Cellular and Molecular Prerequisites for Bone Tissue Engineering

Ramakrishnaiah Siddappa

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Cover page: Front, artistic representation of a polarized image of bone histology and side figures illustrating human mesenchymal stem cells morphology, differentiated adipocytes, extracellular matrix and *in vivo* bone formed by human mesenchymal stem cells. Image on the back is an artistic representation of lamellar bone which could be produced by *Coleus forskohlii* (Makandi, Indian common name) a native plant of India and most commonly used in Ayurvedic medicine for centuries to treat various diseases.

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CELLULAR AND MOLECULAR PREREQUISITES FOR BONE TISSUE ENGINEERING

DISSERTATION

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by

Ramakrishnaiah Siddappa
Born on January 10th 1977
in Bangalore, India.

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ನನ್ನ ತಾಯಿ-ತಂದೆಯರ ಪಾದಾರವಿಂದಗಳಿಗೆ

Dedicated to my parents

Contents

Chapter 1

Cell-Based Bone Tissue Engineering

A Perspective on current limitations and future developments

Bone is the largest portion of the body's connective and physiologically mineralized tissue, which provides structural and mechanical support besides maintaining mineral homeostasis of the body. During embryogenesis, skeletal development takes place by two independent mechanisms: intramembranous ossification and endochondral bone formation. Both processes include mesenchymal stem cell (MSC) condensation of *anlagen* which later develop into skeletal structures with a defined structure and strength[1]. Further, bone remodeling occurs as a dynamic and lifelong process in which old bone is removed from the skeleton and new bone is added by two distinct cell populations called osteoclasts and osteoblasts respectively. Any imbalance in this intricate balance would lead to skeletal disorders such as osteopenia, osteoporosis or osteopetrosis [2, 3]. In addition, skeletal related traumas, which fail to heal themselves, need bone substitutes to restore the skeletal function.

The Need for Bone Substitute

The World Health Authority has declared year 2000 - 2010 as *"The Bone and Joint Decade"* based on the rationale that, firstly, joint diseases account for half of all chronic conditions in people over 65 years old and secondly, there is a large increase in osteoporotic fractures. Thus, over 40 % of the population suffers from either. Over 25 % of health expenditure is spent on trauma-related skeletal deformities and skeletal disorders. Many of these cases require bone graft substitutes to repair the injury or defect. There are over 500,000 bone grafts performed annually in the United States alone, which underlines the great need to improve current procedures for spinal fusion, fracture healing and bone defects reconstruction resulting from trauma and other disorders.

Current Status

Current therapies include ***1. Autografts***, which represent over 50 % of the bone substitutes and involves harvesting bone from one location in the patient's body and transplanting it into another part of the same patient. Autologous grafts produce the best clinical results and successful outcomes, and are thus considered as the *'golden standard.'* ***2. Allografts*** represent about 30 % of bone substitutes, which involves harvesting and processing bone from a cadaver and transplanting it to the patient. ***3. Synthetic materials*** such as metals, plastics and various ceramic materials, which represent approximately 10 % of bone substitutes. Recently, growth factors such as bone morphogenetic proteins, parathyroid hormones and drugs such as bisphosphonates are also being used to treat skeletal related diseases[4-7]. The clinical benefits from autografts are expected since autologous grafts exclude immunogenic related problems and result in a high grafting success rate. However, autografting has several drawbacks including the additional surgical costs for the harvesting procedure, infection and most importantly harvest site morbidity. Allogeneic implants are a-cellular and are less successful than autografts for reasons attributed to immunogenicity and the absence of viable cells in the graft. Another main concern in allografting is disease transmittance. Synthetic materials are subject to various disadvantages such as inferior mechanical properties, toxicity, wear and biodegradability. These drawbacks prompted researchers to develop cell-based bone tissue engineering as an alternative

tool, which aims at developing highly effective bone substitutes for skeletal related disorders.

Cell-based Bone Tissue Engineering

Cell-based bone tissue engineering aims to overcome earlier mentioned drawbacks in current tissue engineering protocols by a multi-disciplinary approach using the knowledge available from biology, medicine, material science and engineering. In short, bone tissue engineering includes isolation and expansion of mesenchymal stem cells (MSCs) from the patient and seeding the cells onto porous biodegradable matrices (scaffolds). During the *in vitro* culture period, the cells are exposed to signaling molecules (e.g. proteins, growth factors and other osteo-inductive molecules) to drive the MSCs into the osteogenic lineage and this tissue engineered graft is subsequently implanted into the defect site to induce and direct the growth of new bone as the scaffold degrades (Figure 1).

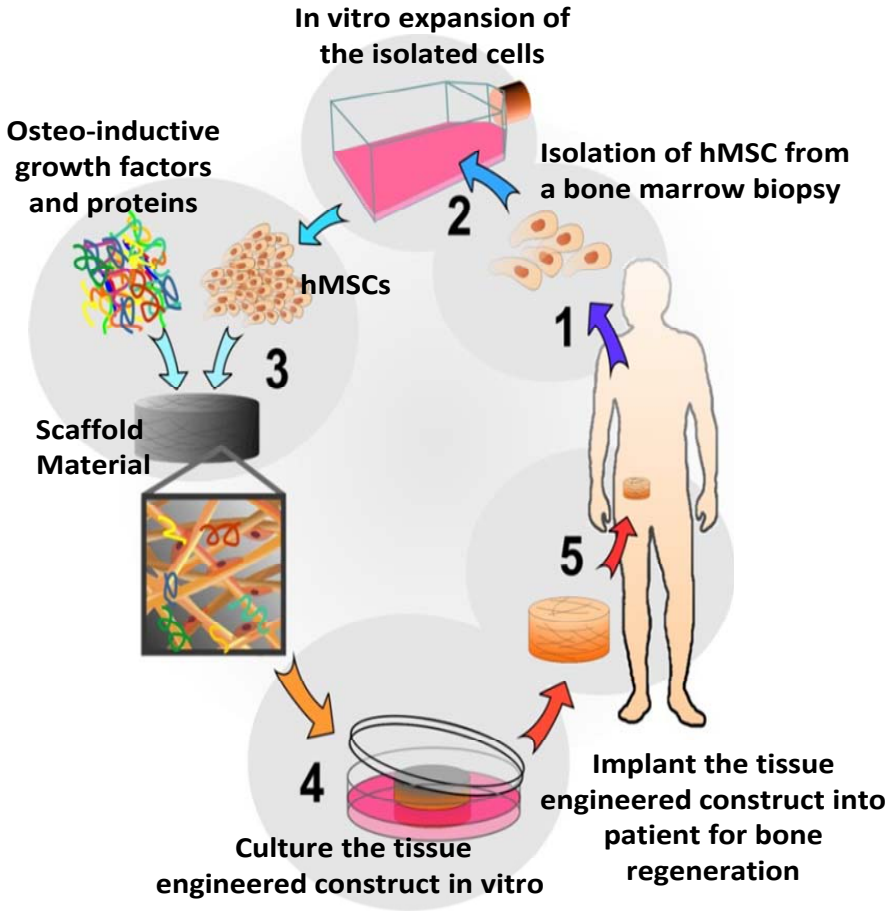

Figure 1. schematic representation of cell-based bone tissue engineering. *A fraction of bone marrow biopsy will be harvested from the patient, in vitro expanded to obtain a large number of cells. The cells will be seeded on to various osteo-inductive materials and cultured further for a number of days with osteo-inductive growth factors. The tissue engineered construct will be implanted back to the patient to heal the bone defect.*

Bone formation by *in vitro* expanded MSCs was first assessed in small animal models by implanting MSCs with porous bioceramics in rats, immunodeficient mice and by repair of small experimentally induced osseous defects[8, 9] Later, large animal models were used to mimic the clinical situation[10, 11]. Although some clinical trials using MSCs showed a favorable outcome in fracture healing[12], a common problem seems to be that the amount of newly formed bone is not sufficient to fully bridge the implant [13]. Current studies demonstrate that pre-differentiation of the isolated MSCs *in vitro* into the osteogenic lineage before implanting, augments the *in vivo* bone forming capacity of the cells[14, 15]. As a consequence, we need to have thorough understanding of MSCs, the materials we use and the molecular and genetic cues which regulate osteogenesis to improve tissue engineering protocols.

Scaffold materials in bone tissue engineering

Currently used bone graft substitutes include metals, ceramics, polymers and composites. Each of these has its own advantages and pitfalls. Ceramics are commonly used for specific application where minimum load bearing strength is needed while metals are used in load-bearing applications. Among ceramics, calcium phosphate is extensively used as bone fillers to heal small defects and these materials are also of prime choice as scaffold for bone tissue engineering for their known osteo-inductive capacity in ectopic sites and posses good cell adhesion properties[16]. One of the most exploited metals for bone regeneration is titanium, which provides instant mechanical support. Since metals exhibit poor integration to the host bone, researchers are trying to tackle this by coating them with a layer of osteo-conductive calcium phosphate [17]. Osteo-conductive properties of calcium phosphates has stimulated researchers to fabricate composites with collagen[18], polymers[19] and coating non osteo-conductive materials such as titanium[20]. Other materials used in bone tissue engineering include chitosan[21], different compositions of hydrogels[22], and multiple types of polymers [23-25]. Thus, it warrants for understanding of MSCs behavior on various materials and their role on differentiation. The research in this field has enabled scientists to develop materials with specific properties by altering micro- and macro-structure, topography, porosity and other properties, which not only enhance cell attachment but also direct the differentiation of MSCs into the osteogenic lineage[26-28].

Mesenchymal stem cells in bone tissue engineering

The term “stromal cells” has been used for the partially defined population of cells which make up the adherent cell layer *in vitro* long-term bone marrow culture, which also includes macrophages and endothelial cells[29]. Bone marrow stromal cells or MSCs are present within the bone marrow and are the source of cells giving rise to skeletal tissues[30, 31]. When a bone marrow biopsy is cultured *in vitro* with fetal calf serum, fibroblastic colonies appear on tissue culture plastic, each derived from a single cell, referred to as colony forming unit-fibroblast (CFU-F). These CFU-Fs are addressed by researchers as marrow stromal fibroblasts, bone marrow stromal cells or mesenchymal progenitor cells. It is estimated that only 15% of the isolated CFU-Fs have stem cell-like properties[32, 33]. CFU-F colonies derived from the bone marrow of virtually all species examined including human, are heterogeneous in size and morphology suggesting that they originate from clonogenic progenitors at various stages

of differentiation[34]. The CFU-Fs derived from the bone marrow are not only heterogeneous in size and morphology but also demonstrate their differential ability in proliferation and bone formation *in vitro*[30]. In addition, CFU-Fs isolated from different donors respond to various osteogenic signals differently and in turn *in vivo* bone formation[35] which will be discussed in depth in upcoming chapters. These cells are now a days most commonly referred to as MSCs, which are multipotent and are able to differentiate into the osteogenic, chondrogenic, adipogenic and many other lineages including myoblasts[31, 36]. MSCs belong to a lineage hierarchy in which only some of the cells are multipotent or primitive progenitors, while other cell populations posses limited differentiation capacity. Besides bone marrow, researchers have isolated multipotent MSCs from many other sources including adipose tissue, tibia, femur, lumbar spine, trabecular bone and placenta[37-39]. The advantages of using MSCs over other readily available, self renewable cell sources such as embryonic stem cells is that MSCs rule out immunogenic rejection, tumor formation and ethical issues associated wtih the use of embryonic stem cells. The effect of MSC isolation methods, source, heterogeneity and donor variation with respect to various osteogenic signals on the efficacy to form bone *in vivo* are important to understand and will be discussed elsewhere in the thesis.

MSCs proliferation

Bone tissue engineering requires a large number of multipotent MSCs for clinical application. This demands extensive *in vitro* expansion of isolated MSCs without compromising their multipotency. Therefore it is important to understand the factors which modulate proliferation of MSCs *in vitro*. Besides, there are many more factors in fetal calf serum used in the culture medium, which regulate the adhesion and proliferation of MSCs. For instance, platelet derived growth factor is known to enhance the colony formation[40] and epidermal growth factor is known to enhance CFU-Fs size in MSC culture[34, 41]. Further, synthetic glucocorticoids have complex effects on proliferation and differentiation of MSCs, which are not very well elucidated. Various isoforms of fibroblast growth factors and transforming growth factors are known to be mitogenic to MSCs[42, 43]. We and other have demonstrated that using Wnt signaling activators, it is possible to enhance *in vitro* proliferation of MSCs without compromising their multipotency and *in vivo* bone forming capacity[44, 45].

Lineage commitment

Once adequate numbers of *in vitro* expanded MSCs are obtained, it is important to direct them into the osteogenic lineage to augment their bone forming ability. Differentiation of multipotent MSCs into a matured osteoblast requires a spectrum of signaling proteins including morphogens, hormones, growth factors, cytokines, matrix proteins, transcriptions factors and their co-regulatory proteins. They induce a temporal expression of many genes (sequential activation, suppression and modulation) which represent phenotypic, structural and functional properties of osteoblasts during the differentiation process. Osteogenic lineage commitment is orchestrated by various developmental signaling pathways and transcriptional regulators which serve as master switches between different lineages (Figure 2). Important transcriptional regulators of differentiation of osteoprogenitor cells to mature osteoblast include various

homeobox proteins such as Msx1, Msx2 and their involvement in bone development has been demonstrated from knockout mice which display severe defects in craniofacial bone development and endochondral bone formation[46-48]. Dlx5 and Dlx6 are expressed in developing limb buds and their role in osteoblast differentiation has been demonstrated[49, 50]. Cbfa1 is considered as a *"master regulator"* of osteoblast differentiation, which belongs to runt family of transcription factors. Cbfa1 knockout mice exhibit impaired chondrocyte and osteogenic differentiation, culminating in complete failure of skeletal ossification[51-53]. Further, Cbfa1 activity is mediated by various down-stream transcription factors such as ATF4, ATF2, AP-1, c-fos, Fra-1, FosB, JunB, JunD osterix[54-63]. Wnt proteins not only regulate proliferation of MSCs but also play a major role in differentiation and skeletal biology. Wnt10 plays a critical role in cell fate decision between osteogenic and adipogenic lineage mediated via regulation of Runx2, Dlx5 and Osterix expression[64, 65]. Osteogenic differentiation of human derived MSCs (hMSCs) is poorly understood. In this regard we and others have focused in depth to study the osteogenic process in hMSCs to understand the differences with MSCs from lower species.

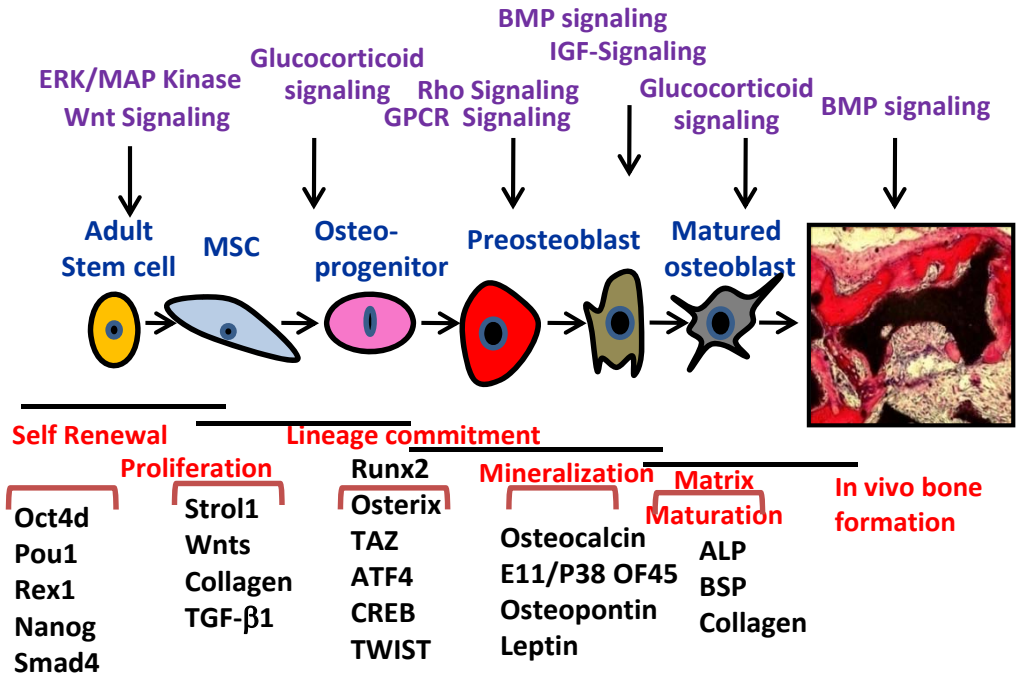

Figure 2. Schematic representation of molecular and genetic signaling cues which regulate proliferation and differentiation of a multipotent MSC into a functional osteoblast.

Matrix mineralization and maturation

Osteoblasts synthesize extracellular matrix containing collagenous and non-collagenous proteins *in vitro* that mineralizes in the presence of an exogenous phosphate source. *In vivo*, calcified bone provides mechanical rigidity and load bearing strength. The matrix synthesis *in vitro* and *in vivo* predominantly starts with type I collagen synthesis that later determines the structural organization, and constitutes

over 90% of the organic matrix. Non-collagenous proteins contribute up to 10 % of the total bone protein content (mainly type X, type III, and type V collagen). Non-collagenous proteins include proteoglycans (albumin, alfa2-HS glycoprotein), glycosylated proteins (alkaline phosphatase, osteonectin, tetranectin and tenascin), glycosylated proteins with potential cell attachment activities (osteopontin, bone sialoprotein and dentin matrix pprotein 1) and gamma-carboxylated proteins (mainly osteocalcin and matrix gla protein). Bone mineral is initially deposited at discrete sites in the collagenous matrix and as it matures, the mineral crystals become larger by aggregation of mineral crystals[66].

Terminal differentiation

The osteocyte is a terminally differentiated stage of an osteoblast which support bone structure and other metabolic functions. Typical morphological features of an osteocyte are its location in lacunae and the numerous cellular extensions, which establish cytoplasmic connections with adjacent cells. Osteocyte maturation and survival is regulated by the formation of gap junction[67] and these gap junctions are formed by connexin 43. Gap-junction disruption by connexin 43-deficiency results in dysfunctional osteoblasts and retarded ossification of the skeleton[68]. Osteocytes seem to have direct communication with each other and lining osteoblasts through cellular processes and the primary function of an osteocyte is demonstrated to be a mechanotransducer in bone remodeling process, evidenced by targeted ablation of osteocytes [69].

Above and beyond to the earlier described parameters which regulate osteogenic differentiation of MSCs *in vivo* and *in vitro*, the process is further controlled systemically by various hormones and growth factors which impact skeletal integrity. Considering everything from the existence of hMSCs in the bone marrow, isolation, proliferation, differentiation, matrix mineralization and *in vivo* bone formation, we are trying to explore and understand the process in hMSCs in depth to modulate these processes at various stages. By understanding the roles of these factors, which regulate the process of osteogenesis, we can augment the current therapeutic treatment for osteoporosis and skeletal related traumas.

Control of osteogenesis by GPCR signaling

A search for druggable genes, defined by the fact that their activity can be modulated using pharmaceutical drugs, shows that over 500 genes belong to the G-protein coupled receptor (GPCR) family which marks them as a potential target to treat osteoporosis and to enhance cell-based bone tissue engineering[70] (Figure 3A). Hormones such as melatonin, epinephrine, calcitonin, calcitonin gene related peptide, prostaglandins, estrogens, parathyroid hormone, parathyroid hormone related peptide and others are implicated in osteogenesis. Most of these hormones transduce their signals via specific GPCRs. GPCRs are transmembrane proteins which transduce extracellular signals generated from various ligands into intracellular levels by coupling to G-proteins. Ligand binding to the receptor 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). The Gα subunit

possesses intrinsic GTPase activity and dissociates from the βγ heterodimer in a GTP-bound form. Depending on the isoform, the GTP-α subunit complex activates one or more of a number of intracellular signaling cascades, such as protein kinase A (PKA) and protein kinase C (PKC) signaling[71] (Figure 3B).

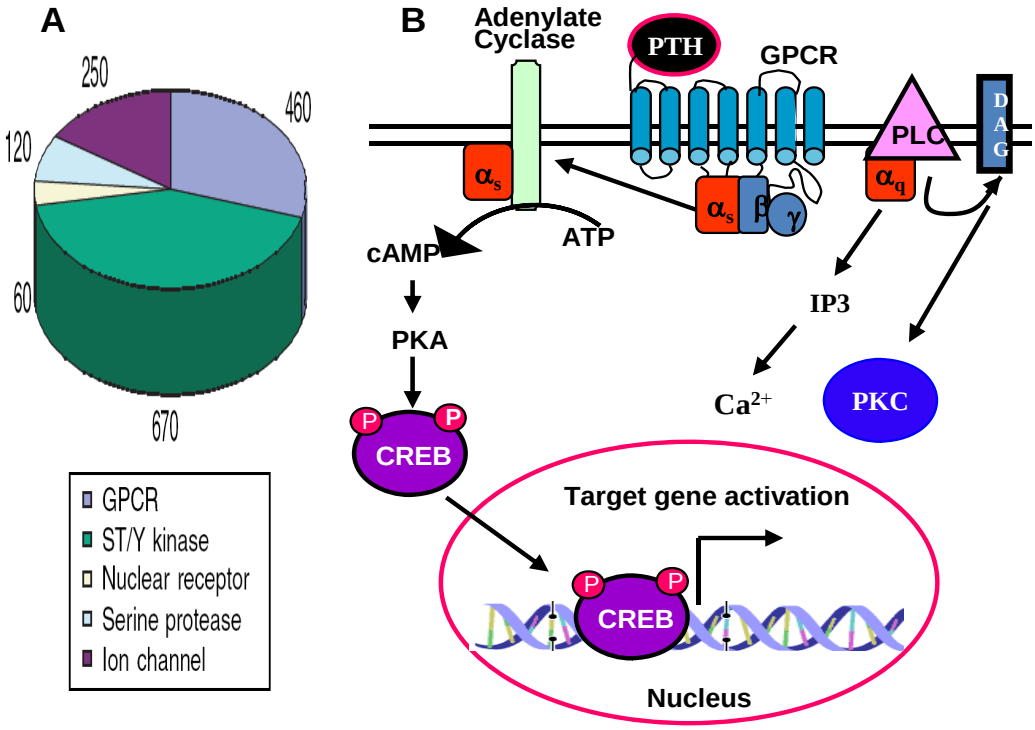

Figure 3. GPCR signaling in mesenchymal stem cell biology. *A. Distribution of druggable genes on human genome, taken from reference 70. B. Schematic representation of GPCR signaling. Binding of a GPCR ligand (such as PTH) activates protein Kinase A (PKA) and Protein Kinase C (PKC) signaling which further activates gene transcription mediated via phosphorylation of transcription factor such as cAMP Response Element Binding Protein (CREB).*

Considering the vast involvement of GPCR signaling in cell proliferation, differentiation and their druggability criteria, the focus of this thesis is to modulate the osteogenic process of hMSCs to enhance current bone tissue engineering protocols. In addition, we focused on understanding the roles of other signaling cues on proliferation and differentiation of hMSCs. Using a multidisciplinary approach to improve bone tissue engineering using hMSCs, the aims of the work described in this thesis is to:

- Unleash their osteogenic response.
- Understand donor variation in response to an osteogenic signal and their *in vitro* expansion capacity for efficient use in bone tissue engineering.
- Understand the role of GPCR signaling in osteogenic differentiation of hMSCs.

- Enhance hMSCs osteogenesis and *in vivo* bone formation by impinging on protein kinase A signaling.
- Use a multidisciplinary approach to further augment the *in vivo* bone forming capacity of hMSCs.
- Use hMSCs as a source of pro-osteogenic cytokines and osteogenic growth factor, to be implemented *in vivo* bone formation by impinging on the host cells.
- Understand how and to what extent these signaling cues are different among the species.

Successful bone tissue engineering using cell-based therapies needs thorough understanding of various factors which directly or indirectly regulate cell growth and differentiation. Further, by bringing together the knowledge from various disciplines, we can control and regulate hMSCs proliferation and differentiation by which we can predifferentiate the hMSCs into the osteogenic lineage before seeding them on to a scaffold material, which will in turn result in successful cell based bone tissue engineering. By contrast, this research was aimed to provide customized and further refined engineered constructs for skeletal related therapies to augment and amplify the patient's clinical capability.

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Chapter 2

The Response of Human Mesenchymal Stem cells to Osteogenic Signals and Its Impact on Bone Tissue Engineering

A Review

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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 efficient use of hMSCs in bone tissue engineering. Recently, there is 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 other cell types with special emphasis on applications in bone tissue engineering.

Key words: *human mesenchymal stem cells, heterogeneity, signaling pathways, osteoblast differentiation, species differences and 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 orthopedic 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] (Figure **1A**). MSCs are a very rare population of cells in bone marrow with a reported incidence of 0.01% to 0.001%[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* (Figure **1B**), and a number of clinical trials have been performed to investigate its feasibility.

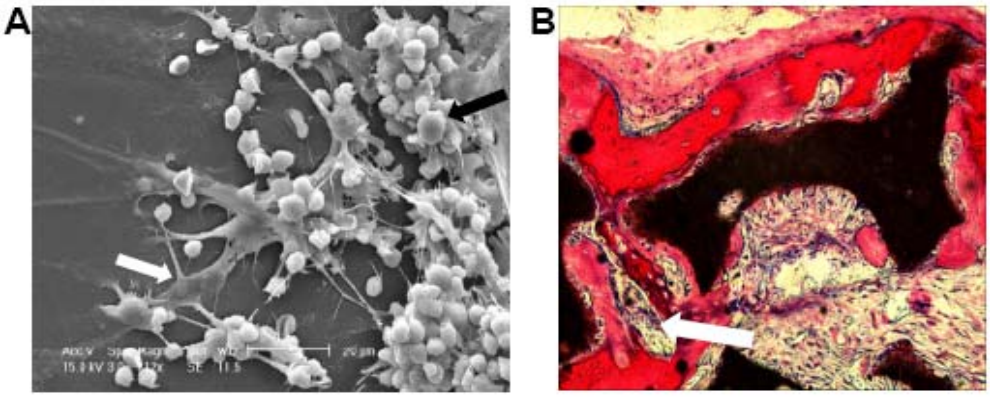

Figure 1. Mesenchymal stem cell isolation and application. A. *Scanning electro micrograph of crude bone marrow aspirate after 24 hours of seeding. Adherent cells can be seen that migrate away (white arrow) from aggregates of non-adherent bone marrow cells (black arrow).* ***B.*** *Ectopic bone formation 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 methelyine blue. Basic fuchsin stains newly formed bone pink and methylene blue stains the remaining fibrous tissue blue (From our unpublished data).*

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 (Figure **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 Thy-1 and higher levels of CD13, Stro1 and stage-specific antigen 1. The higher Stro 1 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 and from various tumors. Bone ALP comprises approximately 50% of total circulating ALP in normal subjects. Measurement of bone ALP is well 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 alkaline phosphatase (ALP) positive cells (an early marker of osteogenic differentiation) 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.

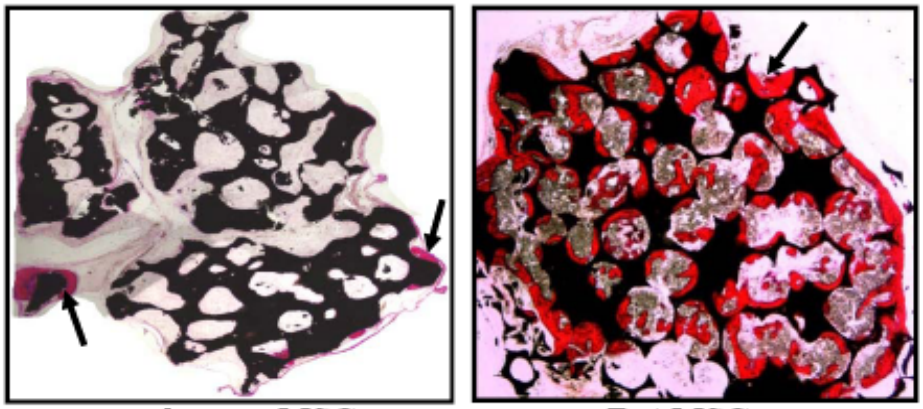

Figure 2. Species differences in biological performance of MSCs. *Ectopic bone formation (red stain, arrows) by an equal number of rat and human MSCs seeded onto calcium phosphate ceramic scaffolds and implanted subcutaneously for 6 weeks in nude mice. Note the enormous difference in the amount of newly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

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 (Figure **3**).

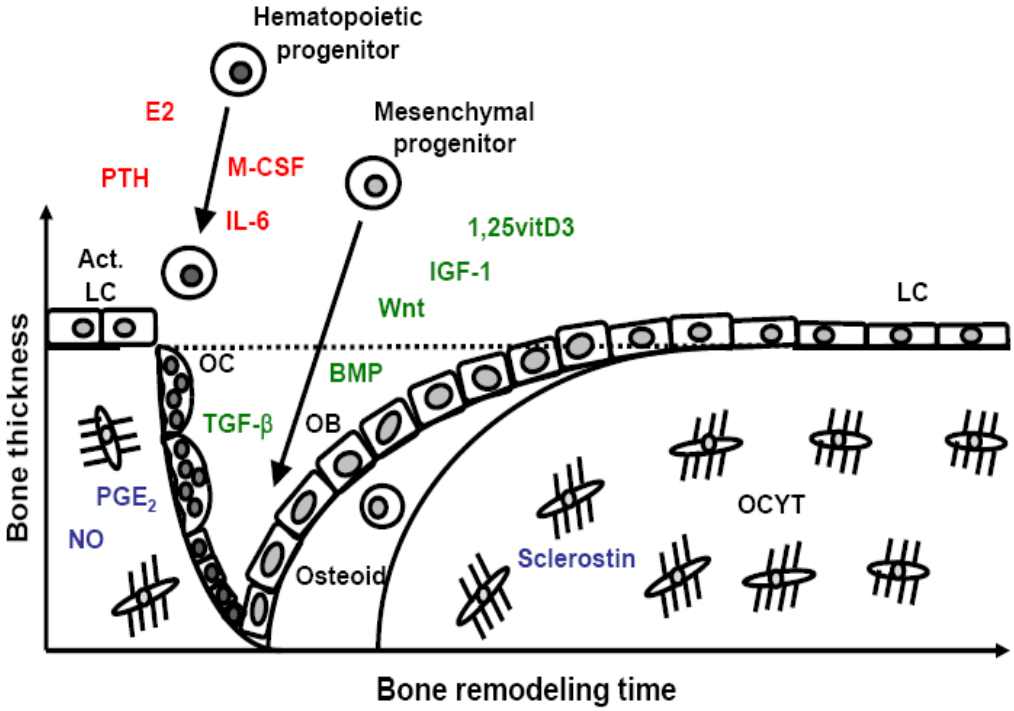

Figure 3. Schematic model of time dependent interactions between bone cells in a basic multicellular unit. *Bone remodeling is initiated by activation of lining cells on the bone surface by signals from osteocytes within bone or from factors in the bone marrow. Nitric oxide (NO) and prostaglandin E_2 (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)*

Mainly, skeletal development is 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]. Further, 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 between hMSCs and other osteogenic model systems. Further, we will discuss possibilities 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 osteo-

genesis 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 efficient 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-β 1 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-β 1 induced expression of osteogenic markers such as ALP, collagen type I and osteocalcin[47, 48]. In contrast, other studies demonstrate that addition of TGF -β 3 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 mineralisation [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 [Ca2+]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 intern 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 treatment 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 super-

family, 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 made 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] (Figure **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 (Figure **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 vitamin D3 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.

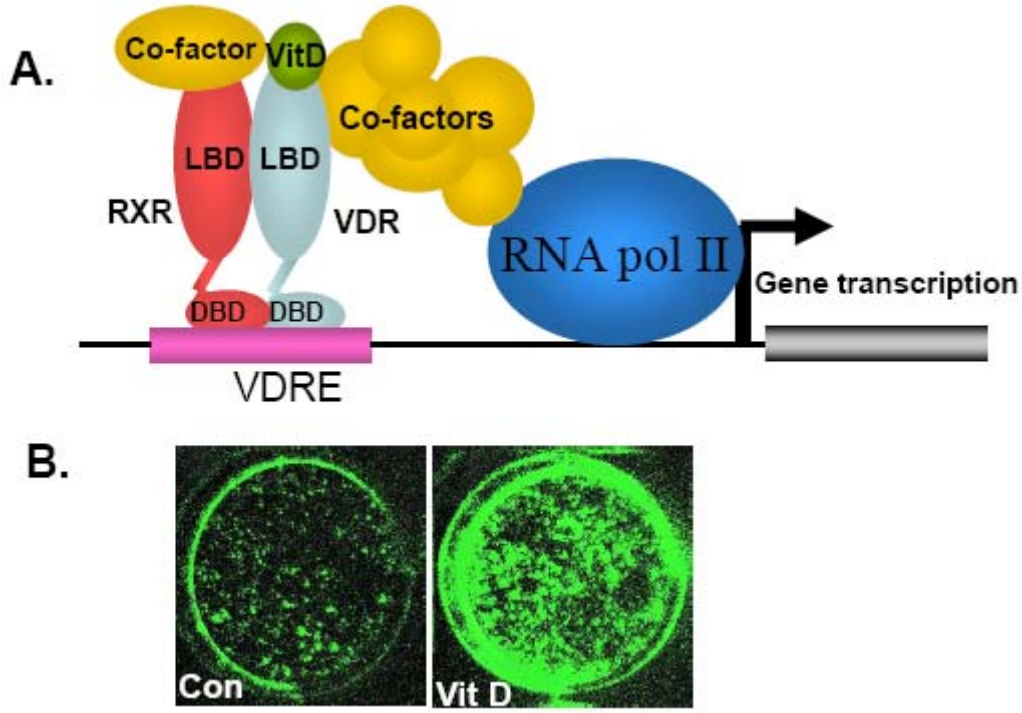

Figure 4. A. Vitamin D receptor-mediated gene activation. *1,25-dihydroxyvitamin D_3 regulated gene transcription. Liganded VDR-RXR heterodimer recruites co-factors and binds to VDRE using the DNA binding domains. Complex formation with basal transcription machinery and histone modifiers enables activation 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).*

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 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 analy-

sis 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 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 (Figure **5**).

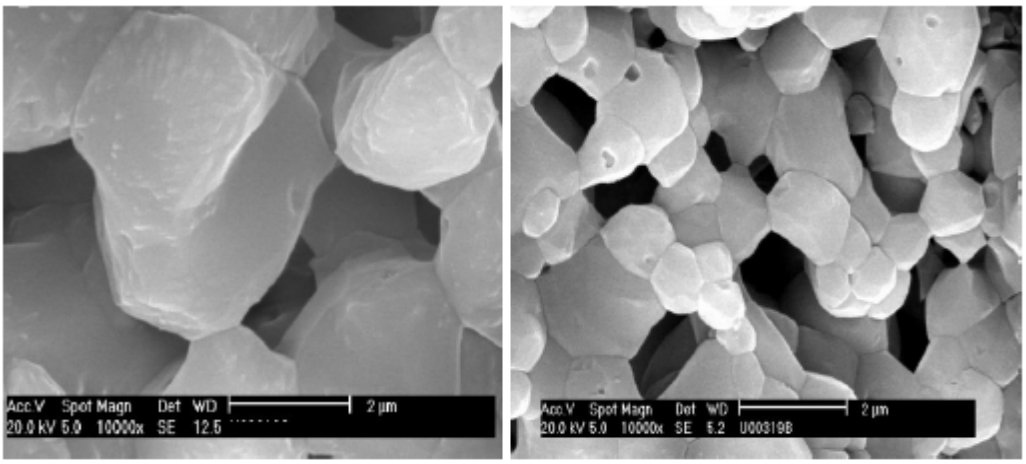

Figure 5. *Scanning electron micrograph of porous biphasic calcium phosphate sintered at either 1300 ºC (left panel) or 1150ºC (right panel). Note the distinct difference in micoporosity. The more micro-porous scaffold is osteo-inductive, whereas the macro-porous is not (From our unpublished data).*

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 inhibited 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 may 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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Chapter 3

Donor variation and Loss of Multipotency during *in vitro* expansion of human mesenchymal stem cells for bone tissue engineering

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Abstract

The use of multipotent human mesenchymal stem cells (hMSCs) for tissue engineering has been a subject of extensive research. The donor variation in growth, differentiation and *in vivo* bone forming ability of hMSCs is a bottleneck for standardization of therapeutic protocols. In this study, we isolated and characterized hMSCs from 19 independent donors, aged between 27 and 85 years and investigated the extent of heterogeneity of the cells and the extent to which hMSCs can be expended without loosing multipotency. Dexamethasone-induced ALP expression varied between 1.2 and 3.7-fold but no correlation was found with age, gender or source of isolation. The cells from donors with a higher percentage of ALP-positive cells in control and dexamethasone-induced groups showed more calcium deposition than cells with lower percentage of ALP positive cells. Despite the variability in osteogenic gene expression among the donors tested, *ALP*, *Collagen type1, osteocalcin* and *S100A4* showed similar trends during the course of osteogenic differentiation. *In vitro* expansion studies showed that hMSCs can be effectively expanded up to 4 passages (approximately 10-12 population doublings from a P0 culture) while retaining their multipotency. Our *in vivo* studies suggest a correlation between *in vitro* ALP expression and *in vivo* bone formation. In conclusion, irrespective of age, gender and source of isolation, cells from all donors showed osteogenic potential. The variability in ALP expression appears to be a result of sampling method and cellular heterogeneity among the donor population.

Key words: *human mesenchymal stem cells, heterogeneity, donor variation and cellular senescence*

Introduction

The contemporary drawbacks of autograft, allograft and xenograft-based bone regeneration methods such as an additional surgical procedure, infection, chronic pain and donor dependencyin successful healing[1-3], has generated large focus on the use of autologous cells for tissue engineering [4-7]. The method utilizes biodegradable materials, which provide an appropriate microenvironment to promote cell-material interaction, adhesion and spreading. Current developments in biomaterial science allow the introduction of bio-active properties to ceramics and biodegradable polymers besides serving as carrier materials[8]. Furthermore, the identification of bone morphogenic protein[9] and other growth factors such as fibroblastic growth factor, epidermal growth factor, transforming growth factors, insulin-like growth factor 1, parathyroid hormone, vitamin D3 and the synthetic glucorticoid dexamethasone improved bone tissue engineering applications since they are known to induce osteogenic differentiation[10-15].

The isolation of hMSCs and their extensive proliferation and ability to differentiate into osteogenic, adipogenic, chondrogenic and myogenic lineages[16, 17] has gained the attention of researchers to use hMSCs for potential clinical use. Multipotent cells have been isolated from many sources including adipose tissue, tibia, femur,

lumbar spine and trabecular bone[18-20]. Traditionally, hMSCs are isolated from an aspirate of bone marrow harvested from the iliac crest or acetabulum. The cells isolated from the latter source are multipotent *in vitro* and form bone *in vivo*[16]. Although hMSCs have superior proliferative capacity *in vitro*, it has been demonstrated that *in vitro* expanded hMSCs show a replicative senescence phenotype culminating in growth arrest and loss of multipotency[21-23]. Cellular senescence and growth arrest are known to occur when telomeres in one or more chromosomes reach a critical length[24]. Since no telomerase activity has been detected in mouse and human MSCs, they show limited proliferative capacity and multipotency in expanded cultures[25, 26]. Retroviral transduction of telomerase into hMSCs extended the proliferative capacity up to 260 population doublings (PD) compared to 26 PD in the control cells while maintaining osteoblast markers, normal karyotype and even enhanced *in vivo* bone formation[27, 28].

Bone tissue engineering could be improved by over-expression of genes such as *BMP2, BMP6, BMP9, Fra-1* and *LIM mineralization protein-3* in MSCs to induces *in vitro* osteogenesis and *in vivo* bone formation[29-34]. However ethical issues restrain the use of genetically modified cells for tissue engineering applications. Therefore more focus has been put on the use of various osteo-inductive stimuli, such as dexamethasone, BMP2, Vitamin D [15] and statins[35] in order to enhance osteogenic differentiation of hMSCs *in vitro*[36, 37]. We recently focused on the use of lithium[38, 39], Trichostatin A[40] and cyclic AMP (manuscript in preparation) as potential compounds to stimulate various steps in the osteogenic process. Besides these developments in bone tissue engineering, the enormous donor variation in growth properties, osteogenic potential and *in vivo* bone formation by hMSCs limits the standardization of therapeutic protocols[41]. Therefore we investigated and characterized hMSCs from 19 independent donors to delineate the heterogeneity among the population and to determine how far the hMSCs can be effectively proliferated *in vitro* while retaining their differentiation abilities for tissue engineering applications.

Materials and Methods

Isolation and culture of hMSCs

Bone marrow aspirates (5-20ml) were obtained from donors with written informed consent. The donors with known skeletal disease or drug history were excluded from the study. hMSCs were isolated and proliferated as described previously[42]. Briefly aspirates were resuspended using 20 G needles, plated at a density of $5x10^5$ cells/cm^2 and cultured in hMSC proliferation medium (PM) containing α-minimal essential medium (α-MEM, Life Technologies), 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), 10 μ g/ml streptomycin (Life Technologies), and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week and cells were used for further sub-culturing or cryopreservation upon reaching near confluence. hMSC basic medium (BM) was composed of hMSC proliferation medium without bFGF, hMSC osteogenic medium (OM) was composed of hMSC basic medium supplemented with 10^{-8} M dexamethasone (dex, Sigma) and

hMSC mineralization medium (MM) was composed of basic medium supplemented with 10^{-8} M dexamethasone and 0.01 M β-glycerophosphate (Sigma). For extensive proliferation of hMSCs, cells were seeded at 1000 cells/cm^2 and cultured in PM until they reached 80% confluence, then they were trypsinized and seeded again at 1000 cells/cm^2. Proliferation rate was calculated by counting the number of cells in triplicate before and after seeding. A fraction of cells from each passage was used for other biological assays.

ALP analysis by flow cytometry

hMSCs were seeded at 5000 cells/cm^2 and allowed to attach for 10 to 15 hours in BM, then cells were incubated with 10^{-8} M dexamethasone for the denoted time periods. Each experiment was performed in triplicate with a negative control (cells grown in BM) and a positive control (cells grown in OM) and one or more experimental conditions. At the end of culture period, the cells were trypsinized and incubated for 30 minutes in block buffer (PBS with 5% bovine serum albumin, BSA [Sigma] and 0.05% NaN2), then incubated with primary antibody (anti-ALP, B4-78 [Developmental Studies Hybridoma Bank, University of Iowa, USA]) diluted in wash buffer (PBS with 1% BSA and 0.05% NaN2) for 30 minutes or with isotype control antibodies. Cells were then washed three times with wash buffer and incubated with secondary antibody (goat anti mouse IgG PE, DAKO) for 30 minutes. Cells were washed three times and suspended in 250 µl wash buffer with 10 µl Viaprobe (Pharmingen) for live/dead cell staining and only live cells were used for further analysis. ALP expression levels were analyzed on a FACS Caliber (Becton Dickinson Immuno cytometry systems). The percentage ALP positive cells were calculated compared to untreated cells and expressed as relative ALP expression compared to respective controls. The data was analyzed using Student's t test ($P<0.05$).

Mineralization and calcium deposition

For mineralization, hMSCs were seeded in MM at 5000 cells/cm2 in T25 culture flasks and cultured for 30 days with cells cultured in BM as negative control (n=4). The total calcium deposition was assayed using a calcium assay kit (Sigma diagnostics; 587A) according to manufacturer's protocol. Briefly, the culture medium was aspirated, washed twice with calcium and magnesium free PBS (Life Technologies) and incubated overnight with 0.5 N HCl on an orbital shaker at room temperature. The supernatant was collected for direct measurement or stored at –20°C. The calcium content was measured at 620 nm (BioTek Instruments) and expressed as µ g calcium/ flask (n=3). The data was analyzed using Student's t test at $P<0.05$. To visualize the mineralized area one flask from each group was stained with von Kossa staining.

Adipogenic assay

To study the adipogenic differentiation ability of expanded hMSCs, cells from each passages were seeded in adipogenic medium (Dulbecco's minimal essential medium [DMEM; Life Technologies], 10% FBS, 0.5 mM isobutylmethylxanthine [Sigma], 1 mM dexamethasone, 10 mM insulin [Sigma], 200 mM indomethacin [Sigma]) at 5000 cells/cm^2 in triplicate and grown for 21 days. Medium was refreshed twice a week and lipid formation was visualized with Oil red O (Sigma) staining. Briefly, the

cells were fixed overnight in formol (3.7% formalin plus $CaCl_2$ $2H_2O$ [1 g/100 ml]), rinsed with water, incubated for 5 min. in 60% isopropanol, and stained for 5 min. in freshly filtered Oil red O solution (stock: 500 mg of Oil red O [Sigma], 99 ml of isopropanol, 1 ml of water; stain: 42 ml of stock plus 28 ml of water). At least three images were taken at different locations of the flasks at same magnification and the numbers of adipocytes were counted in those three images and statistically analyzed using Student *t* test ($P<0.05$).

Gene Expression analysis by qPCR

The effect of dexamethasone on expression of osteogenic marker genes was analyzed by seeding hMSCs at 5000 cells/cm^2 in T75 flasks in BM and OM for 1, 2, 4, 10 and 21 days. Total RNA was isolated using an Rneasy mini kit (Qiagen) and on column DNase treated with 10 U RNase free DNase I (Gibco) at 37°C for 30 minutes. DNAse was inactivated at 72°C for 15 minutes. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. Two µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100x diluted cDNA was used for collagen type 1 (*COL1*) and 18s rRNA amplification and 1 µl of undiluted cDNA was used for other genes. PCR was performed on a Light Cycler real time PCR machine (Roche) using SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes are calculated relative to 18s rRNA levels by the comparative Δ CT method[43] and the statistical significance was found using Student's t test at $P<0.05$. The primers used in the study are listed in Table 1.

In vivo bone formation

To evaluate the donor variation on *in vivo* bone formation by hMSCs, we seeded 200,000 hMSCs in BM onto 2-3 mm biphasic calcium phosphate particles (BCP, 3 particles per condition) prepared and sintered at 1150°C as described previously[44]. The cells were cultured for a further 7 days on the BCP particles. Six immune-deficient male mice (Hsd-cpb:NMRI-nu, Harlan) were anaesthetized by intramuscular injection of 0.05 ml of anesthetics (1.75 ml ketamine 100 µ g/ml, 1.5 ml xylazine 20 mg/ml and 0.5 ml atropine 0.5 mg/ml). Four subcutaneous pockets were made and each pocket was implanted with 3 particles. The incisions were closed using a vicryl 5-0 suture. After 6 weeks the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck) in 0.14 M cacodylic acid (Fluka) buffer pH 7.3, dehydrated and embedded in methyl methacrylate (Sigma) for sectioning. Approximately 10 µm thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). The sections were stained with basic fuchsin and methelyine blue. Basic fuchsin stains newly formed bone pink and methylene blue stains the remaining fibrous tissue blue. The calcium phosphate ceramic material remains unstained and appears black in the sections. At least 5 sections were made from each sample and scanned using Minolta Dimage Scan. The bone formation is expressed as percentage bone areas considering the total available pore area for new bone growth as 100%.

Results

Donor variation in response to dexamethasone.

In vitro differentiation of hMSCs is characterized by change in the morphology of the cells and expression of the early osteogenic marker gene alkaline phosphatase (ALP). We investigated the donor variability in response to a well know osteogenic inducer, dexamethasone.

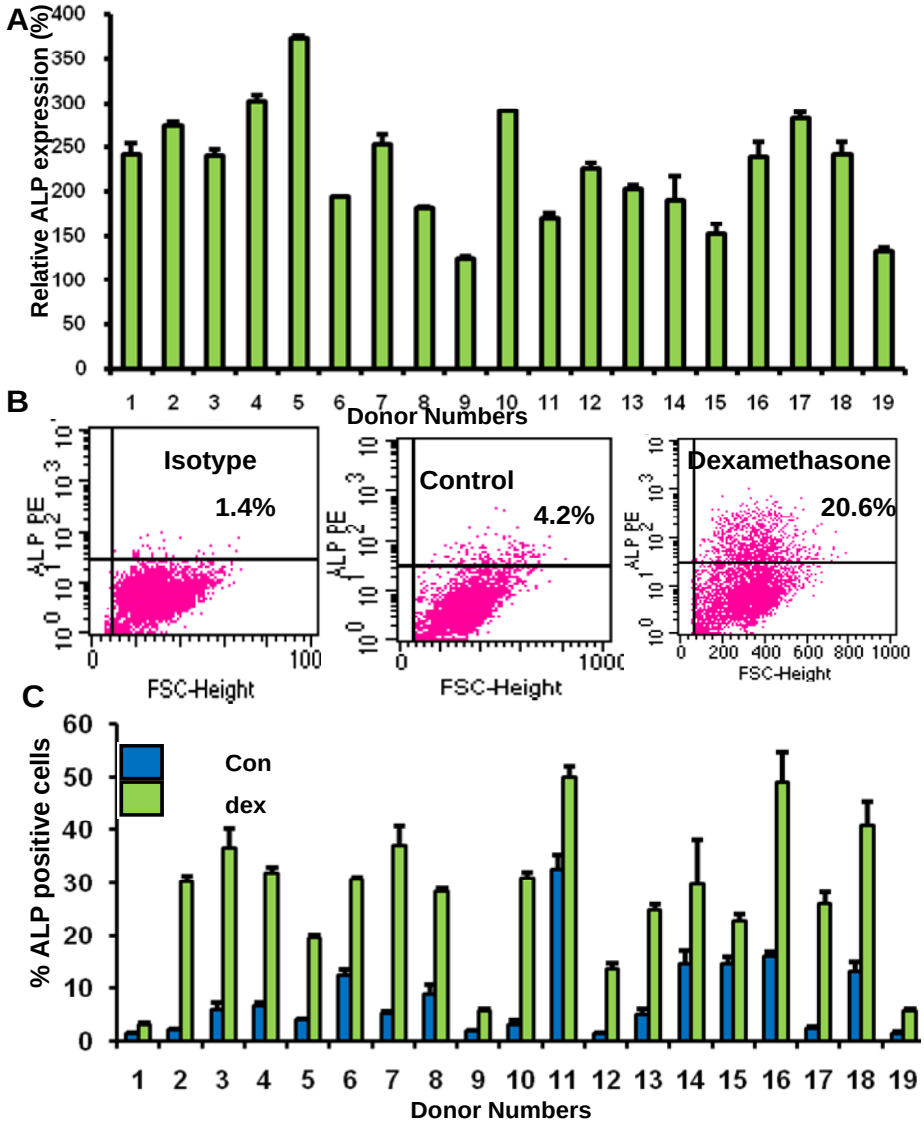

Figure 1. Donor variation in ALP expression. A. *Effect dexamethasone on ALP induction by hMSCs from 19 donors. ALP expression was analyzed by flowcytometry and expressed as percentage relative to untreated cells of the same donor.* ***B.*** *A representative dot plot used to calculate the ALP positive cell fraction in control and dexamethasone-treated groups.* ***C.*** *Percentage of ALP positive cells in untreated (Con) and dexamethasone-treated (dex) cell populations. Error bars represent the standard deviation.*

As shown in Figure 1A, the induction of ALP relative to untreated hMSCs of 19 donors varied between 1.3 and 3.8 fold indicating a vast variation in response to an osteogenic signal. Since we noticed a donor variation in ALP induction we calculated the percentage of ALP positive cells by gating for ALP positive cell fraction in control and dexamethasone-treated cells (Figure 1B). The percentage of ALP positive cells in the untreated group varied between 1 % and 33 % indicating varying amounts of ALP positive cells in the initial culture. Upon dexamethasone treatment, the percentage of ALP positive cells varied between 3 % and 50 % with an average of 27 % (Figure 1C). We continuously monitored ALP expression from D12 of P2 cells over a period of 15 days and we observed induction kinetics reaching peak expression between 5 and 10 days, dropping back to the basal level there after (Figure 2A). This phenomenon of decrease in dexamethasone-induced ALP expression after 7 days is consistently observed in our earlier studies. Further, no statistical correlation was observed when ALP index (ratio of ALP positive cells in dexamethasone-treated and control group) was plotted against age, gender or source of isolation (Figure 2 B-D).

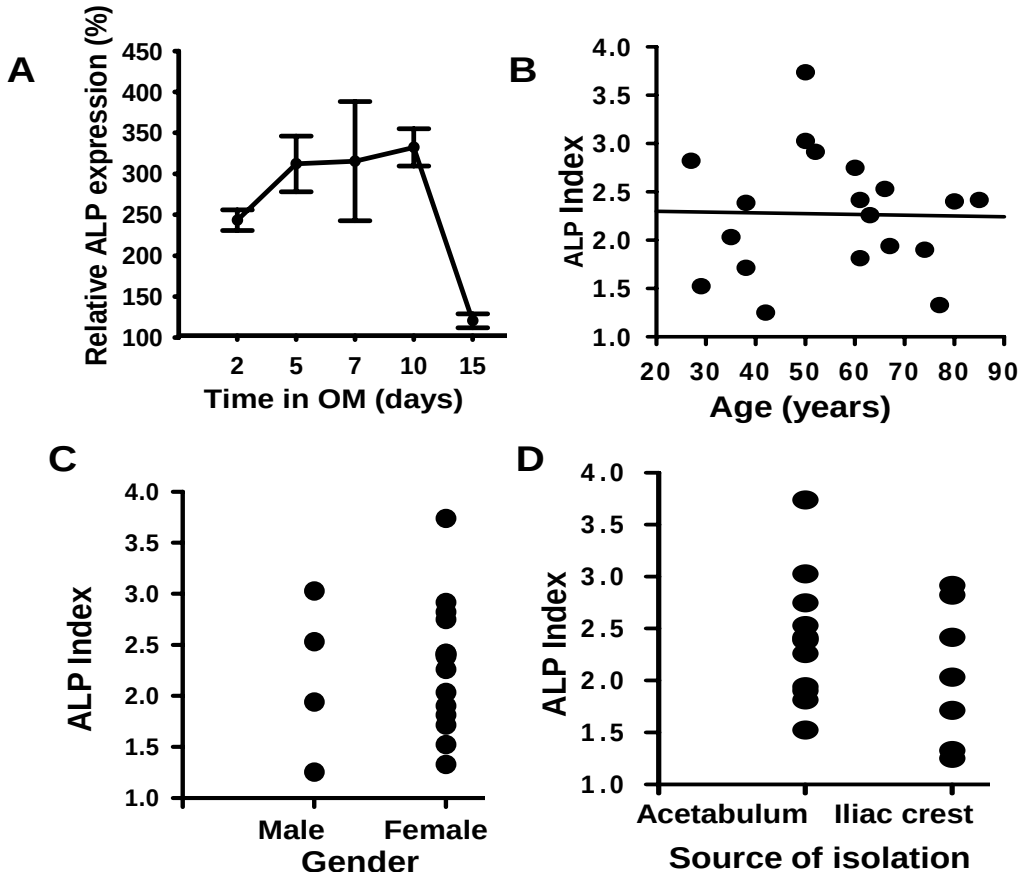

Figure 2. Correlation between ALP and age, gender or source of isolation. A. *ALP induction profile of P2 cells from D12 over a period of 15 days in osteogenic medium (OM). Error bars represent the standard deviation.* ***B-D.*** *Correlation of ALP index (ratio of ALP positive cells in dexamethasone-treated and control group) with donor age (r^2=0.0005), gender (r^2=0.0523) and source of isolation (r^2=0.00056).*

In addition, P2 cells from donor 16, 17 and 18 were tested for their ability to mineralize *in vitro*. As shown by von Kossa staining (Figure 3A), the cells from donors 16 and 18 showed *in vitro* mineralization; however calcium quantification showed no significant difference between the donors. But donor 17 did not show any calcium deposition (Figure 3B), again emphasizing the donor variability. To further understand this variability we compared the percentage of ALP positive cells in untreated and dexamethasone-treated cells from these donors to *in vitro* mineralization ability. The high number of ALP positive cells in D16 and D18 correlated to their *in vitro* mineralization potential. In contrast the absence of mineralization in D17 correlated o a low ALP expression level (Figure 3C).

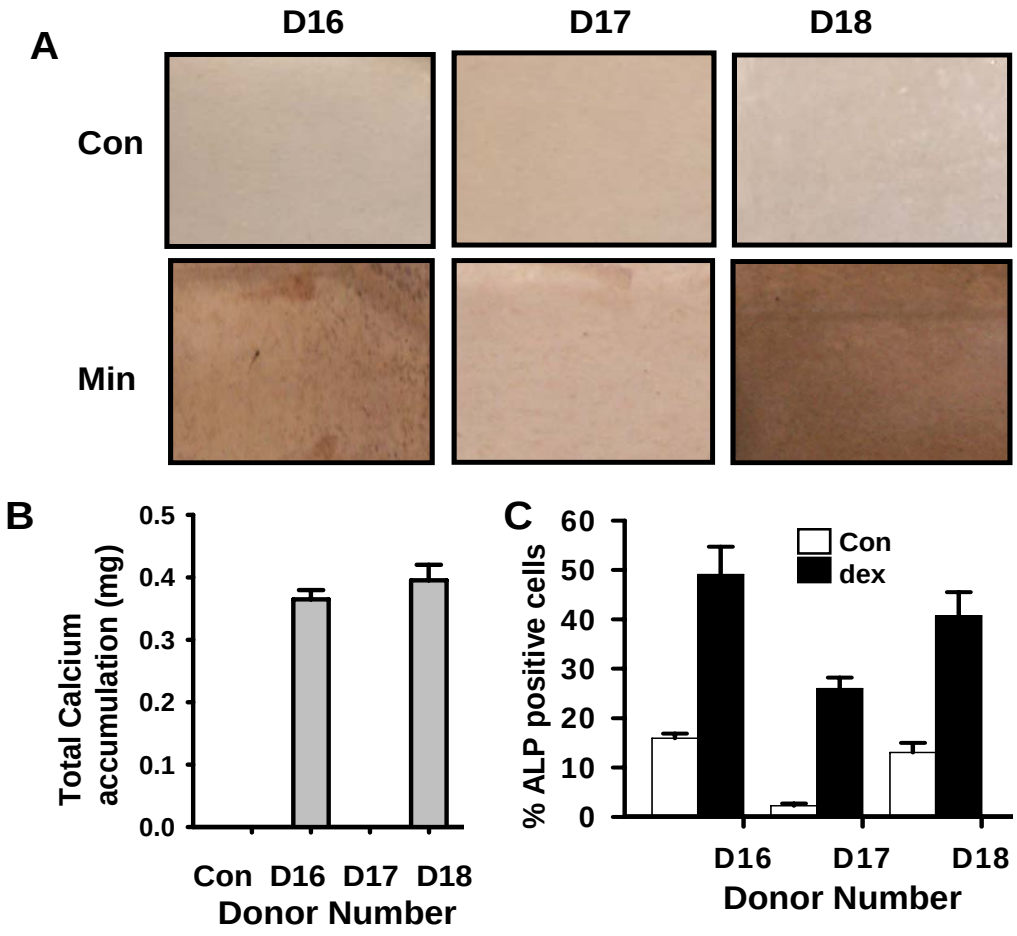

Figure ***3. Donor variation in in vitro mineralization potential.A.*** *von Kossa staining of hMSCs from D16, D17 and D18 grown in basic medium (con) and mineralization medium (min) for 30 days. Note the black staining in dex-treated cells of D16 and D18, indicating mineralization.* ***B.*** *Calcium accumulation by hMSCs from D16, 17 and 18 in MM after 30 days. No detectable calcium was measured in control-treated cells (Con)* ***C.*** *Percentage ALP positive cells in untreated (white bars) and dexamethasone treated (Black bars) cells from D16, 17 and 18. Error bars represents standard deviation (P<0.05).*

Expression profile of osteogenic markers

We further investigated the donor heterogeneity by studying the expression profile of other osteogenic markers such as collagen type 1 (*Col-1*), non collagenous proteins of the extra cellular matrix such as osteopontin (*OP*), osteocalcin (*OC*) and osteonectin (*ON*) from P2 cells of D12, D11, D16 and D18 during a time course of osteogenic differentiation. Among the genes studied, *ALP, COL-1* and *ON* increased progressively during osteogenic differentiation and declined at later stages (Figure 4A).

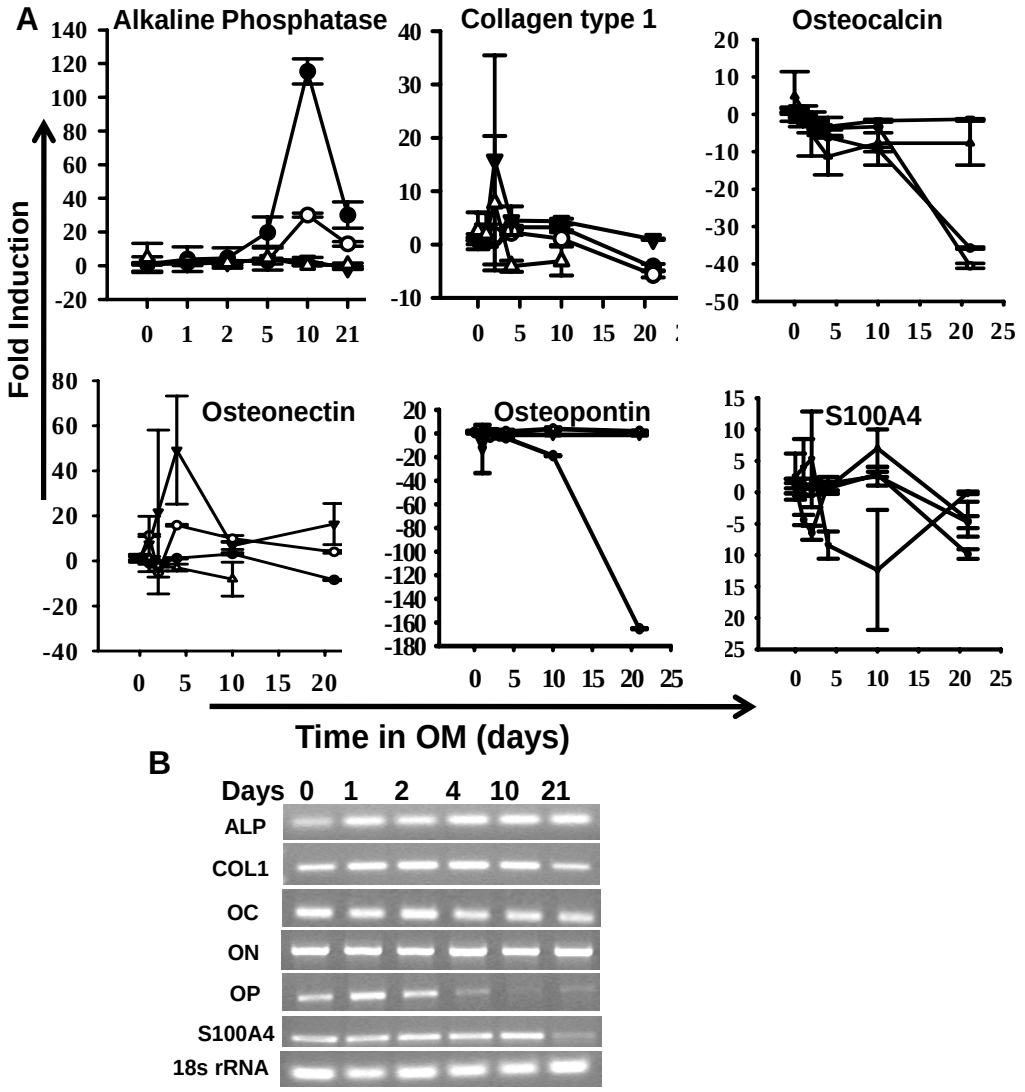

Figure 4. Osteogenic gene expression in dexamethasone-induced hMSCs.A. Gene expression profiles osteogenic markers in Osteogenic Medium (OM). *Expression is indicated as fold induction compared to cells grown in BM and normalized to 18s rRNA. ALP, alkaline phosphatase; col1, collagen type 1; OP, Osteopontin; ON, Osteonectin; OC, Osteocalcin; S100A4, calcium binding protein S100A4. Error bars represent standard deviation.* ***B.*** *Agarose gel of the qPCR samples from a donor in Figure 4A.*

OC expression was stable in the beginning of the culture period and decreased later. Expression of calcium binding protein S100A4, a negative regulator of mineralization persisted in early phase of osteogenic differentiation and declined before the onset of mineralization.

In vitro senescence of hMSCs

Cell-based bone tissue engineering needs enormous amounts of multipotent cells for successful clinical application which requires *in vitro* expansion of the isolated cells. hMSCs isolated from human trabecular bone show a typical phenomenon of cellular senescence including morphological change, decreased proliferation and declined ALP and Col-1 expression[22]. To document how far the isolated hMSCs can be *in vitro* proliferated while retaining their differentiation abilities, we serially passaged hMSCs from D11 up to 9 passages. First we observed a morphological change from thin and spindle-shaped in early passage cells to large, flattened and irregularly shaped in late passage cells (Figure 5A).

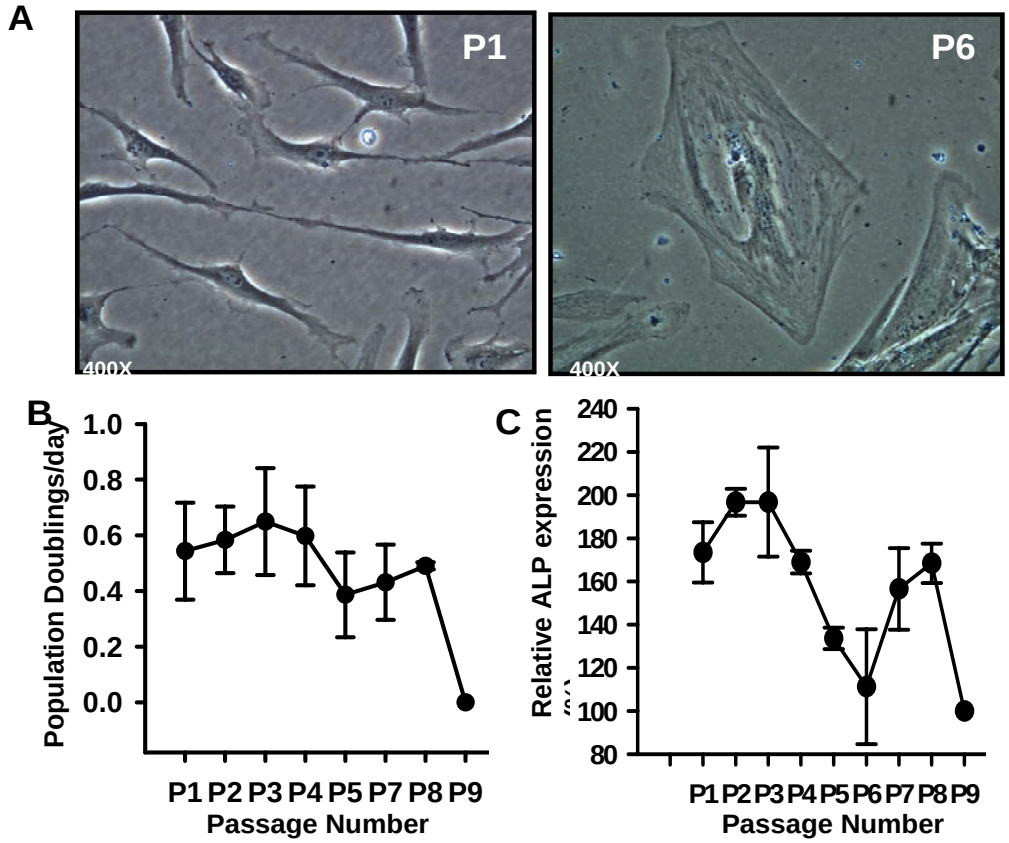

Figure 5. In vitro senescence of hMSCs. A. *Images showing a change in morphology from thin and spindle shaped in passage 1 to large and flattened in passage 6.* ***B.*** *Growth kinetics of serially passaged hMSCs expressed as population doubling per day.* ***C.*** *Relative ALP expression in serially passaged hMSCs compared to the controls of the same passage cells. Error bars represent the standard deviation.*

Growth kinetic analysis showed a rapid increase in proliferation in P2 and P3, followed by a phase of slow growth (Figure 5B). To our surprise, some P6 cells regained proliferative potential and formed independent colonies. We could culture those cells for another 3 passages and then the cells absolutely stopped dividing and died in P9. To confirm this we serially passaged cells from two other independent donors and observed the same phenomenon (data not shown). We are further investigating this unique pattern of hMSC growth in detail by serially passaging hMSCs from a number donors and studying gene expression profile of serially passaged cells by microarray in parallel with their ability to form bone *in vivo*. We also studied the differentiation potential of serially passaged hMSCs by inducing them into osteogenic and adipogenic lineages. ALP expression analyses showed that dexamethasone induced ALP expression in early passages but failed to do so in P6. However, the recovered colonies from P6 showed a slight ALP induction in P7 and P8 (Figure 5C).

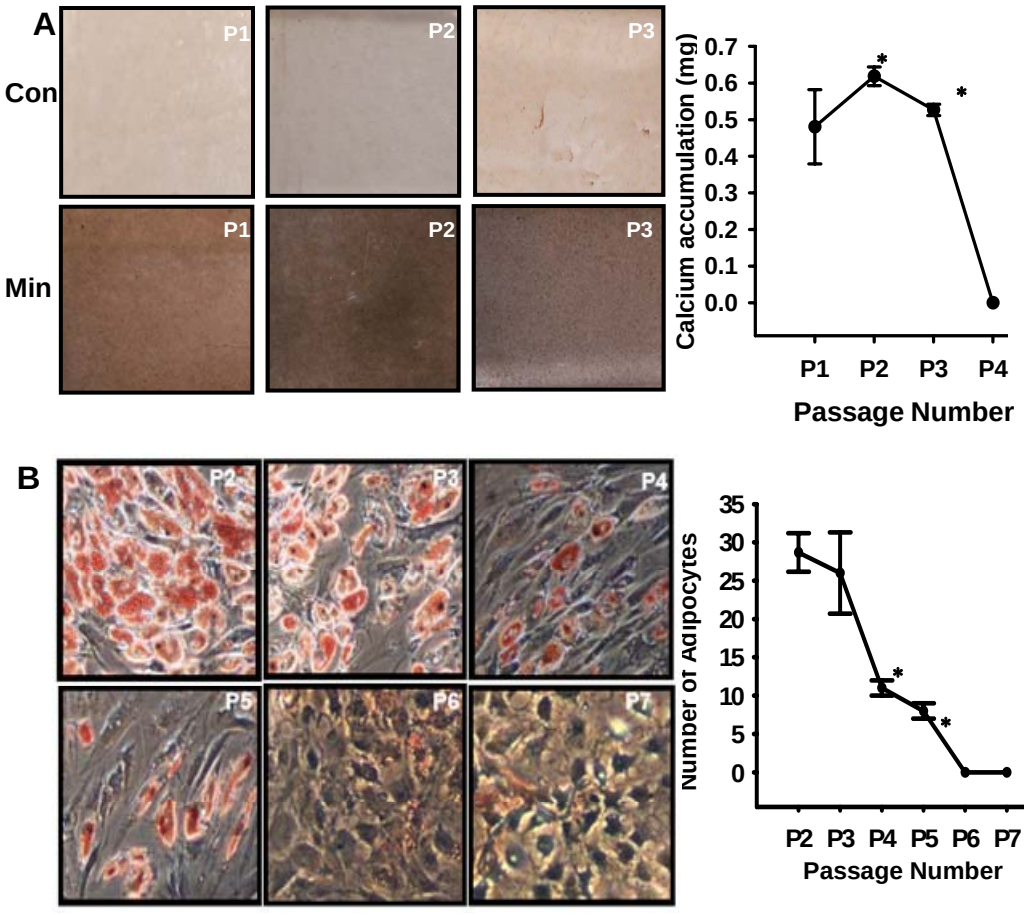

Figure 6. Osteogenic and adipogenic potential of serially passaged hMSCs. A. *von Kossa staining (left upper panel) and quantification of calcium deposition (right upper panel) in serially passaged hMSCs.* ***B.*** *Adipogenic differentiation was visualized by staining with Oil red O (left lower panel) and the number of adipocytes were quantified. (left lower panel). Error bars represent the standard deviation.*

In P9 the hMSCs completely lost responsiveness to dexamethasone. No further analyses could be performed thereafter due to cessation of cell growth and cell death. *In vitro* mineralization studies showed that the cells were able to mineralize *in vitro* up to P3 and no mineralization was observed in P4 and later passages. The calcium deposition by P2 cells was significantly higher than P1 cells. This could be possibly due to the fact that we used cryo-preserved cells of P0 and the cells may have had a lag phase of growth. Further, we observed a significant decrease in the calcium accumulation in P3 and cells from P4 and later passages failed to mineralize as determined by their inability to accumulate calcium (Figure 6A). In contrast, the cells were able to differentiate into adipogenic lineage up to P5. Although no significant differences were observed between P2 and P3 cells, there was a significant drop in adipogenic differentiation ability of P4, P5 cells compared P2 and P3 (Figure 6B).

In vivo bone formation

We further tested whether the large variation between individual donors in ALP expression and *in vitro* mineralization would be reflected on *in vivo* bone formation by hMSCs. We implanted hMSCs from D16, 17, 18 and 19 in immune-deficient mice for 6 weeks. The samples were explanted and stained as explained in materials. We observed bone formation by cells from donor 16, 17 and 18 but no bone was formed by donor 19 (Figure 7).

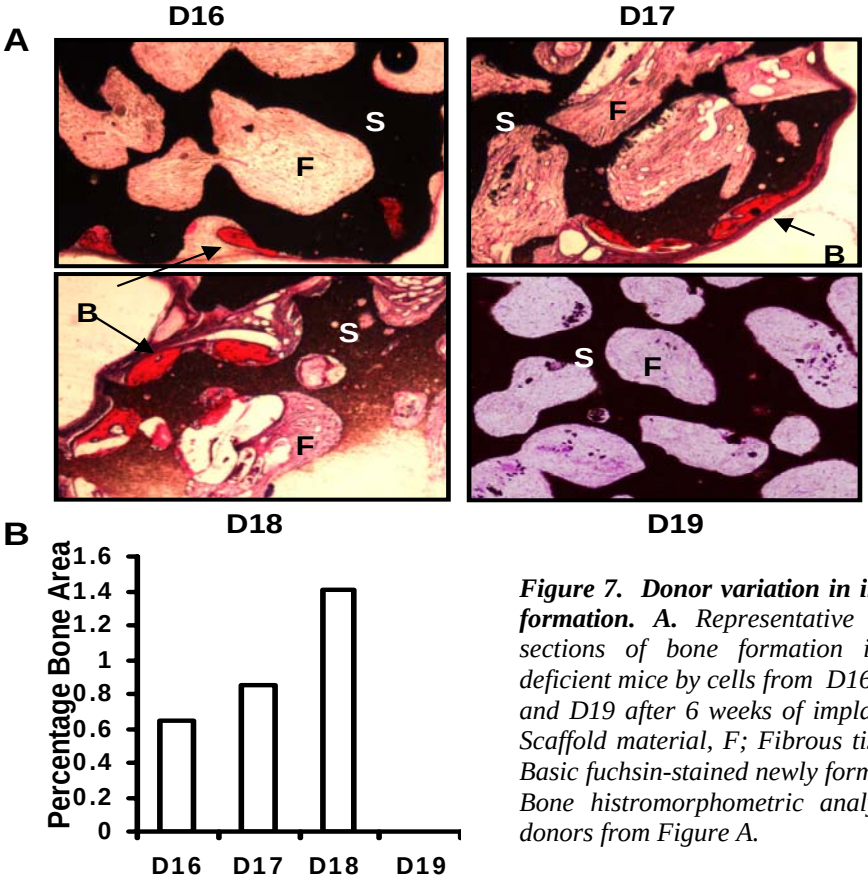

Figure 7. Donor variation in in vivo bone formation. A. *Representative histological sections of bone formation in immune-deficient mice by cells from D16, D17, D18 and D19 after 6 weeks of implantation. S; Scaffold material, F; Fibrous tissue and B; Basic fuchsin-stained newly formed bone.* ***B.*** *Bone histromorphometric analyses of the donors from Figure A.*

The comparative analyses of ALP expression by these donors with *in vivo* bone formation suggest a correlation between *in vitro* ALP expression and *in vivo* bone formation. Although D17 did not mineralize *in vitro* and had lower percentage of ALP positive cells in control and dexamethasone-induced groups, we observed more bone formation compared to D16.

Discussion

Easy isolation, rapid *in vitro* expansion and multipotency of hMSCs attributes to the attractiveness as a candidate for regenerative medicine and tissue engineering. Some clinical trials have shown that hMSCs loaded onto different carrier materials produced clinically relevant amounts of bone[45]. Successful bone tissue engineering using hMSCs mainly depends on the quality of the cells, ability to proliferate, differentiate *in vitro* and to form bone *in vivo*.

In this report, we isolated and characterized hMSCs from 19 independent donors. First we analyzed ALP expression by all the donors because it is known to be a pre-osteogenic marker up regulated during osteoblast differentiation and represents the percentage of committed osteoprogenitor cells[46]. The cells from different donors exhibited a vast difference in ALP induction by dexamethasone. The percentage ALP positive cells in untreated and dexamethasone-treated cells showed marked differences among the 19 donors tested. The presence of higher percentage of ALP positive cells in the initial culture did not always resulted in high ALP induction. This suggests that, the fold induction in ALP expression by dexamethasone is independent of initial number of ALP positive cells which may have influenced by various unknown factors. ALP analyses further suggest no statistical correlation with age, gender or source of isolation although hMSCs from 10 donors out of 19 belong to age group over 60 year and the sample size in male and female group are inadequate which limits on the evident conclusions. Further, we deliberately selected donors of age over 40 years and above since they represent major targets for bone tissue engineering. This could be possibly due to the considerable variation in the composition of the initial aspirate and population of committed progenitors which will effect ALP expression *in vitro*. Another possible explanation is that differential sampling methods by different physicians may result in varying heterogeneity in the final cell composition as reported previously[41, 47]. However, we observed no statistical correlation between ALP index and different physicians who harvested bone marrow (data not shown). Our data suggest that the variability in ALP expression reflects on the functional difference in osteogenic differentiation of hMSCs. The hMSCs with higher percentage of basal and dexamethasone-induced ALP positive cells showed higher *in vitro* mineralization and calcium deposition (Figure 2). However this explanation may not rule out the differences in the physiological status and clinical history of the patients which would account for *in vitro* variations. Collectively our data demonstrate that firstly, irrespective of age, gender and source of isolation of hMSCs, cells from all donors responded to an osteogenic signal by dexamethasone. Secondly, hMSCs isolated from donors by different sampling methods might cause the variation in the heterogeneity of the cell population which would affect *in vitro* cell behavior.

Gene expression analyses of osteogenic markers in hMSCs further demonstrated variation in gene expression levels but the expression profiles of *ALP, COL-1*, *OC* and *S100A4* followed the same trend. For instance, ALP showed peak expression on day 10 but the fold induction varied between 3-fold and 115-fold indicating in the ability of hMSCs from various donors to respond to an osteogenic signal. This variation in hMSC gene expression is consistent with the studies from other group[48]. Efforts have been made to minimize the heterogeneity of hMSCs using monoclonal antibodies to unique cell surface antigens such as SH2, cluster designation (CD)-antibodies specific for hMSCs, PCR with known cell surface, extra cellular matrix and soluble proteins[49-51]. However, the individual clones isolated using these markers still exhibited a differential capacity to form new bone *in vivo*[52]**.**

We investigated whether ALP expression could be used as an indicator of bone-forming capacity. Seeding hMSCs onto porous BCP particles and subcutaneous implantation in immune deficient mice for 6 weeks showed bone formation by 3 donors out of 4. D19, the donor which yielded no bone, had the lowest relative ALP expression of all four donors investigated. Interestingly, D18 had the highest percentage of ALP positive cells, and from this donor we observed the most robust bone formation *in vivo*. This is in line with previous work from our lab demonstrating a correlation between the fold-upregulation of ALP by dexamethasone treatment *in vitro* and bone formation *in vivo*[53]. These results further substantiate the idea that the bone forming capacity of an hMSC culture can be predicted by analyzing its gene expression profile. Because the resolution of ALP analysis is not sufficient to determine the bone-forming capacity of an MSC culture in advance, our current research is focused on identifying new diagnostic markers which predict the bone forming capacity of hMSCs. We approach this by isolating hMSCs from over 80 donors, analyzing their gene expression profiles and in parallel assessing their *in vitro* differentiation abilities and bone forming capacity *in vivo*.

Proliferation of hMSCs is accompanied by loss of multipotency and cellular senescence. This limits the extent to which cells can be expanded. Consistent with previous studies, we observed a typical change in morphology from thin spindle shaped fibroblastic to large, flattened and irregularly shaped during *in vitro* expansion of hMSCs. Furthermore, we show that the serially passaged hMSCs exhibited progressive loss of replicative potential and multipotency. Interestingly, we noticed that some cells in P5 regained growth and formed independent colonies, which was also observed in two other donors. To our knowledge, this is the first observation of this phenomenon in hMSCs. We are currently investigating whether the recovery of proliferative capacity of hMSCs in P6 resembles the escape from senescence during immortalization. We acknowledge the fact that in true immortalization, the cells divide indefinitely, whereas in hMSCs, cells loose proliferative potential after a few extra cell division cycles. We observed peak growth, *ALP* expression, calcium accumulation and adipogenic differentiation in P2 and P3 cells and this could be due to the fact that we used frozen cells of P0 which showed lag phase in growth and differentiation[54]. From our studies we conclude that hMSCs can be expanded *in vitro* up to P3

(which approximates to 10 -12 population doublings starting from a P0 culture) while retaining multipotency for effective use in tissue engineering applications.

Despite the fact that hMSCs reproducibly form bone when implanted in animal models, the bone typically does not bridge the whole implant[55, 56]. To optimize bone tissue engineering, various labs invest in optimizing the proliferation[57] and differentiation of hMSCs *in vitro* [12, 38, 39, 58]. By better understanding of molecular pathways such as MAPK pathway[59], Rho kinase[60], Wnt [39], Notch [61] and receptor tyrosine kinases[12] we could improve bone tissue engineering. We recently discovered that PKA activation in hMSCs during in vitro expansion substantially enhances *in vivo* bone formation (manuscript in preparation). In conclusion, use of hMSCs for cell and tissue engineering applications depends on the ability to *in vitro* expand while retaining their multipotency. Large variation among the donors in composition of cells (progenitor to committed osteoblast), growth and response to osteogenic signals may limit these applications. Careful analyses of multiple samples from the same donor and use of genetic knowledge to enhance *in vitro* and *in vivo* osteogenic differentiation may improve tissue engineering applications.

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Chapter 4

Effect of GPCR ligands on osteogenic differentiation of human mesenchymal stem cells

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In Preperation

Abstract

Previously, we have demonstrated that prolonged PKA activation using cAMP in human mesenchymal stem cells (hMSCs) induces *in vitro* osteogenesis and *in vivo* bone formation. To further investigate the physiological role of PKA in hMSC osteogenesis, we tested a number of G-Protein coupled receptor ligands which signal via intracellular cAMP production. Parathyroid hormone, parathyroid hormone-related peptide, melatonin, epinephrine, calcitonin, calcitonin gene related peptide and prostaglandin E2 failed to induce ALP expression. On the contrary, PGE2 was the only GPCR ligand which inhibited dexamathasone-induced ALP expression and mineralization, which co-incided with the fact that PGE2 was the only ligand able to induce cAMP accumulation in hMSCs, suggesting that physiological levels of cAMP inhibit rather than stimulate osteogenesis. As reported in the literature, we found an additive effect of dexamethasone on PGE2-mediated cAMP accumulation, but surprisingly also on forskolin and cholera toxin-mediated cAMP accumulation. Further studies demonstrated that intermittent exposure of hMSCs to cAMP inhibited dex-induced ALP expression. Taken together, our results demonstrate that cAMP can either stimulate or inhibit osteogenesis in hMSCs, depending on the duration rather than the strength of the signal provided. Our studies further validate our current effort to search for compounds which induce high intracellular cAMP levels to induce osteogenic differentiation of hMSCs for bone tissue engineering applications.

Key words: GPCR signaling, osteogenesis , human mesenchymal stem cells and Protein Kinase A signaling

Introduction

Human mesenchymal stem cells (hMSCs) can differentiate into adipogenic, chondrogenic, osteogenic and myogenic lineages and due to their extensive proliferation abilities they are a potential cell source for clinical use in regenerative medicine and tissue engineering [1, 2]. Besides, hMSCs are increasingly accepted as cell biological model to investigate molecular mechanisms governing signal transduction, differentiation, cell fate decision, senescence and plasticity, which brings the basic research close to clinical level[3-9]. hMSCs are isolated from various sources including tibia, adipose tissue, femur, lumbar spine and trabecular bone, iliac crest and acetabulum[10-12]. The MSCs isolated from these sources are proven to be multipotent *in vitro* and form bone *in vivo* when they are seeded onto various carrier materials and implanted *in vivo*[13-15]. We have recently conducted a phase I clinical trial to treat patients with jaw defects[16]. We successfully demonstrated that bone tissue was derived from *in vitro* cultured hMSCs, but using the current protocol the newly formed bone did not fully bridge the implant. To improve the performance of hMSCs we aim at pre-differentiating hMSC *in vitro* into the osteogenic lineage to augment *in vivo* performance of the cells[17-19]. To further improve the biological activity of hMSCs, we focus on the molecular cues that stimulate *in vitro* proliferation and differentiation. We recently reported that

stimulation of the Wnt signaling pathway and inhibition of histone deacetylase activity can be used as a tool to enhance proliferation and differentiation of hMSCs[20-22]. In addition, we recently discovered that activation of the protein kinase A pathway substantially enhances early osteogenesis *in vitro* with concomitant stimulation of bone formation *in vivo*[23].

Recent studies demonstrate that various signaling pathways such as the MAPK pathway, Rho kinase, Wnt, Notch ,receptor tyrosine kinase and G Protein Coupled Receptor (GPCR) signaling are implicated in regulating osteogenic differentiation of hMSCs[3, 5, 20, 24]. Several different strategies can be taken to steer the osteogenic process, such as the application of small molecules or proteins, through genetic interference and through design of smart scaffolds with bone inducing properties.[25]. In line with this, identification of bone morphogenetic proteins (BMPs), various growth factors and hormones such as parathyroid hormone (PTH), melatonin, calcitonin, epinephrine and prostaglandins have advanced *in vitro* differentiation protocols. In addition, the human genome has over 500 druggable GPCR family genes[26] and most of the earlier mentioned growth factors mediate their signaling via specific GPCRs[27]. In view of the vast involvement of GPCR signaling in cellular processes and to further enhance the osteogenic process, we investigated the effect of a number of GPCR ligands on osteogenic differentiation of hMSCs.

GPCRs are trans-membrane proteins, which transduce extracellular signals generated by their respective ligands into the cell by coupling to G-proteins and activation of intracellular signaling cascades such as protein kinase A (PKA) and protein kinase C (PKC) signaling (see reference [28] and therein). A proto-typical GPCR ligand is PTH, which signals through its specific receptor PTHR1, by activating PKA and PKC signaling pathways. The role of PTH has become evident from PTH-deficient mice which showed diminished cartilage matrix mineralization, reduced metaphyseal osteoblasts and trabecular bone [29]. PTHrP-deficient mice are not viable due to impaired bone formation [30]. PTH has gained remarkable attention due to its ability to stimulate bone formation *in vivo* when administered intermittently. In contrast, continuous adminstration resulted in bone resorption[31, 32]. PTH induces osteogenesis via transcription factors such as cyclic AMP response element binding protein (CREB)[33], AP-1 family members including c-jun, fosB, jun-B, fra-1 and fra-2 [34-36]. Activation of the PTH receptor in MG-63 cell-line induces osteoblast differentiation by stimulating collagen type I synthesis and ALP expression [37]. A recent report demonstrates that intermittent exposure of hMSCs to PTH suppressed adipogenic differentiation and increased ALP expression[38]. Another GPCR ligand, melatonin, is known to induce osteogenesis in MC3T3-E1 and ROS 17/2.8 cells, evidenced by enhanced expression of osteogenic markers such as BSP, ALP, osteocalcin and collagen type 1[39]. Other reports demonstrate that hMSCs express the melatonin receptor and melatonin induces osteogenic differentiation in combination with dexamethasone through the MT2 receptor[40]. Recently, it was described that hMSCs secrete prostaglandin E2 (PGE2) which mediates BMP-2 expression via the EP4 receptor[41]. Furthermore, human osteoblast cell lines and hMSCs express receptors for calcitonin and calcitonin gene related peptide (CGRP) which regulate osteogenic differentiation and

bone formation[42-46]. Together, these studies led us to validate GPCR ligands to induce osteogenesis of hMSCs and to augment present bone tissue engineering protocols.

Materials and Methods

Isolation and culture of hMSCs

Bone marrow aspirates (5-20 ml) were obtained from donors with written informed consent. hMSCs were isolated and proliferated as described previously[47]. Briefly, aspirates were re-suspended using 20 G needles, plated at a density of $5x10^5$ cells/cm^2 and cultured in hMSC proliferation medium (PM) containing a-minimal essential medium (a-MEM, Life Technologies), 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), 10 mg/ml streptomycin (Life Technologies) and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week and cells were used for further sub-culturing or cryopreservation upon reaching near confluence. hMSC basic medium was composed of hMSC proliferation medium without bFGF, hMSC osteogenic medium was composed of hMSC basic medium supplemented with 10^{-8} M dexamethasone (Sigma) and hMSC mineralization medium was composed of basic medium supplemented with 10^{-8} M dexamethasone and 0.01 M b-glycerophosphate (Sigma).

ALP analysis by flow cytometry

hMSCs were seeded at 5000 cells/cm^2 and allowed to attach for 10 to 15 hours in basic medium before addition of any compounds. Each experiment was performed in triplicate with a negative control (cells grown in basic medium) and a positive control (cells grown in osteogenic medium) and one or more experimental conditions. At the end of culture period, the cells were trypsinized and incubated for 30 minutes in block buffer (PBS with 5% bovine serum albumin, BSA [Sigma] and 0.01% NaN2), then incubated with primary antibody (anti-ALP, B4-78 [Developmental Studies Hybridoma Bank, University of Iowa, USA]) diluted in wash buffer (PBS with 1% BSA and 0.05% NaN2) for 1 hour or with isotype control antibodies. Cells were then washed three times with wash buffer and incubated with secondary antibody (rat anti mouse IgG PE, DAKO) for 30 minutes. Cells were washed three times and suspended in 250 µl wash buffer with 10 µl Viaprobe (Pharmingen) for live/dead cell staining and only living cells were used for further analysis. ALP expression levels were analyzed on a FACS Caliber (Becton Dickinson Immuno cytometry systems). The percentage ALP positive cells were calculated compared to untreated cells and expressed as relative ALP expression compared to respective controls. The data was analyzed using Student's t test ($P<0.05$).

Mineralization and calcium deposition

For mineralization, hMSCs were seeded in mineralization medium at 5000 cells/cm^2 in T25 culture flasks and cultured for 30 days with cells cultured in basic medium supplemented with 0.01 M b-glycerophosphate as negative control. The total calcium deposition was assayed using a calcium assay kit (587A, Sigma diagnostics) according to manufacturer's protocol. Briefly, the culture medium was aspirated, washed twice with calcium and magnesium free PBS (Life Technologies) and incubated overnight with 0.5 M HCl on an orbital shaker at room temperature. The supernatant was collected for direct measurement or stored at –20°C. The calcium content was measured at 620 nm (BioTek Instruments) and expressed as mg calcium/flask. The data was analyzed using Student's t test at $P<0.05$.

Microarray analysis

To study the regulation of GPCRs during differentiation, hMSCs were differentiated into the osteogenic lineage using 10^{-8} M dexamethasone for 7 days. RNA was isolated using an RNeasy midi kit (Qiagen) and 8 µg of total RNA was used for probe labeling according to the manufacturer's protocol (Affymetrix). The probe quality was verified using lab-on-chip technology (Agilent Technologies) and samples were hybridized to Human Genome Focus arrays according to the manufacturer's protocol (Affymetrix). Data analysis was performed using Affymetrix GENECHIP 4.0 software. The gene ontology enrichment was performed on the Panther Classification System (http://www.pantherdb.org/tools/genexAnalysis.jsp).

Intracellular cAMP measurements

hMSC were seeded at 5000 cells/cm^2 hMSCs in proliferation medium or osteogenic medium. After a 7 days culture period, hMSCs were washed with PBS and 50 µl α-MEM was added, followed by 50 µl of α-MEM containing 0.02 % BSA and 20 µM Rolipram (Sigma). Cells were next incubated for 2 hours at 37°C, and then lysed using 100 µl of 1 % triton X-100 in PBS on a shaking platform for 30 minutes. Intracellular cAMP was quantified using an Amersham cAMP Biotrack EIA kit. PTH (1-34) and PTHrP was purchased from Calbiochem. Cholera toxin, forskolin, melatonin, PGE2, calcitonin, calcitonin gene related peptide, epinephrine and N6, 2'-O-dibutyryl-cAMP (cAMP) were purchased from Sigma.

Real-time quantitative PCR

The effect of PTH on osteogenic differentiation of the MG-63 osteosarcoma cell line was studied by seeding MG-63 cells at 5000 cells/cm^2 in T25 flasks in basic medium and basic medium supplemented with 10^{-7} M PTH for four days. Total RNA was isolated using an RNeasy mini kit (Qiagen) and on-column treated with 10 U RNase free DNase I (Gibco) at 37°C for 30 minutes. DNAse was inactivated at 72°C for 15 minutes. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. Two µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100x diluted cDNA was used for *collagen type 1 (COL1)* and *18s rRNA* amplification and 1 µl of undiluted cDNA was used for other genes. PCR was performed on a Light Cycler real time PCR machine (Roche) using SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3, using fit point method

by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Gene expression was calculated relative to 18s rRNA levels by the comparative DCT method[48] and the statistical significance was found using Student's t test at $P<0.05$. The primers used for qPCR are listed in Table 1.

Table 1. Primers used for qPCR studies.

Gene		Primer sequence	Product length(bp)
18s rRNA	F	5'cggctaccacatccaaggaa3'	187
	R	5'gctggaattaccgcggct3'	
ALP	F	5'gacccttgacccccacaat3'	67
	R	5'gctcgtactgcatgtcccct3'	
COL1	F	5'agggccaagacgaagacatc3'	137
	R	5'agatcacgtcatcgcacaaca3'	
OC	F	5'ggcagcgaggtagtgaagag3'	138
	R	5'gatgtggtcagccaactcgt3'	
ID1	F	5'gcaagacagcgagcggtgcg3'	346
	R	5'ggcgctgatctcgccgttgag3'	
ID2	F	5'cctcccggtctcgccttcc3'	320
	R	5'ggttctgcccgggtctctgg3'	
SMAD6	F	5'gctaccaactccctcatcact3'	336
	R	5'cgtcggggagttgacgaagat3'	

Results

hMSCs express receptors for various GPCR ligands

Studies have demonstrated that hMSCs express specific GPCRs for various ligands and the expression of some of them is altered upon exposure to dexamethasone[27]. To further understand the global gene expression profile of GPCR related genes in hMSCs, we isolated RNA from hMSCs treated with or without dexamethasone and analyzed gene expression using micro-array technology. Gene Ontology (GO) enrichment for the genes which were regulated over 3-fold in dexamethasone group shows that over 80 genes belong to the GPCR group (Figure 1). For example, Table 2 shows that dexamethasone induces the expression of receptor for prostaglandin E, melatonin, calcintonin, adenylate cyclase activating receptor and many other orphan GPCRs such as *GPR 173, -19* and *-42*. In contrast, *tachykinin receptor, cannabinoid receptor* and other orphan GPCRs such as *GPR 132, -135* and *-44* were down regulated over 3 fold. Dexamethasone-induced regulation of receptors for the ligands used in the study are listed Table 3.

Table 2. GPCR related genes regulated > 3 folds in dexamethasone treated hMSCs

NCBI No.	Gene name	Fold Induction
NM_001059	tachykinin receptor 3;TACR3	-5.49
NM_004122	growth hormone secretagogue receptor;GHSR	-4.93
NM_002548	olfactory receptor, family 1, subfamily D, member 2;OR1D2	-4.66
NM_001716	Burkitt lymphoma receptor 1;BLR1	-3.97
U20760	calcium-sensing receptor	-3.89
NM_013345	G protein-coupled receptor 132;GPR132	-3.82
NM_003555	olfactory receptor, family 1, subfamily G, member 1;OR1G1	-3.76
NM_001702	brain-specific angiogenesis inhibitor 1;BAI1	-3.65
NM_000710	bradykinin receptor B1;BDKRB1	-3.45
NM_030784	G protein-coupled receptor 63;GPR63	-3.40
NM_016083	cannabinoid receptor 1 (brain);CNR1	-3.36
NM_030760	endothelial differentiation, sphingolipid GPCR 8;EDG8	-3.20
NM_022571	G protein-coupled receptor 135;GPR135	-3.16
NM_004778	G protein-coupled receptor 44;GPR44	-3.02
NM_001742	calcitonin receptor;CALCR	2.90
NM_005958	melatonin receptor 1A;MTNR1A	2.99
NM_002377	MAS1 oncogene;MAS1	3.07
NM_019888	melanocortin 3 receptor;MC3R	3.18
NM_000164	gastric inhibitory polypeptide receptor;GIPR	3.18
NM_020633	vomeronasal 1 receptor 1;VN1R1	3.23
NM_030774	olfactory receptor, family 51, subfamily E, member 2	3.26
NM_001051	somatostatin receptor 3;SSTR3	3.43
NM_000956	prostaglandin E receptor 2 (subtype EP2), 53kDa;PTGER2	3.53
NM_004624	vasoactive intestinal peptide receptor 1;VIPR1	3.64
NM_001118	adenylate cyclase activating polypeptide 1 receptor type I	3.66
NM_018969	G-protein coupled receptor 173;GPR173	3.71
NM_020377	cysteinyl leukotriene receptor 2;CYSLTR2	4.13
NM_006143	G protein-coupled receptor 19;GPR19	4.39
NM_006056	neuromedin U receptor 1;NMUR1	4.45
NM_000707	arginine vasopressin receptor 1B;AVPR1B	4.67
NM_005305	G protein-coupled receptor 42;GPR42	4.83
NM_003856	trace amine associated receptor 5;TAAR5	4.97
NM_005314	gastrin-releasing peptide receptor;GRPR	5.96
NM_012375	olfactory receptor, family 52, subfamily A, member 1	6.27
NM_012351	olfactory receptor, family 10, subfamily J, member 1	8.82
NM_001736	complement component 5 receptor 1 (C5a ligand);C5R1	12.87

Table 3. Dexamethasone induced specific GPCR regulation used in the study

NCBI No.	Gene description	Fold Induction
AU154853	calcitonin gene-related peptide-receptor	-3.8
NM_001742	calcitonin receptor	-3.89
NM_005795	calcitonin receptor-like	-1.58
AA747379	calcitonin-related polypeptide, beta	1.157
U20760	calcium-sensing receptor	1.16
J05594	hydroxyprostaglandin dehydrogenase 15-(NAD)	2.99
NM_005958	melatonin receptor 1A	5.54
NM_005959	melatonin receptor 1B	1.29
NM_000315	parathyroid hormone	1.47
NM_000316	parathyroid hormone receptor 1	1.03
NM_005048	parathyroid hormone receptor 2	1.64
AI762344	prostaglandin E receptor 1 (subtype EP1), 42kD	1.21
NM_000956	prostaglandin E receptor 2 (subtype EP2), 53kD	3.52
X83858	prostaglandin E receptor 3 (subtype EP3)	1.05
AA897516	prostaglandin E receptor 4 (subtype EP4)	1.07
AF010316	prostaglandin E synthase	-2.30
NM_000959	prostaglandin F receptor (FP)	-4.59
NM_000960	prostaglandin I2 (prostacyclin) receptor (IP)	3.36
NM_000961	prostaglandin I2 (prostacyclin) synthase	1.80
S36219	prostaglandin-endoperoxide synthase 1	2.89
NM_000963	prostaglandin-endoperoxide synthase 2	1.16
NM_005855	receptor (calcitonin) activity modifying protein 1	-2.65
NM_005854	receptor (calcitonin) activity modifying protein 2	1.14
NM_005856	receptor (calcitonin) activity modifying protein 3	1.7
NM_005630	prostaglandin transporter member 2	1.3

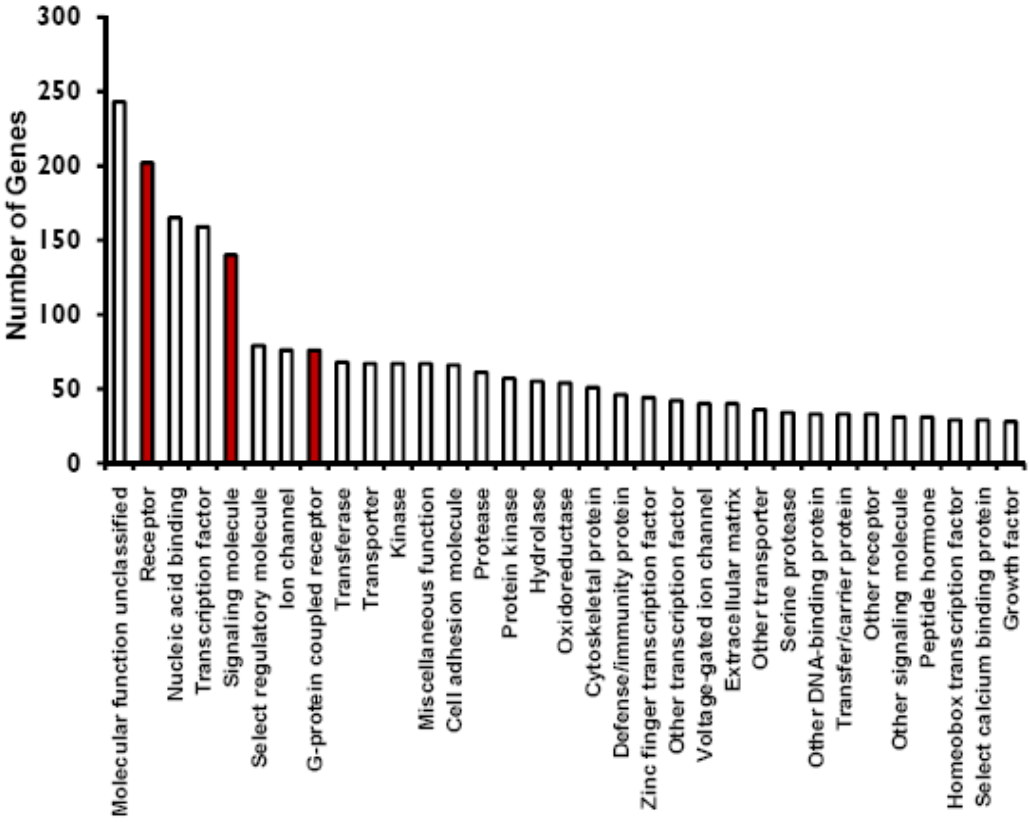

Figure 1. GPCRs regulated in dexamethasone treated hMSCs. *Gene ontology enrichment was performed online at, http://www.pantherdb.org/tools/genexAnalysis.jsp. Note the number of receptor related genes regulated in 10^{-8} M dexamethasone treated hMSCs.*

The effect of PTH/PTHrP on osteogenesis

PTH/PTHrP is an FDA approved drug and currently used to treat osteoporosis. PTH signaling is partly mediated via intracellular cAMP as a secondary messenger. Carpio *et al.* demonstrated that activation of PKA through administration of PTH in MG-63 cells leads to enhanced osteogenesis by inducing *collagen type I* expression[37]. To validate this and to set up a positive control for osteogenesis, we incubated MG-63 cells with 10^{-7} M PTHrP for 4 days and tested the expression of *collagen type 1*. Indeed, PTHrP induced *collagen type 1* expression although *ALP* expression was not significantly affected (Figure 2A). Moreover, our earlier data in hMSCs shows that cAMP treatment for 4 to 7 days significantly enhanced in vitro mineralization and prolonged incubation over 10 days inhibited mineralization. To investigate whether cAMP is able to induce osteogenesis in MG-63, we exposed them to 1 mM cAMP for 3 to 25 days. As shown in Figure 2B, cAMP triggered *in vitro* mineralization with dexamethasone similar to hMSCs. These results validate the positive effect of PTH on osteogenic differentiation through cAMP-mediated activation of PKA in MG-63 cells.

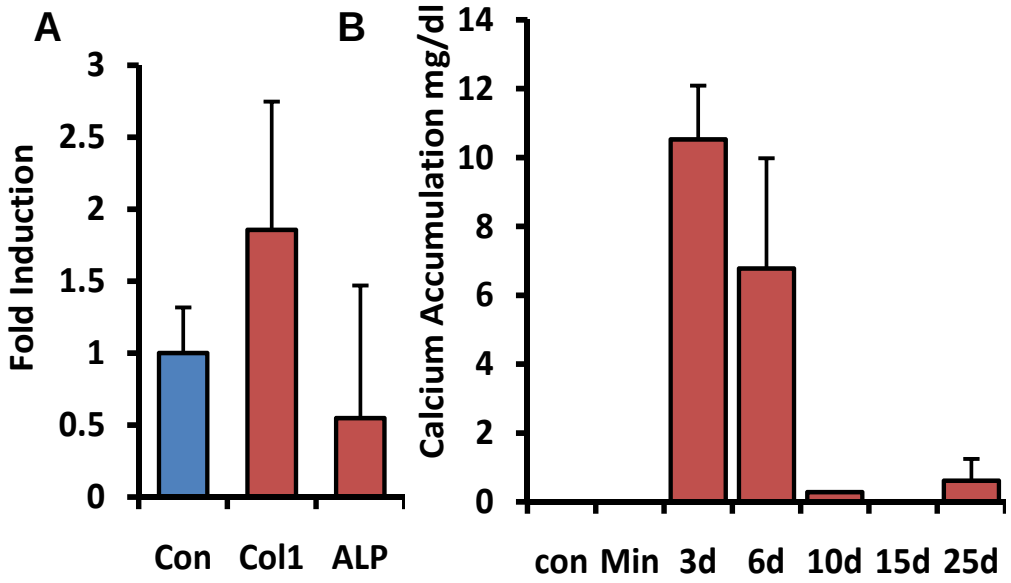

Figure 2. The effect of cAMP and PTHrP on osteogenic differentiation of MG-63 cell-line. ***A.*** *MG-63 cells were incubated with 10^{-7} M PTHrP for 4 days and analyzed the expression of collagen type 1 and ALP compared to untreated cells (Con) and normalized to 18s rRNA expression.* ***B.*** *MG-63 cells were incubated with 1 mM cAMP for first 3, 6, 10, 15 days and cultured without cAMP in mineralization medium (min) for 25 days. Note the enhanced calcium deposition in 3 and 6 days cAMP treated groups. * indicates statistical significance at P<0.001.*

Next, we tested the effect PTH on osteogenic differentiation of hMSCs. Treating hMSCs with 10^{-7} M PTH/PTHrP continuously for 2, 5 or 7 days did not induce ALP expression while dexamethasone did (Figure 3A). In view of the diverse effects of PTH on *in vivo* bone formation depending on the exposure scheme and the reported positive effect of intermittant exposure of hMSCs to PTH (Rickard *et al.*), we treated hMSCs with pulses of PTH, by exposing the cells for 1 to 8 hours per day in a total period of 9 days. After 9 days ALP expression was measured. As depicted in Figure 3B, none of the intermittent exposure schemes altered ALP expression. Next, we tested whether the differentiation stage of hMSCs may influence their response to PTH by first differentiating hMSCs into the osteogenic lineage with 10^{-8} M dexamethasone for 4 days and subsequent incubation with 10^{-7} M PTH in the presence or absence of dexamethasone. As shown in Figure 3C, pre-differentiating hMSCs into the osteogenic lineage also did not influence their response to PTH. In addition, we tested the effect of PTH on the end stage of osteogenic process, i.e mineralization, by incubating hMSCs with 10^{-7} M PTH or PTHrP. Both PTH and PTHrP did neither initiate mineralization when the cells were exposed to these ligands in the absence of dexamethasone, nor did they affect dexamethasone-induced mineralization (Figure 3D). We therefore conclude that under our culture conditions, hMSC osteogenesis is not affected by exposure to PTH.

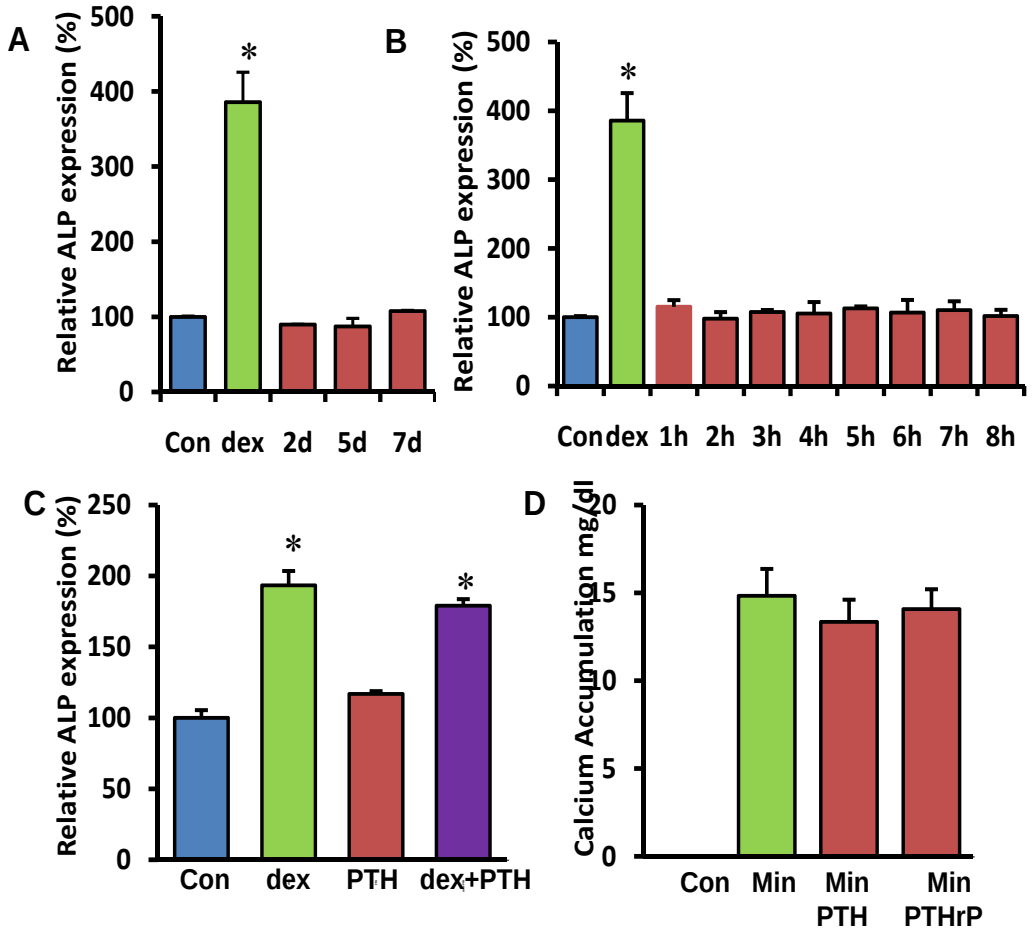

Figure 3. PTH (1-34) and PTHrP don't induce osteogenesis in hMSCs. ***A.*** *hMSCs were incubated with 10^{-7} M PTH (1-34) for 2, 5 and 7 days continuously and analyzed ALP expression at the end of 7 days.* ***B.*** *hMSCs were treated with 10^{-7} M PTH (1-34) for 1 to 8 hours and remaining period in 24 hours the cells were cultured in basic medium. The cycle was repeated for 9 days and ALP expression was measured as explained in materials.* ***C.*** *hMSCs were Predifferentiated into osteogenic lineage with 10^{-8} M dexamethasone for 4 days and subsequently the cells were treated with 10^{-7} M (1-34) for further 4 days with or without dexamethasone and analyzed for ALP expression.* ***D.*** *hMSCs were cultured in mineralization medium with or without 10^{-7} M PTH (1-34) or 10^{-7} M PTHrP for 14 days and cultured for 28 days. At the end of the culture period, the calcium deposition was assayed (see materials). * indicates statistical significance at $P<0.005$. Con; untreated group, dex; dexamethasone, Min; Mineralization medium.*

Inhibition of osteogenesis by prostaglandin E2

Considering the great number of GPCRs expressed in hMSCs, we decided to test a number of commercially available GPCR ligands for their effect on hMSC osteogenesis. Treating hMSCs with either epinephrine, melatonin, calcitonin or calcitonin gene related peptide did not significantly affected ALP expression (Figure 4A). Furthermore, PGE2 also failed to induce ALP expression in hMSCs, however when the cells were exposed to PGE2 in the presence of dexamethasone it significantly inhibited

dexamethasone-induced ALP expression (Figure 4B). These results were confirmed by mineralization results. We analyzed the ability of the above mentioned ligands to induce *in vitro* mineralization by treating the cells with the ligands continuously for 14 days. As shown in Figure 4C, most of the compounds did not affect dexamethasone- induced *in vitro* mineralization except for PGE2, which resulted in a significant reduction in calcium deposition. This suggests that PGE2-induced cAMP accumulation mediates inhibiton of hMSC osteogenesis, which contradicts our observation that sustained treatment with 1 mM cAMP stimulates osteogenesis.

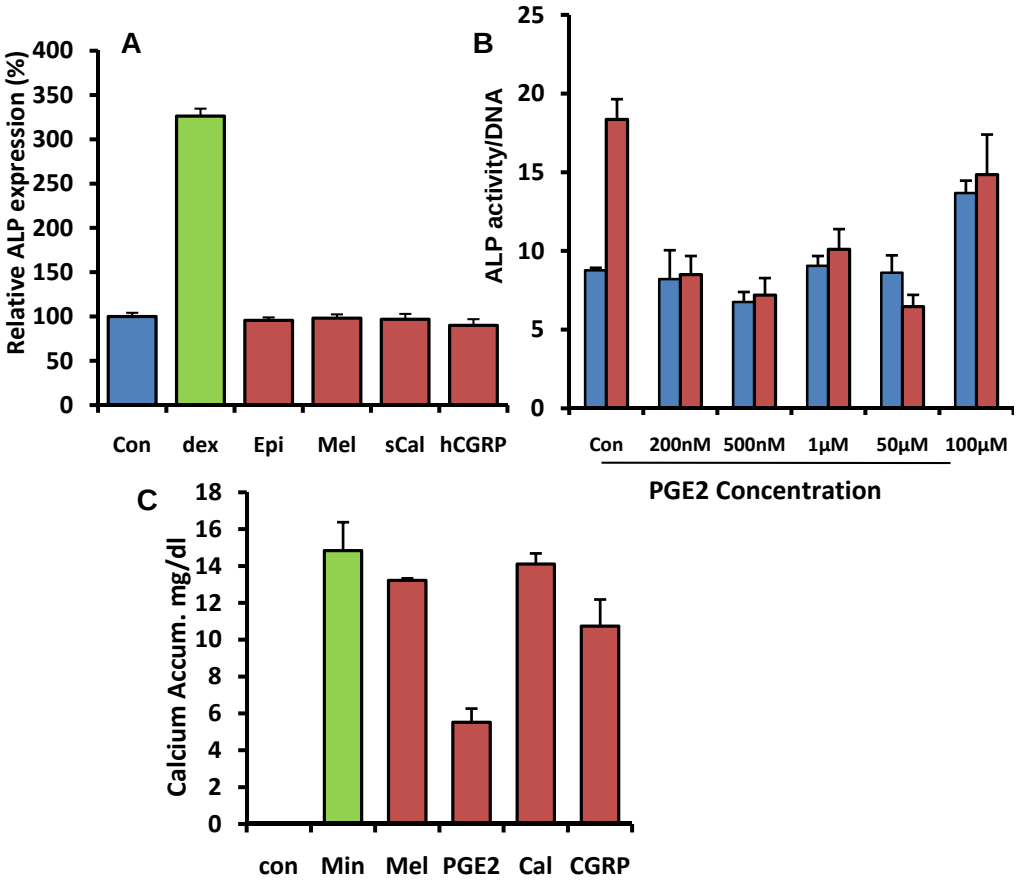

Figure 4. Prostaglandin E2 inhibits osteogenic differentiation of hMSCs. A. *hMSCs were incubated with 10^{-5} M Epinephrine, 10^{-7} M Melatonin, 10^{-10} M calcitonin, 10^{-8} M Calcitonin gene related peptide for 4 days and analyzed for ALP expression by flow cytometry.* ***B .*** *hMSCs were incubated with PGE2 concentrations ranging from 200 nM to 100 µM for 4 days and analyzed for ALP expression. Note the reduction in dexamethasone induced (red bars) ALP expression by PGE2 normalized to total DNA content.* ***C.*** *hMSCs were cultured in mineralization medium with or without 10^{-5} M Epinephrine, 10^{-7} M Melatonin, 10^{-10} M calcitonin, 10^{-8} M Calcitonin gene related peptide for 14 days and cultured further till 28 days in only mineralization medium. Note the reduction in dexamethasone induced calcium deposition by PGE2. * indicates statistical significance at P<0.005. Con; untreated group, dex; dexamethasone, Epi; Epinephrine, Mel; Melatonin, sCal; salmon calcitonin, hCGRP; human Calcitonin gene related peptide, PGE2; Prostaglandin E2, Min; Mineralization medium.*

Intermittent cAMP accumulation is correlated to inhibition of osteogenesis

Most of the GPCR ligands tested failed to induce ALP expression in hMSCs except for PGE2 which inhibited dexamethasone-induced ALP expression and mineralization. Since most of these ligands signal through intracellular cAMP as a second messenger, we further tested if there is a relation between ligand-induced intracellular cAMP accumulation and osteogenesis. For this, we expanded hMSCs with or without dexamethasone for 7 days. As expected, treatment of increasing doses of hMSCs to the well known cAMP-inducers forskolin and cholera toxin resulted in a dose-dependent accumulation of intracellular cAMP. Interestingly, forskolin and cholera toxin-mediated cAMP accumulation was synergistic with dexamethasone treatment, suggesting that dexamethasone modifies components of the GPCR/PKA signaling. After expansion, the cells were treated with various doses of PTH, PTHRP, melatonin, epinephrine, calcitonin, calcitonin gene related peptide and PGE2 for two hours. Among the ligands tested, only PGE2 was able to induce intracellular cAMP production in a dose-dependent manner in dexamethasone-expanded cells, demonstrating that accumulation of intracellular cAMP in hMSCs is associated with inhibition of osteogenesis (Figure 5A). Our previous work shows that prolonged exposure (several days) to a high concentration of di-butyryl cAMP, a stabilized version of cAMP, enhances osteogenesis. To explain the supposed contradiction, we hypothesized that the difference can either be due to the concentration and/or duration of cAMP that should persist in the cell to have a role in cell fate decision. First, we treated hMSCs with a concentration range of cAMP for 3 days and analyzed ALP expression. As shown in Figure 5B, cAMP induced ALP expression in a concentration-dependent manner and as expected cAMP showed a synergistic ALP induction with dexamethasone. In addition, treating hMSCs with 1 mM cAMP enhanced dexamethasone-induced mineralization (Figure 5C). We did not observe a negative effect of cAMP on ALP, suggesting that cAMP concentration does not mediate the observed difference between PGE2 and db-cAMP. Next, we exposed hMSCs to an intermittent cAMP exposure scheme. Cells were treated with 1 mM cAMP for 1 to 8 hours per day for a total period of 3 days and the effect on ALP expression was measured on day 3. As reported before, cells which were continuously incubated with 1 mM cAMP for 3 days showed a significant increase in the percentage of ALP positive cells. In contrast, cAMP treatment for 1-8 hours a day for three days led to a significant decrease in ALP activity (Figure 5D). These results suggest that hMSCs cells need a constant and high concentration of cAMP to stimulate osteogenic differentiation, which cannot be achieved by treatment of hMSCs with GPCR ligands or intermittent exposure to cAMP.

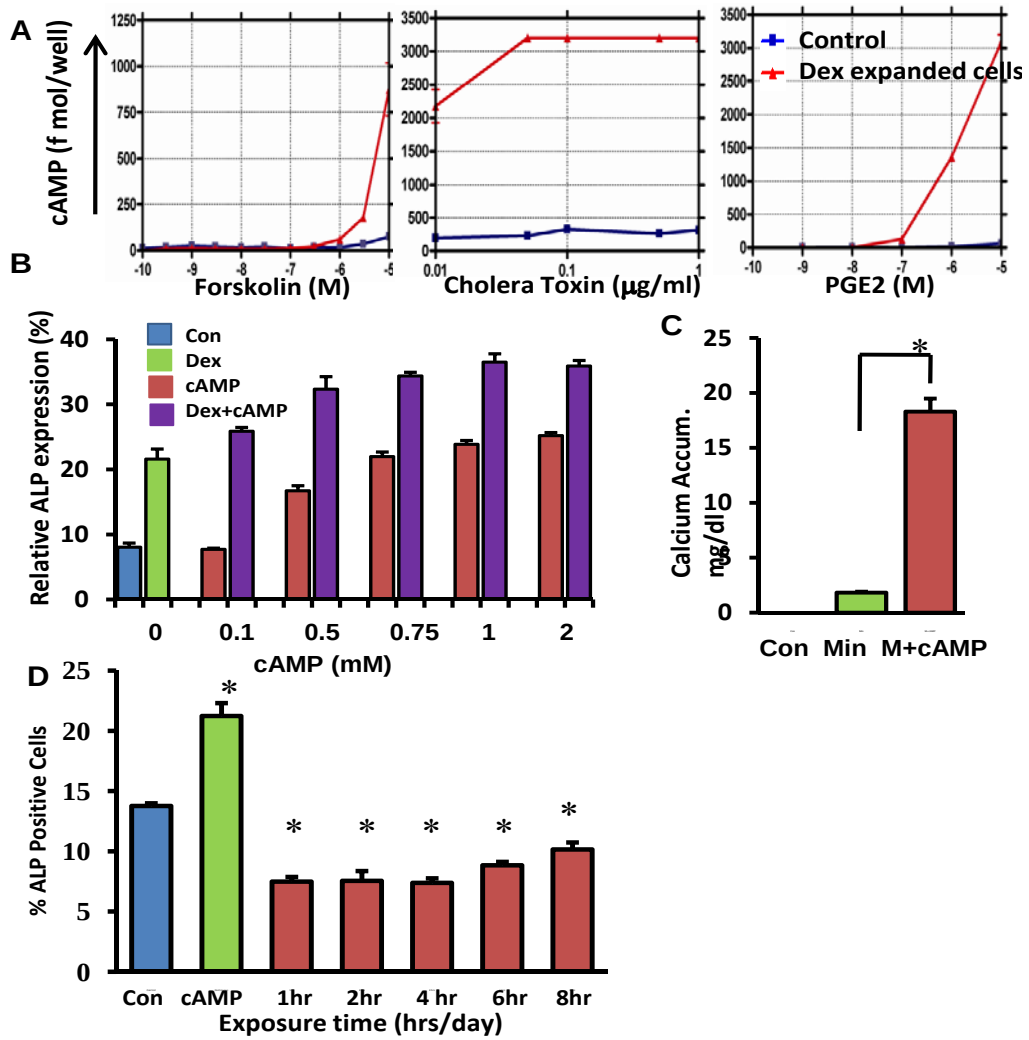

Figure 5. PGE2 stimulates cAMP production in hMSCs. A. *hMSC were expanded in proliferation medium with or without 10^{-8} M dexamethasone and treated with denoted concentrations of cAMP inducing compounds and ligands for 2 hours and intracellular cAMP was measured (see materials). Note the additive effect of Forskolin, Cholera toxin and PGE2 on cAMP induction in dexamethasone expanded cells. * indicates statistical significance at P<0.005.* ***B.*** *hMSCs were incubated with cAMP concentration ranging from 0- 2 mM either with or without dexamethasone for four days. After four days, ALP expression was measured by flow cytometry.* ***C.*** *hMSCs were cultured in mineralization medium supplemented with or without 1 mM cAMP for 5 days and cultured remaining 23 days in mineralization medium only. Total calcium was measured as explained in materials.* ***D.*** *hMSCs were Cells were treated with 1 mM cAMP for 1 to 8 hours per day for a total period of 3 days or continuously incubated with 1 mM cAMP for 3 days and analyzed percentage ALP positive cells by flow cytometry. Note the increase in percentage ALP positive cells in continuously incubated groups with cAMP for 3 days and reduction in intermittently incubated groups. Con; untreated group, dex; dexamethasone, Min; Mineralization medium, M+cAMP; mineralization medium supplemented with 1 mM cAMP, PGE2; Prostaglandin E2.*

Discussion

hMSCs are now of research and clinical interest to utilize their extensive proliferative and multipotent ability in bone tissue engineering applications. *In vitro* pre-differentiation of isolated hMSCs before using them for a clinical application has proven to be more efficient in their survival, function and their ability to form bone *in vivo*[17-19]. In this milieu, researchers have identified a number of compounds, proteins, small molecules, growth factors and synthetic molecules to differentiate MSCs *in vitro* into the osteogenic lineage. The use of these signaling molecules to pre-differentiate hMSCs into the osteogenic lineage can be efficiently used to augment bone tissue engineering protocols. However, this needs a thorough understanding of the differences in the action among species, since most of the knowledge gathered is from primary cells and cell lines derived from lower generic species such as mouse and rat. For instance, BMP, dexamethasone and other compounds have been shown to have diverge effects in different cell types and have a different role *in vivo*[49, 50]. We have identified a number of GPCR related genes regulated during osteogenic differentiation of MC3T3-E1 cells. To extrapolate this to a human situation, we used a number of growth factors/synthetic molecules which activate GPCR signaling to induce osteogenesis of hMSCs.

PTH is a currently used as a drug to treat severe osteoporotic patients. However, clinical studies have demonstrated that the treatment scheme is a vital factor for consideration to achieve the desired positive effect[51]. Furthermore, *in vitro* studies using various cell lines from different species have demonstrated that exposing cells to PTH shows dual effect either enhanced or diminished osteogenesis depending on the exposure scheme[32]. In MG-63 cells, PTHrP induces osteogenic differentiation by enhanced collagen 1 synthesis[37]. Together, although PTH effect on in vivo bone formation has been elucidated, its effect in vitro has been a subject of controversy. In specific, hMSCs do express receptors for various GPCR ligands including PTHR1 and these ligands fail to induce osteogenesis. Rickard *et al.* demonstrate that intermittent PTH exposure of hMSCs inhibits adipogenic differentiation and induces osteogenesis[38]. The authors however, demonstrate that PTH induces intracellular cAMP in HEK293 cells but not in hMSCs. Hence, it is questionable whether the effect observed is mediated via cAMP.. Furthermore, in our study PTH failed to induce either intracellular cAMP levels or ALP expression by itself or in combination with dexamethasone. Our studies and recent observations by Zhao *et al.* demonstrate that a balanced combination of dexamethasone and cAMP is important for cell fate decision either into the osteogenic or adipogenic lineage[52]. The authors further demonstrate that addition of PTH to hMSCs-adipogenic differentiation medium inhibits adipogenic differentiation and induces osteogenesis. hMSCs adipogenic medium contains a cocktail of cAMP-inducing agents, and when PTH is added, cAMP balance is altered and would have an osteogenic effect which is in line with our observation. Zhao *et al*. demonstrate that 10^{-7} M dexamethasone with 0.5 mM cAMP inhibits osteogenic differentiation and induces adipogenesis while we have consistently shown that 1 mM cAMP with 10^{-8} M dexamethasone significantly enhances osteogenesis in vitro and bone formation in vivo[52]. In addition, there is hardly any literature available on

hMSCs for PTH-cAMP driven cell fate decision directing us to unveil the precise role of PTH in cell fate decision. The other possible explanation for PTH's inability to induce ALP expression may be attributed to the fact that it may not produce a sufficiently high concentration of cAMP to trigger the cells into osteogenesis[23]. Furthermore, GPCR signaling is a tightly controlled process and once the required amount of signal is transduced, the cells have a feedback mechanism to internalize the receptors to stop surplus signaling[53]. Our results demonstrate that cAMP can have a strong effect on hMSCs cell fate decision depending on a balanced concentration of dexamethasone, and fine-tuning this duration and concentration is important for bone tissue engineering applications.

Amongst the tested ligands, none affected ALP expression or mineralization *in vitro* either alone or in combination with dexamethasone, except for PGE2 which reduced dexamethasone induced ALP expression and mineralization. The precise role of PGE2 mediated effect on osteogenesis of hMSCs is not studied except a recent study, which describes the role of PGE2 on osteogenesis. PGE2 is shown to inhibit osteogenic differentiation of hMSCs and induce adipogenesis with 10-7 ^{M} dexamethasone. Our results also demonstrate that the only GPCR ligand PGE2 which induced intracellular cAMP production inhibited osteogenesis in vitro. Further, this is substantiated by our intermittent exposure of hMSCs to cAMP to mimic physiological activation GPCR signaling. The role of cAMP in cell fate switch between adipogenic or osteogenic differentiation in hMSCs is demonstrated[52]. The authors demonstrate that cAMP induces adipogenic differentiation in combination with 10^{-7} M dexamethasone. In specific, PGE2 inhibited osteogenic differentiation of hMSCs which is in line with our observations. In contrast, we did not observe adipocytes in PGE2-treated hMSCs possibly because we use a 100 times lower concentration of dexamethasone for osteogenic differentiation[52]. The results suggest that the duration of cAMP treatment is a crucial determinant for cell fate decision. Fine-tuning the equilibrium of these opposing signals to achieve osteogenesis over adipogenesis of hMSCs is important to augment in vitro osteogenesis and in vivo bone forming capacity of hMSCs.

Another intersting observation we made when hMSCs were exposed to cAMP-inducing compounds such as PGE2, Forskolin and Cholera toxin was the enhanced cAMP induction when the compounds were used in combination with dexamethasone. Interestingly, dexamethasone itself exerted no effect on intracellular cAMP induction. The role of dexamethasone in sensitizing the cells to respond to GPCR ligands such as PTH is demonstrated in bovine vascular smooth muscle cells and human osteoblast SOS2 cells[54]. Mori et al. demonstrate that when these cells were incubated with dexamethasone, it had no effect on cAMP induction but, PTH as expected induced cAMP production. The potency of cAMP production by PTH was enhanced over 4 fold in both the cell types when PTH was presented together with dexamethasone[54]. Furthermore, our microarray studies show that receptors for PGE2, melatonin and calcitonin receptors upregulation upon exposing hMSCs to dexamethasone supports the hypothesis that dexamethasone induces the expression of

these receptor in turn sensitizing the hMSCs to these compounds to enhance cAMP induction..

Cell fate decision is a balanced act of signaling molecules. Dexamethasone is currently by far the most used signaling molecule to induce osteogenesis in hMSCs. Researchers are exploring for other molecules, growth factors, proteins and compounds to initiate osteogenesis in hMSCs. It is noteworthy that the osteogenic process can be augmented by using combination of other molecules with dexamethasone. Our results using cAMP and dexamethasone have proven to enhance dexamethasone induced osteogenesis in vitro and bone formation in vivo, underlining the role of cAMP in cell fate decision[23]. However, in this manuscript, our data suggest that physiological levels of cAMP induced by GPCR ligands such as PGE2 or intermittent exposure of hMSCs cAMP inhibited osteogenic differentiation of hMSCs. Taken together, our results demonstrate that cAMP plays a crucial role in osteogenic differentiation of hMSCs which depends on the concentration and duration of cAMP to which the cells are exposed and dexamethasone concentration. hMSCs exposed to 10^{-7} M dexamethasone and 0.5 mM cAMP is shown to inhibit osteogenic process and induce adipogenic differentiation[52], while we demonstrate that 10^{-8} M dexamethasone and 1 mM cAMP significantly enhances dexamethasone induced osteogenesis in vitro and bone formation in vivo[23]. On the other hand, 10^{-7} M dexamethasone with 1 mM cAMP induces adipogenic differentiation of hMSCs which supports the Zhao et al.'s observation (unpublished data). Our data further support the author's observation that physiological levels of cAMP produced by the ligands inhibit osteogenic differentiation of hMSCs with 10^{-7} M dexamethasone, Therefore, understanding this balanced act of concentration- and context-dependent effects of different osteogenic signals is essential to initiate the osteogenic process for their effective use in bone tissue engineering applications.

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Chapter 5

PKA signaling inhibits osteogenic differentiation and bone formation in rodent models

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In Preparation

Abstract

We previously demonstrated that cAMP-mediated Protein Kinase A (PKA) activation induces *in vitro* osteogenesis and *in vivo* bone formation by human mesenchymal stem cells (hMSCs). To analyze the genetical basis for this phenomenon and to translate our findings into a clinical trial, suitable animal models and cell lines are desirable. In this report, we assessed whether in a number of model systems, PKA plays a similar pro-osteogenic role. To this end, we treated MC3T3-E1 cells, mouse calvarial cells, mouse MSCs and rat MSCs with cAMP. Collectively, our data demonstrate that cAMP inhibits osteogenesis in these cell types, evidenced by inhibition of osteogenic markers such as *alkaline phosphatase, osteocalcin* and *collagen type 1*. In contrast, cAMP stimulated adipogenic differentiation in rat MSCs. *Ex vivo* cultured mouse calvaria which were exposed to cAMP showed reduction in bone volume. Taken together, our data demonstrate that cAMP inhibits osteogenesis *in vitro* and bone formation *ex vivo* in rodent models. Evidently hMSCs display a unique response to cAMP, which makes them the only and obviously most relevant cell type for future research.

Key words: *PKA signaling, osteogenesis, bone formation and bone tissue engineering.*

Introduction

Multipotent mesenchymal stem cells (MSCs) can differentiate into several mesenchymal cell lineages such as the adipogenic, chondrogenic, osteogenic and myogenic lineages, which identifies them as a candidate cell source for regenerative medicince[1, 2]. For instance, in cell-based bone tissue engineering, MSCs isolated from the bone marrow are seeded onto various scaffold materials and implanted into a defect site to regenerate damaged or distorted bone tissue. Unfortunately, the newly formed bone does not fully bridge the implant using current protocols[3]. Thus, there it is a prerequisite to enhance the bone forming capacity by hMSCs, which may be achieved by using adequate number of cells with higher osteogenic capacity, appropriate scaffold materials with osteo-inductive and –conductive properties, factors to stimulate osteogenic differentiation *in vitro* and *in vivo* and improved vascular supply for better implant survival. *In vitro* pre-differentiation of MSCs improves their *in vivo* bone forming capacity[4, 5]. Therefore, pre-differentiating the MSCs into the osteogenic lineage *in vitro* using various osteo-inductive signals, proteins, cytokines and growth factors before implantation is a potential strategy to augment *in vivo* bone formation.

Osteogenic differentiation is a complex process controlled and regulated temporally by various signaling pathways[6]. Most studies to delineate the osteogenic process are performed in rodent genetic models and cell lines. MSC differentiation into a matured osteoblast requires a spectrum of signaling molecules including hormones, growth factors, cytokines, matrix proteins, transcriptions factors and their

co-regulatory proteins[7]. Sequential and coordinated activation or inhibition of signaling pathways such as Wnt[8-10], Rho[11, 12], Glucocorticoid[13-15], vitamin D[16], TGF-β[17, 18], BMP[15], MAPK kinase[19, 20] and G protein coupled receptor[21, 22] (GPCR) signaling pathways guide MSCs into a bone-forming cell. For instance, GPCRs are a main class of receptors which modulate proliferation and differentiation of many cell types. The role of GPCR signaling in skeletal morphogenesis has been demonstrated by knockout studies of parathyroid hormone (PTH), parathyroid hormone related peptide (PTHrP) and parathyroid hormone receptor1 (PTHR1). PTH-deficient mice show diminished cartilage matrix mineralization, reduced metaphyseal osteoblasts and trabecular bone [23, 24]. In addition, osteoblasts and primary cells of mesenchymal origin express receptors for adenosine, beta-adrenergic hormone, P2Y2, prostaglandin, calcitonin, melatonin, the calcium sensing receptor and many other orphan receptors[25], which transduce their signals via specific GPCRs. Essentially, most of these ligands, including PTH, mediate their signals through intracellular cAMP production which in turn activates Protein Kinase A signaling (PKA) and partly also via Protein Kinase C (PKC) [26].

There is conflicting data about the role of PKA in osteogenesis. Over six decades, it is known that intermittent PTH administration stimulates bone formation *in vivo,* whereas prolonged exposure leads to bone resorption[22, 27, 28]. PKA activating compounds such as PTH, PTHrP, calcitonin, forskolin, melatonin and prostaglandin E2 induce osteogenesis in different cell types[29-34]. In sharp contrast, a negative role for PKA on osteogenesis is demonstrated in hMSCs and mouse KS483 cells[35-38]. A higher concentration of cAMP with proper dexamethasone stimulation inhibits osteogenic differentiation of hMSCs and induces adipogenesis[39]. The effect of these cAMP-inducing compounds on osteogenesis is further convoluted by exposure schemes, concentration and species differences. We have previously demonstrated that PKA activation in hMSCs using cAMP induces osteogenic differentiation *in vitro* in an autocrine and paracrine fashion[40]. Furthermore, cAMP stimulates the bone forming capacity of hMSCs *in vivo.* To analyze the genetic basis for this phenomenon and to translate our findings into a clinical trial, suitable animal models and cell lines are required. In this view, osteogenic cell lines such as MC3T3 from mouse, ROS from rat and MG-63 from human are extensively used and they tend to show differences in response to signals[6]. We confirmed that PKA signaling induces osteogenesis and bone formation in hMSCs, but in contrast, it inhibits osteogenic differentiation and bone formation in rodent models.

Materials and Methods

Cell culture of MC3T3-E1, C2C12, mouse MSCs and calvarial cells.

The MC3T3-E1 cell line was purchased from Riken Cell bank (RCB 1126) and cultured in MC3T3-E1 basic medium consisting of α-MEM (Biowhittaker), 10% Foetal Calf Serum (FCS, Hyclone), 2 mM L-glutamine (Invitrogen) and 100 U/ml penicillin and streptomycin. MC3T3-E1 osteogenic medium is basic medium supple-

mented with 50 μg/ml ascorbic acid (Sigma) and 10 nM β-glycerophosphate (Sigma). MC3T3-E1 mineralization medium was composed of osteogenic medium supplemented with 100 ng/ml rhBMP2 (R&D systems). The C2C12 cell line was purchased from ATCC (CRL-1772) and cultured in C2C12 basic medium consisting of α-MEM (Biowhittaker), 10% FCS (Hyclone) and 100 U/ml penicillin and streptomycin. Mouse calvarial cells were isolated by dissecting the calvaria aseptically from 2-4 days old C56BL/6 mice (ICR-Harlan). The isolated calvaria were incubated at 37^{o}C for 15 minutes and digested with 3 ml of 2 mg/ml collagenase in PBS for 20 minutes at 37^{o} C. The supernatant was collected and centrifuged for 5 minutes at 1200 rpm to collect the cells. Collagenase digestion was repeated 2-3 times and the isolated cells were cultured in mouse calvarial basic medium consisting of α-MEM, 10% FCS, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. Mouse calvarial mineralization medium was composed of mouse calvarial basic medium supplemented with 50 μg/ml ascorbic acid and 10 nM β-glycerophosphate. To study the effect of cAMP on osteogenic differentiation of mouse calvarial cells, the cells were cultured in mineralization medium with or without 1 mM 8-Bromo cAMP (8b-cAMP, this concentration was used throughout the study unless stated). Mouse MSCs were isolated by dissecting femurs of 8 weeks old mice and bone marrow was flushed into a culture flask with CIM medium which consisted of RPMI 1640 (Invitrogen), 9% horse serum (Hyclone), 9% FBS , 100 U/ml penicillin and streptomycin and 12 μ M L-glutamine. After 3 passages, cells were further expanded in CEM medium consisting of Iscove-modified Dulbecco medium (IMDM, Invitrogen), 9% horse serum (Hyclone), 9% FBS, 100 U/ml penicillin and streptomycin and 12 μ M L-glutamine. For mouse MSC mineralization, CEM medium was supplemented with 100 ng/ml rhBMP2, 200 μ M ascorbic acid, 1 nM dexamethasone and 10 mM β-glycerophosphate with or without 1 mM N6, 2'-O-dibutyryl-cAMP (db-cAMP, Sigma). Rat MSCs were isolated from Albino Wister or Fischer F344 male rats. The femurs were aseptically dissected and transferred into a 50 ml Falcon tube containing rat basic medium consisted of α-MEM (Life Technologies), 10% FBS (Cambrex), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin and 10 μ g/ml streptomycin (Life Technologies). MSCs were isolated by flushing medium through the femur with a 20 G needle. The cells were *in vitro* expanded by growing in rat basic medium. Rat mineralization medium consisted of rat basic medium supplemented with 10 mM beta-glycerophosphate and 10^{-8} M dexamethasone. To study the role of PKA activation on osteogenic differentiation of rat MSCs, the cells were cultured in mineralization medium with or without db-cAMP (Sigma) for denoted time periods.

Alkaline phosphatase assay

To study the effect of cAMP on ALP induction in MC3T3-E1 cells, cells were either treated or untreated with 1 mM 8b-cAMP for denoted time periods. After the culture period, the medium was removed and washed twice with PBS and fixed with 400 μ l of 1% paraformaldehyde for 30 minutes. Then, the cells were washed twice with PBS and stained with ALP staining solution (1 mg of napthol AS-MX phosphate (Sigma) dissolved in 50 μ l of Dimethyl sulfoxide, 5 ml of demineralised water, 5 ml of 0.2 M Tris/HCl (pH 8.9), 100 μ l of MgSO4.7H2O, 6 mg of Fast Blue RR (Sigma). The

cells were incubated with staining solution for 30-60 minutes at 37°C in 5% CO_2, washed with PBS and scanned. For quantitative ALP assay, after the indicated culture condition and time, the cells were washed with PBS and lysed with 75 µl of lysis buffer (Applied Biosystem, RE2111) per well of a 24-well plate for 10 minutes (Galacto-Light Plus, Applied Biosystems). The lysates were either used directly for assay or stored at -20°C. Ten µl of lysate was taken in an optiplate, to which 40 µl of CDP-star was added (Roche) and incubated for 30 minutes after which luminescence was measured on a Victor multi label plate reader (Perkin Elmer). The total protein content was measured using a BCA protein assay kit (Pierce) as described by the manufacturer. The ALP was normalized to total protein content. The data was analyzed using Student's t-test and statistical significance was found at $P < 0.05$.

Mineralization and Calcium assay

For qualitative assessment of mineralization, after denoted culture period, the cells were rinsed with PBS and fixed overnight in 4% paraformaldehyde. Next, the cells were rinsed with demineralized water and stained with 5% silver nitrate (Sigma) under mild UV exposure until distinct black stains were developed in the positive control group. For quantitative measurements, cells at the end of the culture period were washed with 100 µl/well α-MEM. After washing, 150 µl/ well 100 µg/ml calcein (prepared in α-MEM) was added and incubated in the dark at 37°C for 4 hours. Then, the plates were washed three times with 100 µl PBS/well and fluorescence was measured on a Victor counter (Perkin Elmer). The fluorescence was normalized to total protein content measured by a BCA protein assay kit (Pierce). The total calcium deposition was assayed using a calcium assay kit (Sigma diagnostics; 587A) according to manufacturer's protocol. The data was analyzed using Student's t-test and statistical significance was found at $P < 0.05$.

Adipogenic Assay

To study the role of cAMP on adipogenic differentiation of rat or mouse MSCs, the cells were either incubated with or without 1 mM db-cAMP for denoted time periods in mouse or rat mineralization medium. At the end of the culture period, lipid formation was visualized and quantified by Oil Red O staining. Briefly, cells were fixed overnight in formol (3.7 % formalin plus 1 g/100 ml $CaCl_2.2H_2O$), rinsed with water, incubated for 5 minutes in 60 % isopropanol and stained for 5 minutes in freshly filtered Oil Red solution (stock 500 mg Oil Red [Sigma], 99 ml isopropanol, 1 ml water; stain 42 ml stock + 28 ml water). Oil Red stain was quantified by extracting with 5 ml 4% Igepal (Sigma) in isopropanol for 15 minutes by on a shaking platform at room temperature. 100 µl of extract was taken in a 96-well plate and the absorbance was measured at 520 nm.

Quantitative Real Time PCR

To study the role of PKA activation on osteogenic differentiation of MC3T3-E1 cells, cells were treated in triplicate with 1 mM 8b-cAMP for denoted time periods. The total RNA was isolated using Triozol (Life Technologies) as described by the manufacturer. One microgram of total RNA was used to synthesize cDNA using Superscript II Rnase H Reverse Transcriptase (Life Technologies) in a total volume of 20

μ l as described in the manufacturer's protocol. PCR was performed using 5 μ l of cDNA in a total volume of 25 μ l using SYBRGreen (Invitrogen) on ABI ^{TM}PRISM. Expression values were subsequently determined according to threshold cycles (Ct values), indicating the increase of reporter fluorescence above baseline levels. For each gene, PCR was carried out in triplicate and mean expression values were corrected for mean β-tubulin expression levels. The primers used are listed in Table 1.

Table 1. Primers used for qPCR	
ALP	F5'tcagggcaatgaggtcacatc3' R5'gtcacaatgcccacggactt 3'
Cbfa1	F5'gggcacaagttctatctggaaaa 3' R5'cggtgtcactgcgctgaa 3'
Osteocalcin	F5'gaacagacaagtcccacacagc 3' R5'agagacagagcgcagccag 3'
Collagen 1	F5'ccccagcgaagaactcatacag 3' R5'ccattgatagtctctcctaaccagaca 3'
PTHR1	F5'agggatttttgttgccatca 3' R5'gcggctccaagacttcctaa 3'
TBP	F5'agcttctcccaagttacagacaca 3' R5'cacgtgcccgttcttctga 3'

Micro Computer Tomography (μCT)

To study the role of PKA activation on bone formation, 6 mm calvaria were collected from C56BL/6 (ICR-Harlan) mice and trimmed such that substantial sections of parietal bone (pb) and a little section of occipital bone (ob) were present. After each calvarium was dissected, it was immersed in media (α-MEM, BioWhittaker), supplemented with 10% heat-inactivated fetal calf serum (Gibco), 100 U/ml penicillin / 100 μ g/ml streptomycin (Gibco) and transferred to a 24-well plate containing 2 ml of medium with or without added treatments. Mouse calvaria were cultured for 14 days in control medium or supplemented with 10^{-9} M IL1-β (R&D Systems). In the cAMP group, the calvaria were exposed to 1 mM db-cAMP for 4 days and cultured for the remaining 10 days without cAMP. In the IL1-β+cAMP group, the calvaria were incubated always with Il1-β, but for the first 4 days they were exposed together with cAMP and remaining 10 days they were cultured only in the presence of IL1-β. The medium was refreshed three times a week. For scanning, calvaria were positioned as described by Stock *et al.*[41]. Calvaria were imaged on day 0 and bone volume was determined. Treatment groups were assigned based on individual calvarium volume and mean volume. A Skyscan 1076 MicroCT-40 system (Skyscan, Belgium) and associated software was used to collect the data and to reconstruct the slices of the volume containing the calvarium. The X-ray tube was operated with photon energy of 70 kV, current of 140 μ A and a 0.50 mm thick Al filter with a scanning width of 35 mm and pixel size 9 μm. Mean values (n=4) and standard deviations of Bone Volume (mm^3) for control and treated calvaria were compared using Graph Pad Prism. Data was analyzed using one-way ANOVA followed by Dunnett's multiple comparison test and statistically significance was analyzed at $P<0.005$ or $P < 0.001$.

Results

cAMP inhibits osteogenesis in MC3T3-E1 cells.

Previously, we demonstrated that cAMP enhances osteogenic differentiation of hMSCs and their bone-forming capacity[40]. To further investigate the observed pro-osteogenic efect of cAMP in genetically more amenable models, we aimed to delineate the effect of PKA activation in commonly used cell lines and primary cells in the field of osteogenic research. First of all, we cultured MC3TC3-E1 cells in the presence of 1 mM 8b-cAMP for 3 to 28 days, which inhibited both cell growth and ALP expression compared to control groups (Figure 1A). ALP quantification confirms that 8b-cAMP significantly inhibits ALP expression (Figure 1B). PTH, which signals via the second messenger cAMP, is known to have a different effect on ALP expression and osteogenic differentiation depending on the exposure scheme[42].

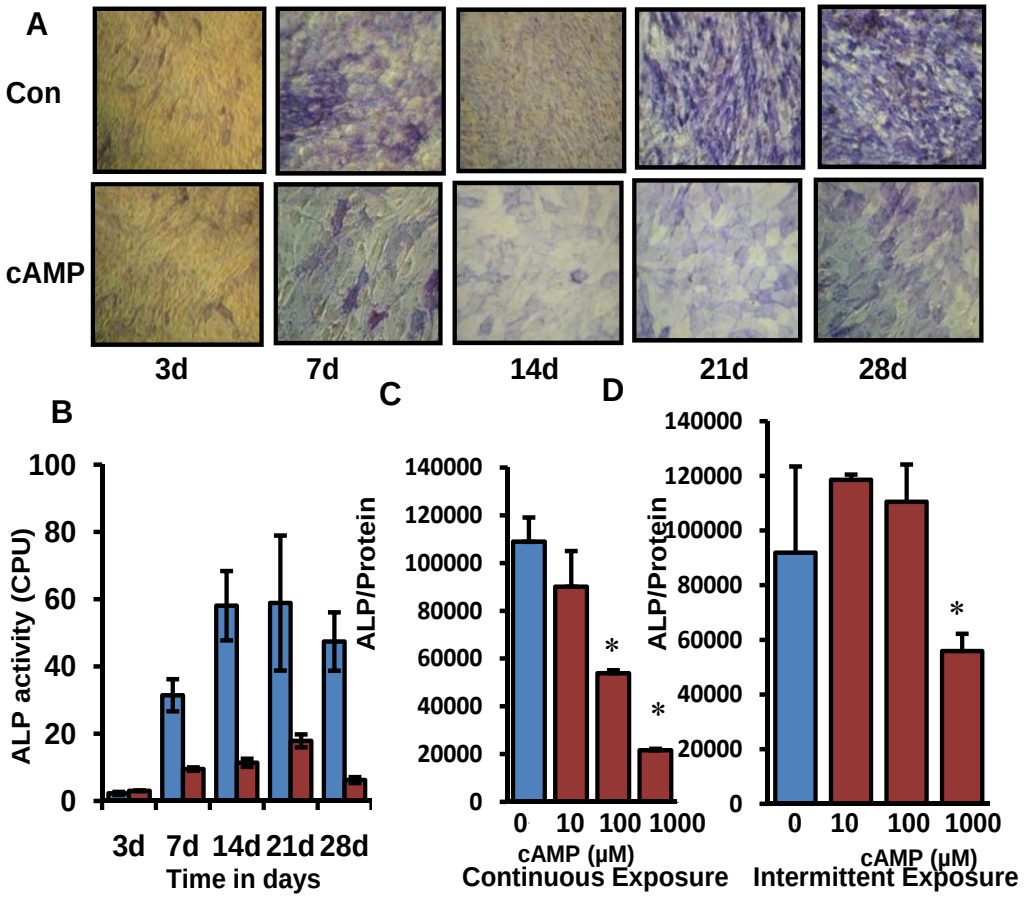

Figure 1. cAMP inhibits ALP expression in MC3T3-E1 cells. A. *MC3T3-E1 cells were exposed to 1 mM 8b-cAMP for the denoted time periods (days) and stained for ALP with napthol AS-MX phosphate and Fast blue RR.* ***B.*** *ALP expression by MC3T3-E1 exposed 8b-cAMP for denoted time periods (days) normalized to total protein content by CDP star assay.* ***C*** *and* ***D.*** *ALP expression by MC3T3-E1 cells treated with various concentrations of 8b-cAMP either intermittently or continuously for 11 days. ALP expression was quantified by CDP star assay.*

To mimic the physiological activation of the PKA pathway more closely, we treated MC3T3-E1 cells either continuously or intermittently with various concentrations of 8b-cAMP ranging from 10 μ M to 1 mM for 4, 7, 11, 15, 18 and 21 days. Continuously exposing MC3T3-E1 cells to 8b-cAMP for 11 days showed a concentration dependent decrease in ALP expression (Figure 1C), which was also observed at the other time points (data not shown). No significant differences were observed when the cells were exposed to 10 μ M 8b-cAMP, however 100 μ M and 1 mM 8b-cAMP significantly inhibited ALP expression (Figure 1C). In the intermittent exposure groups, no significant differences were observed in ALP expression at different time points in the groups when they were exposed to 10 and 100 μ M 8b-cAMP. However, we observed a reduction in ALP expression in cells which were exposed to 1 mM 8b-cAMP for 11 days (Figure 1D).

ALP inhibition by cAMP directed us to investigate the role of effect of cAMP on the final stage of *in vitro* osteogenic differentiation, i.e. mineralization. MC3T3-E1 cells were cultured in mineralization medium supplemented with or without 1 mM 8b -cAMP for 14, 21 and 28 days. von Kossa stains carbonate and phosphate deposits and widespread mineralization was observed when MC3T3-E1 cells were grown in mineralization medium. In contrast, cells in the presence of 8b-cAMP showed no traces of mineralized areas. Exposing cells to 8b-cAMP for 14 days was enough to completely abolish mineralization (Figure 2).

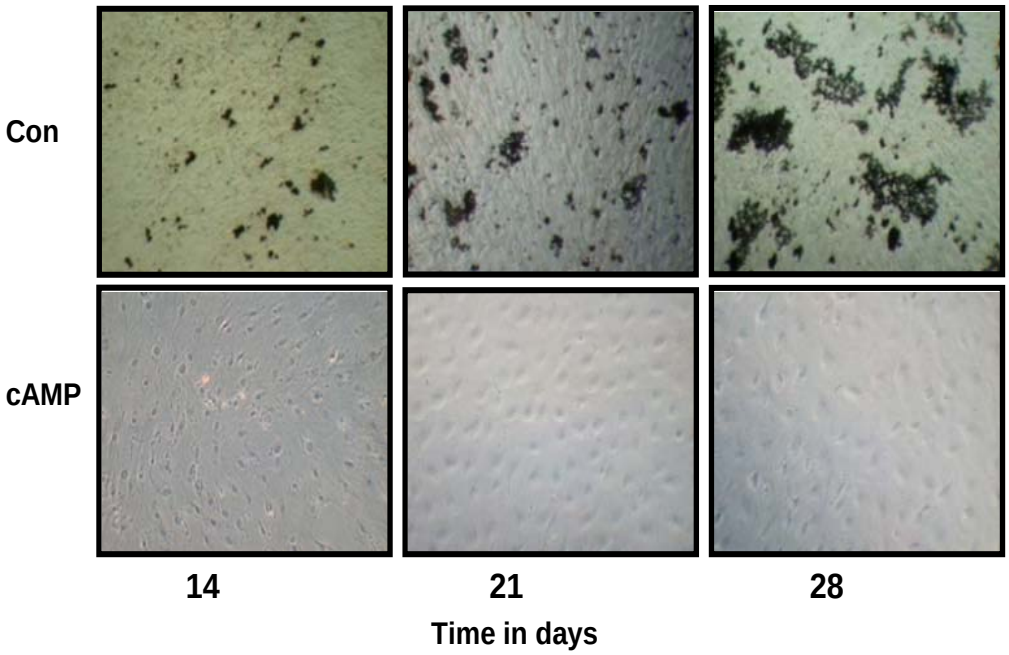

Figure 2. cAMP inhibits in vitro mineralization in MC3T3 cells. A. *MC3T3-E1 cells were exposed to 1 mM 8b-cAMP for only denoted time period and cultured until day 28 in the absence of cAMP. At the end of the culture period, the cultures were stained with von Kossa to visualize mineralized bone nodules. Note, the black mineralized nodules in control group.*

To further elucidate the negative effect of cAMP on osteogenic differentiation of MC3T3-E1 cells, we treated the cells with 1 mM 8b-cAMP and analyzed the expression profiles of some of the osteogenic marker genes such as *ALP*, *cbfa1, PTHR1, osteocalcin* and *collagen type1* at regular intervals during 28 days. Cells which were cultured with 8b-cAMP up to 14 days showed no effects on *ALP* expression. However, cells which were incubated with 8b-cAMP for more than 14 days showed a mild reduction in *ALP* expression. *Osteocalcin* gene expression, which marks late osteogenesis, showed a typical upregulation in the control group. In contrast, *osteocalcin* gene expression was completely abolished in the 8b-cAMP treated group. *PTHR1* which is a known marker expressed by osteoblasts and cells of mesenchymal origin was also completely inhibited by 8b-cAMP, whereas in the control group, *PTHR1* was upregulated in the early phase of osteogenesis. Expression of the osteoblast specific transcription factor *cbfa1* and of *collagen type1* was not markedly affected by cAMP. Taken together, cAMP strongly inhibits *osteocalcin* and *PTHR1* expression but has a milder effect on *collagen type1* and ALP expression (Figure 3).

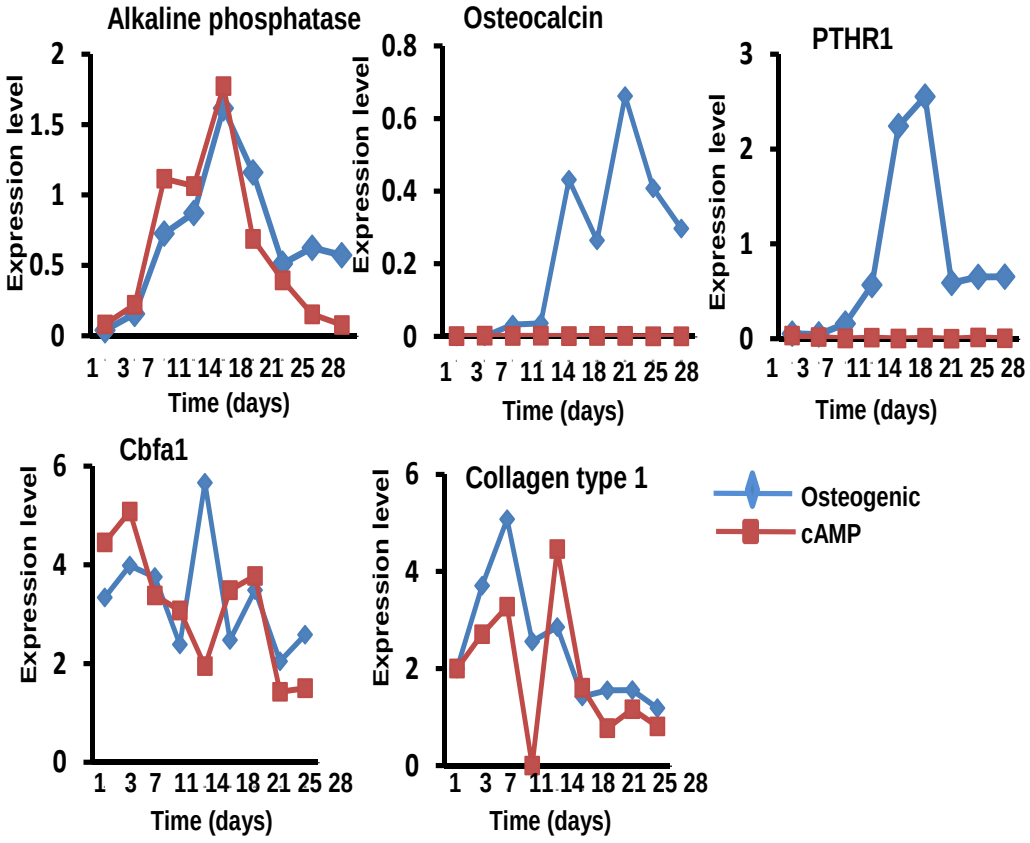

Figure 3. cAMP inhibits osteogenic gene expression in MC3T3-E1 cells*. MC3T3-E1 cells were cultured either MC3T3-E1 osteogenic medium (diamond line) or osteogenic medium supplemented with 1 mM 8b-cAMP (squared line) for denoted time periods (days). Gene expression was analyzed by qPCR on ABI™PRISM and normalized to β-tubulin.*

The effect of cAMP on primary rodent osteogenic cells

In cell-based bone tissue engineering, primary autologous MSCs will be used for clinical application. To investigate the respons of primary cells from rodent models to cAMP treatment, we first exposed mouse calvarial cells to 8b-cAMP for 1 to 28 days. ALP quantification demonstrates that 8b-cAMP did not significantly affect ALP expression (Figure 4A). In agreement with this, mineralization by 8b-cAMP exposed mouse calvarial cells was slightly retarded but precedeed equal compared to the control group (Figure 4B). Next, we tested the effect of cAMP on *in vitro* mineralization of mouse MSCs. Exposing mouse MSCs to mineralization medium resulted in efficient mineralization of the extracellular matrix, evidenced by calcium deposition. In contrast, 1 mM db-cAMP for 3 or 10 days significantly reduced *in vitro* mineralization (Figure 4C). Taken together, our results show that PKA activation not only inhibits osteogenesis in MC3T3-E1 cells and MSCs, but minimally affected mineralization in mouse calvarial cells.

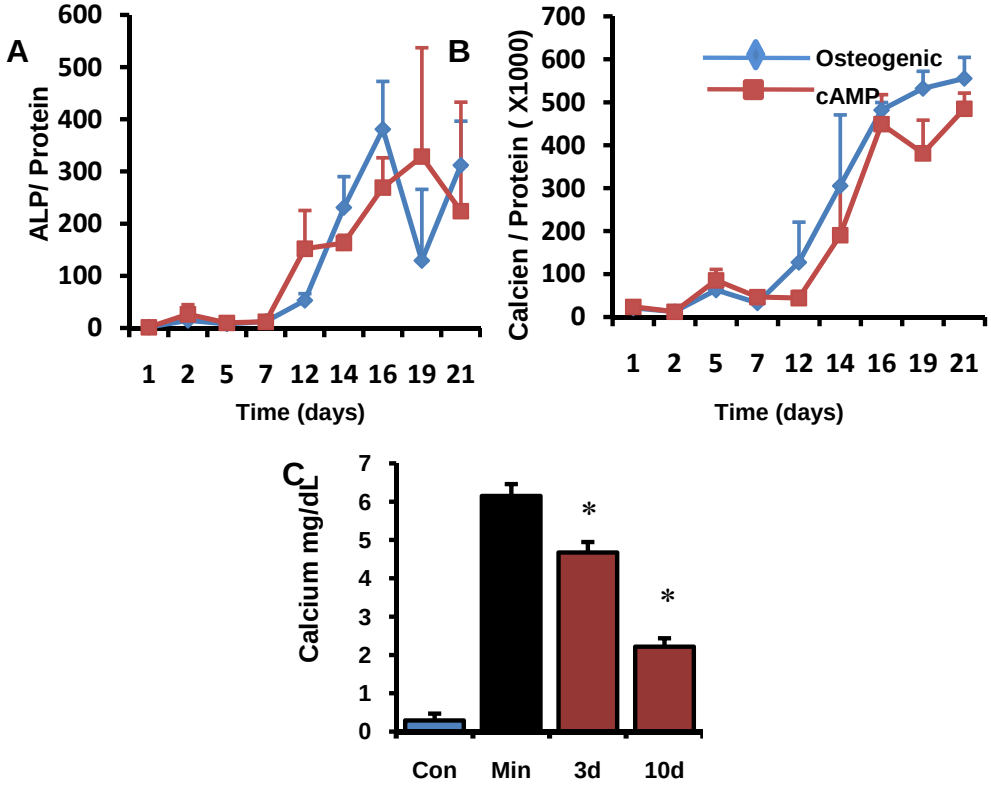

Figure 4. cAMP inhibits osteogenesis in mouse calvarial cells and mouse MSCs. A. *Primary mouse calvarial cells treated with 1 mM cAMP for denoted time periods (days) and ALP expression was quantified by CDP start assay.* ***B.*** *Mouse calvarial cells were cultured in osteogenic medium or osteogenic medium supplemented with 1 mM 8b-cAMP for denoted time periods (days) and then stained with calcein, quantified on Victor counter.* ***C.*** *Mouse MSCs isolated from Col (I)-Luc mice were cultured in mineralization medium (Min), or mineralization medium supplemented with 1 mM db-cAMP for denoted time periods (days) . At the end of 28 days total calcium accumulation was quantified using a calcium assay kit (see materials). * indicates statistical significance at P <0.05.*

Rat MSCs are a frequently used model system for bone tissue engineering and we therefor isolated rat MSCs and treated them with db-cAMP on the first 3, 5, 10 or 15 days of a 25-days culture period. We used this particular scheme of exposure because we have previously demonstrated that when exposure of hMSCs is limited to the first 3-5 days, it enhances *in vitro* mineralization whereas exposure of 10 days and more showed a negative effect on mineralization[40]. To our surprise, cAMP-treatment consistently inhibited mineralization (data not shown) whereas we observed a robust, time-dependent increase in adipogenesis (Figure 5). The lipid droplets formed were stable for up to 20 days without any additional supplements.

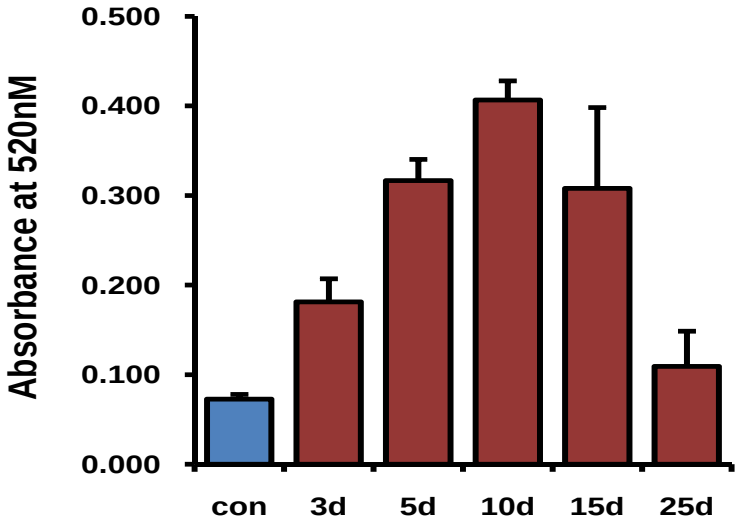

Figure 5. cAMP induces adipocytes differentiation in rat MSCs. A. *Rat MSCs were cultured in basic medium (Con) or basic medium supplemented with 1 mM db-cAMP for denoted time periods (days). Adipocytes were stained with oil red O and quantified on a spectrophotometer.*

PKA activation inhibits ex vivo bone formation

We previously demonstrated that cAMP-mediated activation of PKA strongly enhances the *in vivo* bone forming capacity of ectopically implanted hMSCs. To investigate the effect of cAMP on normal bone formation, we isolated and cultured mouse calvaria *ex vivo*. The calvarium contains both osteoblast and osteoclast and the combined effect of a compound on net bone formation mimics the real *in vivo* situation. The calvaria were exposed to 1 mM db-cAMP for the first 4 days and cultured further 10 days without db-cAMP. IL1-β was used as a positive control in our experimental group because it is known to stimulate bone resorption and inhibits *in vivo* bone formation[43]. As expected, we observed significant decrease in bone volume in the IL1-β treated group. Db-cAMP also decreased the total bone volume compared to control group. In addition, db-cAMP together with IL1-β showed an additive effect on bone volume reduction (Figure 6B), demonstrating that cAMP treatment has a net negative effect on bone formation.

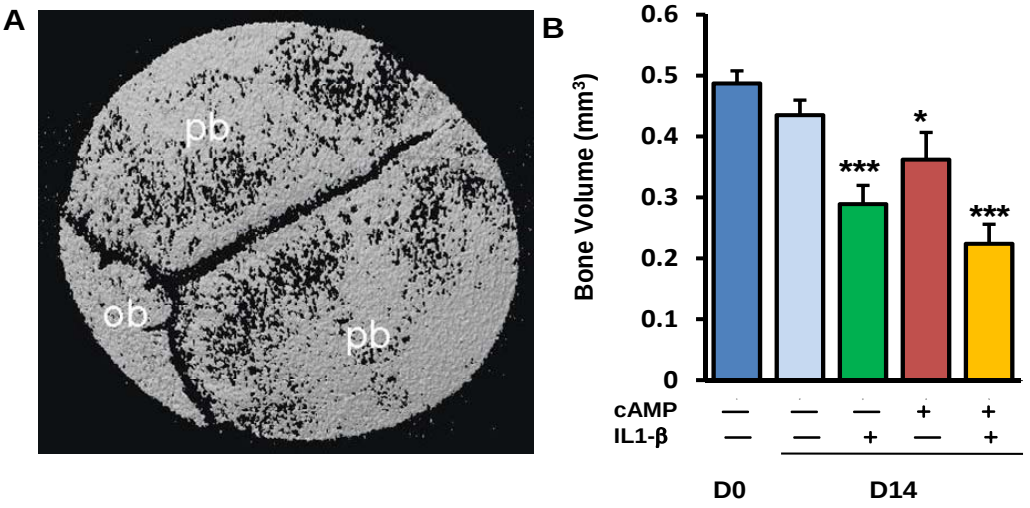

Figure 6. cAMP inhibits ex vivo bone formation. A. *Representative μ CT image of a mouse calverium which was to study the effect of cAMP on ex vivo bone formation.* ***B.*** *Mouse calvaria were cultured for 14 days in control medium (Con) and IL1-β. In cAMP group the calvaria were exposed to 1 mM db-cAMP for 4 days and cultured remaining 10 days without cAMP. In IL1-β+cAMP group, the calvaria were incubated always with Il1-β, but for the first 4 days they were exposed together with db-cAMP and remaining 10 days they were cultured only in the presence of IL1-β. The bone volume was measured by μ CT (see materials). * indicates statistical significance at P <0.05 and *** indicates statistical significance at P< 0.001.*

Discussion

Cell-based bone tissue engineering using MSCs aims to augment bone fracture healing, spinal fusion and other skeleton-related injuries. An enormous effort has been flowing to understand the mechanism of skeletal development right from the embryo to remodeling of the adult skeleton. To gain insight into the genetics of skeletal development, various knockout models for skeletal related genes and many cell lines from various species such as MG-63, MC3T3-E1, ROS from human, mouse and rat respectively, have been developed. However, discrepancy exists between the osteogenic effect of a number of reagents between hMSCs and the model systems mentioned before. 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[44]. Further, the required dosage and action of these signals may vary between cell types[44, 45]. Therefore, genetic and molecular data gathered from various species and cell lines need thorough verification in hMSCs[6]. Vice versa, signaling pathways involved in hMSC osteogenesis cannot be automatically investigated in model systems. In this milieu, we previously demonstrated that PKA activation in hMSCs induces osteogenic differentiation and bone formation *in vivo*. To validate the observed results and to further investigate the molecular mechanism involved in PKA-induced osteogenesis, we decided to screen a number of genetically more amenable cell models and tissues. Thus, we stu-

died the role of cAMP-mediated PKA signaling using pre-osteogenic cell line MC3T3 -E1, primary mouse MSCs and calvarial cells and rat MSCs. Our results unequivocally demonstrate that cAMP inhibits osteogenesis in MC3T3-E1 cells, mouse and and rat MSCs.

There has been a conflict in literature on the role of the PKA pathway in osteogenic differentiation which is further complicated by cell type, molecules used to activate PKA and exposure scheme. PTH and PTHrP are known to induce bone formation when given intermittently while it results in bone resorption in a continuous treatment[28, 46, 47]. The anabolic or catabolic effect of PTH on *in vitro* osteogenic differentiation of MC3T3-E1 cells has been demonstrated to depend on the time and duration of exposure to PTH[42]. In innate situation where cells are exposed to a short pulse of activation by the cAMP inducing ligands such as PTHh, they produce intracellular cAMP in femto molar range. To mimic this more closely, we exposed cells to a range of cAMP concentrations either intermittently or continuously. In both cases we observed a concentration dependent inhibition of osteogenesis. This is possibly due to the concentrations of cAMP that the cells are exposed to. In normal situations, the cells produce intracellular cAMP in femto molar range when they are exposed to intracellular cAMP inducing agonists such as PTH. In addition, species differences in response to an osteogenic signal cannot be neglected. Mouse and rat cells exposed to PTH demonstrated decreased *ALP* and *collagen type 1* expression[48, 49]. Reports show that PTH and cAMP in UMR, MG-63 and vascular cells endothelial cells show enhanced osteogenesis[33, 50, 51]. Additionally, when hMSCs were cultured with cAMP for 4 days, it induced osteogenesis and stimulated the bone forming ability of the cells[40]. The osteogenic impairment by cAMP in these cell types in comparison with the studies done using PTH might also be due to the known fact that PTH/PTHrP signaling is not only mediated via PKA but through multiple signaling pathways such as PKC[52], mitogen activated protein kinase[53] and act together with notch[54], wnt[55] and other signaling pathways to induce a biological effect. In line with the negative effect of cAMP on ALP and mineralization in MC3T3 cells, qPCR studies show downregulation of osteogenic markers such as *collagen type 1, PTHR1* and *osteocalcin* expression. Specifically, *PTHR1* and *osteocalcin* expressions were abolished by cAMP treatment. It was previously demonstrated that PKA activation down-regulates *PTHR1*, probably representing a negative feedback loop on intracellular cAMP production[56]. The reduction in *PTHR1, osteocalcin* expression and *in vitro* mineralization has been observed previously and are in line with our observations[57, 58]. Although enzymatic ALP measurements in cAMP treated MC3T3 cells show a drastic reduction in ALP activity, we observed no significant reduction in *ALP* expression by real-time PCR. Enzymatic ALP assay measures the total ALP activity in cells which include various forms such as liver, kidney and bone specific ALP while by PCR we measure bone specific *ALP* expression only. This may be the cause for insignificant reduction in *ALP* expression by real-time PCR compared to enzymatic ALP assay.

When mouse calvaria were exposed to cAMP *ex vivo*, we observed a reduction in total bone volume. Because calvaria contain both osteoblasts and osteoclasts and considering the possibility that cAMP may rescue IL1-β induced bone resorption,

mouse calvarial were exposed to cAMP with or without IL1-β. We observed an additive effect on bone volume reduction, which is in line with the observed effect of cAMP on osteogenesis *in vitro*. IL1-β is a well known cytokine know to activate osteoclasts and inhibit bone formation *in vitro* and *in vivo*[43]. Besides IL1-β, IL6 is also known to induce osteoclast differentiation and induce bone resorption[59]. Supporting our observation, PKA activation using prostaglandins has been demonstrated to stimulate the production of bone resorbing cytokines such as IL6 and IL1-β, which might synergistically decrease bone volume when the calvaria were exposed to cAMP and IL1-β [60, 61].We have earlier demonstrated that treating *ex vivo* mouse calvaria with Trichostatin A inhibits osteoclast differentiation and strongly stimulates bone formation in this model however, cAMP failed to induce bone formation[62].

The role of PKA signaling in adipocyte differentiation and maintenance has been demonstrated and validates the observation that cAMP induces adipogenic differentiation in rat MSCs[63]. Moreover, to differentiate human MSCs into the adipogenic lineage, researchers use a cocktail of molecules including IBMX, which indirectly enhances intracellular cAMP accumulation by inhibiting phosphodiesterase[64]. Moreover, cAMP-inducing compounds such as prostaglandins and isoproterenol are demonstrated to induce adipogenic differentiation in C3H10T1/2 cells [65, 66]. In contrast, in hMSCs, cAMP inducing compounds such as PTH and PTHRP are known to block adipogenesis by PKA dependent mechanism and induce osteogenic differentiation further highlighting the species differences in the role of a signaling molecule to induce different biological effect[34].

Taken together, our data demonstrate that PKA activation using cAMP in MC3T3-E1, mouse and rat MSCs cells inhibits osteogenic differentiation *in vitro* and bone formation *ex vivo* which is in contrast to hMSCs emphasizing a species dependent effect. Furthermore, cAMP did not significantly affect ALP expression and mineralization of mouse calvarial cells while it inhibited both in mouse MSCs suggesting cAMP has a cell specific effect. The decreased *ex vivo* bone volume was possibly mediated via production of bone resorbing cytokines such as IL1-beta and IL6. In comparison with the known effects of PTH on *in vitro* and *in vivo* bone formation, our data suggest that PKA activation using cAMP in rodent models has a negative effect on osteogenic differentiation.As such, future research on the osteogenic effect of cAMP will be restricted to human mesenchymal stem cells.

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Chapter 6

Protein Kinase A exhibits both paracrine and cell-autonomous control of bone formation by human mesenchymal stem cells

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Submitted

Abstract

Multipotent human mesenchymal stem cells (hMSCs) are increasingly used in the field of regenerative medicine and tissue engineering. To further our knowledge on osteogenic pathways, we studied the effect of PKA signaling on osteogenic differentiation of hMSCs. PKA activation strongly enhanced osteogenic differentiation of hMSCs and microarray and qPCR studies revealed a direct induction of cycloheximide-insensitive expression of BMP target genes *ID2*, *ID4* and *SMAD6* within 6 hours by PKA activation. Further, delayed peak expression of another set of BMP target genes after 5 days implied a second, paracrine mode of activation confirmed by co-incubation with noggin. PKA activation further induced the expression of osteogenic cytokines and growth factors such as *IL-11, IGF1* and other TGF-b members. As a consequence, PKA strongly enhanced the bone forming capacity of hMSCs *in vivo*. These studies demonstrate that PKA displays both cell autonomous and paracrine control of bone formation by hMSCs which can be further applied to improve bone tissue engineering applications.

Key Words: *human mesenchymal stem cells, PKA signalling, osteogenesis, bone formation and bone tissue engineering*

Introduction

The ability of human mesenchymal stem cells (hMSCs) to differentiate into adipogenic, chondrogenic, osteogenic[1] and myogenic[2] lineages has generated a great deal of potential clinical use in regenerative medicine and tissue engineering in the past decade. Concomitantly, hMSCs are increasingly used as a cell biological model system to investigate molecular mechanisms governing signal transduction[3], differentiation[4-6], cell fate decision[7], senescence[8, 9] and plasticity[10, 11] because the step from basic research to clinical application is relatively short. In line with this, a number of pathways have been investigated for their involvement in osteogenic differentiation of hMSCs. In early studies, dexamethasone (dex) and vitamin D3 were used to promote hMSC differentiation *in vitro*. More recent studies include the MAPK pathway[12], Rho kinase[7], Wnt [5], Notch [13] and receptor tyrosine kinases [3]. Our lab is interested to further delineate pathways, which dictate osteoblast differentiation of hMSCs to improve bone tissue engineering protocols. In this light, we focused on one of the classical pathway, PKA. In contrast to glucocorticoid, BMP and vitamin D3, the role of PKA in osteogenesis is conflicting. BMP is known to direct cell fate decision by activation of the osteogenic key transcription factor *RUNX2/CBFA1*[14]. BMP further induces osteogenesis at later stages of differentiation via activating other target genes, such as the Inhibitors of Differentiation genes (*ID*s)[15, 16], which are functionally involved in osteogenic differentiation. Vitamin D3 is known to induce the mineralization phase of osteogenesis *in vitro* by direct activation of, among others, the osteocalcin gene via a vitamin D-response element[17]. Finally, dexamethasone, a synthetic glucocorticoid, drives hMSCs into the osteogenic lineage in which direct activation of *c-FOS* gene expression appears to be involved [18].

In contrast, relatively little is known about the role of PKA in osteogenic differentiation. It is, however, anticipated by the anabolic effect on bone mineral density of certain hormones known to activate PKA. Intermittent administration of PTH increases trabecular and cancelleous bone formation in ovariectomized mice, although continuous administration results in net bone loss[19, 20]. Over expression of another PKA activating hormone, calcitonin gene related peptide, in mice increased bone formation rate and bone mineral density[21]. PTH and calcitonin mediate their anabolic effect via G-Protein coupled receptors (GPCRs), for which both hormones are ligands. Osteoblasts express receptors for many GPCR ligands, such as PTH, parathyroid hormone related peptide (PTHrP), calcitonin, epinephrine, melatonin and prostaglandins[22]. Overall, GPCR seems to have a positive effect on osteogenesis *in vitro*. For instance, melatonin has a positive effect on proliferation and differentiation of osteogenic cells[23]. Melatonin increases expression of alkaline phosphatase (*ALP*), osteopontin, bone sialoprotein (*BSP)* and other bone markers in hMSCs, MC3T3-E1 and rat osteoblast-like osteosarcoma cells, demonstrating that melatonin promotes osteoblast differentiation and matrix mineralization[24]. Similarly, prostaglandin E2 (PGE2) induces osteoblast differentiation[25]. Because binding of both PGE2 and melatonin to their receptors lead to activation of the PKA pathway this implies that PKA is involved in osteogenesis. Upon ligand binding, the GPCR activates guanine nucleotide exchange on intracellular G proteins. One subtype of G proteins, $G\alpha_s$, activates adenylate cyclase, which stimulates the production of the second messenger cyclic 3, 5-adenosine monophosphate (cAMP). cAMP binds to the regulatory subunit of PKA, which liberates the catalytic subunit of PKA. PKA then phosphorylates the cAMP Response Element Binding protein (CREB), in addition to many other proteins. Phosphorylated CREB translocates into the nucleus, binds to cAMP Response element (CRE) sites and stimulates target gene expression[26]. Although GPCR ligands are clearly able to stimulate bone mineral density, it is unknown how and at which point during osteogenesis GPCR ligands regulate osteogenesis.

The effect of PKA activation on osteogenesis has been studied in different cell types with compounds which directly or indirectly activate PKA, although the results are contentious. The most direct evidence on a role of PKA in osteogenic differentiation is from studies in calcifying vascular cells (CVC) [27]. Here, activation of the PKA pathway with cAMP stimulated osteogenic marker genes and *in vitro* mineralization suggesting that the PKA pathway promotes vascular calcification by enhancing osteogenic differentiation of CVC. Furthermore, forskolin increased bone nodule formation at low concentration and inhibited bone nodule formation at higher concentration indicating the biphasic action of forskolin[28]. Further, a recent study shows that PTHrP inhibits *CBFA1* expression through PKA pathway[29]. Diminutive information about the events downstream of PKA activation and osteogenesis in hMSCs directed us to examine the *in vitro* and *in vivo* effects of PKA on osteogenic differentiation and the interaction with other pathways at molecular and genetic level during hMSC differentiation. We delineate a role for PKA at an early stage of differentiation via dual molecular mechanisms involving the BMP pathway. PKA-activated hMSCs demonstrate enhanced *in vivo* bone formation, which opens a promising window for further exploration to improve bone tissue engineering.

Materials and Methods

Isolation and culture of hMSCs

Bone marrow aspirates (5-20ml) were obtained from donors with written informed consent. hMSCs were isolated and proliferated as described previously[49]. Briefly aspirates were resuspended using 20 G needles, plated at a density of $5x10^5$ cells/cm^2 and cultured in hMSC proliferation medium containing a-minimal essential medium (a-MEM, Life Technologies), 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (Asap, Life Technologies), 2mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), 10 mg/ml streptomycin (Life Technologies), and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week and cells were used for further subculturing or cryopreservation upon reaching near confluence. hMSC basic medium was composed of hMSC proliferative medium without bFGF, hMSC osteogenic medium was composed of hMSC basic medium supplemented with 10^{-8} M dexamethasone (dex, Sigma) and hMSC mineralization medium was composed of basic medium supplemented with 10^{-8} M dex and 0.01 M b-glycerophosphate (Sigma). The hMSCs used in the study were from donors of age range between 25 to 80 years.

Proliferation assay

To assess the effect of PKA activation on hMSC proliferation, cells were seeded in triplicate at 5000 cells/cm^2 in basic medium or basic medium supplemented with 1 mM cAMP for three days. Cell numbers were determined using a coulter counter (Beckman Coulter, The Netherlands) and proliferation is expressed as the number of population doublings per day or percentage cell number reduction compared to control. Data was analyzed using Student's t test ($P<0.05$).

ALP analysis by flow cytometry

hMSCs were seeded at 5000 cells/cm^2 and allowed to attach for 10 to 15 hours in basic medium, then incubated with different reagents for the denoted time periods. Each experiment was performed in triplicate with negative control (cells grown in basic medium) and a positive control (cells grown in osteogenic medium) and one or more experimental conditions. In the case of PKA and glycogen synthase kinase-3 (GSK-3) inhibitor groups, cells were incubated in triplicate with 10 mM H89 (Sigma) or with 4 mM lithium chloride (LiCl, CalBiochem) respectively for 10 to 15 hours before addition of cAMP (Sigma) or 5 µg/ml cholera toxin (CTX, Sigma). At the end of the culture period, the cells were trypsinized and incubated for 30 minutes in block buffer (PBS with 5% bovine serum albumin, (BSA [Sigma] and 0.05% NaN2), then incubated with primary antibody (anti-ALP, B4-78 [Developmental Studies Hybridoma Bank, University of Iowa, USA]) diluted in wash buffer (PBS with 1% BSA and 0.05% NaN2) for 30 minutes or with isotype control antibodies. Cells were then washed three times with wash buffer and incubated with secondary antibody (goat anti mouse IgG PE, DAKO) for 30 minutes. Cells were washed three times and suspended in 250 µl wash buffer with 10 µl Viaprobe (Pharmingen) for live/dead cell staining and only living cells were used for further analysis. ALP expression levels

were analyzed on a FACS Caliber (Becton Dickinson Immuno cytometry systems). The data was analyzed using Student's t test ($P<0.05$).

Mineralization and calcium deposition

For mineralization, hMSCs were seeded in triplicate at 5000 cells/cm2 in T25 culture flasks and incubated with 1 mM cAMP for denoted time periods. In each experiment, mineralization medium was used as a positive control and basic medium as negative control. The total calcium deposition was assayed using a calcium assay kit (Sigma diagnostics; 587A) according to manufacturer's protocol. Briefly, the culture medium was aspirated, washed twice with calcium and magnesium free PBS (Life Technologies) and incubated overnight with 0.5 N HCl on an orbital shaker at room temperature. The supernatant was collected for direct measurement or stored at –20°C. The calcium content was measured at 575 nm (Perkin Elmer, Lamda 40) and expressed as mg calcium /flask. The data was analyzed using Student's t test at $P<0.05$.

RNA isolation and quantitative PCR

The effect of cAMP on expression of osteogenic marker genes was analyzed by seeding hMSCs at 5000 cells/cm2 in T75 flasks supplemented with various medium compositions for 3, 5, 10 and 15 days. To analyze the direct induction of BMP target genes by PKA activation, hMSCs were seeded at 5000 cells/cm^2 and supplemented with 1 mM cAMP for 6 hours with or without cycloheximide (Sigma) and RNA was isolated and analyzed for expression of BMP target genes. To analyze the indirect induction of BMP target genes, hMSCs were incubated with 250 ng/ml noggin (R&D systems) for 10 to 15 hours and then supplemented with 1mM cAMP or 100 ng/ml human recombinant BMP2 (R&D Systems). Total RNA was isolated using an Rneasy mini kit (Qiagen) and on column DNase treated with 10U RNase free DNase I (Gibco) at 37°C for 30 minutes. DNAse was inactivated at 72°C for 15 minutes. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. Two µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100x diluted cDNA was used for collagen type 1 (*COL1*) and 18s rRNA amplification and 1 µl of undiluted cDNA was used for other genes PCR was performed on a Light Cycler real time PCR machine (Roche) using a SYBR green I master mix (Invitrogen). Data was analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes are calculated relative to 18s rRNA levels by the comparative DCT method[50] and statistical significance was found using student's t test ($P<0.05$). The primers used in the study are listed in Table 1.

Microarray analysis

To study the genome wide effect of PKA pathway, hMSCs were grown in either basic medium or basic medium supplemented with 1mM cAMP for 6 hours. RNA was isolated using an RNeasy midi kit (Qiagen) and 8 µg of total RNA was used for probe labeling according to the manufacturer's protocol (Affymetrix). The probe quality was verified using lab-on-chip technology (Agilent Technologies) and samples were hybridized to Human Genome Focus arrays according to the manufacturer's protocol

(Affymetrix). Data analysis was performed using Affymetrix GENECHIP 4.0 software.

Table 1. Primers used for qPCR studies.

Gene		Primer sequence	Product length(bp)
18s rRNA	F	5'cggctaccacatccaaggaa3'	187
	R	5'gctggaattaccgcggct3'	
ALP	F	5'gacccttgacccccacaat3'	67
	R	5'gctcgtactgcatgtcccct3'	
COL1	F	5'agggccaagacgaagacatc3'	137
	R	5'agatcacgtcatcgcacaaca3'	
OC	F	5'ggcagcgaggtagtgaagag3'	138
	R	5'gatgtggtcagccaactcgt3'	
ID1	F	5'gcaagacagcgagcggtgcg3'	346
	R	5'ggcgctgatctcgccgttgag3'	
ID2	F	5'cctcccggtctcgccttcc3'	320
	R	5'ggttctgcccgggtctctgg3'	
SMAD6	F	5'gctaccaactccctcatcact3'	336
	R	5'cgtcggggagttgacgaagat3'	

In vivo bone formation

To evaluate the effect of PKA activation on *in vivo* bone formation by hMSCs, we used two current protocols. In the first approach we seeded hMSCs in basic medium at 200,000 cells/particle (3 particles per condition) onto 2-3 mm biphasic calcium phosphate (BCP) particles prepared and sintered at 1150° C as described previously[51]. A day after seeding, the particles with cells were supplemented with basic medium, basic medium with 1 mM cAMP, 10^{-8} M dex or 1 mM cAMP and dex, and cultured for 5 more days. Medium was refreshed twice a week. The cells were cultured for a further 3 days in basic medium. Ten nude male mice (Hsd-cpb:NMRI-nu, Harlan) were anaesthetized by intramuscular injection of 0.05 ml of anaesthetic (1.75 ml ketamine 100 ug/ml, 1.5 ml xylazine 20 mg/ml and 0.5 ml atropine 0.5 mg/ml). Four subcutaneous pockets were made and each pocket was implanted with 3 particles of each condition. Each mouse was implanted with four conditions. The incisions were closed using a vicryl 5-0 suture. After 6 weeks the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck) in 0.14 M cacodylic acid (Fluka) buffer pH 7.3, dehydrated and embedded in methyl methacrylate (Sigma) for sectioning. Approximately 10µm thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). The sections were stained with basic fuchsin and methylene blue to visualize bone formation. In the second approach we cultured hMSCs from four different donors in osteogenic medium supplemented with dex (n=6) or dex and cAMP (n=6) for 5 days. Cells were trypsinized and seeded at 200,000 cells per particle. After 4 hours, the BCP particles were implanted in 12 nude mice for 6 weeks. Each mouse was implanted with two conditions from two donors. The samples were explanted and processed as described

above. At least 3 sections were made from each sample and scored by three different individuals for the incidence of bone formation as either positive or negative for bone formation from those three sections. In an independent experiment (approach IIa in Table 4). We analyzed the effect of PKA activation with cAMP alone on *in vivo* bone formation by culturing hMSCs in basic medium or basic medium supplemented with 1mM cAMP for four days, then implanted in 6 nude mice for 6 weeks and processed as described above. The newly deposited bone was quantified and expressed as % bone area per total pore area of the scaffold.

Results

PKA activation induces osteogenesis in hMSCs

To understand the effect of PKA on hMSC biology, we treated cells with cAMP or cholera toxin (an upstream activator of PKA, see Figure 6 for an overview). We observed a change in morphology from the typical fibroblastic morphology of hMSCs to the cuboidal shape known from differentiating osteoblasts (Figure 1A). cAMP treatment for 3 to 5 days inhibited proliferation by 41% ± 2% and 70% ± 8% respectively compared to controls. However when cAMP was removed from the medium, cells regained a normal growth rate and fibroblastic morphology and reached confluency (data not shown). To analyze the effect of PKA activation on hMSC osteogenic differentiation, we treated cells with 1mM cAMP or 5 µg/ml CTX for 4 days and analyzed ALP expression as an early marker for osteogenesis. Both cAMP and CTX induced ALP expression to the same extent as dex (Figure 1B). A donor dependent variation was observed in ALP expression, both in the dex and cAMP treated group (sea Table 2). Moreover, we observed an additive or synergistic induction of ALP upon co-treatment with dex and cAMP (Figure 1B). To confirm that cAMP and CTX-induced ALP expression is mediated via the PKA pathway, we co-treated cells with cAMP or CTX and H89, a selective PKA inhibitor. H89 inhibited cAMP and CTX-induced ALP expression in hMSCs to basal level (Figure 1C). Interestingly, H89 did not affect dex-induced ALP expression, indicating that dex and cAMP induce ALP expression via different pathways. To further confirm the involvement of the PKA pathway in hMSC osteogenesis, we inhibited phosphorylation of the transcription factor CREB. PKA phosphorylates CREB at serine 133 whereas GSK-3 phosphorylates CREB at serine 129. Serine 129 phosphorylation is a prerequisite for enhanced transcriptional activity of CREB by PKA[30, 31].

Table 2. ALP induction by PKA activators.

dex	cAMP	dex+cAMP	CTX	dex+CTX
222 ± 2[a]	251 ± 14	582 ± 36	251 ± 20	381 ± 5
219 ± 8	325 ± 12	389 ± 3	161 ± 4	364 ± 21
183 ± 16	179 ± 14	265 ± 36	163 ± 5	326 ± 17
279 ± 7	197 ± 9	357 ± 6		
147 ± 15	269 ± 5	387 ± 51		
282 ± 13	216 ± 6	387 ± 51		

[A] Relative ALP expression (%) compared to controls.

Treatment of hMSCs with the GSK-3 inhibitor lithium chloride inhibited cAMP-induced ALP expression from 270 % to 190 % (Figure 1D). Together, our results indicate that phosphorylation of CREB at serine 133 and 129 are important for the enhanced induction of ALP in hMSCs, further supporting the involvement of PKA in hMSCs osteogenesis.

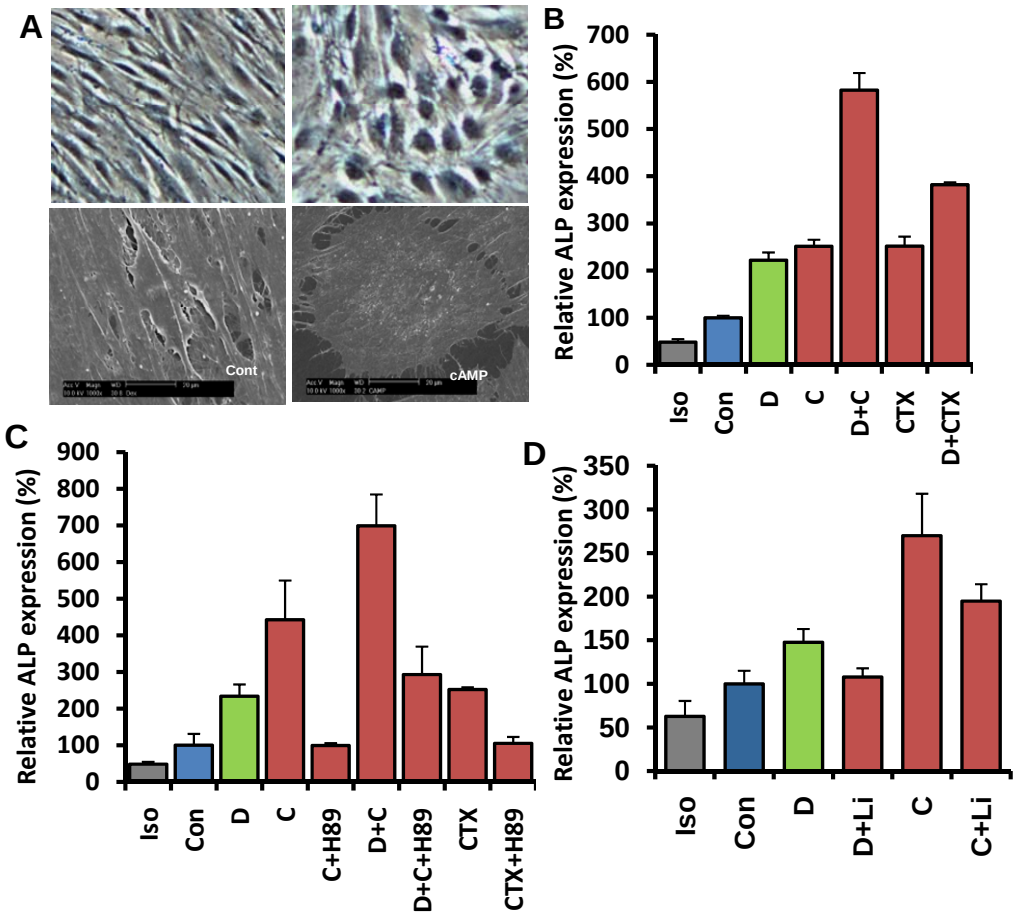

Figure 1. Effect of PKA activation on hMSC biology. A. Effect of PKA activation on hMSC morphology. *Note the change in morphology from fibroblastic in the control (cont) to cuboidal, osteoblast morphology in the cAMP treated hMSCs (upper panel). The cell morphology is further substantiated by scanning electron microscopy (lower Panel).* ***B. Effect of PKA activation on ALP induction.*** *ALP expression by hMSCs treated with cAMP or in combination with dex analyzed by FACS and expressed relative to controls. Error bars indicate standard deviation.* ***C. H89, a PKA inhibitor reverse the PKA induced ALP expression.*** *hMSCs were pre-incubated with H89 for 10 to 15 hours and then co-treated with cAMP for 4 days. Note, that H89 reduces cAMP and CTX induced ALP expression to basal level.* ***D. GSK-3 inhibition partially reduces PKA-induced ALP expression.*** *hMSCs were pre-treated with LiCl for 10 to 15 hours and then co-incubated with cAMP for four days. LiCl partially reduces the cAMP induced ALP expression. Iso, Isotype control; Con, untreated cells; C, cAMP; D, dex; D+C, dex and cAMP; CTX, cholera toxin; CTX+dex, cholera toxin and dex; C+H89, cAMP and H89; CTX+H89, cholera toxin and H89; D+Li, dex and LiCl; C+Li, cAMP and LiCl;*

To further demonstrate that PKA activation stimulates osteogenesis, we treated hMSCs with cAMP for 3 to 15 days and analyzed the expression of the marker genes *ALP*, *COL1* and osteocalcin (*OC*). We found that *ALP* and *COL1* expression increased progressively in both dex and cAMP treated cells, reaching a maximum on day 5 (Figure 2A). *OC* expression did not significantly change in cells treated with dex or dex and cAMP, but we found a 5-fold upregulation in cells treated with cAMP alone for 5 days. In addition, we analyzed the effect of PKA activation on mineralization. We treated hMSCs for 3 to 30 days and noticed that the treatment with cAMP alone did not trigger mineralization (data not shown). However, cAMP was able to induce a drastic increase in dex-induced mineralization (Figure 2B). Exposure of cells to cAMP during the first 3 to 15 days of the culture significantly enhanced dex-induced mineralization and calcium deposition (Figure 2B) with maximum mineralization in cells exposed to cAMP for 5 days. In contrast, continuous treatment with cAMP for the whole 30-day period resulted in profound cell death and pathological mineralization (data not shown).

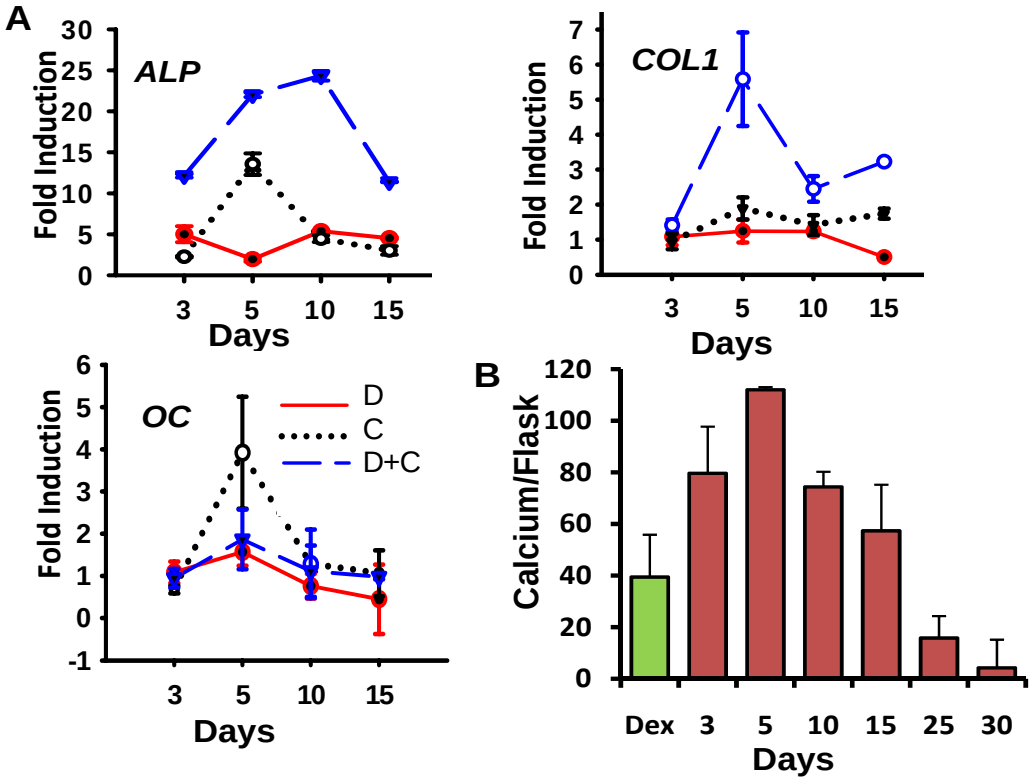

Figure 2. PKA activation induces osteogenic differentiation of hMSCs. A. PKA activation induces expression of osteogenic markers. *hMSCs were either untreated, treated with cAMP, dex or cAMP and dex together for 3, 5, 10 and 15 days. RNA was isolated and expression of ALP, COL1 and OC were analyzed by qCR and expressed as fold induction compared to untreated control cells.* ***B. Short term PKA activation enhances dex induced mineralization.*** *hMSCs were incubated with cAMP for the first 3, 5, 10 15, 25 or 30 days of cultured for the remaining period in osteogenic medium until day 30. At the end of the culture period total calcium deposition was assayed.*

Activation of BMP target gene expression by PKA

Knowing that PKA pathway activation results in enhanced *in vitro* osteogenesis and mineralization of hMSCs, we were interested in the molecular mechanism downstream of CREB activation. To understand the molecular steps leading from PKA activation to mineralization, we performed a microarray experiment on hMSCs which were treated with cAMP for 6 hours. Gene expression data demonstrate the upregulation of typical PKA target genes like *CREM*, a transcriptional activator or repressor by binding cAMP Response Elements (CRE). Previous records show that PKA activation induces the expression of various cytokines including *IL-11* which is consistent with our observation and its role in osteogenic differentiation has been demonstrated[32, 33]. Among the genes regulated by cAMP treatment we were particularly interested in the up regulation of BMP target genes *ID2*, *ID4* and *SMAD6* expression by 8, 11 and 5 fold respectively (Table 3).

Table 3. Selection of genes regulated by cAMP treatment.

Gene	**Fold regulation**
Osteogenic	
Bone morphogenetic protein 2	3[a]
Growth differentiation factor 1	4
Growth differentiation factor 3	14
Growth differentiation factor 10	4
Bone morphogenetic protein 6	8
Bone morphogenetic protein 8	4
IGF1	19
IL-11	15
ALP (Bone specific)	2
ID4	11
ID2	8
ID1	2
SMAD6	5
v-fos osteosarcoma viral oncogene (FosB)	14
cAMP responsive element modulator(*CREM)*	6
Chondrogenic	
growth differentiation factor 5	-3
SOX4	3
Chondroitin sulfate proteoglycan 4	-3
collagen, type IV, alpha 4	-3
Adipocyte markers	
fatty acid binding protein 4	-3
C/EBP	-2
Myogenic markers	
MyoD family inhibitor	2
MADS box transcription enhancer factor 2,	-11

[a]gene regulation expressed as fold up or down (-) compared to untreated cells.

To validate the microarray results we treated hMSCs with cAMP for 6 hours and studied BMP target genes *ID2* and *SMAD6* expression by qPCR and found significant upregulation by 14 and 3-fold respectively (Figure 3A). However upregulation of another BMP target gene, *ID1* was negligible. Together, this suggests that PKA activation leads to direct activation of a subset of BMP target genes. Interestingly, a number of TGF-b family members, such as *BMP2, BMP6, BMP8, GDF1, GDF3* and *GDF10* are up regulated more than 3-fold (Table 3), which provides the alternative possibility of indirect activation by upregulation of TGF-β family members and autocrine activation of BMP target genes.

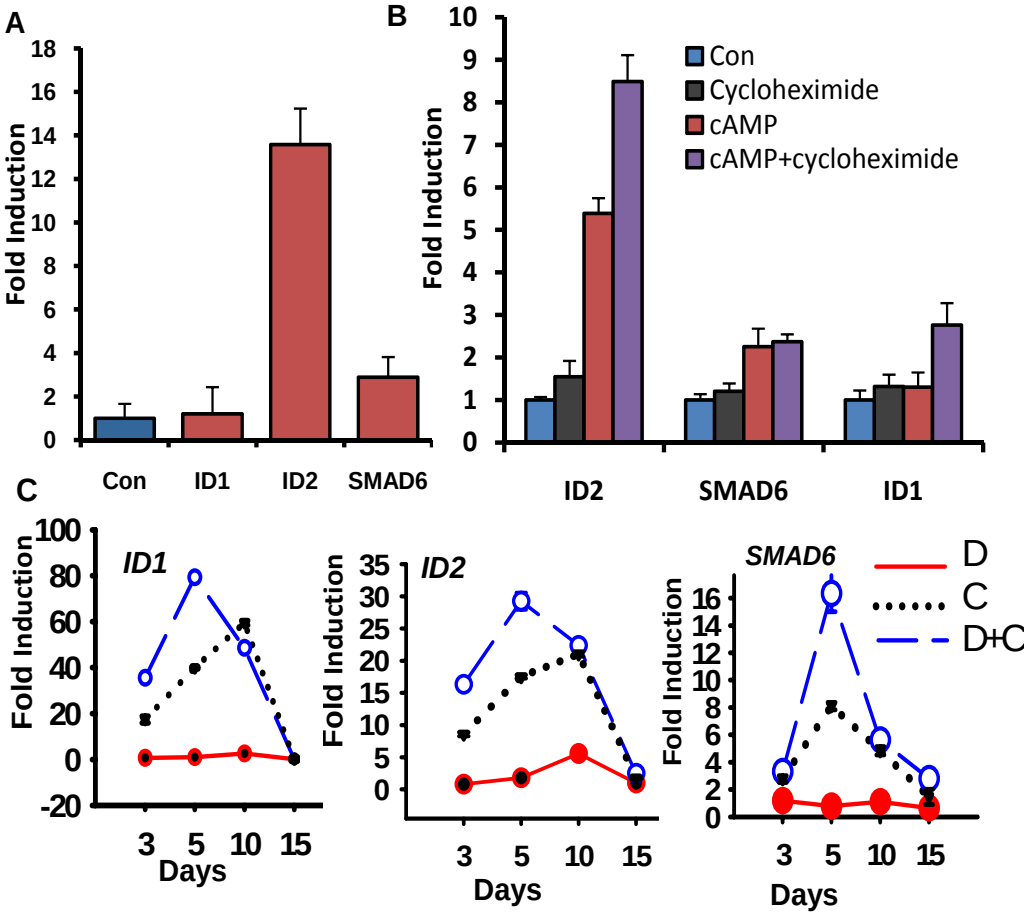

Figure 3. PKA activation induces BMP target genes A. Induction of BMP target genes by PKA activation. *hMSCs were incubated with or without cAMP for 6 hours. Expression of ID1, ID2 and SMAD6 were analyzed by qPCR and expressed as fold induction compared to respective controls.* ***B. PKA induced BMP target genes expression is Cycloheximide (Ch) insensitive.*** *hMSCs were incubated with cycloheximide for 1 hour before addition of cAMP and then co-incubated with cAMP for a further 6 hours. Expression of BMP target genes was analyzed as explained in materials.* ***C. Dynamics of PKA induced BMP target gene expression.*** *hMSCs were incubated with cAMP or 10^{-8} M dex together for 3, 5, 10 till 15 days with respective controls. Expression of ID1, ID2 and SMAD6 were analyzed by qPCR and expressed as fold induction compared to respective controls.*

To investigate whether BMP target genes are activated directly or indirectly, we incubated hMSCs with the inhibitor of translation cycloheximide for 1 hour and then co-treated the cells for another 6 hours with cAMP. We found up regulation of BMP target genes, which were insensitive to cycloheximide indicating that protein synthesis is not required for cAMP-induced transcription of *ID2* and *SMAD6* (Figure 3B). We next studied the dynamics of *ID1*, *ID2* and *SMAD6* expression in the course of a 15 days period upon treatment with cAMP, dex or cAMP and dex together. We found that the expression increases progressively and peaks between 5 and 10 days and then declines to the basal level. Interestingly, we noticed an additive induction of BMP target genes with dex. However we found no effect of dex alone (Figure 3C). The relative late peak of BMP target gene expression suggests that, in addition to direct activation, the genes are activated indirectly as well. Because we mentioned a 3-fold increase of *BMP2* expression after 6 hours of cAMP incubation, we anticipated an involvement of BMPs in late-phase BMP target gene expression. To verify this, we added an inhibitor of BMP, noggin, to cultures of cAMP-treated hMSCs. As shown in Figure 4A, noggin partially reduced *ID2* and *SMAD6* expression. These studies indicate that the PKA pathway induces the BMP target genes both directly mediated via CREB and indirectly by stimulating BMP secretion.

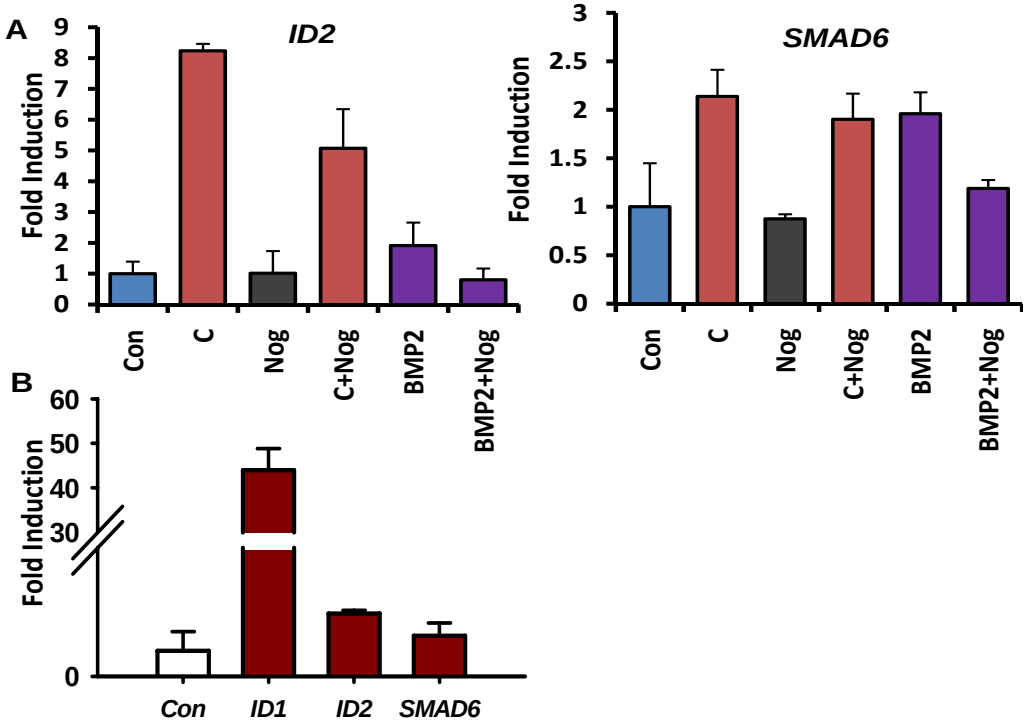

Figure 4. Noggin partially inhibits PKA induced BMP target genes. ***A****. hMSCs were pre-incubated with noggin for 10 to 15 hours then cultured with cAMP or BMP2 for 4 days. Expression of ID2 and SMAD6 was analyzed by qPCR and expressed as fold induction compared to respective controls.* ***B.*** *cAMP treatment of MG-63 a human osteosarcoma cell line for 4 days induces the expression of a subset of BMP target genes. Con, Control; Nog, Noggin; C+Nog, cAMP and noggin; BMP2; BMP2+nog, BMP2 and noggin.*

We also tested the effect of PKA activation in MG-63 cells, a human osteosarcoma cell line and observed that PKA activation for 4 days with cAMP enhanced a subset of BMP target genes in consistence with hMSCs (Figure 4B).

PKA activation enhances in vivo bone formation by hMSCs

Because PKA stimulates hMSC osteogenic differentiation and enhances secretion of pro-osteogenic growth factors, we tested their bone forming capacity by ectopic implantation in immune-deficient mice. We followed two approaches that we currently use in bone tissue engineering. In the first approach, hMSCs were seeded on porous BCP ceramic particles, cultured for 5 days in either basic medium, basic medium supplemented with cAMP, osteogenic medium or osteogenic medium supplemented with cAMP and then implanted subcutaneously in immune-deficient mice. We found a considerably higher incidence of bone formation in cells grown in osteogenic medium with cAMP than in any other group. Bone tissue was observed in 5 out of 10 mice in the control group, 4 out of 10 mice in osteogenic group, 4 out of 5 mice in the cAMP treated group and 8 out of 9 mice in the osteogenic plus cAMP treated group (Table 4 and Figure 5A).

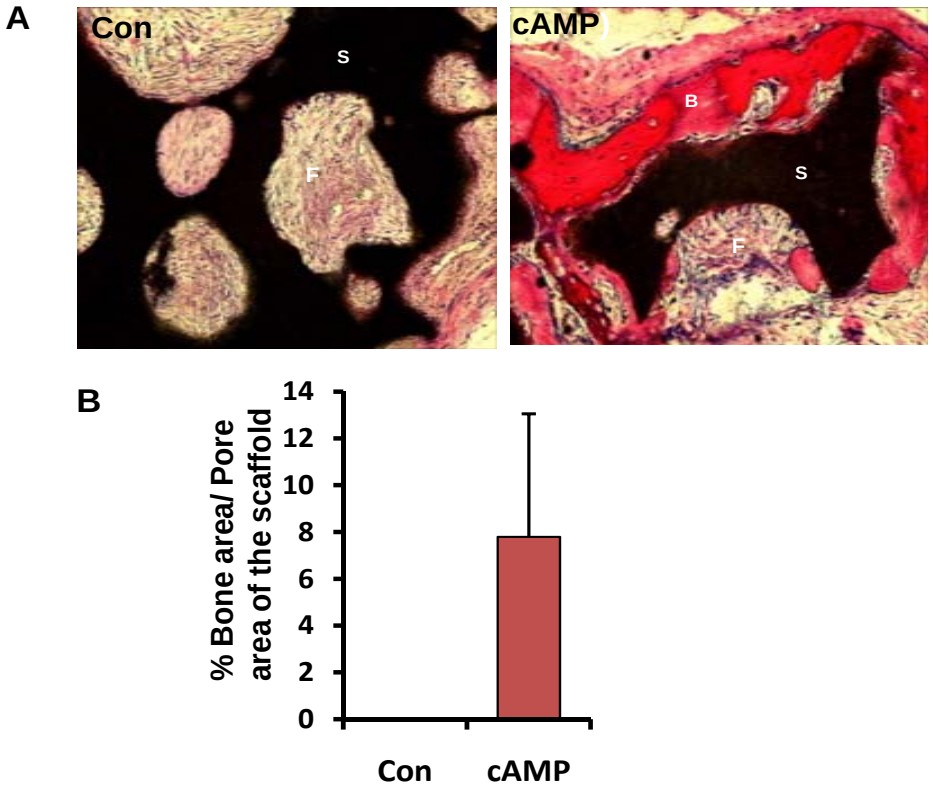

Figure 5. PKA activation induces in vivo bone formation. A. *Representative histological sections of bone formation in nude mice by PKA activated hMSCs grown in basic medium (Con) or PKA activated hMSCs (cAMP) after 6 weeks of implantation. S; Scaffold material, F; Fibrous tissue and B; Basic fuchsin stained newly formed bone.* ***B.*** *Histomorphometric quantification of newly formed bone by cAMP treated hMSCs.*

Table 4. Incidence of bone formation by PKA-induced hMSCs.

Approach	**Approach I**	**Approach II**				**II a**
Donors	1	2	3	4	5	6
Con	5/10[a]					0/6
Dex	4/10	0/6	0/6	0/6	0/6	
cAMP	4/5					6/6
Dex+cAMP	8/9	5/6	0/6	3/6	2/6	

[a]Incidence of bone formation represented as 5 mice out of 10 were positive for bone formation.

This indicates that cAMP treatment enhances the incidence of bone formation. To confirm this, we used a second approach, in which hMSCs from four different donors were cultured in osteogenic medium or osteogenic medium supplemented with cAMP in tissue culture flasks and seeded at 200,000cells/particle 3 hours prior to implantation. The second approach was evidently more stringent on the incidence of bone formation, because this time, none of the cells in the osteogenic group displayed bone formation. Strikingly, 5 mice out of 6 mice implanted with cells from donor 2, 3 out of 6 mice from donor 4 and 2 out of 6 mice with cells from donor 5 displayed bone formation in the cAMP-treated group (Table 4). Cells from donor 3 were unable to produce bone in either condition. In an another approach (Approach II a, Table 4), we tested the effect of PKA activation on *in vivo* bone formation by treating the cells according to the previous protocol but this time, cells were cAMP-treated or untreated. Remarkably we observed bone formation in 6 out of 6 mice and no obvious bone formation in the untreated group. Bone histomorphometric analysis showed 8 to 10% of the total pore area was covered with newly formed bone (Figure 5B). These studies evidently demonstrate that PKA activation in hMSCs enhances bone formation.

Discussion

PKA activation stimulates osteogenic differentiation of hMSCs

Many GPCRs which activate PKA are expressed by different osteoprogenitor and osteoblast cells types[22] and GPCR ligands have an anabolic or catabolic effect on bone formation depending on the dose and duration. A large number of GPCRs are upregulated during osteogenic differentiation of hMSCs (K. Dechering, personal communication) whose *in vivo* and *in vitro* functions, molecular mode of action and intertwining mechanisms with other pathways during osteogenesis remains a challenging area for further research. Therefore we undertook this study to delineate downstream of GPCRs by activation or inhibition of PKA in hMSCs. We studied the effect of PKA activation using cAMP and other PKA activating compounds. The first observation was the appearance of an osteoblast-like morphology. Furthermore, cAMP induces a reversible inhibition of proliferation. Our microarray studies show that cAMP induces the expression of the growth arrest specific 1 (*GAS1*) gene by 17-folds. *GAS1* is known to inhibit cell proliferation when over-expressed in proliferating fibroblasts[34], providing a valid explanation for the reduction in proliferation in PKA-activated hMSCs. The observation that PKA activation as short as 3 days is suf-

ficient to stimulate osteogenesis suggests that PKA has a role in early osteogenesis, which is substantiated by our observation that PKA activates BMP. We are currently investigating the minimal dose and duration of PKA activation to stimulate osteogenesis. Interestingly, short term PKA activation did enhance dex-induced mineralization and calcium deposition but treatment with cAMP alone failed to initiate mineralization. This is consistent with the idea that a role of cAMP in stimulating BMP because BMP2 fails to initiate mineralization of hMSCs but it stimulates dex-induced mineralization[35]. In conclusion, PKA activation enhances dex-induced osteogenic differentiation of hMSCs via interacting cross talk with BMP. On the other side of the spectrum, we observed that long term activation (> 15 days) was lethal to the cells. From these data we anticipate that activation of PKA during osteogenesis is tightly regulated during early phases of osteogenic differentiation. We also tested a range of GPCR ligands such as PTH, PTHRP, melatonin, calcitonin and calcitonin gene related peptide, which are known to influence osteogenesis in different cell types. We found no effect on proliferation or ALP expression in hMSCs (data not shown) suggesting that hMSCs are not responsive to GPCR ligands. We have preliminary evidence that incubation of hMSCs with a selection of GPCR ligands does not stimulate cAMP production. We are currently investigating molecular events upstream of PKA activation.

Synergistic induction of osteogenesis by glucocorticoid and PKA pathways

*D*ex, is a glucocorticoid known to inhibit osteogenesis at higher doses[36] and stimulate osteogenesis at lower concentration in hMSCs[5]. Therefore we analyzed whether dex has any synergism in the induction of osteogenic marker expression with cAMP. Our data clearly demonstrate that dex synergistically induces osteogenesis. The expression profile of some of the osteogenic marker genes such as *ALP*, *COL1* and *OC* showed different expression peaks between day 5 and 10 in different donors, demonstrating donor dependent variation in response to cAMP (data not shown). Consistently, we observed that cAMP treatment alone induced higher expression of *OC* which was inhibited by dex (Figure 2A). Other studies have demonstrated that the *OC* promoter region contains a CREB/ATF site binding site[37, 38] . The data further supports glucocorticoid inhibition of *OC* transcription[36], supporting our observation of cAMP induced expression of *OC*. These studies demonstrate that dex and PKA synergistically enhances the expression of osteogenic marker in which dex provides an essential signal to commence mineralization *in vitro*.

Functional intertwining between PKA and BMP pathways.

BMP2 is a potent inducer of osteoblast differentiation *in vitro* and *in vivo* via inducing its target genes, such as *RUNX2* and the *ID* genes. Our experiments provide evidence that PKA stimulates osteogenesis by impinging on the BMP pathway. First of all we show that the PKA activation directly induces the expression of BMP target genes irrespective of BMP pathway activation. Previous studies show that the promoter regions of BMP target genes, *ID2* and *SMAD6* have CREB binding site[39-41]. Consistently, we show that BMP target genes are directly activated upon PKA activation. Secondly, we show that a number of *BMP*s are upregulated as soon as 6 hours after stimulation with cAMP including *BMP*2. Earlier promoter studies identified

CREB binding sites in the *BMP2* promoter region[42]. Third, we observed that BMP target gene *ID1* was not induced directly by cAMP. However its late peak expression demonstrates indirect induction by cAMP via an intermediate step involving BMP production.

PKA activation induces osteogenic growth factors and pro-osteogenic cytokines.
PKA activation induces *c-FOS* gene expression, an *AP-1* family member of transcription factor and its anabolic effect on bone formation has been demonstrated *in vitro* and *in vivo* models. Over expression of *FosB* has been demonstrated to increase bone formation and inhibit adipogenesis[43-45]. *c-FOS* is upregulated by 14-folds in cAMP treated hMSCs signifies the enhanced osteogenesis *in vitro* and *in vivo*. Microarray analysis further substantiates the regulation of genes and transcription factors of other lineages. Chondrocyte markers such as *GDF5*, Proteoglycon 4, *COL* type 4 were down regulated at least by 3 fold, whilst *SOX4* which is a late marker for hypertropic cartilage is upregulated by 3 times. Adipocyte markers, *C/EBP* and Fatty acid binding protein 4 were down regulated by 2 and 3 folds respectively. A myogenic marker such as MADS box transcription enhancer factor 2 was down regulated by 11 folds (Table 3). These data further support that PKA activation in hMSCs induces osteogenic differentiation whilst inhibiting commitment into other lineages.

In addition, PKA activation induces the expression of other growth factors such as *BMP2, IGF1* and other TGF-β super family members such as *BMP6*, *BMP8*, *GDF1*, *GDF3* and *GDF10* which have been implied in osteogenesis. Besides BMPs, IGF1 is a key growth factor secreted by bone cells and is presumed to act as an autocrine regulator of bone formation. IGF1 increases COL1 synthesis and inhibit collagenase expression by osteoblasts thus increasing matrix apposition and decreasing collagen degradation[46]. Localized IGF1 release from biodegradable scaffolds has been proven to enhance *in vivo* bone formation[47]. Furthermore, PKA activation induces pro-osteogenic cytokines such as *IL-11*. IL-11 induces osteogenic differentiation of mouse MSCs and C3H10T1/2cells[48]. *In vivo* studies further demonstrate that PKA activated hMSCs enhance *in vivo* bone formation substantially covering up to 10% of the total pore area of the scaffold. Taken together, the results demonstrate that PKA not only directs the cells into osteoblast lineage by directly activating BMP target genes but also induces the expression of a range of pro-osteogenic growth factors which in turn triggers osteogenesis in both autocrine and paracrine fashion. Thus, uncommitted neighbouring mesenchymal cells are triggered into osteogenic lineage (Figure 6).

Perspective towards improved bone tissue engineering
From a clinical perspective, understanding the intertwining crosstalk between different pathways during osteogenic differentiation and their *in vivo* effect on bone formation will aid to improve bone tissue engineering applications. Our microarray studies and in *vivo* studies demonstrate that PKA enhances *in vitro* osteogenesis and *in vivo* bone formation. Collectively these studies validate enhanced *in vivo* bone formation by PKA activated hMSCs. In view of the fact that hMSCs are being used to investigate fundamental cell biological processes and signal transduction pathways it short-

ens the gap between basic research and clinical applications. This opens door the door its application in bone tissue engineering.

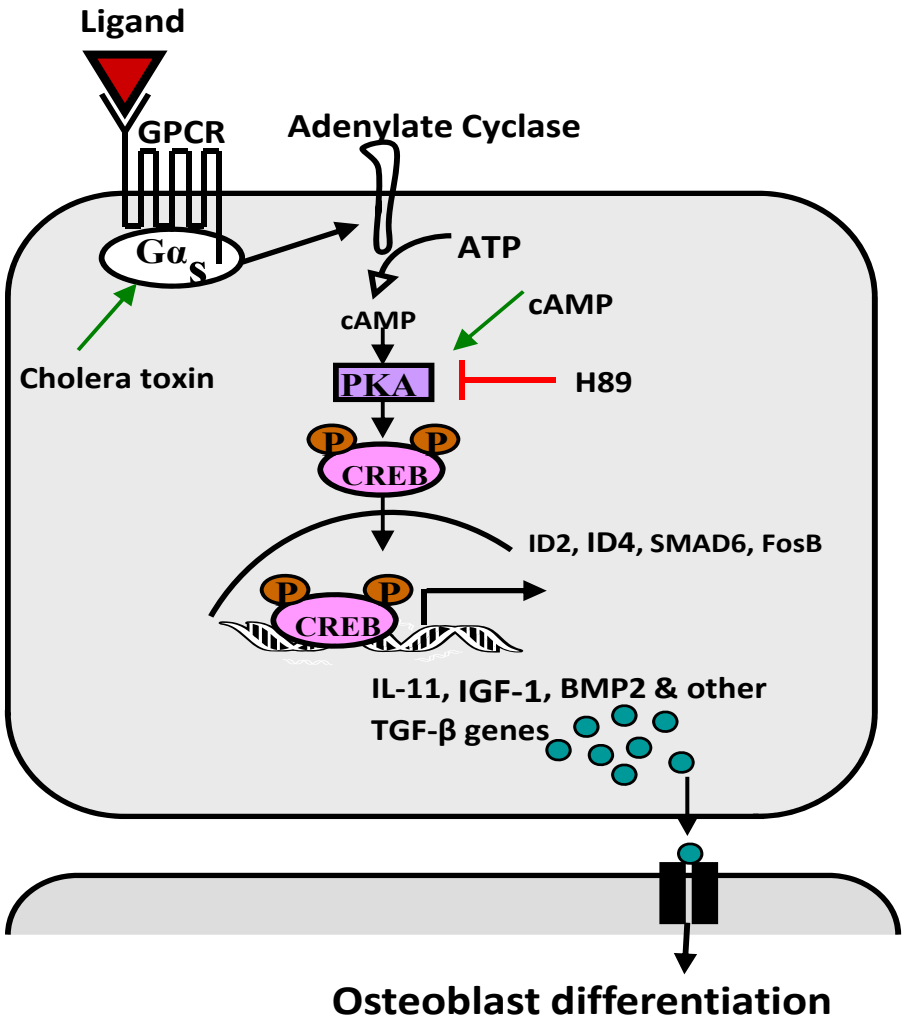

Figure 6. Direct and indirect induction of osteogenesis by PKA activated hMSCs.
Activation of the PKA pathway induces direct expression of BMP target genes such as ID2 and ID4 via CREB resulting in cell autonomous stimulation of osteogenesis. In addition expression of BMP2 and pro-osteogenic cytokines, growth factors is enhanced resulting in paracrine induction of bone formation.

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Chapter 7

A multidisciplinary approach to produce clinically relevant amount of bone by human mesenchymal stem cells

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In preperation

Chapter 7 A multidisciplinary approach for bone tissue engineering

Abstract

A key concern in cell-based bone tissue engineering is the limited amount of bone formation in tissue-engineered constructs. We report here a multidisciplinary approach to augment the in vivo bone forming capacity of hMSCs by culturing them in a perfusion-based dynamic bioreactor to optimize nutrient availability during culture. Furthermore, we optimized osteogenic differentiation of the cultured cells, by supplying them with cyclic AMP at the end of the culture period. As expected, cAMP inhibited proliferation both in static and dynamic conditions. cAMP efficiently upregulated the early osteogenic marker *ALP* in both static and dynamic condition. When the cells were implanted subcutaneously in immuno-deficient mice, newly formed bone covered up to 25 % of the total pore area available for bone growth in the dynamic-cAMP group compared to 2-8% bone formation in the other conditions. The improved bone formation by a combination of dynamic culturing and addition cAMP seems a highly efficient method to boost the bone-forming capacity of human mesenchymal stem cells and brings us two steps closer to clinical evaluation of bone tissue engineering.

Key Words: *Perfusion bioreactor, human mesenchymal stem cells, PKA signaling, and bone tissue engineering.*

Introduction

The easy isolation procedure and the potency of human mesenchymal stem cells (hMSCs) to differentiate into adipogenic, chondrogenic, myogenic, neurogenic and osteogenic lineages has generated a remarkable interest for their effective use in regenerative medical applications[1, 2]. Cell-based bone tissue engineering includes isolation of hMSCs, *in vitro* expansion, seeding onto osteo-inductive scaffold materials and implanting tissue engineered construct back into the patient to heal a bone defect. hMSCs are typically isolated from bone marrow and other sources such as adipose tissue and placenta[3-5]. When hMSCs are seeded onto porous bioceramics and implanted in animal models, they are able to repair small experimentally induced osseous defects[6, 7]. Furthermore, the bone forming ability of these cells were also tested in large animal models to mimic a clinical situation[8, 9]. Although some clinical trials using hMSCs showed a favorable outcome in fracture healing[10], a common problem seems to be that the amount of newly formed bone is insufficient to fully bridge the implant [10-12]. Current studies demonstrate that pre-differentiation of MSCs *in vitro* into the osteogenic lineage before implanting, augments the *in vivo* bone forming capacity of the cells[13, 14].

The differentiation of multipotent MSCs into a matured osteoblast requires a spectrum of signaling proteins including morphogens, hormones, growth factors, cytokines, matrix proteins, transcriptions factors and their co-regulatory proteins[15-17]. Currently, dexamethasone is commonly used to initiate the osteogenic process in hMSCs, thus ignoring the multiple signaling pathways that control osteogenesis.

Therefore, understanding the osteogenic process regulated by various signaling cues in time is important to augment the biological activity of hMSCs. In this milieu, we focus on the molecular cues that stimulate *in vitro* proliferation and differentiation, which in turn improve vivo bone formation. We have reported that stimulation of the Wnt signaling pathway and histone deacetylase inhibitors such as Trichostatin A can be used as a tool to enhance proliferation and differentiation of hMSCs, respectively[18-20]. In addition, we recently demonstrated that protein kinase A (PKA) activation in hMSCs using 1 mM cyclic adenosine mono phosphate (cAMP) consistently enhances *in vitro* osteogenesis and *in vivo* bone formation by hMSCs[21]. Another way to stimulate osteogenesis is through mechanical strain and fluid shear stress [22, 23]. *In vivo*, osteoblasts and osteocytes experience interstitial fluid shear stress upon mechanical loading of bone through fluid flow inside the canalicular–lacunar and trabecular spaces within bone tissue[24, 25]. To mimic the *in vivo* mechanical stimulation that cells feel, researchers have developed various kinds of 3D perfusion bioreactors with defined mechanical stimulations. Fluid shear force caused by a perfusion bioreactor system enhances osteogenic differentiation and mineral deposition, suggesting that the mechanical stimulation provided by fluid shear forces in 3D flow perfusion culture induces the osteoblast phenotype. Increased fluid shear forces also resulted in the generation of a better spatially distributed extracellular matrix emphasizing the importance of mechanosensation on osteoblast differentiation in a 3D environment[26].

We have recently reported that cell growth can be effectively monitored in time in a perfusion bioreactor system [27, 28]. The outstanding effect of fluid shear stress on osteogenic differentiation has been also demonstrated in various other cell types. Culturing rat primary calvarial cells in 3D dynamic flow conditions enhanced cell distribution, early osteogenic marker alkaline phosphatase (ALP), osteocalcin, osteopontin expression and *in vitro* mineralization compared to static condtions[29]. Moreover, when rat MSCs where cultured in a perfusion bioreactor they showed enhanced osteogenic differentiation and calcium deposition compared to counterpart static groups[30]. The earliest report about hMSCs in a perfusion bioreactor is by Koller et al[31] followed by a number of studies showing ectopic bone formation by hMSCs implanted subcutaneously in nude mice[32]. As mentioned earlier, the limited capacity of hMSCs to produce clinically relevant amount of bone and our phase I clinical trial with inadequate bone formation by hMSCs emphasizes the need to improve bone forming ability of hMSCs by supporting osteogenesis at multiple stages of differentiation[11].

Besides efficient control of osteogenic differentiation, the production of bone grafts of clinically relevant size faces another problem. The cells which are deep inside the construct would have insufficient gas and nutrient supply leading to limited survival of the cells in culture and construct failure. In this view, reports demonstrate that culturing these cell-seeded constructs or materials in dynamic conditions with controlled gas and nutrient increases nutrient availability and stimulates cel survival. Besides, bioreactor based technology cuts down the cost and labor consuming *in vitro* cell-culture protocols[27-29].

In this work, we aimed to combine the beneficial properties of fluid shear stress on osteogenic differentiation with the previously reported effect of cAMP on *in vitro* differentiation and bone formation *in vivo*. For clinical application of cell-based bone tissue engineering, the physician would require the readily available tissue engineered constructs at the site of the surgery. As an initial step to bring the technology from bench to bedside, we combined the molecular mechanisms to regulate osteogenesis of hMSCs with controlled culture conditions. We report here the synergistic effect on hMSCs osteogenesis and *in vivo* bone formation of culturing hMSCs in perfusion flow with cAMP. This opens a window towards successful clinical application of bone tissue engineering using hMSCs by combining mechanical and molecular cues.

Materials and Methods

Isolation and culture of hMSCs

Bone marrow aspirates (5-20 ml) were obtained from donors with written informed consent. hMSCs were isolated and proliferated as described previously[33]. Briefly aspirates were re-suspended using 20 G needles, plated at a density of $5x10^5$ cells/cm^2 and cultured in hMSC proliferation medium containing a-minimal essential medium (a-MEM, Life Technologies), 10% fetal bovine serum (FBS, Cambrex), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), 10 mg/ml streptomycin (Life Technologies), and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week and cells were used for further sub-culturing or cryopreservation upon reaching near confluence. The frozen P0 cells were expanded and seeded in proliferation medium at 200,000 cells/particle (3 particles per condition) onto 2-3 mm biphasic calcium phosphate (BCP) particles prepared and sintered at 1150° C as described previously[34]. A day after seeding, the particles with cells were either cultured in static conditions or transferred to bioreactor. First 5 days the cells were cultured on BCP particles in proliferation medium and further 4 days the cells were treated with or without 1 mM cAMP (sigma).

Bioreactor and bioreactor system

A direct perfusion flow bioreactor was used as described previously[28]. Briefly, the bioreactor comprises an inner and outer housing, which are configured as coaxially disposed, nested cylinders. The bioreactor system consisted of a bioreactor, a sterile fluid pathway (made of g sterilized PVC tubing with low gas permeability) that includes a medium supply vessel, a pump, an oxygenator and a waste vessel. The fluid pathway contains a temperature sensor and two dissolved oxygen sensors, which are placed at the medium inlet and outlet of the bioreactor. The whole bioreactor system is placed in a temperature controlled unit at 37°C. The incubation units lacks a gas-controlled atmosphere and to supply the cells with oxygen and carbon dioxide an oxy-

genator was developed. The oxygenator comprises a closed chamber containing a gas -permeable silicon tube. The gas environment in the chamber is kept at a constant level of 21% O_2 and 5% CO_2 and medium is pumped through the gas-permeable tube. This system enables a medium flow over and through the cell-seeded biomaterials with constant pH and a constant oxygen concentration. The bioreactor system is depicted in Figure 1.

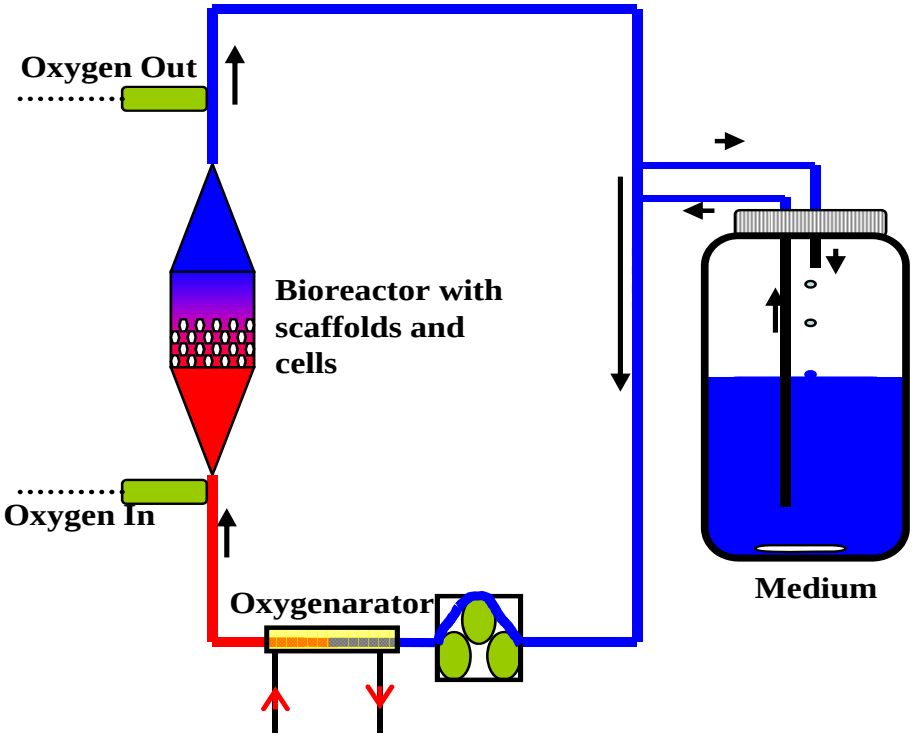

Figure 1. Schematic representation of a perfusion bioreactor used in the study.

Seeding and culturing of hMSCs in static and Bioractor systems

The frozen P0 cells were expanded and statically seeded in proliferation medium at 200,000 cells per three 2-3 mm biphasic calcium phosphate (BCP) particles prepared and sintered at 1150° C as described previously[34]. After 4 hours, 2 ml of proliferation medium was added and the hybrid constructs were incubated statically overnight in a CO_2 incubator. The scaffolds were then divided into two groups: a dynamic and a static group. In the dynamic group, the hybrid constructs are cultured in the bioreactor system described above, whereas in the static group the hybrid constructs are cultured in a non-tissue culture treated 25-well plates at 37°C in a humid atmosphere with 5% CO_2. Subsequently, the cell-seeded constructs were transferred into 2 separate bioreactor systems and medium recirculation was started at 4 ml/min (108 mm/s) using 200 ml of proliferation medium for 3 days. This is referred to as the dynamic condition. Then, medium was refreshed with new medium and one bioreactor was supplemented with 1 mM cAMP whereas the other bioreactor was treated as a control. The cells were further cultured for 4 more days. At the same time, in the static group, hy-

brid constructs were cultured for 3 days under static conditions. At this time point, the hybrid constructs were divided into two groups one of which was supplemented with 1 mM cAMP, whereas the other group was treated as a control and cultured for further 4 days.

RNA isolation and quantitative PCR

The effect of static and dynamic culture systems supplemented with or without cAMP on expression of osteogenic marker genes was analyzed by isolating RNA at the end of the culture period. The RNA was isolated by a Trizol RNA kit (Qiagen) and DNase treated with 10U RNase free DNase I (Gibco) at 37°C for 30 minutes. DNAse was inactivated at 72°C for 15 minutes. Two µg of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. One µl of 100x diluted cDNA was used for collagen type 1 (*COL1*) and 18s rRNA amplification and 1 µl of undiluted cDNA was used for other genes PCR was performed on a Light Cycler real time PCR machine (Roche) using a SYBR green I master mix (Invitrogen).

Table 1. Primer sequences used for qPCR studies.

Gene	Sequence	Product length (bp)
18s rRNA	F -5'cggctaccacatccaaggaa3' R- 5'gctggaattaccgcggct3'	187
Collagen 1	F -5'agggccaagacgaagacatc3' R- 5'agatcacgtcatcgcacaaca3'	138
BSP	F -5'aggttagctgcaatccagc3' R- 5'ccatcatagccatcgtagcc3'	555
Osteopontin(OP)	F -5'ccaagtaagtccaacgaaag3' R- 5'ggtgatgtcctcgtctgta3'	348
Osteonectin(ON)	F -5'actggctcaagaacgtcctg3' R- 5'gagagaatccggtactgtgg3'	438
Osteocalcin (OC)	F -5'ggcagcgaggtagtgaagag3' R- 5'gatgtggtcagccaactcgt3'	138
CBFA1	F -5'ttacttacaccccgccagtc3' R- 5'cagcgtcaacaccattc3'	536
S100A4	F -5'agcttcttggggaaaaggac3' R- 5'ccccaaccacatcaagagg3'	200
Alkaline Phsphotase (ALP)	F -5'gacccttgacccccacaat3' R- 5'gctcgtactgcatgtcccct3'	70
Id1	F-5'gcaagacagcgagcggtgcg3' R-5'ggcgctgatctcgccgttgag3'	346
Id2	F-5'cctcccggtctcgccttcc3' R-5'ggttctgcccgggtctctgg3'	320
Smad6	F-5'gctaccaactccctcatcact3' R-5'cgtcggggagttgacgaagat3'	336

Data was analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes are calculated relative to 18s rRNA levels by the comparative DCT method[35] and statistical significance was found using student's t test ($P<0.05$). The primers used in the study are listed in Table 1.

Real-time oxygen measurement

The oxygen concentration was measured real-time in the medium at the inlet and outlet of the bioreactor as explained in Figure 1. The oxygen electrodes were sterilized before placing them in the system (Applikon, the Netherlands). We calculated the difference in oxygen concentration between the medium inlet and medium outlet (Δ DO) and assumed that with constant specific oxygen consumption (q_o), liquid volume of the bioreactor (V_l) and perfusion flow rate (F_l) it is directly proportional to the biomass concentration.

Cell distribution, load and viability

Cell distribution and cell load on the scaffolds in the bioreactor were qualitatively assesed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) staining method. A solution of 1% MTT was applied on the scaffolds containing cells. After 4 hours, the MTT solution was removed the scaffolds were rinsed with PBS. Scaffolds and cells were visualized using light microscopy. Metabolites in the cultivation medium (glucose, lactate and ammonia) were measured using the Vitros DT 60 medium analyzer (Ortho-Clinical Diagnostics, Johnson and Jahnson).

In vivo bone formation

To evaluate the effect of cAMP and culture condition on *in vivo* bone formation, the tissue engineered constructs which were seeded and cultured in 4 different conditions as explained earlier were implanted subcutaneously in 10 nude male mice (Hsd-cpb:NMRI-nu, Harlan). The mice were anaesthetized by isolflurine inhalation, four subcutaneous pockets were made and each pocket was implanted with 3 particles of each condition. Each mouse was implanted with four conditions namely static control, static cAMP, and bioreactor control and bioreactor cAMP. The incisions were closed using a vicryl 5-0 suture. After 6 weeks the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck) in 0.14 M cacodylic acid (Fluka) buffer pH 7.3, dehydrated and embedded in methyl methacrylate (Sigma) for sectioning. Approximately 10µm thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). The sections were stained with basic fuchsin and methylene blue to visualize bone formation.

Results

cAMP inhibits hMSC proliferation

We have previously demonstrated that cAMP inhibits proliferation, induces osteogenic differentiation *in vitro* and bone formation *in vivo*. To further apply the *in vivo* bone forming capacity of the hMSCs to a clinical setting, we used a multidisciplinary approach by culturing tissue engineered constructs in static and 3D perfusion bioreactor systems in the presence or absence of 1 mM cAMP. We seeded hMSCs onto BCP particles and exposed them to either static or dynamic conditions. First, we allowed hMSCs to proliferate on the scaffolds in proliferation medium for 3 days to allow the cells to completely cover the scaffold and next the cells were further cultured for 4 days in the presence or absence of cAMP. After 7 days, we stained the scaffolds with MTT. As anticipated, cAMP inhibited proliferation as demonstrated by MTT staining of the particles in both static and dynamic conditions while hMSCs cultured in the absence of cAMP showed typical homogeneous cell distribution throughout the scaffolds (Figure 2A). This observation is supported by measurement of oxygen consumption by the cells. The inlet oxygen concentration was kept at a constant level (red line) by saturation of the medium in the oxygenator and the outlet oxygen concentration decreased in time (blue line). The difference in the ingoing and outgoing oxygen concentration shows the oxygen consumption by the cells (DDO, Black line). In dynamic control group, the oxygen consumption increased first three days and due to a technical problem cAMP supplemented bioreactor did not show an increase in oxygen consumption as seen in its inability to maintain a constant inlet oxygen concentration and hence the oxygen consumption is graphically concealed. After addition of cAMP to the bioreactor, DDO in the cAMP group was significantly lower than control bioreactor, confirming cAMP inhibits cell growth. In contrast, DDO increased exponentially in the control group, corresponding to cell growth (Figure 2B). The sporadic peaks indicated by an arrow shows the disturbances caused when the bioreactors are opened for medium refreshments. In line with our earlier observations, MTT assay staining and DDO measurements confirm that cAMP inhibits proliferation of hMSCs. Glucose consumption, lactate production and ammonia production showed no significant differences until the addition of cAMP. After 3 days of cultivation, the medium was refreshed and one bioreactor was supplemented with 1 mM cAMP and the other one was treated as control. cAMP addition resulted in growth inhibition which is reflected by decreased glucose consumption by hMSCs (Figure 3). As a consequence of cell confluence on the scaffolds, the oxygen in the bioreactor was insufficient and the cells took the anaerobic pathway to metabolise glucose as reflected by the higher lactate production in the control group compared to the cAMP-supplemented bioreactor (Figure 3).

Glutamine is an essential amino acid required for protein sytnhesis, nucleic acid biosynthesis and cell growth[36]. Glutamine metabolism in the cells results in the formation of glutamate and ammonia[37]. The total ammonia produced by the cells is an indication of glutamine consumption by the cells which depends on the cell mass. However, if the medium is not refreshed the produced ammonia may have a secondary effects such as growth inhibition and cytotoxic[38]. The higher ammonia production in the control bioreactor indicates the higher cell number compared to cAMP

supplemented group (Figure 3). Taken together, it is obvious that cAMP inhibits proliferation of hMSCs.

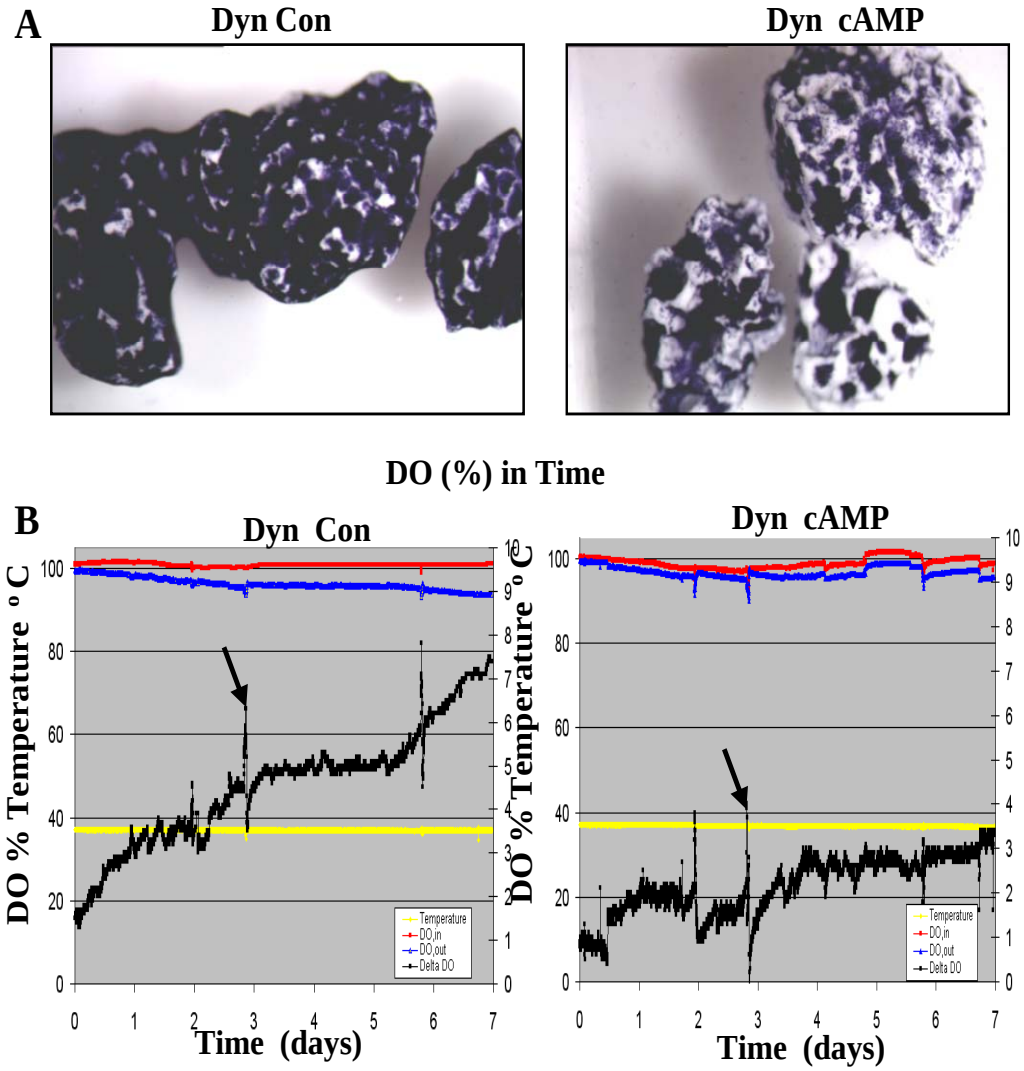

Figure 2. cAMP inhibits hMSC proliferation. A. *MTT staining of the tissue engineered constructs which were cultured in static and bioreactor supplemented with or without 1 mM cAMP. Note the cell number difference in the control (Dyn Con) and cAMP (Dyn cAMP) treated groups. Similar differences were observed in statically cultured groups (not shown here).* ***B.*** *Real –time O2 consumption measurements in the bioreactors. The inlet oxygen concentration was kept at a constant level (red line) by saturation of the medium in the oxyenerator and the outlet oxygen concentration decreased in time (blue line). The difference in the ingoing and outgoing oxygen concentration shows the oxygen consumption by the cells (DDO, Black line). Note the oxygen consumption increases in the control bioreactor (Dyn Con) while DDO stays constant after addition of cAMP to the bioreactor (Dyn cAMP) indicating inhibition of cell growth. The arrows indicate the disturbances caused by medium refreshment regime.*

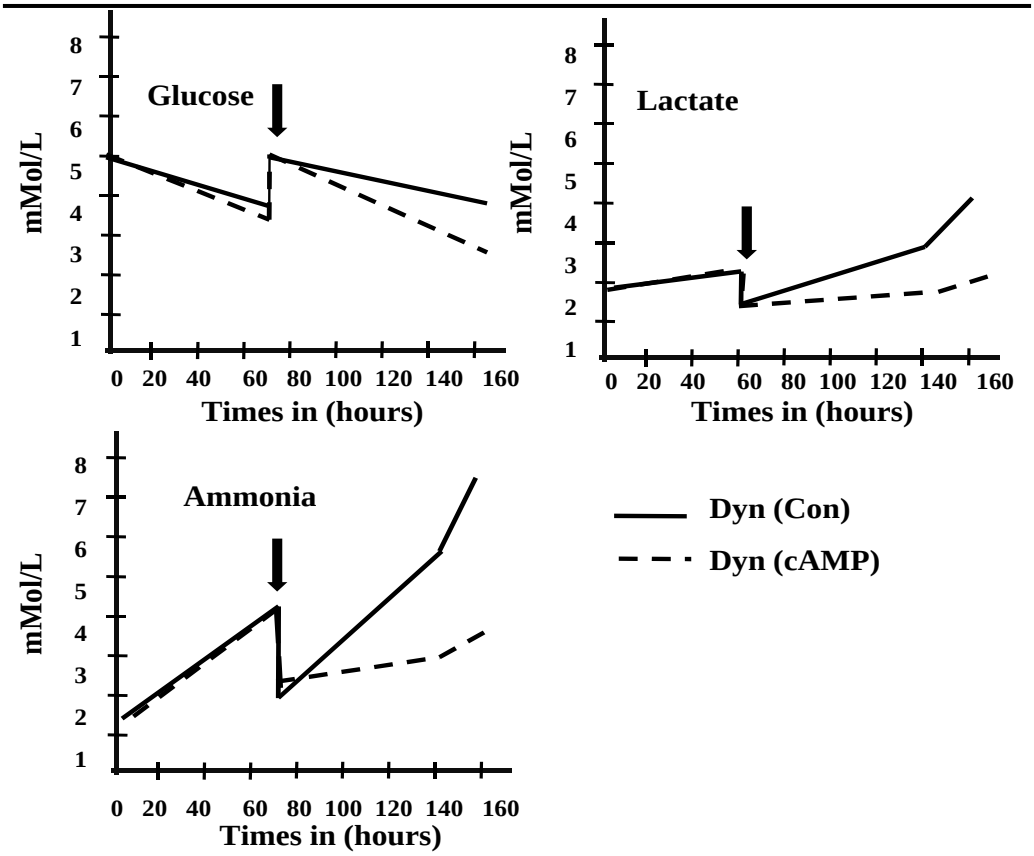

Figure 3. *Nutrient consumption and waste production of hMSCs in control bioreactor (Dyn Con) and cAMP supplemented bioreactor (Dyn cAMP). The black arrow indicates the addition of cAMP to the bioreactor.*

cAMP enhances osteogenesis in vitro

To study the effect of cAMP on *in vitro* osteogenesis, we isolated RNA from the cells in all condition at the end of the culture period and analyzed gene expression by qPCR. As expected, cAMP induced ALP expression in both static and dynamic conditions compared to their respective groups. However, we did not observe a significant difference in ALP expression between static and dynamic conditions, both in control and cAMP-supplemented groups. Furthermore, we observed a significant increase in BMP2 expression in cAMP supplemented conditions. Consequently, the BMP target genes such as *Id1, Id2* and *Smad6* were upregulated which is in line with our earlier observations. Osteogenic specific transcription factor *Cbfa1, collagen type 1, osteocalcin,* calcium binding protein *S100A4* and *osteopontin* expressions were unaffected either by culture conditions or cAMP addition (Figure 4). cAMP seem decreased *osteonectin* expression in static condition, however this decrease was not observed in dynamic condition. In summary, as anticipated, cAMP induced expression of *ALP*, *BMP2* and BMP- target genes *Id2, Id2* and *Smad6,* however no significant differences were found in these gene expression profiles between different static and dynamic culture conditions. In addition, we analyzed extra cellular matrix (ECM)

formation on the tissue engineered constructs before implantation in nude mice. Electron microscopic analysis demonstrated that ECM was formed in all the tissue engineered constructs cultivated in different conditions. No gross differences in ECM formation were observed by electron microscopy (Figure 5).

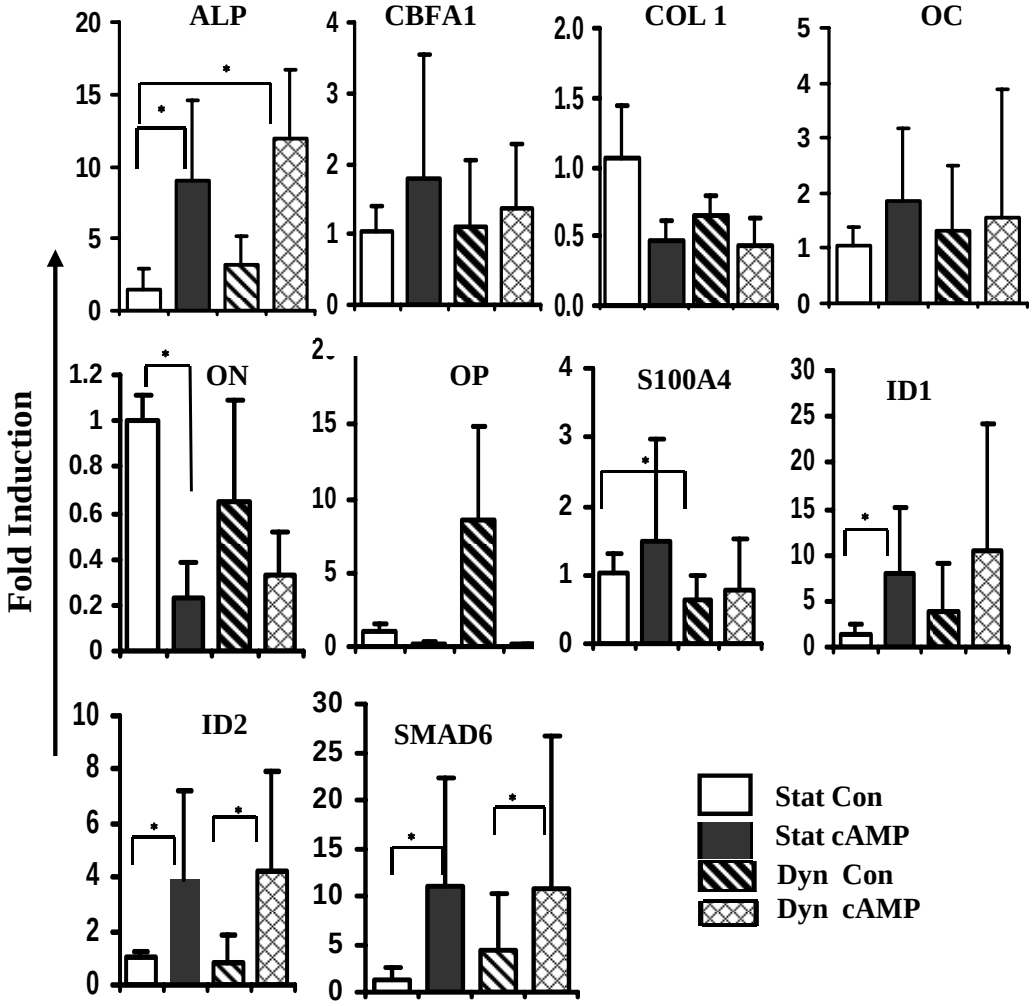

Figure 4. cAMP induces osteogenic differentiation of hMSCs. *Osgeogenic gene expression in tissue engineered constructs cultured in static con (Stat Con), static cAMP (Stat cAMP), dynamic control (Dyn Con) and dynamic cAMP (Dyn cAMP) groups. RNA was isolated from these tissue engineered culture real-time PCR was performed (see materials). The gene expression is indicated as fold induction compared to static control (Stat Con) group and normalized to 18s rRNA. Error bar represent standard deviation.*

cAMP in perfusion bioreactor enhances in vivo bone formation

To further investigate the combined effect of 3D perfusion culture system and cAMP-induced osteogenic differentiation, we implanted the tissue engineered constructs of

all four conditions in nude mice for 6 weeks. Histological analysis demonstrated that addition of cAMP in static condition did not enhance bone formation *in vivo* which is in contrast to our earlier observations.

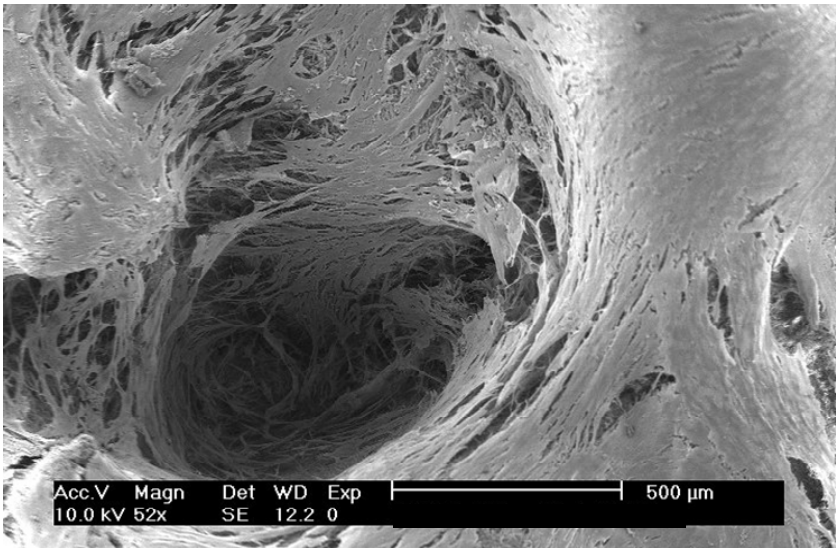

Figure 5. A representative electron microscopic image showing extracellular matrix formation on the tissue engineered constructs.

Culturing hMSCs in a perfusion bioreactor system did not either enhance *in vivo* bone forming capacity significantly compared to static control group. Interestingly, tissue engineered constructs which were cultured in a bioreactor supplemented with 1 mM cAMP significantly enhanced bone formation covering up to 25 % of the available pore area for bone growth (Figure 6A). Another imperative observation is that cAMP in dynamic culture conditions resulted in formation of multiple bone marrow-like structures in most of the sample and such structures were virtually absent in other conditions (Figure 6A right panel, white hollow arrow). In the deposited bone tissue, we could typically see osteocytes embedded in the mineralized bone and osteoblast lining periphery of the newly formed bone (Figure 6A right panel, black arrows). These results indicate that culturing hMSCs in the presence of 1 mM cAMP in a perfusion bioreactor significantly enhances bone formation *in vivo*.

Discussion

After successful isolation of MSCs from the bone marrow and discovering their multipotential ability to differentiate into various lineages[1, 39], there are number of scientific attempts to prove the proof of concept to regenerate bone tissue in small rodent, sheep, dog and goat models[40-45]. Despite, revealing the ability of the MSCs to regenerate bone tissue in animal models, to date there are only few human clinical trials to treat tibial fracture, augmentation of maxilla using hMSCs with moderate outcomes[10, 12, 46].

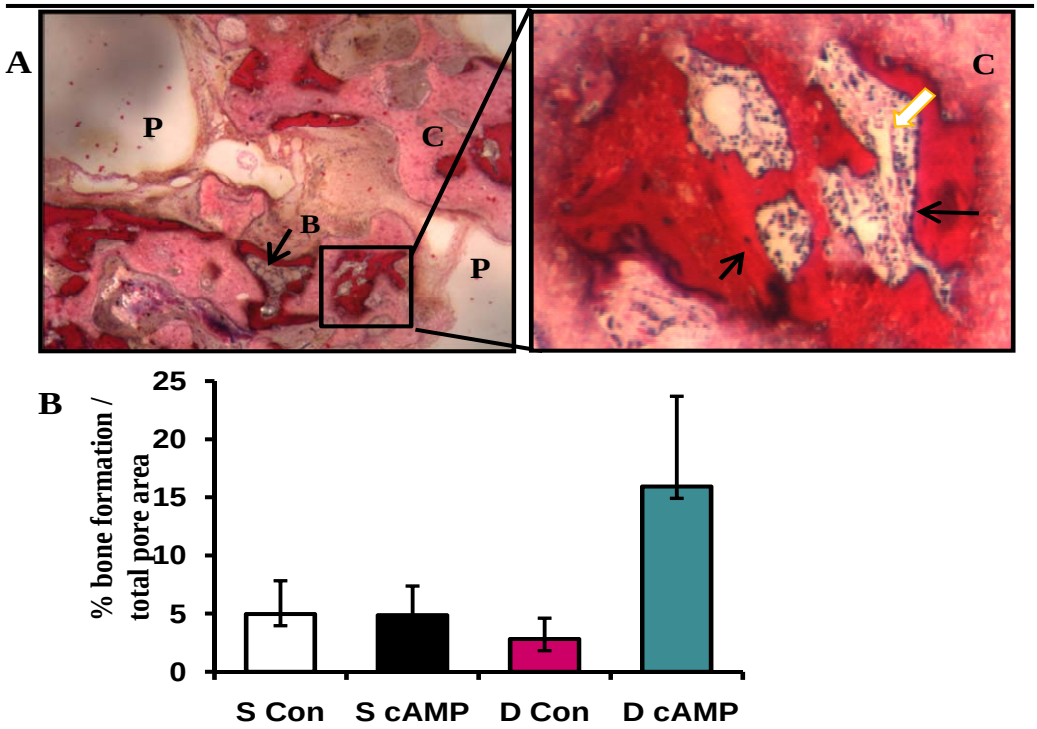

Figure 6. cAMP enhances in vivo bone formation in a perfusion bioreactor culture system. *Tissue engineered constructs cultured in static con (S Con), static cAMP (S cAMP), dynamic control (D Con) and dynamic cAMP (D cAMP) conditions were implanted subcutaneously in nude mice for 6 weeks (seem materials).* ***A.*** *A representative histological sample showing bone formation in a tissue engineered construct (Figure A, black arrow with letter B). C indicates ceramic BCP, P indicates available pore area for the bone growth. Magnified image of a bone marrow formation in Dyn cAMP treated condition (right panel0. Note the osteocytes embedded in the mineralized matrix, lining osteoblasts on the newly formed bone (black arrows) and the bone marrow-like structures (white arrow).****B.*** *Histo-morphometrical analyses for the newly formed bone in various conditions. The newly formed bone was quantified and expressed as percentage bone growth compared to the total available pore area for the newly formed bone.*

Recently, Maracci et al. successfully treated patients with large diaphysis defects and the follow up study for 7 years has demonstrated the clinical success of bone tissue engineering using hMSCs. However, the authors had no negative control and did not demonstrate that the bone tissue formed was by implanted cells[47]. Our recent clinical trial to treat patients with maxillary defects using autologous hMSCs and hydroxyapatite scaffolds demonstrated that the implanted cells were incapable of producing bone to a clinically relevant state[11]. Overview on the clinical attempts using hMSCs suggest that the key point to consider to augment present bone tissue engineering is to enhance the *in vivo* bone forming capacity of the implanted cells. As the isolated MSCs are multipotent, unless the cells get proper inductive signal to differentiate into a particular lineage, they would take a default pathway which would limit the bone forming ability of the cells. Therefore, a simple speculation is that by *in vitro* differentiating the isolated hMSCs into osteogenic lineage would augment the *in*

vivo performance of the cells. Currently, there are a number of osteo-inductive molecules which directs the hMSCs to differentiate into osteogenic lineage such as dexamethasone[48, 49], vitamin D[50-52], Trichostatin A[53] and indeed many bone morphogenetic proteins[13, 54, 55]. Furthermore, we are currently screening over 20,000 molecules for their ability to induce osteogenic differentiation of hMSCs. Recently, we have demonstrated that PKA activation using cAMP induces osteogenic differentiation of hMSCs and consistently induces *in vivo* bone forming ability of hMSCs in nude mice model[21]. hMSCs isolated from various donors tend to show discrepancy in their *in vitro* differentiation and *in vivo* bone forming ability.

Our phase I clinical trial to augment jaw defects using tissue engineered approach yielded insignificant amount of bone to a clinical situation[11], since then we are aiming to improve the *in vivo* performance of hMSCs. Mechanical strain and fluid shear stress are shown to induce osteogenic differentiation[22, 23] which also depicts the *in vivo* conditions that osteoblasts and osteocytes feel[24, 25]. Scientists have developed various kinds of 3D perfusion bioreactors with defined mechanical stimulations and their ability to induce osteogenic differentiation *in vitro* has been demonstrated in comparison with the static conditions[26]. In this milieu, we attempted to produce clinically relevant amount of bone in a perfusion bioreactor effectively controlling and monitoring cell growth and differentiation using goat bone marrow stromal cells[27, 28]. However, with the known over performance of goat and rat MSCs compared to hMSCs limits the extrapolation of the obtained results to a real clinical situation using autologous hMSCs. Therefore, in this study we aimed to augment bone tissue engineering in a multidisciplinary approach by combining cAMP and perfusion bioreactor using hMSCs to closely mimic clinical situation. In line with our earlier observations, cAMP inhibited proliferation both in static and dynamic conditions which typically depicts the inverse relation between proliferation and differentiation[56]. Interestingly, even with less number of cells in cAMP treated tissue engineered constructs, they produced significantly higher amount of bone indicating the amount of the committed cells into the osteogenic lineage is important rather than just the cell load on the tissue engineered constructs. On the hand, it can be further speculated that the presence of a higher load of differentiated functional osteoblasts on the tissue engineered constructs would even enhance the in vivo bone forming capacity of the cells. We are currently investigating to optimize a practical balance of cell load before addition of cAMP to achieve utmost bone formation. Furthermore, we observed no significant differences in osteogenic gene expression profiles and matrix formation between static and dynamic conditions suggesting that mechanical stimuli in combination with cAMP played the key role in enhanced the *in vivo* bone forming capacity. There seem to be a slightly higher but statistically insignificant ALP expression in dynamic condition and mostly other osteogenic genes such as *Cbfa1, collagen type I, osteocalcin, osteonectin*, negative regulator of mineralization *S100A4* showed no significant difference between static and dynamic conditions. Although, we observed a slightly higher induction of BMP2 expression in dynamic conditions, it did not reflect on its target gene expression such as *Id1, Id2* or *Smad6*. Dynamic culturing of hMSCs did not show an increased in vivo bone formation compared to static condition. The mechanical stimulation of the cells by fluid flow has been suggested to impact on intra-

cellular calcium levels[57, 58], nitric oxide signaling[59-61] and intracellular messengers and transcription factors[62, 63]. The improved bone formation by a combination of dynamic culturing and cAMP seems to be very unique effect, since just culturing hMSCs in dynamic condition did not show an added effect on either osteogenic gene expression profile or bone formation. The effect of cAMP on in vivo bone formation in static conditions is although apparent from our earlier studies; our results suggest that there is distinct combination effect which cAMP otherwise able to deliver its effect on in vivo bone formation in static condition.

The donor variation in response to an osteogenic signal is a well-known phenomenon. We have demonstrated that hMSCs from a number of donors respond differently to osteogenic signals[15, 64]. Our earlier studies using hMSCs isolated from a number of donors showed consistently enhanced bone formation by cAMP treatment[21] however, in this case cAMP failed to enhance in vivo bone forming capacity of hMSCs which is most likely a donor dependent effect. On the other hand, when cAMP was presented in dynamic condition, together it enhanced in vivo bone forming ability of the cells to a greater extent which expands the application of cAMP as an osteogenic inducer for bone tissue engineering purposes.

A balanced concentration and context effect of osteogenic stimuli are important induce osteogenesis in hMSCs. 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[65]. Further, the required dosage and action of these signals may vary between cell types[65, 66]. Moreover, the response of hMSCs should always be considered in the light of the heterogeneous nature of this cell population and donor variation[67, 68]. The crucial role of cAMP in cell fate decision has become apparent from the current studies and the induction depends on the concentration and duration of cAMP and dexamethasone to which hMSCs are exposed[21, 69]. 10^{-7} M dexamethasone and 0.5 mM cAMP is shown to inhibit osteogenic process and induce adipogenic differentiation[69], while we demonstrate that 10^{-8} M dexamethasone and 1 mM cAMP significantly enhances dexamethasone induced osteogenesis *in vitro* and bone formation *in vivo*[21] and in agreement with the authors a balanced context, concentration and species dependent effect of osteogenic stimuli need to be investigated for their effective use in bone tissue engineering. In conclusion, our data reveals a multidisciplinary approach using a perfusion bioreactor system in combination with a precise blend of osteogenic signals to augment *in vivo* performance of hMSCs to form bone far beyond any current tissue engineering protocols would produce and we are currently structuring clinical trials.

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Chapter 7 A multidisciplinary approach for bone tissue engineering

Chapter 8

General Discussion and Conclusions

The requirement for new bone to replace or restore the function of traumatized and degenerated tissue and for the replacement of lost mineralized tissue as a consequence of increasing age is a major clinical need. To date, bone tissue engineering regimes are attractive, but have yet to demonstrate their clinical efficacy. Bone is unique with a vast potential for regeneration from cells with stem cell characteristics. Mesenchymal stem cells (MSCs) are multipotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament and fat. These primitive progenitors exist postnatal in low incidence with extensive proliferation capacity[1-4]. In essence, MSCs can be expanded *in vitro* and seeded onto various biomimetic scaffolds to generate the appropriate tissue[5]. These properties in combination with their potential to differentiate into multiple tissues have generated tremendous interest in the use of MSCs to replace damaged tissues. In particular, their ability to differentiate into the osteogenic lineage *in vitro* and to form bone *in vivo* has allowed us to utilize them for cell-based bone tissue engineering. Cell-based bone tissue engineering includes isolation, expansion and modulation of MSCs in combination with osteo-conductive or -inductive scaffolds to support and guide regeneration together with proper selection of osteo-inductive growth factors. These approaches are often referred to as bone tissue engineering and provide alternative solutions for skeletal tissue reconstruction and replacement. The bottleneck in current bone tissue engineering protocols is that the newly formed bone does not fully bridge the implant[5]. In addition to this, there are a number of factors which ought to be addressed such as species difference and donor variation in response to osteogenic stimuli for successful application of hMSCs for cell-based bone tissue engineering. We structured our research to answer these questions and to augment the biology of hMSCs for their effective use to enhance current bone tissue engineering protocols.

Skeletal development and osteogenic differentiation is mainly studied using mouse genetic models and osteogenic cell lines such as MC3T3, C2C12, ROS17/2.8, UMR 108, MG-63 and SAOS-2. Extrapolation of the knowledge gathered from lower species to the human situation is feasible, but needs thorough understanding of differences in the mechanism between species in response to different signals[6]. 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[7]. Dexamethasone plays a crucial role in differentiation of hMSCs into a specific lineage in a concentration dependent way. Dexamethasone is commonly used to induce *in vitro* osteogenic and adipogenic differentiation of hMSCs. At a concentration of 100 nM, it induces osteogenic differentiation and at a higher concentration of 1 μM it is used to induce adipogenic differentiation of hMSCs. In contrast, dexamethasone does not induce osteogenesis in the two most frequently used cell lines, MC3T3 and C2C12. Many BMPs are known to induce ALP expression and osteogenic differentiation in various cell lines and MSCs isolated from mice and rat. However, most BMPs fail to induce ALP expression as well as mineralization in hMSCs, unless they are presented in combination with dexamethasone. In chapter 2, we discussed in-depth the major signaling pathways studied in other biological models such as glucocorticoid, TGF-β, BMP, GPCR, Rho, Vitamin D signaling, MAP kinase signaling, which are known to regulate osteogenesis in

relation to hMSCs. Furthermore, chapter 6 and 7 show that cAMP induces *in vitro* osteogenic differentiation of hMSCs and cAMP-treated hMSCs consistently enhanced *in vivo* bone formation. In sharp contrast, cAMP inhibited osteogenesis in MC3T3 cells and primary cells isolated from mouse and rat. Surprisingly, cAMP-treated rat MSCs showed adipogenic differentiation. In the process of cell fate decision, dexamethasone concentration apparently seems to play a crucial role in combination with cAMP. At 10^{-7} M, dexamethasone with cAMP inhibits osteogenic differentiation and induces adipogenic differentiation[8]. In contrast, we show that hMSCs exposed to 1 mM cAMP for 4 -7 days with 10^{-8} M dexamethasone significantly enhanced ALP expression and *in vitro* mineralization, indicating a concentration and context dependent effect of cAMP on cell-fate decision. Species difference is also highlighted in chapter 4, where we tested a number of GPCR ligands such as PTH, PTHrP, melatonin, epinephrine, PGE2, calcitonin, calcitonin gene related peptide, which are known to induce osteogenesis in various cell types. However, all these ligands were unable to induce osteogenic differentiation in hMSCs further emphasizing the species difference in response to many osteogenic stimuli. Taken together, we conclude that hMSCs respond differently to many osteogenic signals. Therefore, literature-driven differentiation protocols which are mostly done in lower generic species to apply for hMSCs need a thorough investigation in hMSCs before bringing it to a clinical level. Our results further validate the species, concentration and context dependent effect of cAMP-PKA signaling on osteogenic differentiation and the unique response that hMSCs display to cAMP, which makes them the only and obviously most relevant cell type for future research. From the literature and from our studies it is apparent that cell fate decision is a balanced act of signaling molecules. For instance, our results using cAMP have proven to enhance dexamethasone induced osteogenesis in vitro and bone formation in vivo, underlining the role of cAMP in cell fate decision[9]. However, when the cells were induced with physiological levels of cAMP by PGE2 or intermittent exposure of hMSCs, cAMP inhibited osteogenic differentiation of hMSCs. This is further complicated by the concentration and context-dependent effect of osteogenic molecules on cell lines from different species. By studying the basic mechanisms in hMSCs, it not only shortens the clinical application gap but also overcomes the species dependent effects of these signaling molecules. All together, hMSCs present themselves as the best cell source to study molecular mechanism underlying the differentiation processes into various mature cell types, which would not only provide insights into the causes of disorders originating from MSCs but also helps to develop safer, more specific and effective therapeutic applications in regenerative medical field.

For successful cell-based bone tissue engineering, we need to know the *in vitro* osteogenic potential and *in vivo* bone forming ability of the cells from a particular donor. To identify the markers which regulate these processes, we are currently isolating and characterizing hMSCs from over 80 donors for their *in vitro* differentiation and *in vivo* bone forming capacity. In parallel, we are performing gene expression profiling to identify predictive markers for *in vivo* bone forming capacity of the cells. In this process, we analyzed a small segment of hMSCs from 20 donors aged between 20 and 90 years and we investigated the heterogeneity in response to dexa-

methasone and the extent to which hMSCs can be expanded without losing multipotency. In Chapter 3, we describe that hMSCs can be expanded *in vitro* up to 12 population doublings while retaining their multipotency to differentiate into various lineages. Dexamethasone induced ALP expression in hMSCs from all the donors although the extent of induction varied among the donors. The other very important observation is that the initial percentage of ALP positive cells varied between 1 and 33% and upon dexamethasone treatment percentage of ALP positive cells varied between 3 and 50%, with an average of 27%. In addition, we also observed a donor-dependent effect of cAMP on ALP expression. cAMP induces ALP expression in hMSCs isolated from many donors, yet again the extent of induction varied between donors. Besides *in vitro* discrepancy among the donors in response to an osteogenic signal, hMSCs isolated from a number of donors show variation in the amount of bone formation *in vivo*. In conclusion, the hMSCs isolated from the bone marrow show intrinsic heterogeneity with respect to multipotency, growth rate and response to osteogenic signals which might be attributed to facts such as sampling method, initial percentage of ALP positive cells in the biopsy and their ability to respond to osteogenic stimuli. No initial descriptive statistics of the marrow biopsies can assist in estimating the differentiation potential *in vitro* and *in vivo* bone forming capacity. Although, ALP is accepted as predictive marker for *in vitro* osteogenic differentiation, ALP expression cannot be used as marker to predict the *in vivo* bone forming capacity of the cells. hMSCs offer high hopes in clinical applications, however, the lack of common standards and a precise definition of MSC preparations remains a major obstacle in research and application of hMSCs. Whereas surface antigen markers have failed to precisely define this population, a combination of proteomic and microarray data would provide a new dimension for the definition of hMSCs. Currently, in our microarray screen, we aimed to identify markers which better foretell the *in vivo* bone forming capacity.

Pluripotent embryonic stem (ES) cells can differentiate into any cell type of an organism and self-renew indefinitely. However, legal and moral controversies concerning their use for therapeutic and clinical application have prompted active examination of the reservoirs of MSCs in the bone marrow. On the other hand, MSCs can differentiate into a variety of mesenchymal cells such as adipocytes-, chondrocytes- and osteoblasts[1], and can also give rise to non-mesenchymal cells like neural cells[10], hepatocytes,[11] skeletal muscle[12], smooth muscle[12], astrocytes[13] and into cardiac muscle cells[14]. Thus, MSCs are remarkably malleable and exhibit a high degree of plasticity and given the enormous promise of these cells to the development of new therapies. There is no doubt that in the near future most fundamental questions will be resolved by MSCs research.

The fact that insufficient amounts of *in vivo* bone formation occurs upon implantation of hMSCs merits research to augment their biological activity *in vitro* and *in vivo*. In consideration of the fact that pre-differentiation of hMSCs enhances *in vitro* osteogenesis and might also improve *in vivo* bone formation, we are currently screening over 20,000 small molecules and compounds for their ability to induce osteogenic differentiation in hMSCs. Further, by microarray analysis we have identified a number of G-Protein Coupled Receptor (GPCR) signaling-related genes regulated

during osteogenesis. Hence, to augment *in vitro* differentiation, we tested a series of GPCR signaling-related molecules to enhance hMSC osteogenesis. Some of the well-known GPCR ligands which induce osteogenic differentiation in various cell types such as PTH, PTHrP, melatonin, epinephrine, PGE2, calcitonin, calcitonin gene related peptide fail to induce osteogenesis in hMSCs. Comprehensive understanding of GPCR signaling allowed us to activate or inhibit GPCR signaling using various molecules. PKA activation using cAMP strongly enhanced *in vitro* osteogenic differentiation and *in vivo* bone formation by hMSCs from a number of donors. In chapter 6, we describe that cAMP enhances osteogenesis in hMSCs by autocrine and paracrine fashion by direct induction of BMP target genes such as *ID2* and *SMAD6* within 6 hours of cAMP treatment. Further, cAMP treatment of hMSCs induced expression of many growth factors such as BMP2 and other TGF-β members and expression of many pro-osteogenic cytokines and growth factors such as *IL-11, IL-8* and *IGF1.* As a consequence, PKA strongly enhanced the bone forming capacity of hMSCs *in vivo*. As demonstrated in chapter 3, hMSCs from some donors do not form bone *in vivo* in normal culture conditions. However, when these cells were treated with cAMP, it induced bone formation by these cells. In summary, we have identified a cost effective and highly potent osteogenic inducer *in vitro* and enhancer of bone forming capacity of hMSCs *in vivo.* A well known *in vitro* osteogenic inducer dexamethasone has disputed effect on in vivo bone forming capacity[15, 16]. When cAMP is presented to hMSCs in combination with dexamethasone, it enhances both *in vitro* osteogenesis and *in vivo* bone forming capacity of hMSCs isolated from a number of donors which would further offer an outstanding opportunity for clinical application. We are currently stepping towards clinical application by use of these balanced mixtures of osteogenic signals to augment bone tissue engineering procedures.

In chapter 6, we have demonstrated that cAMP enhanced osteogenesis in hMSCs *in vitro* and bone formation *in vivo*. However, cAMP-induced bone formation is clinically insufficient and therefore it warranted us to use a multi-disciplinary approach to even further enhance the bone forming ability of the cells to a clinical situation. 3-dimensional dynamic flow conditions and fluid shear strain enhance osteogenic differentiation and bone formation[17-21]. In chapter 7, we show that by culturing hMSCs in a perfusion bioreactor supplemented with cAMP, the bone forming ability of the cells was greatly enhanced to a level where the newly formed bone covered up to 20- 25 % of the total pore area available for new bone growth. Recently, hMSCs are explored for immune modulation purposes and they also represent a source to replace marrow microenvironment damaged by myelo-ablative chemotherapy or to correct acquired or inherited disorders of bone, muscle, or cartilage or used as vehicles for gene therapy[22]. hMSCs are shown to inhibit T cell proliferation caused by the production soluble factors from hMSCs[23]. In addition, MSCs have been shown to provide cytokine and growth factor support for expansion of hematopoietic and embryonic stem cells[24-27]. Likewise, when hMSCs are exposed to cAMP, it induces the production pro-osteogenic cytokines and growth factors such as IL-11, IL-8, IGF1 and BMP2 and this offers to use them as a factory to produce osteogenic factors which would not only direct hMSCs into the osteogenic lineage but they would attract host MSCs and differentiate them into the osteogenic lineage. These outstanding results

open a window of opportunity to treat skeletal related injuries and bone reconstruction protocols in a multi-disciplinary approach by pragmatic selection of materials, cells, growth factors, osteo-inductive molecules and culture conditions.

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Summary

Recent advances in medicine and other biological disciplines have considerably enhanced the life expectancy of human and consequently, resulting in age related health problems including skeletal complications. In addition, bone substitute to regenerate fractures resulting from trauma, congenital and degenerative diseases adds up to the total clinical need. During the last decade a number of bone tissue engineering strategies have been implemented to overcome the limitations of the current therapies. Cell -based bone tissue engineering using Mesenchymal Stem Cells (MSCs) isolated from the bone marrow seeded on to various osteo-inductive biomaterials with osteo-inductive or –conductive growth factors offers a new promise. The success of bone tissue engineering rather depends on various factors such as scaffold materials, the quality of MSCs and their survival and differentiation in the implantation site.

To understand *in vitro* behavior of hMSCs, we isolated and characterized hMSCs from a number of donors. Donor variation is a well known phenomenon and MSCs isolated from different donors behave differently *in vitro* and *in vivo*. Our efforts to understand this has demonstrated that hMSCs show a large variation in response to a well known osteogenic stimulus dexamethasone. The percentage of ALP positive cells in the initial culture and after dexamethasone treatment varied enormously among the donors. In addition, the resolution of ALP induction by dexamethasone *in vitro* did not seem to be a predictive marker for *in vivo* bone formation which limits the standardization of therapeutic protocols. Serial *in vitro* expansion of hMSCs to test their efficacy to retain their multipotency demonstrated that hMSCs can be effectively expanded up to 10 -15 population doublings and the cells seem to loose osteogenic potential before they loose adipogenic differentiation capacity. All together, the most striking observation is the very low amount of *in vivo* bone formation by all the donors. Typically, when hMSCs are seeded on to 3-4 mm ceramic materials, they were able to form bone which covered a maximum of 3 % of the available pore area with newly formed bone which might limit integration of the implant and success of bone tissue engineering. The inability of MSCs to form clinically relevant amount of bone in the tissue engineered construct is therefore one of the current limitation in bone tissue engineering. The available knowledge on MSC biology shows that the process of proliferation and differentiation of MSCs is driven by various molecular signaling pathways. We structured our research to osteo-instruct hMSCs into osteogeic lineage considering the fact that pre-differentiation of hMSCs *in vitro* into osteogenic lineage enhances *in vivo* bone formation.

The vast involvement of G-Protein Coupled Receptor (GPCR) signaling in osteogenesis in various cell types directed us to reveal its role in hMSCs biology. Exposing hMSCs to various GPCR ligands such as PTH, PTHrP, Melatonin, Calcitonin, Calcitonin gene related peptide and Prostaglandin E2 (PGE2) did not enhance osteogenic differentiation. However, PGE2 inhibited *in vitro* mineralization and interestingly, only PGE2 was able to induce intracellular cAMP in hMSCs. Further, our detailed research has demonstrated physiological amounts of cAMP produced by GPCR ligands inhibit osteogenesis while a continuous exposure for 4 days enhances *in vitro* osteogenesis and *in vivo* bone formation by hMSCs. We demonstrate that cAMP en-

hances osteogenesis of hMSCs by autocrine and paracrine fashion by direct induction of BMP target genes such as *ID2* and *SMAD6* within 6 hours of cAMP treatment. In addition, cAMP-treated hMSCs induced expression of many growth factors such as BMP2 and other TGF-b members and expression of many pro-osteogenic cytokines and growth factors such as *IL-11*, *IL-8* and *IGF1*. As a consequence, PKA strongly enhanced the bone forming capacity of hMSCs *in vivo*. To even further augment the *in vivo* bone forming capacity of hMSCs, we have successfully employed a multidisciplinary approach by combining positive effects of perfusion bioreactor and cAMP. In normal situation without *in vitro* pre-differentiation, the ectopic bone forming ability of hMSCs varies between less than 1% to a maximum of 3% available pore area covered with newly formed bone. Pre-differentiating hMSCs with cAMP, we could consistently augment bone forming capacity up 10-15% of the available pore area covered by newly formed bone. In a perfusion bioreactor supplemented with cAMP up to 25- 30% of the available pore area was covered with newly formed bone. In summary, we have identified a cost effective and highly potent osteogenic inducer *in vitro* and enhancer of bone forming capacity of hMSCs *in vivo* which offers an outstanding opportunity for clinical application. We are currently stepping towards clinical application by using balanced mixtures of osteogenic signals to augment bone tissue engineering procedures.

Samenvatting

Recente ontwikkelingen in de geneeskunde en andere biologische disciplines hebben de levensverwachting van mensen aanzienlijk verbeterd. Dit brengt echter wel leeftijdgerelateerde problemen met zich mee, waaronder aandoeningen aan het bot. Bovendien is er vraag naar botvervangende materialen voor de behandeling van fracturen als gevolg van trauma's en aangeboren afwijkingen. Het afgelopen decennium zijn er een aantal bot-weefselkweek strategieën ontwikkeld om de beperkingen van de huidige behandelmethodes te ondervangen. De strategie waarbij mesenchymale stamcellen (MSCs) worden geisoleerd uit het beenmerg en vervolgens gezaaid op osteoinductieve of osteoconductieve dragermaterialen, in aanwezigheid van osteoinductieve groeifactoren, biedt nieuwe perspectieven. Het succes van bot-weefselkweek is afhankelijk van factoren zoals het dragermateriaal, de kwaliteit van de MSCs en hun overleving en differentiatie na implantatie.

Om een beter beeld te krijgen van het *in vitro* gedrag van humane MSCs, hebben we hMSCs van een aantal donoren geïsoleerd en gekarakteriseerd. Donorvariatie is een bekend fenomeen en MSCs van verschillende donoren gedragen zich anders *in vitro* en *in vivo*. Ons onderzoek heeft aangetoond dat hMSCs erg verschillend reageren op de bekende osteogene stimulus dexamethasone. Het beginpercentage ALP positieve cellen in kweek en het aantal na behandeling met dexamethasone varieert enorm tussen verschillende donoren. Bovendien lijkt de toename in ALP expressie als gevolg van dexamethasone-behandeling *in vitro* geen voorspellende factor te zijn voor *in vivo* botformatie, wat de standaardisatie van behandelingsprotocollen bemoeilijkt. Expansie van hMSCs heeft laten zien dat de cellen 10-15 keer verdubbeld kunnen worden zonder dat ze hun multipotentie verliezen, en dat de capaciteit tot osteogene differentiatie eerder afneemt dan die tot adipogene differentiatie. De meest opvallende observatie echter, is de enorm lage hoeveelheid bot die in elke donor *in vivo* wordt gevormd. Wanneer hMSCs worden gezaaid op keramische materialen met een diameter van 3-4 mm, bezet het nieuw gevormde bot hooguit 3% van het totale porievolume, wat de integratie van het implantaat beperkt. Het gebrek aan klinisch-relevante hoeveelheden nieuw gevormd bot in implantaten is dan ook een van de huidige beperkingen van bot- weefselkweek. De huidige kennis van de MSC-biologie laat zien dat de differentiatie en proliferatie van MSCs gedreven wordt door verscheidene moleculaire signaaltransductie routes. Het is bekend dat osteogene pre-differentiatie van hMSCs *in vitro* de botformatie *in vivo* verbetert, en dit onderzoek had daarom als doel hMSCs te differentiëren in de osteogene richting.

Het is bekend dat G-protein gekoppelde receptoren (GPCR) in verschillende celtypen een rol spelen in osteogenese en we hebben daarom onderzocht wat de rol van deze receptoren is in hMSCs. Blootstelling aan liganden voor deze receptoren, zoals parathyroid hormoon, parathyroid hormoon related peptide, melatonine, calcitonine, calcitonin gene related peptide en prostaglandine E2 (PGE2) bleek geen effect te hebben op osteogene differentiatie. Alleen PGE2 bleek een negatief effect te hebben op mineralisatie en was tevens de enige ligand waarbij de intracellulaire concentratie cAMP toenam. Ons onderzoek heeft verder aangetoond dat physiologische hoeveelheden cAMP, zoals geproduceerd door GPCR ligands, osteogene

differentiatie negatief beïnvloeden, terwijl continue behandeling gedurende 4 dagen de differentiatie *in vitro* en botvorming *in vivo* stimuleert. We laten hier zien dat cAMP osteogenese stimuleert via autocrine en paracrine mechanismen, middels directe activatie van BMP doelgenen zoals Id2 en Smad6 binnen 6 uur. Ook worden er in cAMP-behandelde hMSCs aanzienlijk meer bot-specifieke groeifactoren aangemaakt, zoals BMP-2 and andere TGF-β-leden, en bot-bevorderende cytokines zoals Il-11, Il-8 en IGF-1. Als gevolg hiervan is *in vivo* botvorming aanzienlijk verbeterd in PKA-geactiveerde hMSCs. Om het botvormende vermogen van hMSCs nog verder te verbeteren hebben we met succes de positieve effecten van cAMP en perfusiebioreactoren gecombineerd (in een multidisciplinaire aanpak). In een standaard situatie, zonder *in vitro* pre-differentiatie, varieert de hoeveelheid door hMSCs ectopisch gevormd bot tussen de 1% en 3% van het totale porie volume. Wanneer hMSCs worden behandeld met cAMP voor implantatie is dit al 10-15%, en wanneer cAMP wordt gecombineerd met een perfusie bioreactor is 25-30% van het totale porie-volume gevuld met nieuw gevormd bot. We kunnen concluderen dat we in cAMP een rendabele en krachtige stimulator van osteogene differentiatie *in vitro* en botvorming *in vivo* hebben gevonden, met uitstekende mogelijkheden voor klinische toepassingen. We werken momenteel aan toepassingen waarbij gebruik wordt gemaakt van een combinatie van osteogene signalen om de procedures voor bot -weefselkweek te verbeteren.

Acknowledgments

From experience I can tell you that these last pages of a Ph.D thesis are the most widely read pages of the entire publication. It is here where you think that you will find out whether you have meant something in the life of the PhD candidate…me! While this may be true to some level, you have to weigh my verdict with the disturbingly low level of sanity left in this Ph.D candidate after several years of studying toxic molecule's effect on stem cell differentiation and to make "Bone" out of it, which even changed my nick name to "The Bone Maker"!

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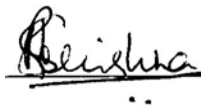

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Curriculum Vitae

Ramakrishnaiah Siddappa was born on January 10th 1977 in Bangalore, India. He obtained his B.Sc and M.Sc (Microbiology) degrees at University of Agricultural Science, Bangalore, India with distinction. Later, he did another M.Sc (Biotechnology) at Wageningen university, The Netherlands with a Nuffic Scholarship and graduated on 2003. He continued his education as a Ph.D at University of Twente, The Netherlands under supervision of Dr. Jan de Boer and Prof. Clemens van Blitterswijk. His research was aimed to understand the cellular and molecular signals which regulate the in vitro osteogenesis and in vivo bone formation by human mesenchymal stem cells for cell-based bone tissue engineering applications. Currently, he is working as a research scientist at Dept. of Tissue Regeneration, University of Twente.

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