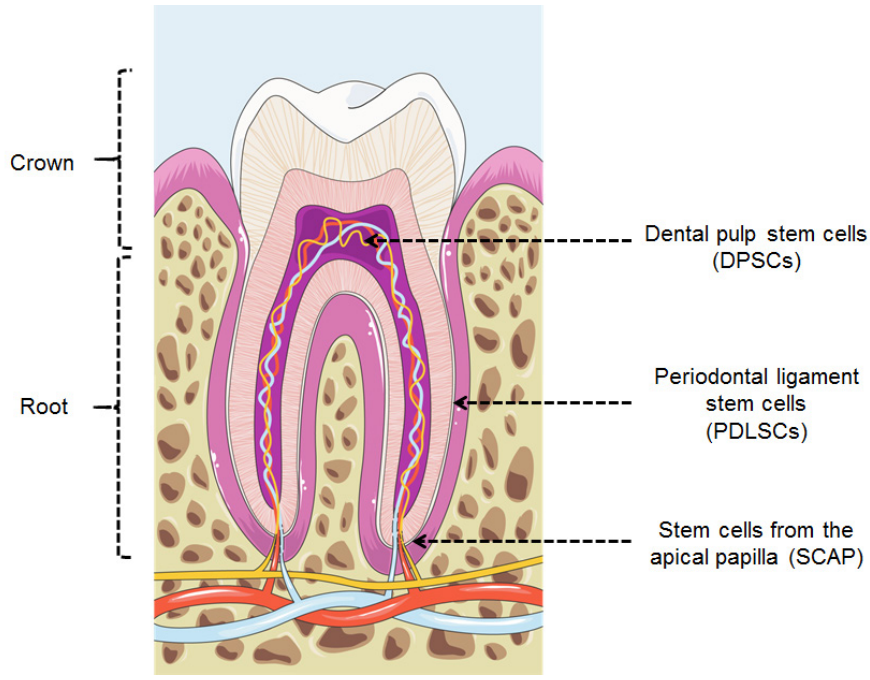

Figure 3. Cross section of a molar tooth and location of stem cell subpopulations identified in dental tissue structures.

embryonic development), expresses Sox10 resulting from binding of Sox9, Ets1 and cMyb transcription factors to one of the two enhancers for this gene – Sox10E2. The CNC cells migrate by well-defined pathways, colonizing the corresponding skull fragments (Gong, 2014; Kaukua *et al.,* 2014). Their targeted migration is ensured by chemoattraction to the Stromal cell-derived factor-1 (SDF-1), as well as by mechanisms combining the stream of migrating cells – contact inhibition of locomotion (CIL), and preventing separation of individual neural crest cells from the stream – co-attraction (Theveneau & Mayor, 2012). As a result of this precise mechanism of migration, CNC cells originating in the forebrain and upper part of the interbrain, colonize the frontonasal and periocular region of the skull; CNC cells originating in the lower part of the interbrain inhabit the maxillary part of the first branchial arch – corresponding to the mandible and jaw bones in humans; and CNC cells originating from the cerebellar area form the hyoid bone (Gong, 2014). Within the areas of the skull, which they inhabit, CNC cells differentiate into many tissues, such as the bones and cartilage of the neck or jaw, smooth muscles of the head or tooth elements – including dental pulp (Gong, 2014). Irregularities in migration of CNC cells within individual skull structures may contribute to the occurrence of numerous diseases – e.g. the Treacher-Collins syndrome, whose symptoms include malformations within the head (Kasat & Baldawa, 2011). Hence, the study of the biology of cells originating from the neural crest can contribute to a better understanding of the etiology of such diseases, as well as creation of future therapies (Gong, 2014; Trainor, 2010). A very important aspect related to participation of CNC in the formation of skull bones is the ossification mechanism. It should be emphasized that it differs from the mechanism of ossification of long bones (Kini & Nandeesh, 2012). A separate mechanism of bone formation makes the cells derived from the neural crest attractive in terms of their potential use in regeneration of the skull damage (Kini & Nandeesh, 2012; Laino *et al.,* 2006; Laino *et al.,* 2005; Javed *et al.,* 2010).

OSTEOGENIC DIFFERENTIATION

SCs, with particular emphasis on MSCs, have been increasingly used in the treatment of human skeletal system damage (Saeed *et al.,* 2016). Nevertheless, despite the initial successes of clinical trials, further optimization of methods is still required for both, the *ex vivo* preparation of these cells for cell therapy and the methodology of the cell therapy applications – including bone defects (Veronesi *et al.,* 2013). In this context, understanding the mechanisms underlying the ossification process, and thus osteogenic SCs differentiation, plays a particularly important role. Due to the bone type, two separate processes are responsible for bone formation during individual development.

The first process represents intramembranous ossification, characterizing flat bones, with particular emphasis on flat bones of the skull, but also of the jaw bones (Kini & Nandeesh, 2012; Bartel, 2004). The second is endochondral ossification, which is characteristic for long bones, but in practice applies to most peripheral bones (Bartel, 2004). Ectomesenchymal tissue originating from the neural crest plays a key role during intramembranous ossification (Kaucka *et al.,* 2016). MSCs residing within the neural crest specialize in actively proliferating osteoprogenitor cells (Heino & Hentunen, 2008). These cells – expressing the Sox9 and Runx2 transcription factors, exhibit bipotent characteristics and can differentiate into both, the bone and the cartilage cells. At a later stage of osteogenic differentiation, progenitor cells differentiate towards osteoblasts, which is associated with an increase in the expression level of genes characterizing osteogenic differentiation, such as Runx2 and Osterix transcription factors, and extracellular matrix (ECM) proteins, such as type I collagen, fibronectin and growth factor Bone morphogenetic protein 2 (BMP2) (Javed *et al.,* 2010). Synthesis of ECM proteins is particularly important at this stage of osteogenic differentiation. They form an osteoid – an organic bone matrix constituting about 20% of its mass and consisting in about 90-95% of collagen I (Kini & Nandeesh, 2012). ECM, which at this stage becomes enriched in collagen I, promotes activation of signal cascades affecting transcriptional activity of cells in the niche. The effects of these interactions are: maintenance of expression of genes responsible for the osteogenesis process, gradual inhibition of cell proliferation resulting from reduction in histone protein synthesis, and induction of synthesis of additional ECM proteins characteristic for the bones, such as the osteopontin, osteocalcin, bone sialoprotein and osteonectin (Javed *et al.,* 2010). The final stage of differentiation at the cellular level is formation of osteocytes – cells located in the bone cavi-

ties with a characteristic morphology determined by the network of tabs (Bartel, 2004). These tabs allow osteocytes to contact each other with the help of gap joints. It is the osteocytes that are responsible for the synthesis of approximately 70% of bone mass inorganic matrix, consisting in 99% of hydroxyapatite (Kini & Nandeesh, 2012). It should be emphasized that only about 15% of the initial osteoblast pool undergoes terminal differentiation to osteocytes, the others undergo programmed death accompanied by high expression of proapoptotic genes from the Bax family and inhibited expression of the Bcl-2 gene (Javed *et al.,* 2010).

The second mechanism of bone formation is endochondral ossification, which is a complex process and consists of the following five successive stages:

1. development of the cartilaginous model,
2. growth of the cartilaginous model,
3. production of primary ossification centers,
4. production of secondary ossification centers,
5. final production of articular cartilage and epiphyseal plate.

In the first stage, the cartilaginous model develops from the mesenchyme. Built from vitreous cartilage, the model lengthens as a result of intensive proliferation of chondrocytes with simultaneous deposition of ECM. In the next stage of long bone formation, the cartilage surrounding the bone model is transformed into the periosteum (Kini & Nandeesh, 2012). It comes about as a result of blood vessels penetration into the cartilaginous tissue, which promotes the transformation of primitive cartilage cells into osteoblasts synthesizing components of the osteoid (Kini & Nandeesh, 2012; Bartel, 2004). This process begins in the central part of the cartilaginous model, corresponding to the middle of the shaft of the future bone. The place where ossification is initiated is called the primary ossification center. Within it, chondrocyte overgrowth occurs – hypertrophic chondrocytes are formed (Kini & Nandeesh, 2012). They take part in the synthesis of the mineralized matrix and gradually undergo apoptosis during bone formation, creating empty spaces inside which will be occupied by blood vessels at a later stage (Yang *et al.,* 2016). Nevertheless, the latest research with transgenic animal models indicates that a certain pool of hypertrophic chondrocytes is not apoptotic, but instead it is transdifferentiated into osteoblasts, showing progressive expression of both, the Osterix transcription factor and Collagen 1A1 (Yang *et al.,* 2016). Secretion of the vascular endothelial growth factor (VEGF) plays a very important role in hypertrophic chondrocytes during the ossification process (Yang *et al.,* 2012). It results in invasion of the blood vessels that are being transformed into perichondrial periosteum (Kini & Nandeesh, 2012). In this way, a vascular bud is created that allows osteoblast precursors to penetrate into the bone, which then colonize niches formed by dead hypertrophic chondrocytes and undergo further osteogenic differentiation (Maes *et al.,* 2010). Secondary ossification centers are formed within the epiphysis of the bones using an analogous mechanism as the primary centers, resulting in epiphyseal ossification (Kini & Nandeesh, 2012). The epiphyseal cartilage is present between the bone shaft and its root until about 20 years of age, which allows continuous bone growth in length (Bartel, 2004). It is worth mentioning that processes similar to those described above involve natural bone repair mechanisms (Kini & Nandeesh, 2012). Hence, the awareness of the existence of two separate ossification mechanisms is particularly important from the point of view of regenerative medicine. Considering the ontogenetic origin of the skull bones, membranous ossification appears to be a natural process whose exact reconstruction is associated with the possibility of the most perfect bone tissue regeneration for the treatment of skull lesions (Kini & Nandeesh, 2012; Laino *et al.,* 2005; Javed *et al.,* 2010; Bartel, 2004; Kaucka *et al.,* 2016).

THE INFLUENCE OF THREE-DIMENSIONAL CULTURE ON OSTEOGENIC DIFFERENTIATION OF STEM CELLS

The osteogenic differentiation of SCs described in the previous section, resulting in bone formation, is always a process embedded in the three-dimensional (3D) niche of a developing organism (Bartel, 2004; Mohyeldin *et al.,* 2010). Despite this fact, many studies on SCs differentiation are carried out in a two-dimensional (2D) culture, directly on a culture plastic or on a surface protein-coated with ECM proteins (Kim *et al.,* 2011). Such conditions will never be able to fully reflect the native conditions prevailing in the body, which may even be associated with the loss of the parenthood potential of cells grown *ex vivo* (Yang *et al.,* 2014). Such a phenomenon was observed in the case of skeletal muscle SCs, which very quickly lose their native characteristics during standard *ex vivo* cultivation on a polystyrene cell culture surface. In contrast, those grown on a flexible hydrogel retain the features of SCs and are able to reconstitute in a niche after re-implantation within the muscles of the limbs of mice (Gilbert *et al.,* 2011) The fate of SCs, and above all their behavior, is defined *in vivo* by their niche. The niche of SCs is not only their location, but a whole range of factors affecting these cells, such as: the spatial arrangement of different types of cells relative to each other, the interaction of cells with ECM proteins, and intercellular interactions resulting from their paracrine activity (Li & Xie, 2005). Therefore, *in vitro* reproducing of conditions as close as possible to those in a native niche is very important from the point of view of tissue regeneration. 3D cultures allow simulation of a number of environmental properties defined by the SCs niche, such as the physical properties (stiffness, deformation) (Yang *et al.,* 2014; Humphrey *et al.,* 2014), or the presence of substances forming ECM (Lee *et al.,* 2007). In the case of osteogenic differentiation where niche-derived factors exert a great influence on its course, the use of 3D cultures brings beneficial *in vitro* effects. MSCs derived from the rat bone marrow, cultured in 3D constructs in the *in vitro* rotational system, showed significantly higher expression levels of osteogenic differentiation markers (e.g. Osterix, Osteopontin, Collagen 1 A1) when compared to cells differentiated under 2D conditions. Also, cells derived from the 3D constructs differentiated in the rotational system showed significantly greater deposition capacity of the mineralized ECM when compared to cells derived from the 2D culture, as demonstrated by the Von Koss staining (Tang *et al.,* 2017). Osteogenic differentiation of human MSCs derived from bone marrow also occurred more efficiently when grown in alginate beads. This process was more successful than under 2D culture conditions, both when the 3D culture was supplemented with ALP, as well as when unmodified beads were used. An interesting finding from this study was that the beads in which ALP was immobilized under conditions of lower calcium concentration, were destabilized due to disintegration of calcium-alginate bonds used for the bead cross-linking. Such a phenomenon may contribute to the release of differentiated cells from beads after transplantation *in vivo,* when the availability of free calcium ions will decrease due to the ALP activity (Westhrin *et al.,* 2015). The use of 3D scaffolds to regenerate skeletal tissues has been also used *in vivo*. Os-

teoblasts obtained by differentiating mouse induced pluripotent SCs (iPS cells) were implanted in mice after placing them in gelatin scaffolds. After 12 weeks of implantation, cells within the 3D scaffolds retained osteoblast features, such as the ability to form a mineralized cell matrix, as well as expressing osteogenic markers, such as osteocalcin and bone sialoprotein (Bilousova *et al.,* 2011). Another strategy used with 3D constructs is their independent (without cells) implantation within the damaged bone. After implantation into the damaged femur of the rabbit, ceramic – akermanite scaffolds produced by a 3D printing method allowed partial repair without causing an excessive immune response (Liu *et al.,* 2016). This repair was confirmed by histological analysis of the constructs, as well as by gene expression analysis (Col1, Runx, Osteopontin, Osterix) of cells colonizing the scaffolds after their implantation. For akermanite scaffolds, the level of transcript expression for all four genes associated with osteogenic differentiation had increased between 6 and 12 weeks after implantation of the ceramic constructs (Liu *et al.,* 2016). The examples of various applications of both, the 3D cell cultures and 3D scaffolds, show that they can serve as versatile tools in the field of research on regeneration of bone defects and damages (Westhrin *et al.,* 2015; Bilousova *et al.,* 2011; Liu *et al.,* 2016; Vecchiatini *et al.,* 2015). It is worth emphasizing that the authors of publications presenting the process of differentiating encapsulated cells indicate that the big advantage of this method is the relatively small volume of capsules in which the cells are embedded, which allows maintaining an equal distribution of both, nutrients and oxygen in the culture medium (Westhrin *et al.,* 2015), and this effect is compounded by the use of bioreactors (Vecchiatini *et al.,* 2015). In this context, an important issue is the effect of oxygen concentration alone on osteogenic differentiation of MSCs, as well as the impact of different oxygen concentrations on a 3D culture.

THE EFFECT OF OXYGEN CONCENTRATION ON STEM CELLS

An atmospheric oxygen concentration of 21% is now recognized as a standard in cell culture (Tiede *et al.,* 2011). Nevertheless, it is known that many types of stem cells reside in a niche with a significantly lower oxygen concentration, oscillating within physiological level of 1–9% (Mohyeldin *et al.,* 2010; Simon & Keith, 2008). The hypoxic niche plays a very important role in the context of maintaining the SC behaviors, including hematopoietic stem cells (HSCs) and MSCs. For instance, in the bone marrow niche, the HSCs reside in a location distant from the blood vessels (Eliasson & Jönsson, 2010). This results in a lower oxygen concentration surrounding HSCs than e.g. for pericytes directly adjacent to the blood vessels. It should be emphasized that despite the fact that the bone marrow is richly vascularized, the partial oxygen concentration in the blood found in the bone marrow blood vessels is relatively low when compared to the other body tissues (comparable to oxygen concentration in the jugular vein). As a result, the oxygen concentration gradient in the bone marrow varies in the range of 1–6%, with the lowest concentration corresponding to the HSCs' location (Mohyeldin *et al.,* 2010). Under such conditions, the proliferation of HSC cells is inhibited, which allows them to be inactive in their proliferation (quiescent); this in turn reduces the risk of accumulation of harmful mutations, particularly dangerous for cells with parental characteristics (Zychowicz, 2012). Inhibition of proliferation is associated with the action of hypoxia-inducible factor 1 (HIF-1), which inhibits activity of the proliferation promoting transcription factor c-Myc (Eliasson & Jönsson, 2010). A similar role for HIF-1 has been demonstrated in case of MSCs, where 1% oxygen-induced HIF-1 activity (induced in an *in vitro* culture) resulted in increased expression of the p27 protein, and thus a blockade of DNA replication and proliferation (Kumar & Vaidya, 2016). This hypoxia-induced factor is a key element in regulation of the SCs behavior by the availability of oxygen. HIF-1 is a heterodimeric protein consisting of HIF-1α and HIF-1β subunits (Ito & Suda, 2014). Under conditions of lower oxygen concentration, HIF-1α is not degraded and is dimerized with HIF-1β forming a transcription factor affecting expression of a very large group of genes (Simon & Keith, 2008). However, in several studies on MSC behavior in hypoxia, it has been shown that the permanent exposure of these cells to a low oxygen concentration (1–5%), generally enhances their proliferative, as well as colony-forming potential, which may be utilized for their effective expansion *in vitro* (elegantly summarized in Burakova *et al.*, 2014). Thus, the exact conditions regarding duration of the MSC exposure to hypoxia should be considered depending on the required functional outcome expected from these cells in an *in vitro* culture.

One of the most important effects of hypoxia is the switch of cellular metabolism from mitochondrial respiration to glycolysis. Under conditions of low oxygen concentration, activation of HIF-1α-dependent pyruvate dehydrogenase kinase (PDK) occurs (Murray *et al.,* 2006). As a result, oxidation of pyruvate to acetyl-CoA is significantly limited because PDK inhibits the pyruvate dehydrogenase activity (Ito & Suda, 2014), which in turn blocks the formation of acetyl-CoA and hence the Krebs cycle. Mitochondrial respiration - and especially the electron transport chain, are the main sites for the formation of reactive oxygen species in the cell. Under conditions where the cell metabolism is based on glycolysis, their quantity decreases (Murray *et al.,* 2006), and thus the risk of DNA, RNA, lipid and protein damage is reduced. Proliferation is also inhibited, which prevents the uncontrolled growth of the population of the most primitive SCs, and also reduces the risk of mutation accumulation resulting from random polymerase errors within the genome of these cells (Murray *et al.,* 2006; Ito & Suda, 2014). The location of the BM-MSCs within the niche they occupy is the subject of many debates (Bianco *et al.,* 2013), but it is known that they are located in the bone marrow regions richer in oxygen than HSCs cells. However, taking into account the range of oxygen concentration assumed for bone marrow (1–6%) and the conditions necessary for HIF-1α activity (oxygen concentration approx. 5%), there is no doubt that in their native niche these cells are exposed to lower oxygen concentration than the one used in standard cultures (Mohyeldin *et al.,* 2010; Simon & Keith, 2008).

The results of studies on the effect of hypoxia on the behavior of human MSCs derived from bone marrow do not provide unambiguous answers about its effect. A comparison of osteogenic differentiation of human MSCs at 21% and 1% oxygen concentrations shows conflicting observations, depending on the work being analyzed (Hsu *et al.,* 2013; Hung *et al.,* 2012). Depending on the analyzed work, hypoxia resulted in a significantly lower expression of genes associated with osteogenic differentiation (e.g. Osteopontin, Osteocalcin) (Hsu *et al.,* 2013) or a higher level of expression of genes associated with osteogenic differentiation (e.g. Osteopontin, Osteocalcin, Collagen1a1) (Hung *et al.,* 2012). The results of

studies on osteogenic differentiation of human MSCs were also characterized by large individual variability. This has manifested itself, among others, through a large diversity in the deposition ability of the mineralized matrix, intra-group fluctuations in the proliferative index and the ability to form colonies for cells from different patients under both, the normoxia and hypoxia conditions (Ciapetti *et al.,* 2016). Interestingly, using an animal system *in vivo,* it was observed that a decrease in blood flow within the dental pulp results in increased mineralization and differentiation of DPSCs in the rat odontoblast layer (Ito *et al.,* 2015). On the other hand, human DPSCs grown at 3% oxygen concentration had exhibited an essentially lower ALP activity and also a significantly lower degree of mineralization when compared to DPSCs cultured at a 21% oxygen concentration (Iida *et al.,* 2010). However, research in this area using DPSCs is still being optimized. Therefore, further research on the effect of hypoxia on the process of differentiation of MSCs, including DPSCs, as well as on their proliferative capacity as described above, is still required.

In this context, in order to elucidate a more complete and accurate mechanism of the impact of hypoxia and other factors on osteogenic differentiation of MSCs, with particular emphasis on future applications of these results in regenerative medicine, it is important to optimize the methods used for the cell isolation and *ex vivo* propagation. Discrepancies in the methodology often resulted in contradictory data obtained by different groups studying MSC potential (Kumar & Vaidya, 2016; Hsu *et al.,* 2013; Hung *et al.,* 2012; Ciapetti et al., 2016; Ito *et al.,* 2015; Iida *et al.,* 2010; Ahmed *et al.,* 2016). Thus, further optimization of the protocols for the effective harvesting and expansion of MSCs with high differentiation potential as well as strategies for examining their biological properties and proregenerative mechanisms, are still required to fully understand the application potential of these cells .

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