The Response of Human Mesenchymal Stem Cells to Osteogenic Signals and its Impact on Bone Tissue Engineering

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Abstract: Bone tissue engineering using human mesenchymal stem cells (hMSCs) is a multidisciplinary field that aims to treat patients with trauma, spinal fusion and large bone defects. Cell-based bone tissue engineering encompasses the isolation of multipotent hMSCs from the bone marrow of the patient, *in vitro* expansion and seeding onto porous scaffold materials. *In vitro* pre-differentiation of hMSCs into the osteogenic lineage augments their *in vivo* bone forming capacity. Differentiation of hMSCs into bone forming osteoblasts is a multi-step process regulated by various molecular signaling pathways, which warrants a thorough understanding of these signaling cues for the efficient use of hMSCs in bone tissue engineering. Recently, there has been a surge of knowledge on the molecular cues regulating osteogenic differentiation but extrapolation to hMSC differentiation is not guaranteed, because of species- and cell-type specificity. In this review, we describe a number of key osteogenic signaling pathways, which directly or indirectly regulate osteogenic differentiation of hMSCs. We will discuss how and to what extent the process is different from that in other cell types with special emphasis on applications in bone tissue engineering.

Keywords: Human mesenchymal stem cells, heterogeneity, signaling pathways, osteoblast differentiation, species differences, bone tissue engineering.

BONE TISSUE ENGINEERING: A NEED FOR IMPROVEMENT

Bone tissue engineering has developed as a multidisciplinary field that applies the principles of biology, medicine and engineering to develop tissue substitutes for restoration, maintenance or improvement of diseased or damaged bone tissue [1, 2]. Every year, more than one million patients in the US alone undergo orthopaedic bone surgery and there is a great demand for treatment of non-healing bone defects. Bone autografts and allografts have been successfully applied for many years, but there are a number of disadvantages such as multiple surgeries, failure of the graft materials, rejection by the host, insufficient material availability and infection [3-5]. An alternative method is cell-based bone tissue engineering, in which mesenchymal stem cells (MSCs) are isolated from bone marrow of the patient, expanded *in vitro* and seeded onto scaffold materials such as porous calcium phosphate ceramics. The cell-seeded graft is next transplanted into the defect area for tissue regeneration.

MSCs are adult stem cells of mesodermal origin and are referred by many terminologies such as colony forming unit-fibroblast, marrow stromal fibroblasts, bone marrow stromal cells or mesenchymal progenitor cells. MSCs are multipotent, and are able to differentiate into the osteogenic, chondrogenic, adipogenic and many other lineages including myoblasts [6, 7]. The earliest reports of MSCs date back about four decades when fibroblast-like colonies from bone marrow were isolated by Friedenstein and coworkers by virtue of their adherence to tissue culture plastic [8] (Fig. (**1A**)). MSCs are a very rare population of cells in bone marrow with a reported incidence of 0.001% to 0.01% [8-10]. Besides bone marrow, researchers isolated MSCs from many other sources including adipose tissue, tibia, femur, lumbar spine, trabecular bone and placenta [11-13]. MSC-based bone tissue engineering reproducibly forms bone *in vivo* (Fig. (**1B**)), and a number of clinical trials have been performed to investigate its feasibility. The outcome is that bone is produced but the amount of bone tissue formed upon implantation of hMSCs is insufficient and typically does not fully bridge the implant [14-16]. These data are in sharp contrast to results obtained with goat and rat mesenchymal stem cells, which completely bridge the implant with newly formed bone (Fig. (**2**), unpublished data). Evidently, greater emphasis is required on augmenting the performance of hMSCs. Therefore, researchers are trying to improve the performance of hMSCs by pre-committing the isolated cells into the osteogenic lineage and consequently the *in vivo* bone forming ability of the cells. A large body of literature is available describing that *in vitro* expansion and differentiation of hMSCs can be manipulated by means of small molecules, proteins, genetic interference and scaffold design and some of these interventions enhance their *in vivo* bone forming capacity.

MESENCHYMAL STEM CELL HETEROGENEITY

MSCs show a vast heterogeneity with respect to multipotency, colony size, growth rate and cell morphology, ranging from fibroblast-like spindle shaped to large flat cells [17]. Attempts have been made to isolate a pure fraction of MSCs to overcome the heterogeneity in the cell population using different markers. MSCs are negative for CD34, CD44, CD45, c-Kit and express low levels of FLk-1 and

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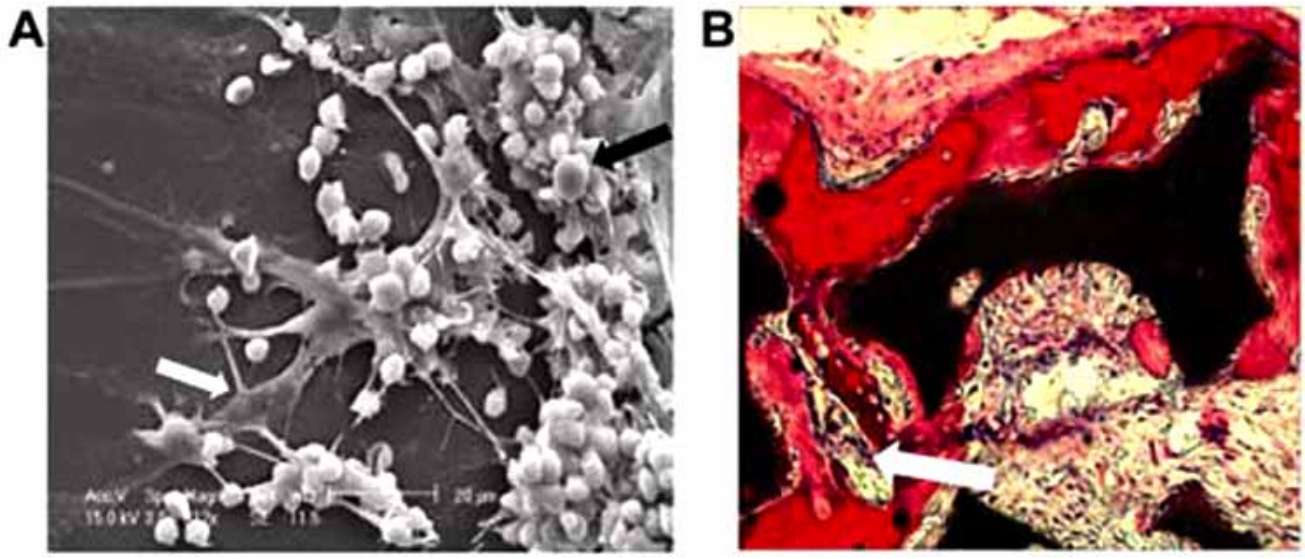

Fig. (1). Mesenchymal stem cell isolation and application. A. Scanning electro micrograph of crude bone marrow aspirate 24 hours after seeding. Adherent cells can be seen that migrate away (white arrow) from aggregates of non-adherent bone marrow cells (black arrow). **B.** Ectopic bone formation by human mesenchymal stem cells (red staining) seeded onto porous ceramic scaffolds (black). Note that the presence of bone induces ectopic bone marrow formation (white arrow). The sections are stained with basic fuchsin and methylene blue. Basic fuchsin stains newly formed bone pink and methylene blue stains the remaining fibrous tissue blue (From our unpublished data).

Thy-1 and higher levels of CD13, Stro1 and stage-specific antigen 1. The higher Stro1 positive homogeneous cells are shown to have better proliferative and differentiation abilities [18]. Others have used a combination of these markers to enrich undifferentiated MSC populations [19, 20]. However, even clonally isolated hMSCs showed differential capacity to form bone *in vivo* [21]. Moreover, the gene expression profile of a single MSC colony was assessed, revealing the expression of a plethora of markers typical for osteo-, chondro-, adipo- and even neurogenic lineages [22]. This suggests that MSCs display phenotypical plasticity, which is skewed when inductive signals are given [23]. Bone specific alkaline phosphatase (ALP) is used as an early marker for differentiation of MSCs into osteogenic lineage. ALP is a glycoprotein localized in the plasma membrane of osteoblasts. The precise role is unclear although it is essential for mineralization. Total circulating ALP is derived from liver, intestine, spleen, kidney, and placenta. Bone ALP comprises approximately 50% of total circulating ALP in normal subjects. Measurement of bone ALP is well

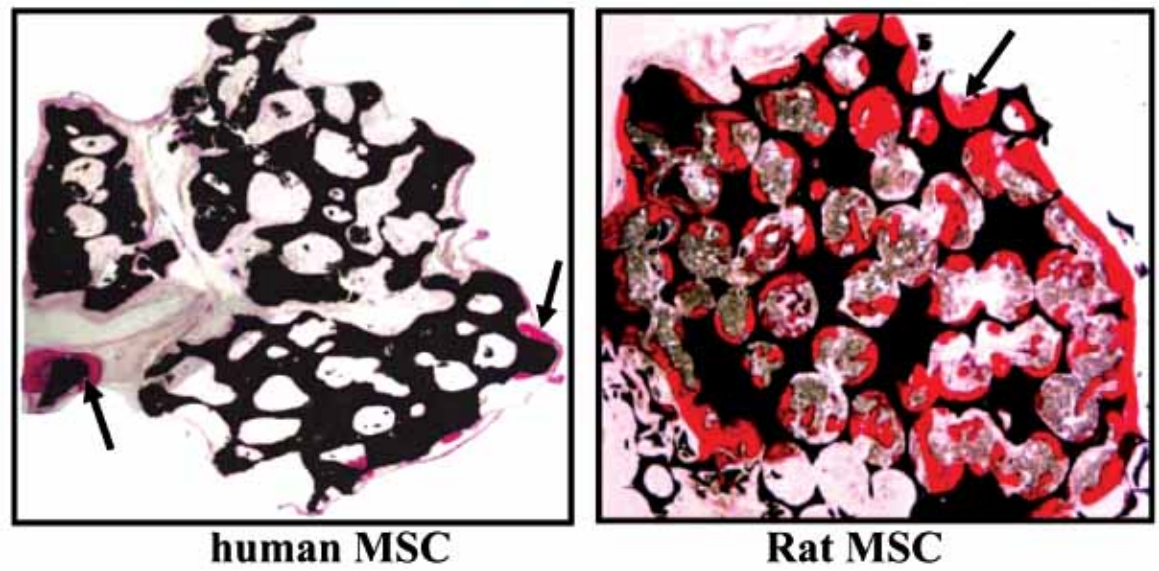

Fig. (2). Species differences in biological performance of MSCs. Ectopic bone formation (red stain, arrows) by an equal number of rat and human MSCs seeded onto calcium phosphate ceramic scaffolds and implanted subcutaneously for 6 weeks in nude mice. Note the enormous difference in the amount of newly formed bone between rat and human MSCs**.** The sections are stained with basic fuchsin and methylene blue. Basic fuchsin stains newly formed bone pink and methylene blue stains the remaining fibrous tissue blue (From our unpublished data).

accepted as a marker for bone turnover and a marker for *in vitro* osteogenic differentiation [24]. The current challenge lies in finding conditions to optimize the differentiation of MSCs. Although typically, the percentage of ALP positive cell in early passage hMSCs varies between 1% and 33%, it goes up to 50% upon dexamethasone treatment. Using combinations of dexamethasone with various inductive signals such as Trichostatin A [25], Vitamin D and cAMP it is possible to increase the ALP positive cell fraction up to 70-80 % (R.S, H.F, unpublished data). It is our belief that current *in vitro* differentiation recapitulates only a small segment of the complex signaling hierarchy of bone formation *in vivo* and as such, we are not yet able to fully unleash the osteogenic potential of hMSCs. Therefore, it is of prime importance to understand the genetic and molecular cues which regulate osteogenic differentiation of hMSCs for their efficient use in bone tissue engineering.

UNDERSTANDING hMSC BIOLOGY: STEPS TOWARDS DEVELOPMENT

Bone is a dynamic tissue which is constantly being remodeled by catabolic osteoclasts and anabolic osteoblasts, which are kept in balance by an intricate regulatory network of hormones, growth factors, cytokines, chemokines and mechanical cues (Fig. (**3**)). Skeletal development is mainly studied using mouse genetic models, osteogenic cell lines and skeletal disorders. For instance, there is ample literature available focusing on osteogenic differentiation of immortalized osteogenic cell lines such as MC3T3, C2C12, ROS17/2.8, UMR 108, MG-63 and SAOS-2. Extrapolation of knowledge gathered from lower species and cell lines to the human situation is feasible, but needs thorough understanding of differences in the mechanism between species in response to different signals [26]. For instance, it is known that hMSCs respond differently to key osteogenic signals such as bone morphogenetic proteins (BMPs) and dexamethasone compared to some of the most frequently used osteogenic model cell lines [27]. Furthermore, the required dosage and action of these signals may vary between cell types [27, 28]. Moreover, the response of hMSCs should always be considered in the light of the heterogeneous nature of this cell population and donor variation [29, 30]. This review will highlight a number of signaling pathways which have been implicated in bone formation and have been used to control proliferation and differentiation of hMSCs. We will emphasize the overlap and difference in response to activation of the pathway

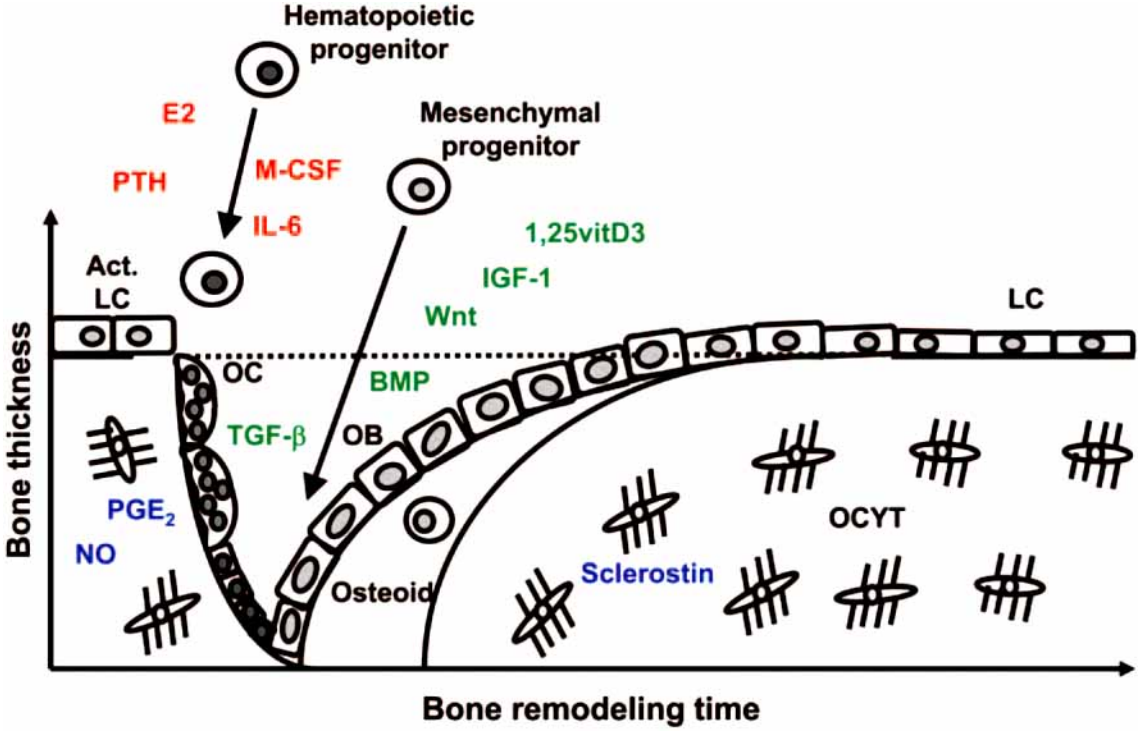

Fig. (3). Schematic model of time dependent interactions between bone cells in a basic multicellular unit. Bone remodeling is initiated by activation of lining cells on the bone surface by signals from osteocytes within bone or from factors in the bone marrow. Nitric oxide (NO) and prostaglandin E_2 (PGE_2) are examples of such factors produced by osteocytes in response to, for example, mechanical loading. Parathyroid hormone (PTH) and estrogen (E_2) are systemic factors affecting osteoclastogenesis *via* activated lining cells. As a result of osteoclastic resorption, factors such as transforming growth factor-β (TFG-β) stored in the bone matrix are released and contribute to the initiation of osteoblastic bone formation and inhibit bone resorption. Bone formation is regulated by many locally produced factors such as bone morphogenetic proteins (BMPs), Wingless-type MMTV integration site family of proteins (Wnts), insulin-like growth factor-1 (IGF-1), as well as systemic factors such as 1,25 dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$). Bone forming osteoblasts are incorporated into bone, become resting lining cells, or die by apoptosis. Upon mineralization of osteoid, the incorporates cells, now called osteocytes, secrete sclerostin and, thereby, provide a negative feedback on bone formation and prevent overfilling of the resorption pit. OB, osteoblast; OC, osteoclast; OCYT, osteocyte; M-CSF, Macrophage Colony Stimulating Factor; LC, Lining Cells; Act LC, Active lining cells. (Courtesy Dr. van Benzooyen).

between hMSCs and other osteogenic model systems. Further, we will discuss the possible ways to manipulate the pathways for bone tissue engineering.

GLUCOCORTICOID SIGNALING

Glucocorticoids are the most popularly used osteogenic factors in bone tissue engineering but are clinically better known as anti-inflammatory drugs, which act by binding to a specific cytoplasmic glucocorticoid receptor (GR). Glucocorticoids can either switch "on" the expression of anti-inflammatory genes, such as secretory leukocyte protease inhibitor (SLPI) or, switch "off" inflammatory gene expression by targeting pro-inflammatory transcription factors such as Activator protein-1 (AP-1) and members of the mitogen activated protein kinase (MAPK) pathways [31, 32]. Glucocorticoid receptors bind to DNA as a homodimer at consensus glucocorticoid response elements (GREs) in the promoter region of glucocorticoid-responsive genes, resulting in the induction or repression of genes. The number of GREs and their relative position are important determinants of the magnitude of the transcriptional response to glucocorticoids (for detailed reviews see [33, 34]). Although glucocorticoids are the most commonly used molecules in osteogenic differentiation of MSC, ironically, extensive use of glucocorticoids as anti-inflammatory drugs causes accelerated bone loss, osteopenia and an increased incidence of fractures [35]. Numerous *in vitro* studies demonstrate that the popularly used synthetic glucocorticoid, dexamethasone enhances osteogenesis and mineralization in hMSCs [29, 36]. Differential display experiments using hMSCs show that dexamethasone induces osteogenic differentiation by regulating genes such as TGF-β-induced gene product (big-h3), calphobindin II, cytosolic thyroid-binding protein, 22-kDa smooth muscle protein (SM22) and the extracellular matrix proteins osteonectin/SPARC, type III collagen, and fibronectin [37]. Further, other studies demonstrate that dexamethasone treatment of hMSCs resulted in a change in cytoskeletal organization during osteogenic differentiation, suggesting that cytoskeletal organization is required for osteogenic differentiation by dexamethasone [29]. It is evident that dexamethasone induces *in vitro* osteogenic differentiation of hMSCs, but not by induction of typical osteogenic transcription factors such as Cbfa1, Osterix or fosB. As mentioned earlier, dexamethasone consistently has an additive or synergistic effect on ALP expression and osteogenic differentiation in combination with several other osteo-inductive molecules such as Trichostatin A [25], Vitamin D and cAMP (unpublished data), suggesting that dexamethasone induces osteogenesis *via* a unique molecular pathway. The concentration of dexamethasone is a crucial factor in differentiation of hMSCs into a specific lineage [38]. At a concentration of 100 nM, dexamethasone is used to induce osteogenic and chondrogenic differentiation of hMSCs, whereas higher concentrations are known to inhibit osteogenic differentiation [29, 39, 40]. In contrast, as much as 1 μM is needed for adipogenic differentiation of hMSCs. Mendes *et al.* show that the presence of dexamethasone in culture was not required to obtain *in vivo* bone formation. However, in cultures without bone-forming ability or with a low degree of *in vitro* osteogenesis, dexamethasone increased the *in vivo* bone-forming capacity of hMSCs [39, 41]. In contrast, dexamethasone does not induce osteogenesis in the two most frequently used osteogenic cell lines, MC3T3 and C2C12 [42-44]. These studies not only demonstrate the inconsistency of various osteogenic molecules *in vitro* and *in vivo* but also show that the right concentration and combination of various cues are important for their effective use in bone tissue engineering.

TGF-β AND BMP SIGNALING

The transforming growth factor-β super family of proteins (TGF-β) includes the TGF-βs, activins and bone morphogenetic proteins (BMPs), which are known to mediate a wide range of biological functions including cell proliferation, differentiation and extracellular matrix formation [45]. The proteins signal through serine-threonine kinase receptors, mediating the phosphorylation of the mothers against decapentaplegic (Smad) family of transcription factors [46]. Heterodimers of Smads-1, -2, -3, -5 or -8 with Smad4 translocate into the nucleus and activate gene transcription [46]. A large number of target genes are activated by TGF-β specific Smad2/3, such as plasminogen activator-1 (PAI-1), type I collagen, cell cycle regulators p15 and p21 and transcription factor junB. TGF-β signaling has been extensively studied in the field of cartilage biology but less is known about the role of TGF-β in bone biology. TGF-β1 has been shown to stimulate osteogenesis in MG-63 and hMSCs, resulting in the formation of three-dimensional cellular condensations referred to as *bone spheroids*. Further, TGF-β1 induced expression of osteogenic markers such as ALP, collagen type I and osteocalcin [47, 48]. In contrast, other studies demonstrate that addition of TGF-β3 to hMSCs markedly reduced ALP expression indicating disparity in the role of TGF-β signaling in osteogenic differentiation of hMSCs [49].

In contrast, the critical importance of the BMPs is widely recognized in the field of bone biology. BMPs are secreted growth factors that were originally identified by their ability to induce ectopic bone [50]. Over 20 BMPs have been identified and characterized to date and have been implicated in various developmental processes [51]. The functions of these BMPs are studied extensively by creating transgenic mouse models for BMPs and their receptors. BMP2- and BMP4-deficient mice are nonviable and show abnormal development of the heart and mesodermal tissues. Furthermore, BMP receptor 1-deficient mice die at E9.5 due to the impairment in mesoderm development. [52]. BMPs are known to have divergent effects on cellular differentiation, which is further complicated by species differences. Among various BMPs studied, BMP-2, -4, -6, -7, and -9 induce ALP activity in C2C12 cells [53]. The best studied target gene of BMP signaling is the transcription factor runx2/cbfa1, which controls the osteogenic differentiation program [54-56]. Other target genes are the so-called Inhibitors of differentiation (Id) [57, 58]. Further, BMP-2, -4, -6, -7, and -9 strongly induce osteocalcin expression and mineralization in C2C12 and C3H10T1/2 cells [59]. In C3H10T1/2, BMP-2 induces osteogenic differentiation by activation of zinc finger transcription factor ZNF450 and in C2C12 *via* activating osteoblast specific transcription factors such as Runx2,

osterix and TAZ [60, 61]. In addition, over expression of various BMPs using retroviral and adenoviral vectors or administration of recombinant BMPs effectively induced orthotopic and ectopic ossification [62-64].

While BMPs have been studied extensively in other cell lines, their mechanism of action in hMSCs is peculiar. As described in the earlier paragraph, many BMPs are known to induce ALP expression and osteogenic differentiation in various cell lines and MSCs isolated from mice and rats [27]. Exogenous addition of BMP-6 to hMSCs induced the up regulation of osteoblast-related genes such as collagen type I, osteocalcin, bone sialoprotein and transcription factors Cbfa1/Runx2 and Osterix, demonstrating that hMSCs do have BMP receptors and are able to respond to BMPs [65]. However, most BMPs fail to induce ALP expression as well as mineralization in hMSCs. When presented to the cells in combination with dexamethasone, BMPs synergistically up regulate ALP expression. This suggests that dexamethasone is required to remove a molecular barricade in hMSCs, which prevents BMPs to induce ALP. PI3 kinase represents a candidate for the barricade function [66]. Although rhBMP2 alone is unable to induce *in vitro* osteogenic differentiation of hMSCs, supplementing hMSCs with rhBMP2 significantly enhances their *in vivo* bone forming ability [67].

WNT SIGNALING

Wnts (Wingless-type MMTV integration site family of proteins) are secreted growth factors with pivotal roles in a variety of cellular activities, including cell fate determination, proliferation, migration, polarity and differentiation [68]. Wnt signaling occurs upon binding of secreted Wnts to frizzled receptors and their co-receptors low-density lipoprotein receptor–related protein 5 and 6 (LRP5/6). The canonical Wnt signaling pathway acts *via* the bipartite transcription factor β-catenin/ T cell factor (TCF), which binds to the promoter of Wnt-responsive genes and thus initiates their transcription. In the absence of Wnt signaling, β-catenin degradation occurs as a result of phosphorylation by a protein complex consisting of adenomatous polyposis coli (APC), axin, and glycogen synthase kinase 3 (GSK3) and subsequent degradation by the proteosome. Wnt signaling inactivates the axin–APC–GSK3 complex resulting in the accumulation of cytoplasmic β -catenin, which translocates into the nucleus and activates Wnt-responsive genes [69]. A role of Wnt signaling in skeletal development has been demonstrated [69]. An inactivating mutation in the Wnt co-receptor LRP5 is involved in osteoporosis/pseudoglioma syndrome whereas activating mutations in LRP5 are associated with high bone mass syndromes [70]. A more detailed analysis of mice with a defect in LRP5 suggested an effect of Wnt signalling on proliferation of osteoprogenitors rather than on osteoblast differentiation or mineralization [70]. On the other hand, studies suggest that Wnt signaling stimulates osteogenic differentiation by activating Runx2, Dlx5 and osterix and by suppression of adipogenic transcription factors C/EBα and peroxisome proliferator activated receptor (PPARγ) [71]. Furthermore, Gong *et al*. reported that pre-osteogenic C3H10T1/2 cells show increased expression of bone-specific ALP upon overexpression of Wnt3A or a stabilised form of β-catenin [72].

While a lot is known about the role of Wnt signaling in skeletal development, relatively little is known about it in hMSC biology. hMSCs express Wnts -2, -4, -5a, -7a, -10a, and Wnt co-receptor LRP5 [73]. Exposure of hMSCs to low levels of the Wnt mimic lithium enhances their proliferation without affecting the multipotency. Higher concentrations of lithium severely inhibit hMSC proliferation [74-76]. Enhanced proliferation by Wnt activation is not only observed in bone marrow-derived hMSCs but also in MSCs derived from adipose tissues [75]. In contrast to the earlier reported positive effect on osteogenesis in cell lines, Wnt signaling consistently inhibits *in vitro* differentiation and mineralization of hMSCs. [76, 77] Although Wnt signaling inhibits dexamethasone-induced *in vitro* osteogenic differentiation, it does not seem to affect *in vivo* bone forming ability of these cells [74]. Interestingly, addition of Wnt3a or LiCl resulted in transmigration of hMSCs through filters coated with extracellular matrix indicating that Wnt signaling regulates the migratory behavior of hMSCs [78]. The positive effect on proliferation at lower Wnt levels could be used for bone tissue engineering purposes by exposing hMSCs to Wnts during the proliferative stage. Further, specific inhibitors of GSK, such as lithium and BIO [79], may also have a therapeutic benefit by enhancing proliferation *in vitro* [80, 81]. During differentiation, Wnt signals could be removed and cells can be directed to the osteogenic lineage by providing other osteogenic signals to augment bone formation.

G-PROTEIN COUPLED RECEPTOR SIGNALING

The G-protein coupled receptors (GPCRs) are an important family of receptors which transduce extracellular signals by coupling to catalytic heterotrimeric G-proteins and activation of further downstream signaling cascades. GPCRs have seven integral membrane spanning domains and are known to regulate many cellular processes. Ligand binding induces a conformational change in the receptor, resulting in the formation of a high affinity receptor-G-protein complex, which catalyses guanine nucleotide exchange on the alpha subunit of the G-protein. G-proteins are composed of three subunits (alpha, beta and gamma) [82]. The Gα subunit possesses intrinsic GTPase activity and dissociates from the βγ heterodimer in GTP-bound form. About 20 mammalian G protein α subunits have been identified, which can be divided into four families based on their primary sequence similarity: Gs, Gi, Gq, and G12. These G protein α subunits regulate the activity of several second messenger-generating systems [83]. For example, the Gq family controls the activity of phosphatidylinositolspecific phospholipases, such as phospholipase C-β (PLC-β), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate two second messengers, inositol 1,4,5- trisphosphate (IP3) and diacylglycerol (DAG). IP3 and DAG in turn lead to an increase in the intracellular concentrations of free calcium $[Ca^{2+}]i$ and the activation of a number of protein kinases, including protein kinase C (PKC) [84]. The members of the Gs family activate adenylyl cyclases which in turn activate protein Kinase A (PKA) signaling *via* intracellular adenosine 3´,5´- monophosphate (cAMP). In contrast, Gi family members can inhibit a subset of these enzymes, thereby controlling the intracellular

concentrations of cAMP [85]. Among many GPCRs, the receptor for parathyroid hormone (PTHR), which activates both PKA and PKC, has been studied in depth with respect to osteogenic differentiation. The role of PTH in fetal skeletal development has been demonstrated by knockout studies of PTH, PTHrP and the receptor PTHR. PTH-deficient mice showed diminished cartilage matrix mineralization, decreased neo-vascularization, reduced metaphyseal osteoblasts and trabecular bone [86]. PTHrP deficient mice died due to impaired bone formation [87]. In line with this, mice over expressing constitutively active receptor for PTH/PTHrP with bone specific Collagen type 1 promoter promoted increased bone formation [88]. These studies indicate the crucial role of GPCR/PTH signaling in skeletal morphogenesis and may explain the post-natal anabolic effects of PTH. Over six decades, it has been known that intermittent PTH administration stimulates bone formation *in vivo*, whereas prolonged exposure leads to bone resorption [89, 90]. PTH is known to induce osteogenesis by inducing the activity of many transcription factors including cyclic AMP response element binding protein (CREB) [91], AP-1 family members including c-jun, fosB, jun-B, fra-1 and fra-2 [92-94]. Studies have shown that hMSCs express PTHR and PTH [95, 96]. Exposure of hMSCs to PTH stimulates the expression of bone active cytokines such as IL-6 and IL-11, which may play a role in activation of osteoclasts resulting in osteoporosis [97]. In contrast, activation of the PTH receptor in the osteosarcoma cell line MG-63 induces osteoblast differentiation by stimulating collagen type I synthesis and ALP expression [98]. A recent report demonstrates that intermittent exposure of hMSCs to PTH suppressed the adipogenic differentiation by inhibiting PPAR-γ and glycerol 3-phosphate dehydrogenase activity and led to an increased ALP expression [99]. In contrast, we could not demonstrate a positive effect of PTH on osteogenesis of hMSCs *in vitro*, neither by intermittent nor continuous exposure of hMSCs to PTH (1-34) and PTHrP (unpublished data). The reason for this discrepancy is unknown but highlights the often conflicting data on the role of PTH on *in vitro* osteogenesis.

Other GPCRs expressed in osteoblast cell lines and primary cells of mesenchymal origin include the receptors for adenosine, beta-adrenergic hormone, P2Y2, prostaglandin, calcitonin, melatonin, the calcium sensing receptor and many other orphan receptors [100-102]. hMSCs respond to calcium oscillation by IP3 signaling [103]. Others have shown that hMSCs express prostaglandin E2 and respond to recombinant PGE-2 resulting in intracellular cAMP production [104]. Our recent studies demonstrate that PKA activation in hMSCs significantly enhances *in vitro* osteogenic differentiation (unpublished data). Further, we consistently demonstrate that short term PKA activation in hMSCs for 4 days *in vitro*, induces bone formation by hMSCs *in vivo*. These studies demonstrate that PKA-activating small molecules such as cAMP, cholera toxin and forskolin can be applied to enhance *in vitro* osteogenic differentiation and *in vivo* bone formation.

RHO-GTPase SIGNALING

Rho-GTPases belong to the Ras super-family of small GTPases and are known to control a wide variety of cellular processes such as actin cytoskeleton rearrangement, microtubule dynamics, cell adhesion and polarity. Like all members of the Ras superfamily, Rho GTPases function by a conformational switch from inactive GDP to active GTP. GTP bound GTPases are able to bind a variety of downstream target proteins called effectors, which can in turn initiate a variety of cellular responses. The involvement of Rho signaling has been demonstrated in the differentiation of MSCs into neuronal cells [105]. *Pasteurella multocida* toxin (PMT), a bacterial toxin that activates GTPase stimulates proliferation of primary mouse calvarial cells and markedly inhibits the differentiation of osteoblast precursors into bone nodule, which was reversed by the Rho inhibitor [106]. These results show that Rho activation using PMT inhibits osteoblast differentiation through a mechanism involving the Rho-ROCK pathway in mouse calvarial cells. Conversely, ROCK inhibitors stimulate osteoblast differentiation [106]. In sharp contrast, a recent study by McBeath *et al.* demonstrate the involvement of Rho GTPase signaling in hMSC cell fate decision [107]. hMSCs which were allowed to adhere, flatten and spread underwent osteogenesis, while unspread, round cells became adipocytes. Further, dominant negative RhoA triggers hMSCs to become adipocytes, while constitutively active RhoA expression induced osteogenic differentiation. Another study by Meyers *et al.* confirmed that constitutively active RhoA induces the osteoblastic phenotype and suppresses adipogenic differentiation of hMSCs when cultured in modeled microgravity [108]. These molecular cues could be used to improve hMSC differentiation into the osteogenic lineage by modifying the microenvironment of the scaffold materials used in tissue engineering or by gene therapeutical or pharmaceutical intervention on the pathway with molecules such as LPA and PMT [106, 109].

VITAMIN D SIGNALING

Vitamin D is a secosteroid is produced in the skin by the action of sunlight and undergoes successive hydroxylations in liver and kidney to become biologically active 1,25-dihydroxy-vitamin D (VitD3). The major biological function of VitD3 is to maintain the serum calcium levels within the normal ranges by increasing the efficiency of intestinal absorption of dietary calcium. VitD3 binding to its receptor results in a conformational change, which results in heterodimer formation of the vitamin D receptor and the retinoid X receptor. VitD3 response elements (VDRE) in the promoter region of many genes are recognized by an active heterodimer [110] (Fig. (**4A**)). One of the most well known target gene is osteocalcin, which has a VDRE in its promoter. VitD3 exposure of hMSCs transduced with a luciferase gene driven by a 1.4kb fragment of the human osteocalcin promoter leads to rapid induction of reporter gene expression (Fig. (**4B**)) and injection of VitD3 into mice transgenic for the same construct give high expression throughout the body [111, 112]. Moreover, VitD3 enhances transcription of osteopontin, bone sialoprotein [113], collagen type I and osteoprotegerin [114]. In MG-63 cell line, VitD3 exposure results in an enhanced expression of ALP, collagen type I, osteocalcin, Runx2 and osterix. Similar results were observed in primary hMSCs [115]. Other studies have shown that VitD3, besides inducing the

expression of osteocalcin, also enhanced the expression of other osteogenic growth factors such as Insulin like growth factor-1 (IGF-1) and IGF-binding protein 2, 3 and 4 in hMSCs [116]. These investigations clearly outline a positive effect of vitD3 on osteogenesis of hMSCs although the effect on *in vivo* bone formation by VitD3 has recently been questioned [117]. Future studies have to focus in more detail on the combined effect of vitamin D3 and other pro-osteogenic signals.

MITOGEN ACTIVATED PROTEIN KINASE SIGNALING

Mitogen-activated protein kinase (MAPK) signaling is involved in various cellular functions such as proliferation, differentiation and migration and is activated by a number of growth factors such as basic fibroblast growth factor (basic FGF), IGF-1 and platelet-derived growth factor [118-120]. Presently, four MAPK members have been identified: ERK1/2, c-Jun-amino-terminal kinase (JNK), p38 and ERK5 [121]. ERK5 and ERK1/2 are known to induce immediate early genes, such as c-Fos and c-Jun [122, 123]. Jaiswal *et al.* investigated the role of MAPK family members ERK, JNK, and p38 on osteogenic differentiation of hMSCs. First, treating hMSCs with osteogenic supplements resulted in sustained ERK activation from day 7 to day 11 that coincided with differentiation. In contrast, JNK activation occurred much later (day 13 to day 17) in the osteogenic differentiation process, which was associated with extracellular matrix synthesis and increased calcium deposition. Inhibition of ERK activation by PD98059, a specific inhibitor of the ERK signaling pathway, blocked osteogenic differentiation in a dose-dependent manner which was further confirmed by transfection of hMSCs with a dominant negative form of MAP kinase (MEK-1). These observations provide a potential mechanism involving MAP kinase activation in osteogenic differentiation of hMSCs and suggest that commitment of hMSCs into osteogenic lineages is governed by activation or inhibition of ERK [124].

Another example of MAPK-driven differentiation is provided by melatonin, which is a hormone produced by the pineal gland and known to induce osteogenic differentiation of hMSCs *via* MT2 melatonin receptors and the MEK/ERK signaling cascade [125]. Furthermore, Simmons *et al.* demonstrate that application of cyclic strain to hMSCs

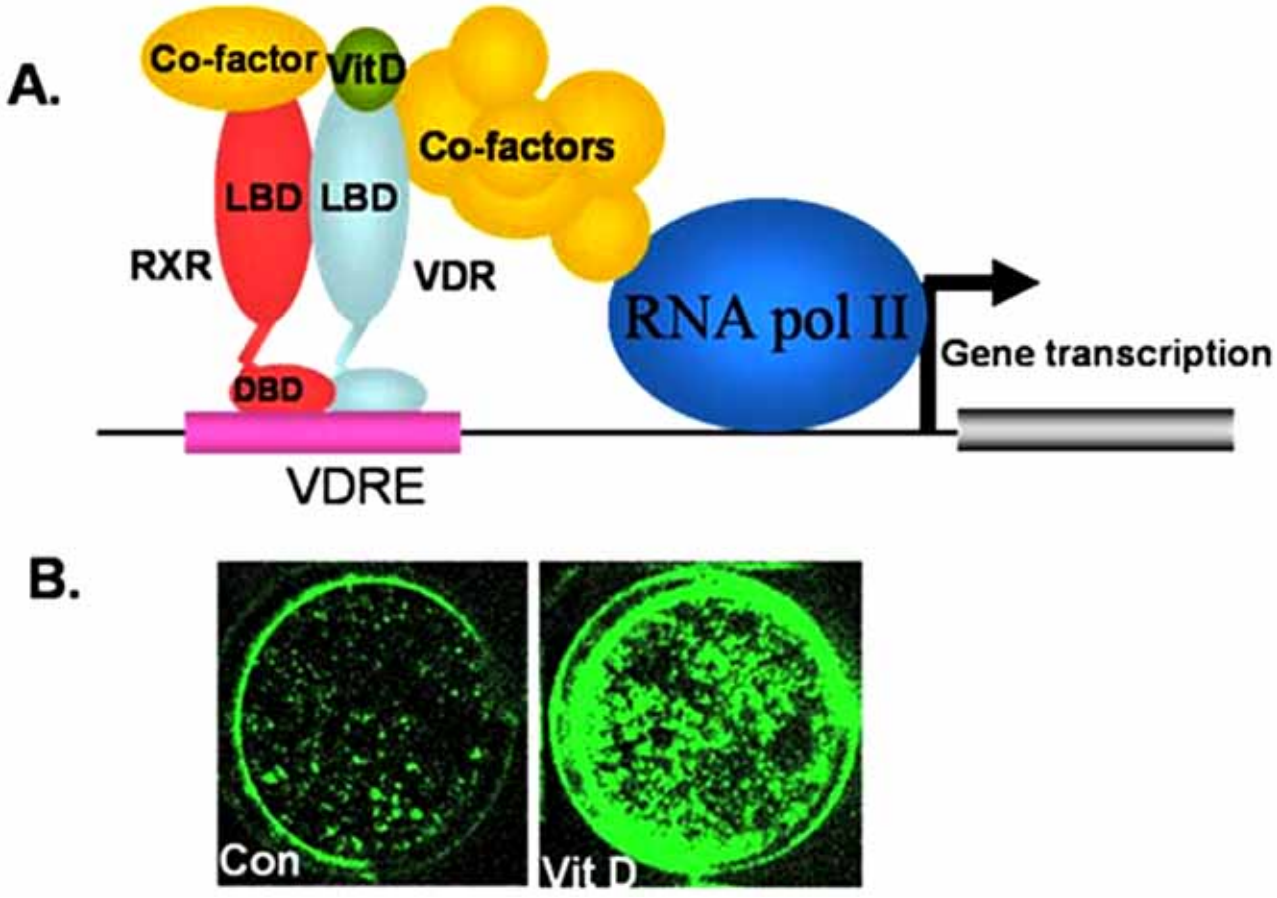

Fig. (4). A. Vitamin D receptor-mediated gene activation. 1,25-dihydroxyvitamin D_3 regulated gene transcription. Liganded VDR-RXR heterodimer recruites co-factors and binds to VDRE using the DNA binding domains. Complex formation with basal transcription machinery and histone modifiers enables activation of gene transcription. DBD, DNA binding domain containing the two zinc fingers; LBD, ligand binding domain; VDR, vitamin D receptor; VDRE, vitamin D responsive element; VitD, 1,25-dihydroxyvitamin D_3. (Courtesy Dr. van Benzooyen) **B.** Bioluminescent image of hMSCs transduced with a lentiviral vector carrying the human osteocalcin promoter driving the luciferease gene in control medium (con) or medium supplemented with vitamin D3 (From our unpublished data).

enhanced matrix mineralization compared to untreated cells through activation of ERK1/2 and p38 MAP kinase pathways, which was reversed by ERK inhibitors suggesting that mechanical signals regulate hMSC function [126]. These recent developments demonstrate that osteogenic differentiation can be controlled by providing proper extracellular cues and mechanical stimuli to the cells. Other non-collagenous proteins, such as laminin-5 and dentin matrix protein-3 induced osteogenic differentiation of hMSCs *via* ERK1/2 signaling by inducing expression of Cbfa-1 and ALP, resulting in enhanced matrix mineralization [127, 128]. Overall, these data demonstrate a pivotal role of MAPK signaling in osteogenic differentiation of hMSCs [129] and provide a potential tool to enhance bone tissue engineering.

CURRENT LIMITATIONS AND FUTURE DIRECTIONS

The past decade has seen a surge in publications on hMSCs and more and more is known about the biological properties of this fascinating cell type. Despite these advances, clinical efficacy of hMSCs in bone tissue engineering is still not within reach because we are still faced with a number of questions to be answered and problems to be solved.

First of all, *in vitro* osteogenic differentiation can still be optimized. As outlined in this review, hMSCs respond to many different external signals but studies to demonstrate the extrapolation of *in vitro* differentiation to bone formation *in vivo* are still underrepresented and deserve more attention. Moreover, research on osteogenic differentiation of hMSCs is mainly driven by literature on osteogenesis in model cell lines and animals. However, it is clear that hMSCs sometimes respond different to osteogenic molecules than what was expected from the literature. In this light, a more discovery-driven approach could be anticipated in which high throughput screening of hMSC differentiation can be performed with banks of small molecules, proteins or RNAi. Molecules identified in screens can be directly applied in osteogenic protocols *in vitro* but also shed a light on the ins and outs of hMSC osteogenic differentiation.

A second point of concern in bone tissue engineering is the survival and proliferation of hMSCs after implantation. Even though *in vitro* expansion of hMSCs on ceramics is as efficient as expansion of goat or rat MSCs, implantation of the latter two results in widespread bone formation, whereas the former does not. The reason for this is currently unknown and it is not trivial to find out, because elaborate analysis tools are required to investigate the fate of the cells after implantation. Promising applications of non-invasive imaging technology are entering the literature such as µCT and MRI [111, 130]. Using another imaging modality, bioluminescent imaging of luciferase transgenic cells, we recently obtained evidence that goat MSCs survive implantation and proliferate whereas human MSCs do not. Thus, this warrants further investigations into the mechanism of cell survival of hMSCs. Evidently, nutrient availability is one of the prime suspects when it comes to cell death in tissue grafts. Non-invasive imaging can be of great support to monitor cell survival. Non-invasive imaging can also be applied in another area of concern in bone tissue engineering, which is the control of the differentiation process *in vivo*. In contrast to the manipulative possibilities *in vitro*, differentiated hMSCs are released into a black box upon implantation, hoping for the best. From *in vitro* studies it is known that hMSCs express osteogenic markers as long as they are exposed to osteogenic stimuli, but will switch fate upon exposure to another stimulus [107]. Thus, carefully instructed hMSCs should also receive instruction after implantation. To manipulate the signaling context at the graft site, several options can be considered. For instance, osteogenic compounds can be released from the

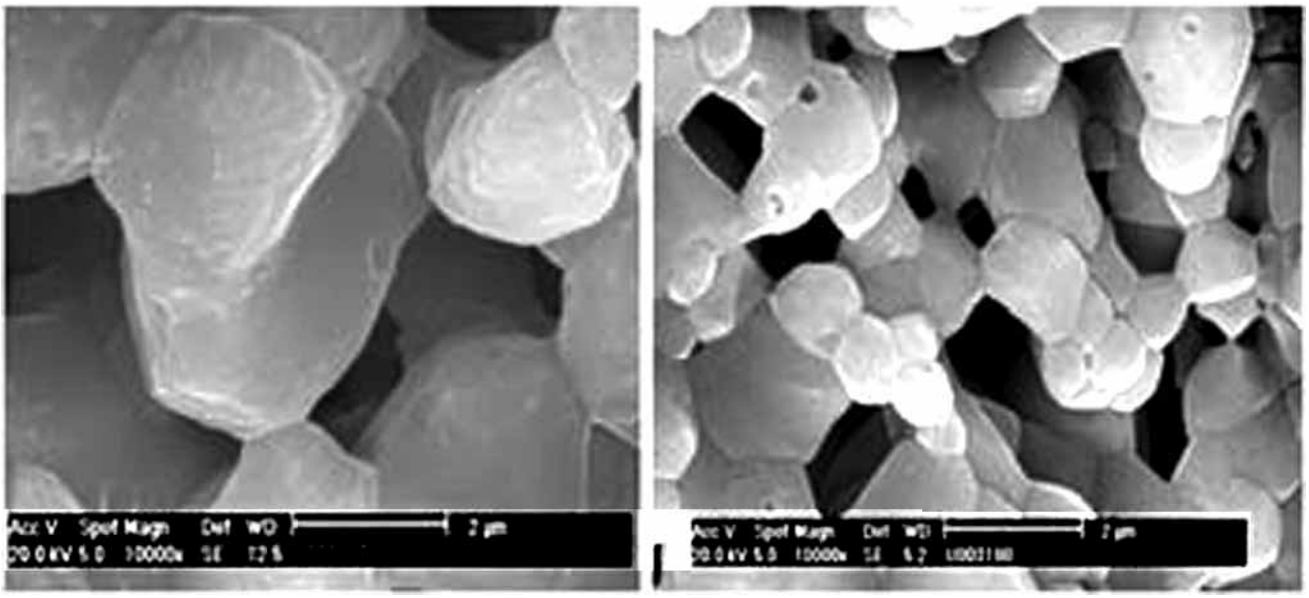

Fig. (5). Scanning electron micrograph of porous biphasic calcium phosphate sintered at either 1300 ºC (left panel) or 1150ºC (right panel). Note the distinct difference in micoporosity. The more micro-porous scaffold is osteo-inductive, whereas the macro-porous is not (From our unpublished data).

scaffold material, for which a number of successful applications have been described in the literature. Another option is to use materials with an intrinsic property favoring osteogenic differentiation, e.g. osteo-inductive ceramics, scaffolds coated with natural extracellular matrix proteins, or materials with favorable mechanical properties (Fig. (**5**)).

Finally, in autologous bone tissue engineering, we are faced with the fact that bone marrow biopsies from different individuals vary enormously with respect to their biological performance [29, 30]. This makes standardization of the technique difficult and as such, we should get a finger behind these differences. Various animal models are used for orthopaedic related research such as rats, rabbits, dogs and goats. Other species such as mice, sheep, horses and primates are also been used for *in vivo* bone studies. Mostly to test the osteogenic potential and *in vivo* bone forming ability of the MSCs, immune deficient nude mice (NMRI nu/nu) are very commonly used and well accepted among the scientists in this field. These mice have deteriorated thymus resulting in impaired immune system due to greatly reduced number of T cells and can be used to study variety of tumor, tissue grafts and xenografts as it mounts no rejection response. Further, to study the clinically applicability of the bone tissue engineered grafts dogs, goats, horse and even primates are used and the results obtained between different species should not be neglected. Outlining the differences in cell survival, response to osteogenic molecules and bone formation *in vivo* does not only help in the identification of critical parameters for bone tissue engineering but may also help us in identifying the true nature of the somewhat enigmatic population of cells referred to a human mesenchymal stem cells.

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