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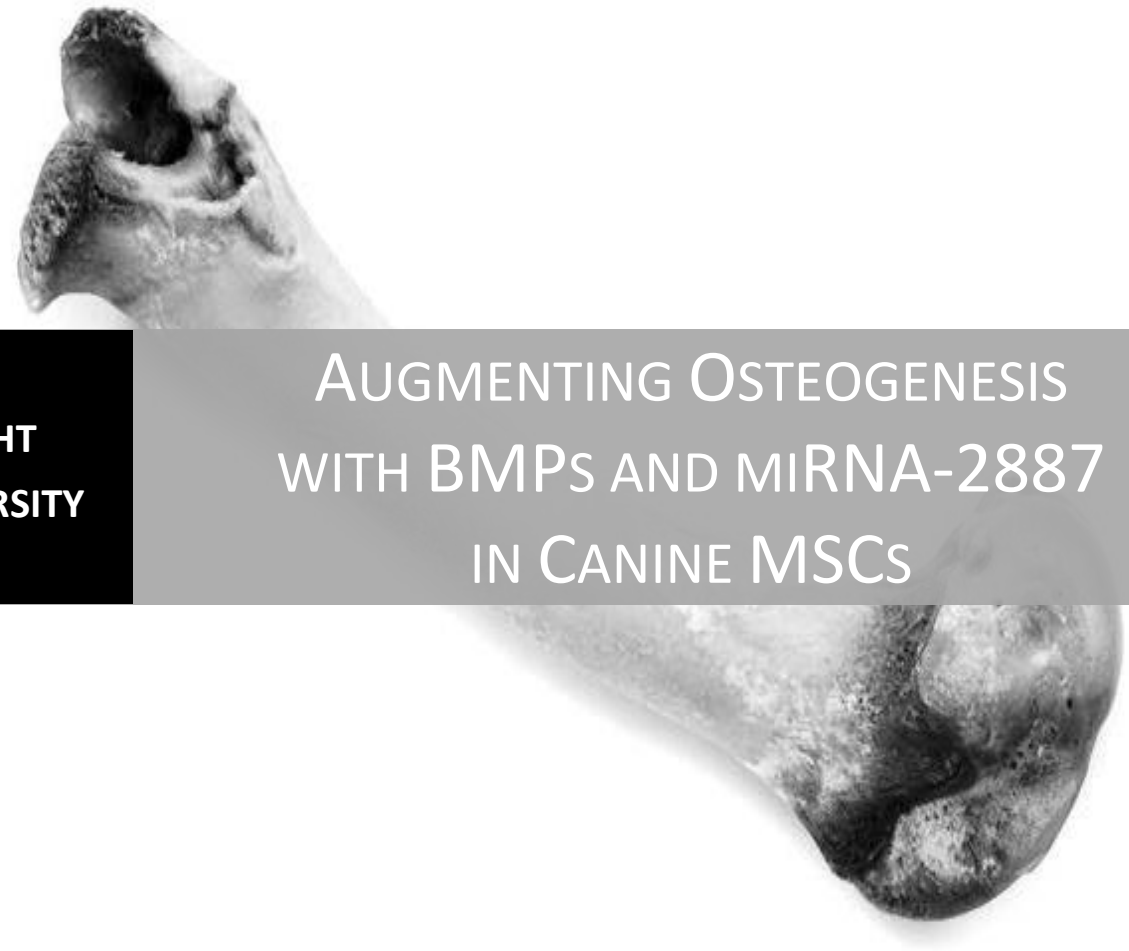

UTRECHT UNIVERSITY

AUGMENTING OSTEOGENESIS WITH BMPS AND MIRNA-2887 IN CANINE MSCS

Honours Program project

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Dedicated to:

Papa

“Always Look On The Bright Side Of Life”

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Summary

Clinicians are currently still challenged with large bone defects and their treatment, due to absent or delayed vascular ingrowth, which causes hypoxic conditions at the fracture site. Several therapeutic strategies have been developed, involving growth factors, grafts, scaffolds and stem cells. Complications still exist in these therapies and therefore new therapy strategies are tested in vitro with the use of mesenchymal stem cells (MSC) that undergo osteogenic differentiation. Important inducers of osteogenesis are dexamethasone, a glucocorticoid, and bone morphogenetic proteins (BMP), which are growth factors. Dexamethasone shows to have a negative effect in human MSCs when given the entire culture period, due to inhibition of proliferation and decreased viability and therefore dexamethasone should only be added the first 7 days of culture. However, this study demonstrated that canine MSCs cultured with osteogenic medium during the entire culture period of 21 days reveal the best osteogenic differentiation when compared with culture with osteogenic medium without dexamethasone and osteogenic medium supplemented with dexamethasone only for the first 7 days. Under optimal osteogenic conditions, BMP-2 and BMP-6, the most potent inducers of all BMPs in rodents and human, were assessed for their additive effect on osteogenesis. Both in monolayers and aggregates, BMP-2 100ng/ml revealed to have the best osteogenic potential in comparison with BMP-6 and the highest noggin expression in aggregates. The greater potential of BMP-2 in inducing osteogenesis is in again in contrast with humans and rodents and could be caused by species-specific BMP-receptor expression differences and noggin. Noggin in rodents functions as an antagonist of BMPs, except for BMP-6. Therefore, BMP-6 is found to be more potent in mice and rats compared with other BMPs. In the final study performed, noggin was supressed using a microRNA (miRNA), a type of RNA involved in gene regulation, and it was expected that this would enhance osteogenesis as reported in rodents. miRNA-2887 at a concentration of 50nM was transfected in canine MSCs treated with osteogenic medium supplemented with BMP-2 100ng/ml and resulted in suppression of noggin. Surprisingly, noggin suppression did not lead to an increase of osteogenic markers, but a decrease. In conclusion, in canine MSCs BMP-2 was found to be more potent in augmenting osteogenesis compared with BMP-6, even though noggin was highest expressed in BMP-2 conditions, while suppression of noggin expression negatively affected osteogenesis. Altogether, this indicates that noggin functions in a similar fashion as in humans by agonizing BMPs.

1 General introduction

Large bone defects are still a major problem in regenerative medicine, which could occur after extensive trauma, tumours and infection. Normally, fracture healing can be seen as either direct or indirect. Direct, or primary, fracture healing takes place when the fracture sides are close together and there is a rigid internal fixation. In the much more common indirect (or secondary) fracture healing a combination of two types of bone formation is seen with two types of callus formation (1). Indirect fracture healing occurs through either endochondral or intramembranous ossification. Endochondral bone formation takes place via committed osteoprogenitor cells in the periosteum and involves five stages, starting with haematoma and inflammation, and moving to angiogenesis, cartilage calcification, cartilage removal and bone remodelling (2). During this process, soft callus is formed that will thereafter be calcified. Intramembranous ossification occurs when mesenchymal stem cells immediately differentiate into osteoblasts and form bone without the transitional chondrocytes. During this process, at a greater distance from the fracture side, hard callus is formed. Woven bone is formed and thereafter remodelling takes place and lamellar bone is made. This bone formation and remodelling is carried out by osteoblasts, responsible for bone formation, and osteoclasts, which resorb bone.

Fracture healing consists of endochondral and intramembranous ossification and can be subdivided into three different phases, starting with inflammation and followed by chondrogenic and osteogenic phases (figure 1). In mice tibia, the chondrogenic phase is characterized, on day 7 of healing, by a peak in collagen type II and type X expression (3). During endochondral ossification the amount of type II collagen will reduce more quickly than type X. Osteogenesis starts at day 1, indicated by the upregulation of collagen type I and osteocalcin expression. Maximum osteogenesis is reached at day 14 till day 21.

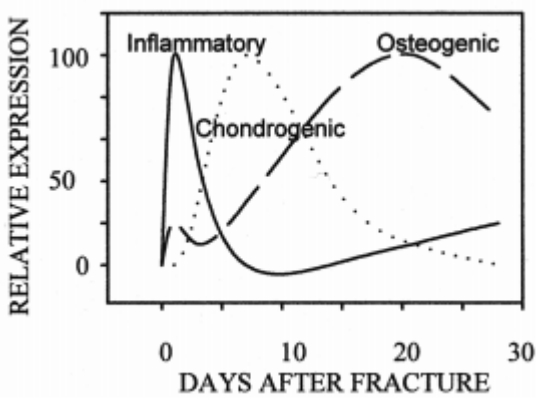

Figure 1. Fracture healing can be subdivided into three stages, namely inflammation, chrondgrogenesis and osteogenesis **(3)**

Systemic

Bone formation and remodelling is continuously orchestrated systemically by different hormones and factors. It involves growth hormone, insulin-like growth factors, parathyroid hormone and Vitamin D.

Growth hormone and insulin-like growth factors

Insulin-like growth factors (IGF) are present in bone matrix, endothelial cells, osteoblasts, and chondrocytes (4). They are regulated by somatotropin, the growth hormone (GH). After growth hormone releasing hormone (GHRH) is secreted by the hypothalamus, GH is released from the anterior lobe of the pituitary gland (5). GH stimulates the liver and growth plates to produce IGF. IGF-I (somatomedin-C) stimulates osteoblasts to form bone matrix (6), IGF-II is involved in calcification during endochondral ossification (7). Research in dogs has revealed the influence of IGF-I and GH on body size development. The level of IGF-I is linear correlated with body size in adult dogs, whereas the basal plasma GH levels do not differ between breeds (8). In young animals however, the GH does differ between large and small breed dogs. Great Danes (GD) have been shown to undergo a period of gigantism with higher levels of GH compared with beagles and miniature poodles (MP) of the same age (9) (10). IGF-I levels did not significantly differ between beagles and GD. It did differ in GD and MP, however no correlation between GH plasma levels and IGF-I was found. It is therefore believed that hypersecretion of GH at a young age is more of influence on body size than IGF-I levels.

Parathyroid Hormone

Parathyroid hormone (PTH) is a hormone involved in the control of osteoclastogenesis and is excreted by the parathyroid gland in case of hypocalcaemia. PTH signalling in osteoblasts is mediated by transcriptional factor cAMP response binding protein (CREB). PTH could cause catabolic or anabolic effects (11). PTHs catabolic activity involves shrinking of osteoblasts, allowing osteoclast to make contact with bone matrix to release phosphate and calcium and thereby maintaining a steady calcium plasma concentration. PTH also influences absorption of calcium in the intestine and renal reabsorption directly. Indirectly, PTH influences the calcium and phosphate absorption in the gut and the calcium reabsorption and mobilization in bone through calcitriol stimulation. Low levels of plasma PTH have an anabolic effect, increasing the amount of osteoblasts, alkaline phosphatase and collagen synthesis.

Vitamin D

Vitamin D (Vit D) influences the absorption of calcium in the gut, the reabsorption in the kidneys and increases the amount of osteoclasts and their activity. Vit D could originate from animal origin, cholecalciferol (Vit D_3), or from plant origin, ergocalciferol (Vit D_2) (11). Vit D is an essential part of a dogs nutrition; dogs cannot synthesise enough Vit D_3, because of low levels of 7-dehydrocholesterol (7-DHC) in the skin (12). After absorption in the gut, vitamin D is hydroxylated in the liver by 25-hydroxylase into 25-hydroxyvitamin-D (25-OHD). Thereafter, hydroxylation of 25-OHD in the renal mitochondria takes plays via 1α-hydroxylase, to form 1,25 dihydroxy-cholecalciferol ($1,25(OH)_2D$ or calcitriol) or $24,25\text{-}(OH)_2D$ via 24-hydroxylase. Calcitriol stimulates intestinal absorption as well as renal reabsorption and increases the amount of osteoclasts and their activity. $24,25\text{-}(OH)_2D$ was thought to be a by-product with no biological effect, but it is now known that it stimulates bone formation without an increase in bone resorption (13). The calcitriol synthesis is influenced by levels of calcium and phosphate, PTH and calcitonin. Calcitriol formation is stimulated by 1α-hydroxylase (directly and via PTH) in case of low plasma levels of calcium and phosphate. Additionally, the

amount of 24,25-$(OH)_2D$ formed by 24-hydroxylase will be reduced because of these low plasma levels. In contrast, calcitriol stimulates 24-hydroxylase activity. Calcitriol forms its own feedback loop by reducing PTH.

Cytokines and growth factors

Several cytokines and growth factors play a role during osteogenesis, at different stages in both endochondral ossification and intramembranous ossification (figure 2). For example, interleukins, members of the transforming growth factor β superfamily, Fibroblastic growth factor, Platelet-derived growth factor and Wnt signalling. Angiopoietin and VEGFs are important in regulating angiogenesis to support an adequate blood supply (1).

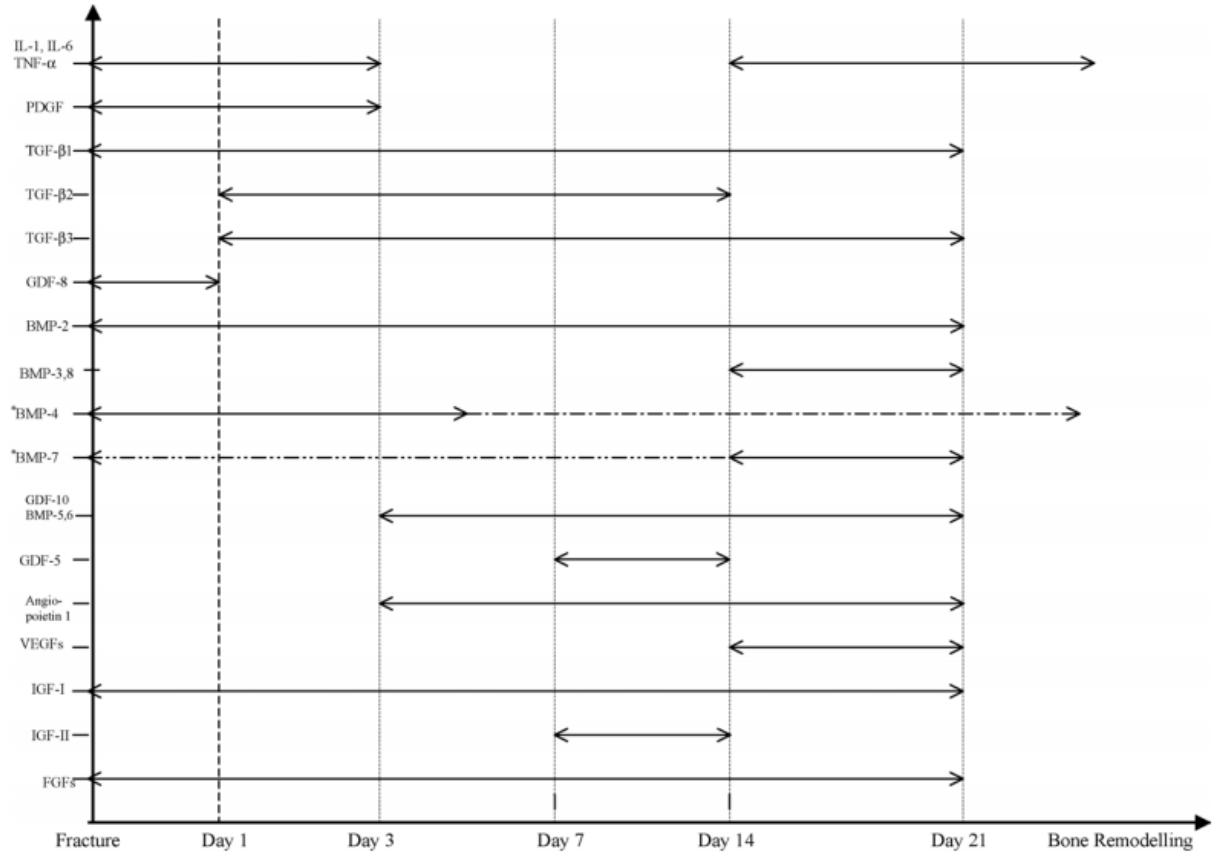

Figure 2. Timeline of different growth factors expressed during bone repair. Dotted line represents disagreement in literature about timing of BMP-4 and BMP-7 **(4)**.

Interleukin and tumour necrosis factor

Interleukin-1 (IL-1) and IL-6 play a role in initiating bone formation (14). IL-1 and Tumour necrosis factor α (TNF-α) attract immune cells to the fracture site during the first 24 hours. TNF-α is important in recruiting MSCs and their skeletogenic differentiation and in endochondral ossification it causes chondrocyte apoptosis and regulates the resorption of mineralized cartilage by the production of different factors (15). Il-1 and TNF-α play an important role in initiating bone repair, intramembranous bone formation and trabecular bone remodelling. They are expressed by

macrophages, recruited inflammatory cells and mesenchymal cells in the periosteum (16). IL-1 and TNF-α are also increasingly expressed during bone remodelling from day 21 until day 28.

Transforming growth factor β superfamily

Transforming growth factor β (TGF-β) superfamily consists of structurally related polypeptides, and some of these play an important role in bone formation. Their effect depends on the factor itself and on cell responsiveness (17). TGF-β1 stimulates the matrix production and osteoblast differentiation (18). TGF-β1 is normally present in unfractured bone in mice tibia and will elevate in the first 24-hours after fracture healing, and then will return back to baseline levels (3). TGF-β2 and TGF-β3 are only elevated during fracture healing, with a peak during the chondrogenic phase. TGF-β mediated osteogenesis involves two different pathways, namely the canonical pathway and the non-canonical pathway (**figure 3**). The canonical and non-canonical pathways could start with the activation of TGF-β receptor type I (TGF-β-RI), which leads to commitment of progenitor cells to the osteoblastic lineage, osteoblast proliferation and differentiation (19). The canonical pathway is activated after a heteromeric complex of specific type I and type II serine/threonine kinase receptors is formed (17). TGF-β binds to the TGF-β receptor type II (TGF-β-RII), which will then phosphorylate TGF-β-RI. Next, Smad2 and Smad3, two types of signal transducing receptor-activated Smads (R-Smads), will be activated (20) (21). Smad2 and Smad3 will interact with Smad4, a Collaborating Smad (Co-Smad) and will then move to the nucleus to initiate the formation of transcriptional complexes with specific DNA-binding ability (17). Smad7 is an antagonist that will prevent the phosphorylation of Smad2, its interaction with Smad4 and the accumulation in the nucleus (22). Smad7 can cause degradation of the TGF-β-RI after interaction with Smurf1 (23). Furthermore, Smad6 has the ability to bind Smad2 and TGF-β-RI and subsequently Smad4 can't bring the R-smads to the nucleus (24).

Additionally, the non-canonical pathway is stimulated. TGF-β-activated kinase 1 (TAK1) is stimulated by TGF-β-RI and next MKK3/6 and p38 mitogen-activated protein kinase (MAPK) will be activated. Overexpression of TAK1 with TAK1-binding protein 1 (TAB1) will lead to enhanced TGF-β-induced type I collagen production (25). Additionally, TAK1 regulates the steady state of MKK3 and p38 MAPK.

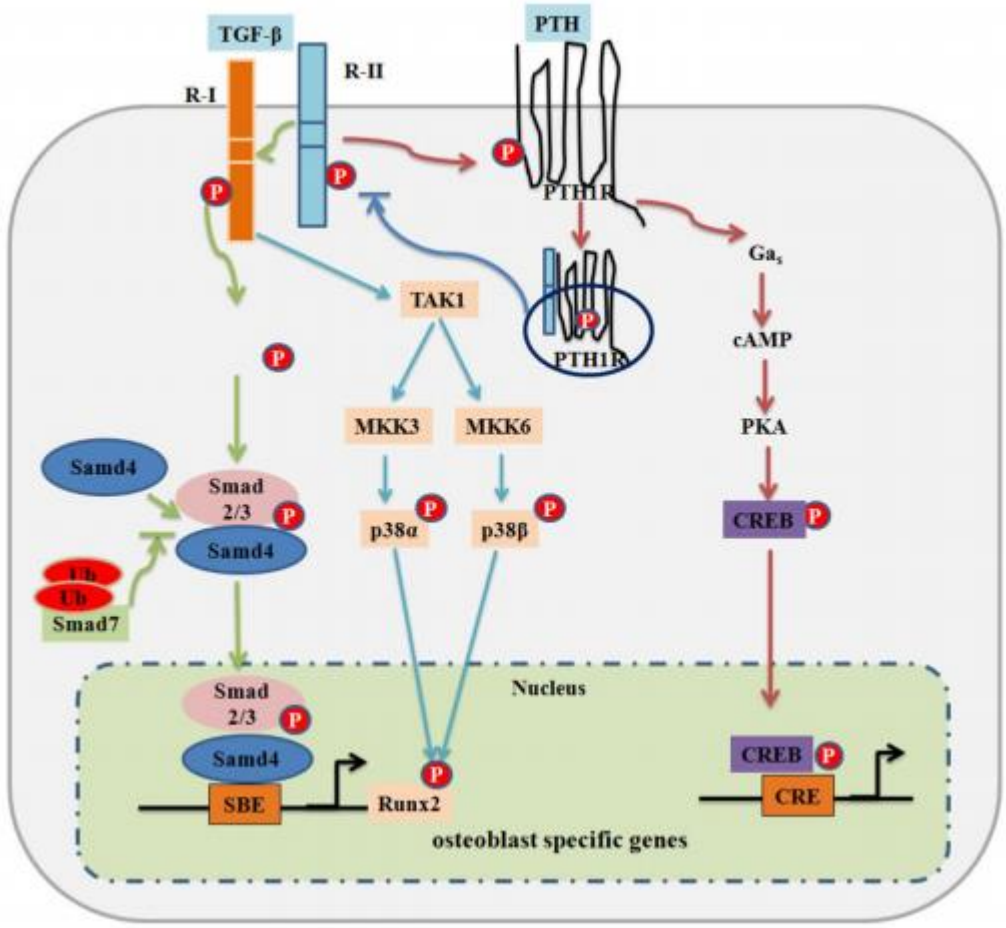

Figure 3. A schematic representation of TGF-β mediated osteogenesis. After binding TGF-β-RII, TGF-β-RI will be activated and the Smad and TAK1 pathway will start. Smad2/3 will bind Smad4 and move to the nucleus to interact with transcription factors and will cause gene expression of several osteogenic genes, such as RUNX-2. Smad7 is an antagonist of Smad4 which will prevent a translocation to the nucleus. PTH signalling is downregulated in case of TGF-β-RII activity. PTH binding will lead to TGF-β pathway damping and activity of CREB, which will mediate PTH signalling in osteoblasts **(18)**

Another set of members of the TGF-β superfamily are the bone morphogenetic proteins (BMPs). BMPs are important during embryonic development of bone formation (26). They act in a similar way as TGF-β as will be discussed in chapter three. During fracture healing in mice tibia, BMP-2 was the first of all BMPs to be upregulated and during osteogenesis a second peak is present (3). BMP-4, -7, -8 and -3 are only upregulated during the osteogenic phase of fracture healing from day 14 till day 21, while BMP-5 and BMP-6 are continuously upregulated. The combination of TGF-β and BMP-2 causes a 5 times larger bone volume than when BMP-2 is given alone during ectopic bone formation in mice (27). TGF-β and BMP-2 doubled the amount of osteoblasts and halved the number of osteoclasts during calcification of bone matrices. The effect of BMP-2 also increases in combination with other growth factors, such as FGF-2 and PDGF-AB (28), suggesting that a combination of BMP-2 with other growth factors will stimulate osteogenesis more than BMP-2 alone.

Growth and differentiation factors (GDFs) are also members of the TGF-β superfamily and involved in fracture repair. GDF-8 is only upregulated during the first 24-hours in fracture healing of mice tibia (3). GDF5 is particularly expressed during the chondrogenic phase of fracture healing. As a contrast, GDF10 (or BMP-3b) is upregulated throughout the fracture repair, but mainly on day 7 and 21, suggesting it plays a part in chondrogenesis and intramembranous ossification.

Additionally, PTH interacts with TGF-β-RII. PTH receptor-I (PTH-RI) phosphorylation by TGF-β-RII leads to downregulation of PTH signalling (29). The opposite occurs with the damping of TGF-β signalling by PTH. PTH signalling will be stimulated when TGF-β-RII is knocked down in mice. The phenotype of these animals will look similar to animals with a continued expression of PTH-RI.

Fibroblastic growth factor

Fibroblastic growth factor (FGF) is a family that consist of nine polypeptides that are structurally related (1). FGF promotes growth and differentiation of several cell types, including osteoblasts. FGF-2, -4 and -6 are osteoblast proliferation inducers and ALP activity and mineralisation reducers (30). FGF-2 is mostly expressed by osteoblast and showed the highest proliferation activity in human osteoblasts. FGF-2 is more potent than FGF-1 expressed in chondrocytes (31). FGF is expressed during early stages of fracture healing. It also plays a role in angiogenesis and mitogenesis in mesenchymal cells.

Platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a homo- or heterodimeric polypeptide that is expressed during early stages of fracture healing by platelets. PDGF is a stimulator for chemostatics in inflammatory cells and in MSCs and in osteoblast it stimulates proliferation and migration (4) (5). PDGFs are used during platelet rich plasma therapies in which patient receive a concentrated suspension of platelets in a small amount of plasma (32). During this therapy, PDFGs and other platelet-derived growth factors will stimulate fracture healing.

Wnt

Wnt promotes osteogenic differentiation and normally accumulates in high levels in the nucleus of potential osteogenic cells in developing long bone. Wnt binding to the Wnt co-receptors LRP5 or LRP6 will activate the β–catenin signalling, involved in the canonical pathway (33). Binding of Wnt to LRP5/6 will antagonize the adenomatous polyposis coli (APC)-Axin- glycogen synthase kinase 3β (GSK3 β) complex (34). As a consequence the free β-catenin pool will increase. After β–catenin is stabilized, the molecule will be move to the nucleus where it accumulates and interacts with the lymphoid enhancer factor-1/T-cell factor (LEF-1/TCF) family of transcription factors. This will lead to transcriptional activity of downstream target genes. β–catenin could also be stabilized by PTH in absence of Wnt (33). Furthermore β-catenin signalling pathway activity in human mesenchymal stem cells could occur through TGF-β-RI activation (35). Wnt signalling also partly mediates Hedgehog protein induced osteogenesis. β–catenin knock-out mice show disrupted osteoblast differentiation (36)

Current therapies

When bone defects are too large, the body is unable to regenerate the bone by itself. To solve this problem, several therapies have been developed, including the use of growth factors, grafts, scaffolds, miRNAs and the use of stem cells. Currently, two types of growth factors, BMP-2 and BMP-7, are employed to initiate bone formation. These BMPs have problems that need to be addressed, concerning safety, cost and ectopic bone formation (37).
Other treatments concentrate on bone grafts, which could be either autologous grafts (the golden standard) or allogeneic grafts from donors. The first reduces the risk of immunoreactions and infections and therefore rejection and has a comparable effect as BMP treatments (4) (38). However, to obtain the graft, an extra surgery must be done, with the disadvantage of complications (infections, hematoma, and pain) and high costs (39) (40). Scaffolds, consisting of synthetic or biomaterial, could also be used, promoting migration, proliferation and differentiation (4). Additionally, delivery systems can enable bone ingrowth in this scaffold by employing BMP-2 or BMP-7 locally (37). The concentration of BMP decreases within weeks. At the same time, mesenchymal stem cells are gravitated towards the fracture site. However, it is not yet clear which dose is optimal, and negative effects, such as infections and high costs, are still present.

Bone marrow mesenchymal stem cells and adipogenic stem cells

Because these treatments do not give the desired results, in vitro studies are performed to find new therapeutic strategies. Stem cells are increasingly used, because of their great potential. Stem cells have the ability to differentiate in different tissues and could originate from different cell lineages, such as skeletal muscles, adipogenic tissue, nerve tissue, liver and bone marrow. Additionally, stem cells have a self-renewal potential and are viable for a long time (41). Mesenchymal stem cells (MSCs), which are osteoprogenitors, are used in therapies in which they are delivered directly at the fracture site with its surrounding growth factors, after they are aspirated from the iliac crest (42). Before the cells are injected, cell expansion and differentiation could take place in vitro, although this will increase the risk of infection and cost (4). Expansion in vitro before injection could cause tumours due to mutations and epigenetic changes, however this risk is minimal and no case reports are known in human studies (43). Future therapy strategies will probably involve a combination of a scaffolds, mesenchymal stem cells and growth factors.

Bone marrow mesenchymal stem cells (BMSCs) and adipose-derived stem cells (ASCs), both derivatives from mesenchyme, are successfully differentiated to the osteogenic, adipogenic and chrondrogenic lineage in humans (44) (41), pigs (45) and dogs ((46), unpublished data). BMSCs and ASCs are commonly used during in vitro experiments to study osteogenic differentiation. ASCs are easy to obtain in high quantities and are therefore commonly suggested as an alternative for BMSCs. However, the two cell types react differently towards the osteogenic medium, despite the corresponding surface markers in humans (CD13, CD29, CD44, CD,90, CD105, SH3 and STRO-1) and in dogs (W8B2, 39D5, W3C4, W5C4 and 58B1), possibly due to differences in cell adhesion markers that are important in homing and mobilizing hematopoietic stem cells (47) (48). ASCs in humans can

differentiate to osteoblasts, although to a lesser extent than BMSCs (49). The same is seen in BMSCs differentiation towards adipocytes. In canine stem cells, a similar effect seems to appear (46).

MicroRNA

A more recent form of therapy is based on gene therapeutic strategies. Cells will be transfected with a virus or non-virally transduced with a gene involved in osteogenesis to ensure continuous protein expression. This could occur in vivo, with direct delivery of the genetic material in the patient, or ex vivo, where cells will be transfected/transduced in vitro after expanding them. Genetic material could consist of certain osteogenic genes, but microRNAs could also trigger gene expression.

MicroRNAs are small pieces of RNA, approximately 22-24 nucleotides long. miRNAs control gene expression in somatic cells and thus maintain the phenotype of the cell. miRNA formation starts with DNA transcribing of independent genes by RNA polymerase II (50). This results in a nascent primary miRNA (figure 4). This nascent primary RNA will fold into hairpin bends and is then called primary miRNA (Pri-miRNA). Subsequently, the Pri-miRNA will be attached by the RNase-III endonuclease DROSHA in combination with DGCR8 (a dsRNA-binding protein (dsRBP)). The pri-miRNA will be cleaved by DROSHA molecules and the remaining ~70-nucleotide combination, named Pre-miRNA, will be exported out of the nucleus by Ran-GTPase Exportin-5. miRNAs could also be formed using very short introns (mirtrons) of protein coding genes. Mirtons are spliced and debranched and subsequently pass the DROSHA step. In the cytoplasm, a ~20-basepair miRNA duplex is made through binding of the enzyme DICER and transactivation-responsive (TAR) RNA-binding protein (TRBP). Next, one strand of the miRNA duplex will be incorporated in the RNA-induced silencing complex (RISC). The other (passenger) strand of the miRNA duplex will be released and degraded. The remaining guide strand will connect with the RISC, and this complex will exert its function by blocking or upregulating a specific gene. During RISC formation and functioning, Argonaute proteins (AGO) and glycine-tryptophan protein of 182kDA (GW182) are

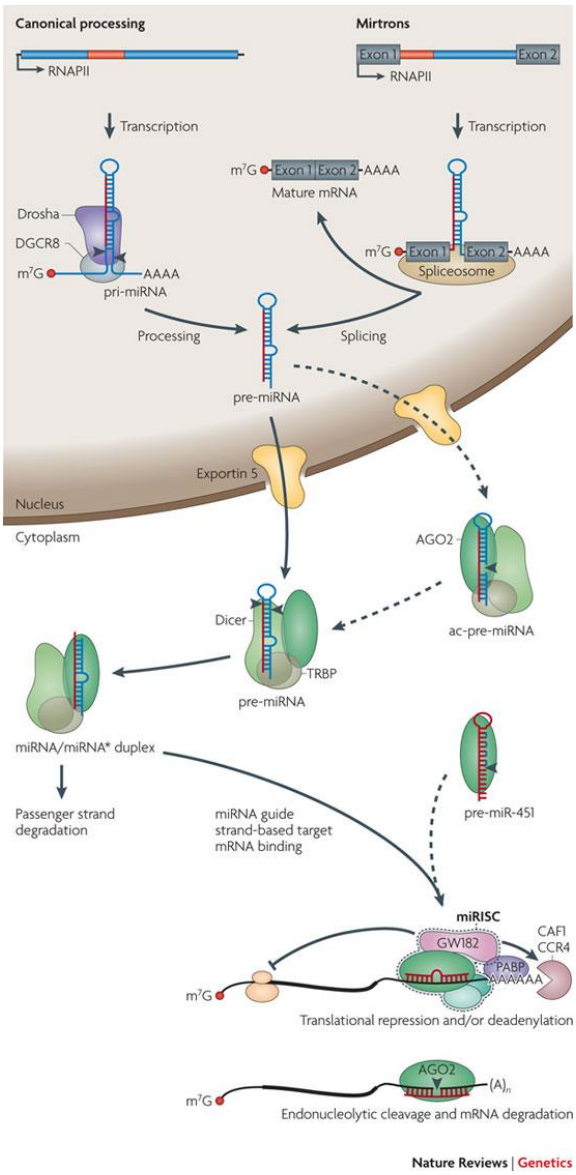

Figure 4. The formation of microRNA. After transcription, DROSHA splices the transcript and pre-miRNA is formed. This could also occur through intron splicing. In the cytoplasm the pre-miRNA is cleaved by AGO2 and/or DICER and the miRNA duplex is split, the passenger strand gets degraded and the guide strand will block or upregulate a gene in cooperation with RISC, GW182, PABP, CCR4 and CAF1 **(50)**.

important. AGO interacts with miRNAs and GW182 is a downstream effector during repression. AGO2 in mammals can form an additional intermediate, AGO2-cleaved precursor miRNA (ac-pre-miRNA), by cutting of the 3'arm of some pre-miRNAs, which will contribute to strand selection and helps the removal of the passenger strand and unwinding of the miRNA duplex (51). GW182 binds poly(A)binding protein (PABP) and attracts CCR4 and CAF1 (50).

Recently several studies have been performed to determine which miRNAs are involved in osteogenesis. Fifty-eight miRNAs were upregulated in preosteoblastic mice cells (MC3T3) from day 7 until day 28 with a peak expression during mineralisation, and ten miRNAs were found to be downregulated (52). Of these, miRNA-29b was found to be the most upregulated, with miRNA-29b inducing RUNX-2 (runt-related transcription factor 2) and Alkaline phosphatase protein and mRNA expression after 48 hours. It targets anti-osteogenic factors and modulates proteins in the extracellular matrix. Another miRNA, miRNA-2861, has been found to inhibit histone deacetcylase 5 (HDAC5), which is involved in degradation of the osteogenic marker RUNX-2 in mice osteoblasts (53). Silencing of miRNA-2861 in mice caused reduced bone formation and a decreased bone mass, due to reduced RUNX-2 expression. Overexpression of miRNA-2861 stimulates BMP-2 induced osteogenesis. miRNA-196a is also involved in osteogenesis (54). Overexpression of miRNA-196a by Lentiviral transfection causes osteogenic differentiation in ASCs without affecting adipogenic differentiation.

Aim of the HP project

The overall aim of this Honours Program study is to study the role of BMPs on osteogenesis and evaluate whether downregulation of noggin, an antagonist of BMP signalling by means of miRNA technology, would augment osteogenesis. In order to do so, the HP project started with further optimization of the osteogenic culture conditions in canine mesenchymal stem cell cultures by fine tuning the supplementation with dexamethasone. In follow up studies, using the optimized protocol of osteogenic differentiation, the additive effect of two bone morphogenetic proteins, BMP-2 and BMP-6 will be studied for the first time in canine MSCs. Finally, modulation of noggin expression by a novel miRNAs in osteogenesis will be studied.

The effect of dexamethasone on osteogenic differentiation of mesenchymal stem cells

2

Abstract

Clinicians are still confronted with fracture healing of large bone defects. To establish new treatment strategies, new strategies are tested in vitro with mesenchymal stem cell (MSC) that differentiate into osteoblasts. An important inducer of in vitro osteogenic differentiation is dexamethasone, a glucocorticoid. In human MSCs, continuous dexamethasone supplementation throughout the whole culture period has negative effects, such as inhibition of osteoblasts proliferation and decreased viability. However, there are no reports specifically for canine MSCs. Therefore, the aim of this study was to determine if dexamethasone supplementation only during the initiation of osteogenic differentiation would have a beneficial effect on in vitro osteogenesis of canine MSCs. Thirteen donors were cultured for 21 days with osteogenic medium without dexamethasone supplementation and with dexamethasone for 7, and 21 days. At 21 days, osteogenesis was determined with Alizarin red staining and gene expression profiling of osteogenic markers. Mineralized noduli were larger and more frequently present in monolayer cultures treated with dexamethasone for 21 vs. 7 days and no dexamethasone. Osteogenic medium without dexamethasone supplementation showed similar results with 7 days of dexamethasone supplementation. Relative gene expression of osteogenic markers was in line with the observations in Alizarin red staining: 21 days of supplementation resulted in the highest expression of osteocalcin as compared to no supplementation and 7 days of dexamethasone. Altogether, this indicates that the best osteogenic differentiation is realized by adding dexamethasone to the osteogenic medium for 21 days in canine MSCs.

Introduction

New therapeutic strategies to help the body regenerate bone are tested during in vitro studies. During these in vitro experiments osteogenic differentiation is established in mesenchymal stem cells (MSCs) with the use of dexamethasone, which is a glucocorticoid. Dexamethasone causes osteogenesis by activating Wnt/B-catenin signaling-dependent RUNX-2 pathway (55) and eventually activates Runt-related transcription factor 2 (RUNX-2) transcription.

In this way, RUNX-2 is influenced by three different factors activated by dexamethasone. Firstly, the presence of dexamethasone causes the transcription of FHL2 (Four and a half LIM domains protein 2) possibly by binding to a glucocorticoid response element in the promoter of FHL2 (figure 1) (56). FHL2 binds to β-catenin in the cytoplasm and moves to the nucleus, where it binds to LEF-1/TCF causing RUNX-2 expression. Secondly, dexamethasone causes upregulation of MKP-1 (mitogen-activated protein kinase (MAPK) phosphatase), which dephosphorylates and activates RUNX-2 (57). Finally, dexamethasone causes the transcription of TAZ (transcriptional coactivator with PDZ-binding motif), which will then bind RUNX-2 and a TAZ/RUNX-2 complex is formed (58). The TAZ/RUNX-2 complex becomes active by a pathway that is controlled by ascorbic acid (AsA). The presence of AsA results in a higher secretion of collagen type I in the extracellular matrix (ECM) (59). Collagen I will bind to α2β1-integrin and causes the activation of a MAPK signalling pathway. This pathway will end with the translocation of P-ERK1/2 to the nucleus, activating the TAZ/RUNX-2-complex. The TAZ-RUNX-2-complex will then transcript other osteogenic markers, such as osteocalcin.

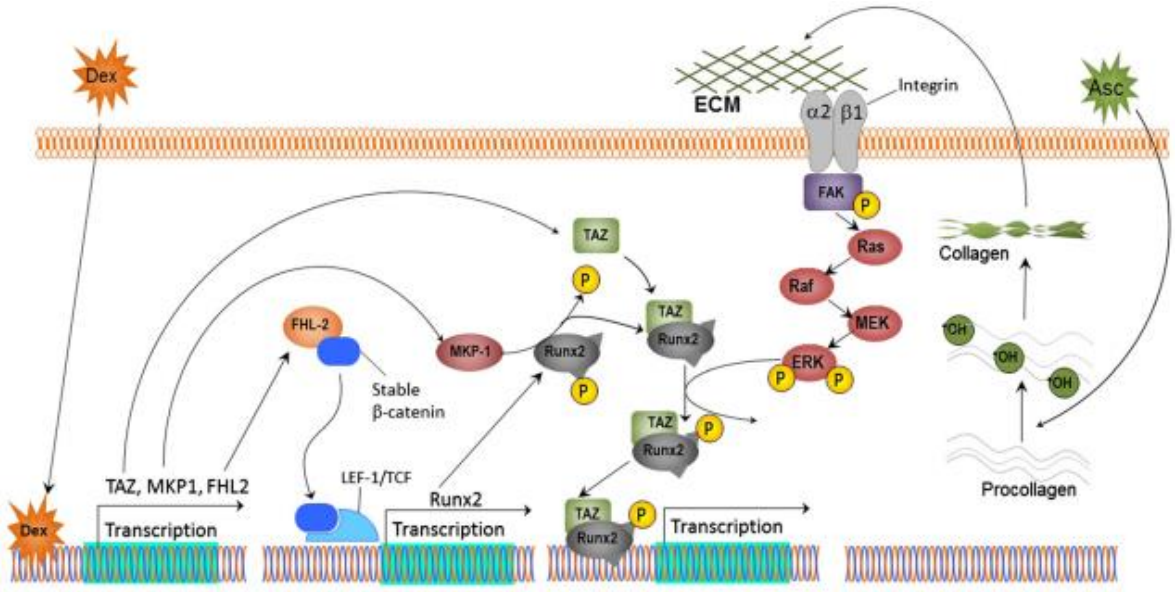

Figure 1. RUNX-2 is activated in the presence of dexamethasone by the transcription of TAZ, MKP1 and FHL2. At the same time Ascorbic acid (represented in this figure by Asc) causes collagen I synthesis, which is responsible for activation of the TAZ/RUNX-2-complex by α2β1-integrin and the MAPK signalling pathway **(55)**

The timing of dexamethasone addition is very important for the induction of osteogenesis. As was shown in human MSCs, less osteogenic differentiation takes place when dexamethasone is not present during the first fourteen days of culture (60). Additionally, a beneficial effect on

osteogenesis was reached when a shorter time period of dexamethasone supplementation took place. The best osteogenic differentiation was observed when cells received dexamethasone only the first 7 days of culture for both concentrations of 10nM and 100nM (60). An equal amount of differentiation was achieved in the presence of lower concentrations of dexamethasone (i.e., 10nM) throughout the whole culture period. However, the presence of 100nM of dexamethasone for 21 days had adverse effects. Giving higher concentrations of dexamethasone had negative effects on cell proliferation and viability, suggesting that the beneficial effect of dexamethasone supplementation only during the first 7 days on osteogenesis is achieved by preservation of cell proliferation and viability. Interestingly, it was found that 100nM dexamethasone is related to less inter- and intra-individual differences compared with 10nM dexamethasone.

Thus far, it is not known whether canine MSCs respond to the timing of dexamethasone in the same way that human MSCs do. Species-specific differences appear in literature regarding the effect of glucocorticoids. For example, in mice and rats dexamethasone increases the mRNA and protein production of aryl hydrocarbon receptor (AHR), a receptor involved in rapid proliferation, osteoblast differentiation and bone remodelling, via the glucocorticoid receptor (GR) (61) (62). Additionally cytochrome P4501A (CYP1A), involved in drug metabolism during the phase I biotransformation, is upregulated in rat-derived H4IIe cells. In humans however, dexamethasone reduces the AHR transactivation and CYP1A1. We cannot exclude the possibility that canine MSCs may respond differently to dexamethasone compared with human MSCs. Therefore, canine MSCs of 7 donors were cultured in this study for 21 days with dexamethasone for 21 days, 7 days and osteogenic medium without dexamethasone and it was found that indeed the canine MSCs response is different from human MSCs response. It was expected that, as in humans, 7 days of supplementation would initiate the best osteogenic differentiation. However, this study shows that, based on the alizarin red staining and qPCR results, the highest amount of osteogenesis was established with the continuous presence of dexamethasone.

Material and Methods

Bone marrow derived stem cells (BMSCs)

Bone marrow was obtained from 7 different donors ranging in age from 4 to 19 months, all of whom were used in unrelated experiments; this was permitted by the Ethics Committee on Animal Experimentation (DEC) of Utrecht University. Bone marrow was collected immediately from the humerus of both forelegs after the dogs were euthanized. The limbs were cut off and washed twice with Chlorexidine. At the medial side of the leg and parallel to the femur an incision was made to expose the bone. The diaphysis of humerus was removed with a saw and collected in a 50 ml tube with α-MEM (Invitrogen, 22561-021), 10% FCS(PAA Cell Culture Company), 1% penstrep (PAA, P11-010) and 15-20 UI/ml heparin (Leo Pharmaceutical Products BV, DG7794). Subsequently, the bone was washed with the medium and a sterile dish was used to collect the bone marrow. The cells were then counted with an automatic cell counter (Biorad TC10 automated cell counter) and plated in a T175 flask. Plating was done in expansion medium (10% FC, α-MEM, 1% penicillin/streptomycin

(p/s), 0.1 mM ascorbic acid (Sigma A8960), 10^{-9}M dexamethasone (Sigma D1756)) and FGF (AbD Serotec, PHP105, 1ng/ml). Per flask, a cell density of 1,3-2*10^6 live cells/cm^2 was reached. After 24 hours of incubation at 37 °C, 5% CO_2 and 20% O_2. The cells were washed twice with Hanks solution (PAA) which causes the dead and unattached cells and debris to be washed of. The remaining, living cells were trypsinizised and cryopreserved in α-MEM, 10% DMSO and 10% FCS in liquid nitrogen until they were used for the experiment.

Adipose tissue derived stem cells

Subcutaneous fat was obtained from the lower back after the animal was shaved and disinfected. It was collected in 50 ml tubes containing α-MEM and 1% p/s. The fat was dissected with a knife no 10 to remove blood vessels and fibrous tissue-connections. The fat was weighed, 0.04% collagenase type 1 (C9891, Sigma) was added in a 1:1 ratio and the mixture heated on an orbital shaker with 250 rpm at 37°C for 60 minutes until the fat was viscous. This suspension was then centrifuged at 1500 rpm for 15 minutes to obtain cells. The supernatant was removed and cells were suspended in α-MEM containing 10% FCS, 1% p/s and 0.05% Fungizone. After filtering with a 70µm cell strainer, the suspension was centrifuged again at 1500 rpm for 5 minutes at room temperature. The mononucleated cells obtained were plated in a T175 flask with a cell density of 7*10^6 live cells per flask. After 24-hours of incubation at 37°C, the cells were washed with 2% Hanks BSS and 2% FCS to remove dead and unattached cells. Thereafter, cells were cryopreserved in α-MEM, 10% DMSO and 10% FCS liquid nitrogen, until they were used in experiments.

Expansion medium

Cells receiving expansion medium served as a negative control in this experiment. Cells were thawed and used when passage 2 was reached. The cells were plated in a 12-wells plate with a cell density of 3,000 cells per cm^2 and supplemented with expansion medium, consisting of α -MEM with 10% FCS and 1% p/s, 10^{-4}M ASAP and 5 µL/mL fungizone (Invitrogen, 15290-018). The cell medium was changed every 3 to 4 days.

Osteogenic differentiation of MSCs and ASCs

MSCs were seeded in a density of 3,000 cells per cm^2 in 12-well plates and they received expansion medium the first 24-hours to adhere. Next, cells received osteogenic medium (D-MEM high glucose (Invitrogen, 31966-021), 10% FCS, 1% penicillin/streptomycin, 10mM β-Glycerolphosphate (Sigma, G6376), 0.1 mM ASAP and 5 µL/mL fungizone with 10^{-7}M of dexamethasone for 7 or 21 days or osteogenic medium without dexamethasone. Media were changed every 3 to 4 days and after 21 days the experiment was ended. For every condition and donor two wells were used for Alizarin red staining and RNA isolation.

Alizarin red staining

After 21 days, an Alizarin red staining was performed to stain calcium deposits. The cells were washed with Hanks and neutral buffered formalin (4%, Klinipath) was added for 30 minutes to establish fixation. The cells were washed twice with distilled water after the formalin was removed.

Each well was incubated for 20 minutes after receiving 0.75 ml Alizarin red (Sigma A5533-256, pH 4.1-4.3, final concentration 2%). After incubation, the cells were washed with distilled water until the water stayed clear and distillate water was added for analysis. Analysis of the cells was performed using a light microscope.

RNA isolation and cDNA

Cells were collected in 350µl RLT per well and stored at -70°C until isolation was performed. RNA isolation of the cells was achieved by the usage of a RNeasy minikit (Qiagen, 74134) according to manufactures protocol. To quantify the amount of RNA, a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands) was used. Next, cDNA was made using 400ng of RNA and iScript™ cDNA Synthesis Kit (Biorad), according to manufactures protocol.

qPCR

qPCR was executed with a BioRad CFX-384 cycler and IQ SYBRGreen SuperMix (BioRad, Veenendaal, The Netherlands). For all primers, non-Template controls were also taken in to account. Specific dog primers were used and analyses were performed using BioRad CFX Manager 3.0. Five reference genes were carefully chosen, based on earlier research with Genorm (45). Two ribosomal genes were selected; ribosomal protein L8 and ribosomal protein S5 (RPL8 and RPS5) and succinate dehydrogenase complex, subunit A (SDHA), glucoronidase beta (GUSB) and beta 2 microglobulin (B2MG) (primers represented in appendix 1). Three osteogenic markers, osteonectin (SPARC), osteopontin (SPP1) and osteocalcin (BGLAP) were analysed to determine the amount of osteogenic differentiation.

Statistics

A cox proportional hazard regression model with a survival analysis was performed, using R 3.0.2. CT values were used that were corrected for the mean reference genes CT value. CT values that did not come up were censored. The best model was fitted, considering the reference genes, cell type, donor, condition and cell type:condition interaction for each gene. A confidence interval not containing 1 was considered as significant. The p-value is additionally represented for those who prefer this.

Results

Alizarin red staining

The adipose tissue-derived and bone marrow-derived stem cells were stained after 21 days of culture with alizarin red staining to indicate calcium depositions (figure 2). Cells that received expansion medium did not show red staining. All adipogenic cell donors and 6 out of 7 bone marrow cell donors that received 7 days dexamethasone showed red noduli. Cells that received no dexamethasone showed the same amount of noduli and intensity as those that had received 7 days

of dexamethasone. Three adipogenic and four BMSC donors showed more intense red staining after 21 days of dexamethasone as compared to the other conditions.

During culture, cells detached in all the conditions, forming aggregates on day 7 when over-confluency was reached, but enough cells remained in monolayer to allow staining after 21 days. Additionally, the formed aggregates were cut and stained, after deparaffinization, with alizarin red. The aggregates that did receive dexamethasone showed red noduli. There was no red staining present when cells received osteogenic medium without dexamethasone and in the expansion receiving cells.

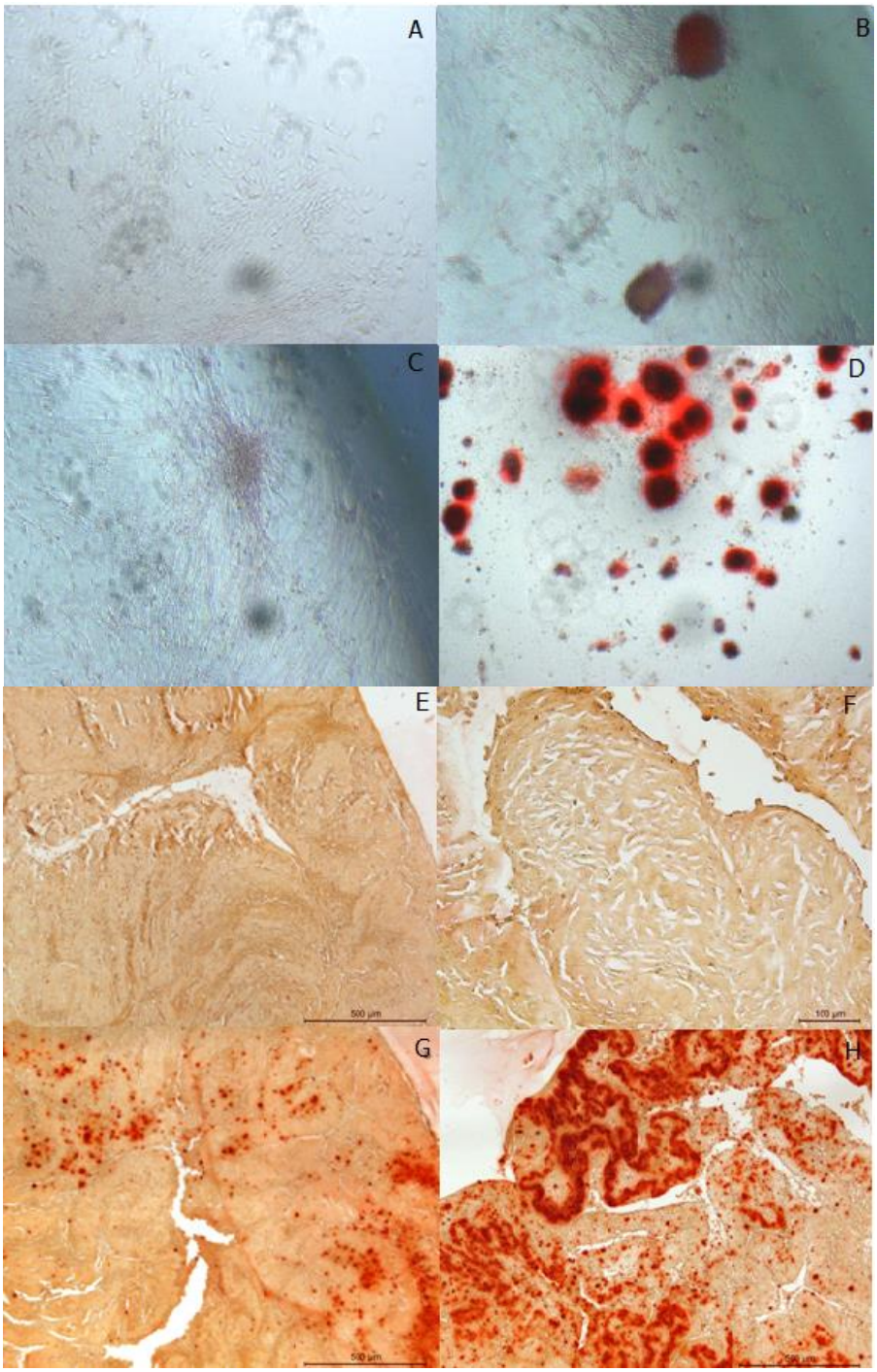

Figure 2. Light microscopy of alizarin red staining. Bone marrow derived MSC cultured in expansion medium have an elongated morphology (A). MSCs receiving osteogenic medium without dexamethasone do show differentiation, indicated by two red noduli (B). More mineralized noduli are present after 21 days (D) of dexamethasone supplementation as compared to 7 days alone (B). Tissue sections of 3D cell-aggregates (E-H) show no red noduli in expansion (E) and osteogenic medium without dexamethasone (F). Osteogenic differentiation is achieved after 7 days of culturing with complete osteogenic medium (G and H). (A-E, G and H) X4, bar 500µm, (F) X10, bar 100 µm

qPCR;

Gene expression of target genes was corrected for 5 reference genes and the average, relative gene expression (N-fold) was calculated for three osteogenic markers; osteonectin (SPARC), osteopontin (SPP1) and osteocalcin (BGLAP).

SPARC is an early osteogenic marker. This marker showed a downregulation for all conditions in both ASCs as BMSCs (figure 3). SPARC was significantly downregulated for 21 days of dexamethasone (p-value: 0.0064, Confidence Interval (CI): 0.02324-0.948) and almost for 7 days of dexamethasone (p-value: 0.0081, CI: 0.99832-48.247) in BMSCs. The difference in expression with osteogenic medium without dexamethasone was also significant for both conditions, with a p-value of 0.0024, CI: 0.01588-0.757 in 21 days of dexamethasone and p-value: 0.0022, CI: 0.01522-0.743 for 7 days. Osteonectin revealed only one ASC donor with a small upregulation.

SPP1 is expressed by osteoblasts, osteocytes, and osteoclasts and influences bone mineralisation rate and matrix resorption (63). SPP1 was significantly upregulated in cells that did not received dexamethasone for ASCs (p-value: 0.0011, CI: 1.719-173.08) and insignificant for BMSCs (figure 4) and showed the highest expression in 5 BMSC donors and 3 ASC donors. SPP1 was significantly downregulated in 21 days of dexamethasone in ASCs on average (p-value: <0.0005, CI: 2.08-194.81), whereas 7 days of dexamethasone showed an expression level which was similar with the control, and therefore was significantly different from 21 days dexamethasone (p-value: 0.0053, CI: 1.109-60.2). The expression of SPP1 was comparably upregulated in BMSCs for 7 and 21 days (13.40 and 12.57 respectively), however this was not significant. Additionally there was a significant difference (p-value < 0.00001, CI: 0.00093-0.164) in expression for 21 days of dexamethasone between the ASCs (-11.24) and BMSCs (12.57).

The mean relative gene expression of osteocalcin showed a non-significant downregulation of ASCs for all three conditions, with 21 days of dexamethasone being the least negative (figure 5). This is in contrast with the BMSCs, which revealed an average of 35 N-fold change in 21 days of dexamethasone. It was significantly upregulated compared to expansion (p-value < 0.0001, CI: 0.000521-0.117), no dexamethasone (p-value: 0.0063, CI: 0.022423-0.944) and 7 days of dexamethasone (p-value: 0.001 CI: 0.010382-0.615). Additionally, osteocalcin was significantly upregulated for no dexamethasone supplementation (p-value <0.001, CI: 1.8152-192.05) and 7 (p-value: 0.0034 CI: 1.2429- 84.53) days of dexamethasone in BMSCs as compared to the expansion medium.

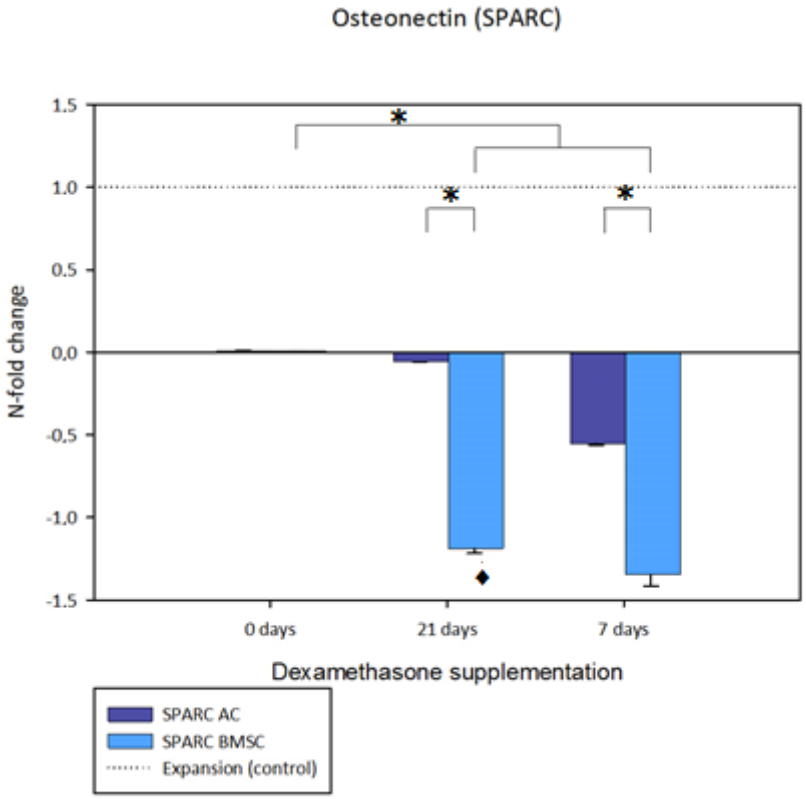

Figure 3. Gene expression of Osteonectin (SPARC). A significant downregulation is present for 21 days dexamethasone in BMSCs as compared to the expansion (dotted line), osteogenic medium without dexamethasone (0 days) compared to 21 and 7 days of dexamethasone in BMSCs and between the two cell types for 21 and 7 days of dexamethasone. ♦= p<0.05 in comparison with expansion (dotted line), *= p<0.05

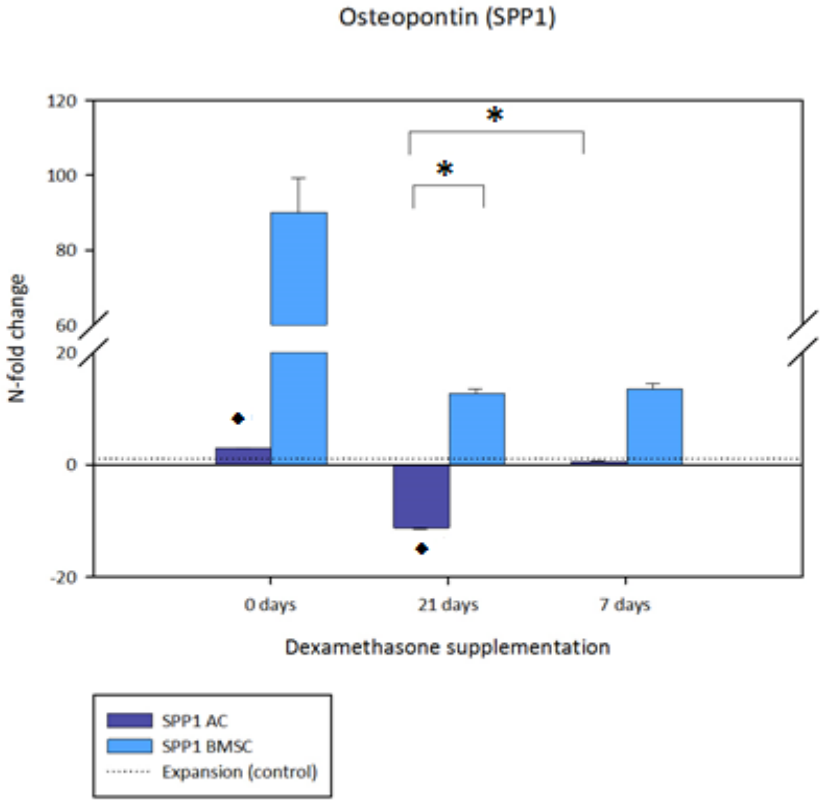

Figure 4. Gene expression of Osteopontin (SPP1). Osteogenic medium without dexamethasone (0 days) and 7 days of dexamethasone are significantly up-and downregulated in ASCs as compared to expansion (dotted line). The expression between 21 days and 7 days of dexamethasone is also significant in ASCs. 21 days of dexamethasone showed a significant difference in expression between the two cell types. ♦= p<0.05 in comparison with expansion (dotted line), *= p<0.05

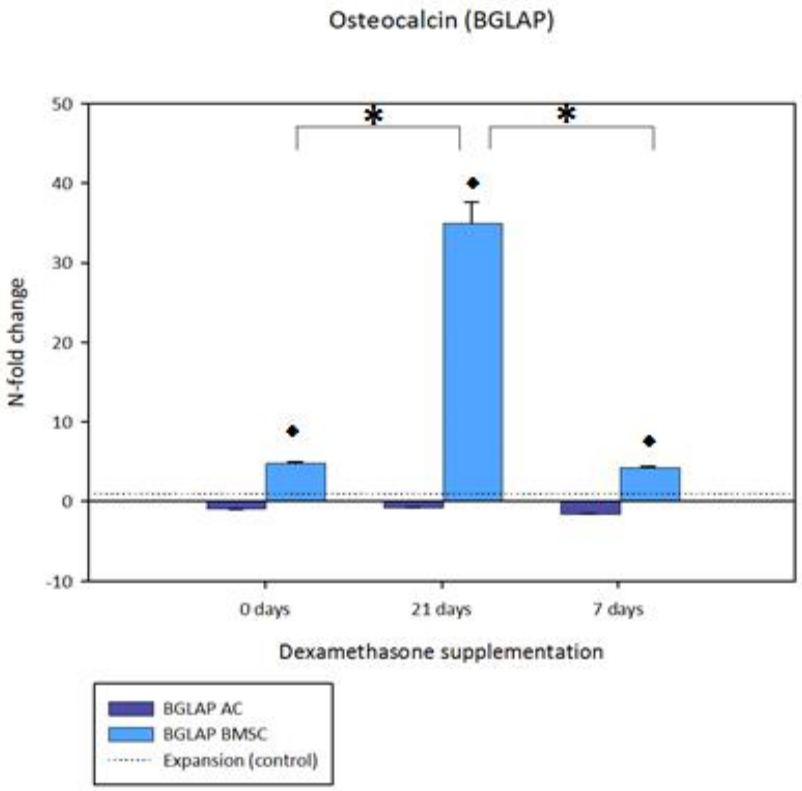

Figure 5. Gene expression of Osteocalcin (BGLAP). All three conditions are significantly upregulated in BMSCs. Additionally, 21 days of dexamethasone is significantly upregulated as compared to osteogenic medium without dexamethasone (0 days) and 7 days of dexamethasone. ♦= $p<0.05$ in comparison with expansion (dotted line), *= $p<0.05$

Discussion

Dexamethasone is not only used as a treatment in hormonal diseases, but is also important in initiating osteogenic differentiation in vitro and in vivo (64). However, the optimal culture conditions concerning supplementation with dexamethasone are not yet clear for every animal species. Dose and timing of dexamethasone supplementation during osteogenic differentiation are still a point of discussion. In this study, the timing of dexamethasone supplementation to obtain the best osteogenic differentiation in canine BMSCs and ASCs was tested, on the basis of alizarin red positive noduli and relative gene expression of osteocalcin. In canine MSCs it was found that Alizarin red staining showed the most noduli and intense staining after 21 days of dexamethasone and BGLAP showed the highest expression in 21 days of dexamethasone. This is contrary to the reported results in human MSCs, where it was found that 7 days of dexamethasone gave the best osteogenic differentiation (60) indicating that there are species-specific differences within the osteogenic lineage.

Reliability of osteogenic markers

In this study we examined three osteogenic markers to establish if osteogenic differentiation took place. Interestingly, the relative gene expression of SPP1 revealed the highest expression in BMSCs cultured in osteogenic medium without dexamethasone supplementation, although this was insignificant. Despite the high expression of SPP1 in the absence of dexamethasone, only a few noduli stained with the alizarin red staining. It is possible that in the absence of dexamethasone osteogenic differentiation is delayed as compared to dexamethasone supplementation during the first 7 days of culture or throughout the whole culture period (21 days). At 21 days, the expression of SPARC was decreased in all osteogenic conditions, compared with expansion. Given SPARC is an early marker of osteogenesis, we cannot exclude that SPARC is no longer upregulated at day 21 in cultures receiving osteogenic medium. Altogether, both SPP1 and SPARC did not corroborate osteogenic differentiation at day 21 even though there were clearly mineralized noduli present. SPP1 is expressed throughout the mineralisation process by osteoblasts and osteoclasts and is upregulated in small amounts after 3 hours up to 4 weeks (65) (66). These low levels are also found in vivo in developing rat bone with a maximum expression of SPP1 at day 14 (67). Altogether this indicates that SPP1 and SPARC are markers that may be useful during earlier stages of osteogenic differentiation (68). In contrast to SPP1 and SPARC, higher expression of BGLAP correlated with the presence of the mineralized noduli, indicating that this is an appropriate marker for the later stages of osteogenesis.

Osteogenic differentiation potential of adipose-tissue derived stem cells

The results of the ASCs are contradictory regarding osteogenic differentiation. While positive alizarin red stained noduli were present, the qPCR results did not support osteogenic differentiation. Relative gene expression of all three osteogenic markers was downregulated compared with controls. At this stage, one explanation would be that ASCs may need an additional stimulus, such as a growth factor, to differentiate. In line with our results, human ASCs have been reported to have less potential to differentiate in vitro towards the osteoblast lineage (49). However, ASCs are commonly used for during in vitro experiments and in in vivo therapy strategies. This is probably due to the fact that ASCs are easy to obtain in high amounts and have lower donor side morbidity (69).

Species-specific differences in osteogenesis

Interestingly, we demonstrated here that canine MSCs perform better in the osteogenic lineage in the presence of dexamethasone during the whole process than do human MSCs, which are preferably supplemented with dexamethasone only during the first 7 days of culture. This difference can be attributed either to a species-specific difference or study design differences. Thus while menopausal women may suffer from osteoporosis due to decreased levels of oestrogen (70), and ovariectomised rats suffer from the same symptoms and so can serve as an animal model for menopausal women (71), ovariectomised dogs do not suffer from secondary osteoporosis, possible due to a different oestrus cycle (72). Additionally, in humans it is known that a loss of bone mass, due to osteopenia and osteoporosis, is a common side effect after endo- or exogenous hypercorticism (73). In dogs, osteopenia is inducible with prednisone, however uncommon (74). This

indicates an intrinsic difference between bone homeostasis in humans and dogs, probably due to differences at least on the hormonal level. These differences could indicate that canine and human cells also respond differently to dexamethasone during in vitro experiments. As mentioned previously, rodents respond differently to dexamethasone than humans, with the upregulation of AHR and CYP1A1 after binding of a glucocorticoid. It cannot be excluded that there also exists a difference between dogs and rats or humans.

The differential response of canine MSCs to dexamethasone compared to previous reports on human MSCs could also be explained by differences in the experimental approach, including the length of the culture period and the donors used. In canine MSC, it is common to culture for 21 days, however the human MSCs were cultured for 28 days. We cannot excluded that dexamethasone only has negative effects on canine MSC proliferation and viability after day 21. Furthermore, the studies in human are limited by the number of donors used. Two donors are used in Jessica Alms' study to determine the optimal supplementation period with dexamethasone. This is a very small number and hence might not represent the total population. Moreover, these human donors suffered from osteoarthritis (OA). Mesenchymal stem cells in osteoarthritic patients have, due to the proliferative response of MSCs in OA patients, increased mRNA expression for TGF-β, TGF-β1 isoform, TGF-βR-II, and TGF-βR-III (75). TGF-β plays a role in osteogenic differentiation by its direct influence on RUNX-2 transcription through a canonical and non-canonical pathway, and indirectly through its interaction with PTH, Wnt, FGF and BMP signalling (18). In contrast, the canine donors in this study were healthy laboratory animals that did not suffer from OA, and therefore it is possible that their MSCs would react differently to the same treatment.

Pellets

During this study, in which a difference between dogs and humans was observed, a problem occurred after one week of culturing; monolayer cultures detached. The same problem with different detaching time points, ranging from 7 days until day 18, has been seen in previous studies performed by our group (data not shown) (46). To establish if the detached monolayers did differentiate, an Alizarin red staining was performed. The formed aggregates were stained and showed red noduli, suggesting that mineralisation did occur. In order to overcome this technical limitation, we opted to use a pellet culture system during osteogenic differentiation instead of monolayers. Typically, pellet cultures are used for chondorgenic differentiation of MSCs based on the assumption that condensation of MSCs within the fracture site, the first step, is followed by induction of chondrogenesis. Interestingly, 3-Dimensional culture methods with osteogenic differentiation are recently performed with positive results in human MSCs (76) and human cord blood-derived unrestricted somatic stem cells (77) (78).

Conclusions

Bone-marrow derived MSCs have a better osteogenic potential than adipose-tissue derived MSCs under similar culture conditions. Furthermore, canine MSCs demonstrate optimal differentiation towards the osteoblastic lineage when dexamethasone is supplemented during the entire 21-day culture period. This is in contrast with human MSCs where only 7 days of dexamethasone supplementation at the initiation of osteogenic differentiation gives the best result. Altogether this indicates that there may be species-specific differences in the activation of the GH pathway. Furthermore, pellet culture of MSCs to induce osteogenic differentiation might be more effective in inducing osteogenesis and better simulating the in vivo situation.

BMP-2 and -6: effects on osteogenic differentiation of canine MSCs in monolayer and aggregate culture

3

Abstract

Bone morphogenetic proteins (BMPs) are growth factors that are able to stimulate osteogenic differentiation through binding of BMP type II and BMP type I serine/threonine kinase receptors. BMP-signalling is antagonist by several factors, including noggin, which antagonizes all BMPs except BMP-6. In humans and mice, BMP-2 and BMP-6 are found to be the most potent inducers of bone formation of the BMP-family. In this study, BMP-2 and BMP-6 were supplemented over a wide dose range (1, 10, 50 and 100ng/ml) in canine bone marrow derived mesenchymal stem cells (MSCs) in monolayers and a 3-dimensional culture method of aggregates in order to define the optimal dose. Thereafter, osteogenic differentiation was assessed during a three week culture period in aggregates, with and without BMP-2 and BMP-6 100ng/ml supplementation. After 7 days of culture, 100ng/ml of BMP-2 and BMP-6 resulted in optimal osteogenic differentiation in monolayers. Additionally, BMP-2 and BMP-6 supplemented at a dose of 100ng/ml stimulated osteogenic differentiation during a three week culture experiment in aggregates, with BMP-2 being more potent in comparison with BMP-6. This is in contrast with reports in rat osteosarcomas, mice myoblasts and mice mesenchymal osteoprogenitor cells, and could be caused by BMP-receptor expression differences of BMP-RII (preferred by BMP-6) and BMP-RIB (preferred by BMP-2) and possibly noggin. It is tempting to hypothesize that noggin does not act as an antagonist of BMP-2, but as an agonist, as described for human MSCs.

Introduction

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and the majority of BMPs is known to induce osteogenesis to a certain extend. Most of the BMPs, except BMP-3 and BMP-8, possess the ability to upregulate Runt-related transcription factor 2 (RUNX-2). RUNX-2 is an important transcription factor during osteogenic differentiation and is essential for osteoblast maturation. In RUNX-2-null mice no endochondral and intramembranous bone formation occurs (79). BMP-2 and BMP-7 are currently the only BMPs that are commercially available as a treatment (rh-BMP-2 INFUSE® bone graft system (Medtronic, Inc.), rh-BMP-7Osteogenic Protein-1™ (OP-1™ Stryker Biotech). In dogs, BMP-2 has been reported to increase the Alkaline phosphatase (ALP) activity and the ALP, RUNX-2, and osteocalcin mRNA expression in mesenchymal stem cells (MSCs), suggesting that BMP-2 is able to initiate bone formation in these MSCs (80). BMP-7 has been used in several studies and demonstrated its potential in fracture healing in vivo in rabbits, humans, and dogs (81) (82) (83). In vitro, BMP-7 transfection increases osteocalcin (BGLAP) expression in relatively mature mouse osteoblasts (84). Despite the success of BMP-2 and BMP-7, BMP-6 has been reported to be more potent in initiating bone formation in human MSCs compared with BMP-2, -4, -7, and -14 (85). Additionally it was found in adenovirus transfected mice cells that BMP-2, BMP-6 and BMP-9 obtain the greatest potential in initiating osteogenesis in comparison with eleven other BMPs (86) (84).

BMPs cause osteogenesis to occur through binding to the BMP type II and BMP type I serine/threonine kinase receptors. These receptors are binding ligands, which act cooperatively. This means that the ligand is bound by both receptor types at the same time. There are three type II receptors (BMP receptor II (BMPR-II), activin receptor II (ACTR-II), and ACTR-IIB) and three type I receptors (BMPR-IA, BMPR-IB, and activin receptor-like kinase2 (ALK2)). After phosphorylation of BMP type II and BMP type I receptors, Smad transcriptional modulators (R-Smad1/5/8) are recruited and phosphorylated (87). Thereafter, Smad4, which is also present in the TGF-β signalling pathway of bone formation, is phosphorylated by the BMP receptors and becomes active by binding of Smad1/5/8 (88).

BMP signalling is regulated by several antagonists. Smad6 has the ability to block the BMP signalling by binding Smad1 and thus compete with Smad4 (89). Additionally, Smad6 inhibits Smad1 phosphorylation through BMPR-IA. Smad6 has furthermore the ability to bind TGF-β-RI, but also ACTR-II and BMPR-IB (24). Another antagonist is Smurf1. Smurf1 can interact and degradate R-Smads and thereby negatively regulate the BMP signalling (23). Upregulation of mRNA of Smad6 and Smurf1 in response to BMP-2 causes degradation of the BMPR-IB receptor, therefore BMP-2 ligand does not increase the BMPR-IB activity and no positive feedback occurs. Furthermore, there is another BMP antagonist, noggin, which is upregulated when the BMP signalling pathway is activated. BMP-2 is responsible for increasing noggin mRNA expression in a time-dependent and dose-dependent (up till 1 µg/ml) manner in human bone marrow derived MSCs (90). BMP-4 is most sensitive to noggin antagonism, followed by BMP-2 and thereafter BMP-7 in rat osteosarcomas, mice myoblasts and mice mesenchymal osteoprogenitor cells (91). BMP-6 is the least affected by noggin as compared to BMP-2, -4 and -7 and noggin does not interfere with BMP-6 receptor binding.

The latter most probably explains why BMP6 has been shown to be more potent in mice myoblast (C2C12) and multipotent mesenchymal cells (C3H10T1/2).

BMP is mostly added in a dose of 100ng/ml or more to stimulate osteogenic differentiation in vitro (80) (18) (85). In humans and mice comparative studies between several BMPs and their osteogenic potential has been performed (85) (86) (84). However, in dogs this has not yet been described. In this study, a wide range of BMP-2 and BMP-6 (1 to 100ng/ml) were evaluated and compared for their effects on osteogenesis in canine MSCs (figure 1). In order to do so, the osteogenic potential of BMP-2 and BMP-6 over a wide dose range was evaluated at first during a short term culture period (7 days). Thereafter, follow up studies were performed for 7 and 21 days of culture. The golden standard of in vitro osteogenic differentiation is performed in monolayer cultures. However, culturing in monolayers by no means represents the in vivo situation. Recently, stem cells cultured in a 3-dimentional (3D) culture revealed osteogenic differentiation in human MSCs (76) and human cord blood-derived unrestricted somatic stem cells (77) (78). Therefore in this study, a 3D culture method of aggregates was tested in a 7 day culture experiment with the addition of BMP (figure 1, blue circles) and in a 21 day culture period for expansion and osteogenic medium (figure 1, green circles). A comparison between monolayer culturing and 3D culturing was made for the first time in canine MSCs. It was found that the presence of 100ng/ml BMP-2 and BMP-6 gives the best osteogenic differentiation and that osteogenic differentiation did occur in aggregates. Subsequently this experiment was followed by a long term culture period experiment (21 days) with the optimal dose of BMP-2 and BMP-6 in aggregates.

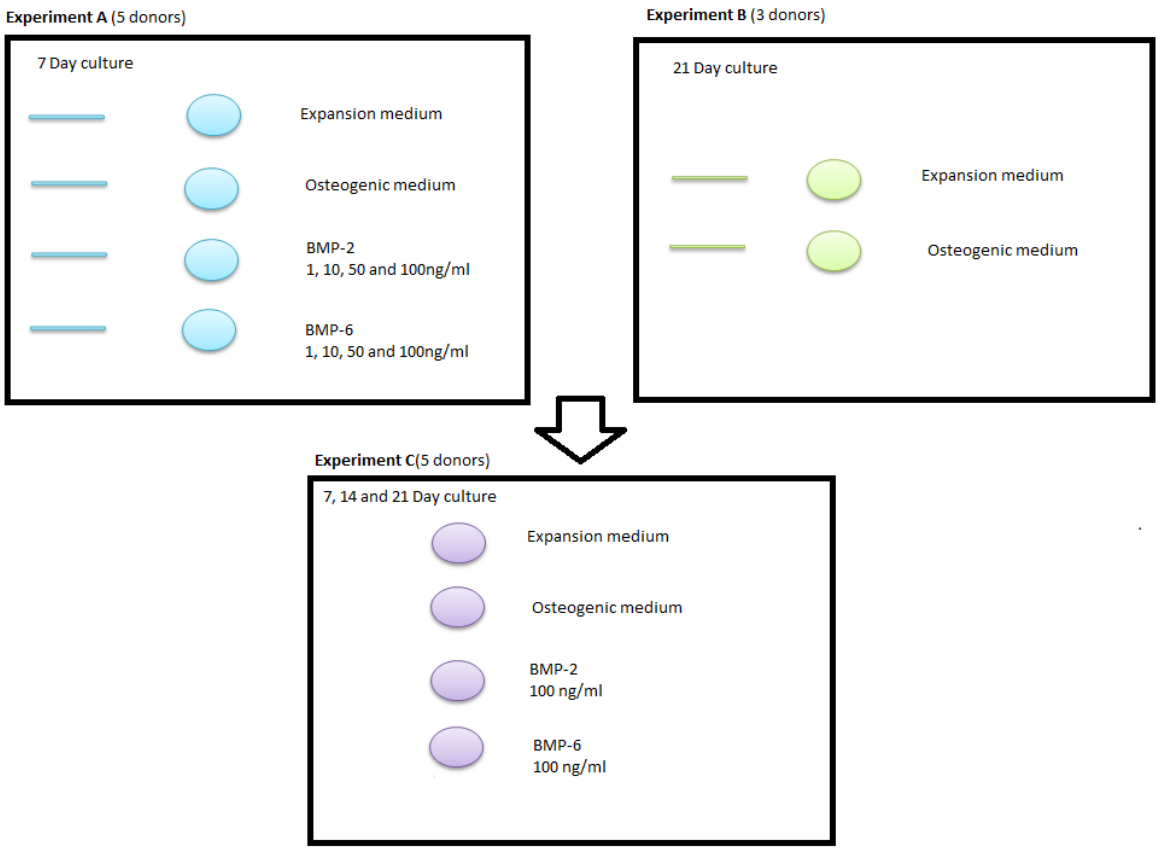

Figure 1. a schematic representation of the study design. The bars represent monolayer culture, the circles 3D pellet culture of cell-aggregates. A 7-day culture experiment was performed using both monolayers and aggregates with the addition of expansion, osteogenic medium and BMP-2 or BMP-6 1, 10, 50 and 100ng/ml in monolayers and 10, 50 and 100ng/ml in aggregates (blue). Additionally, monolayers and aggregates were cultured with expansion and osteogenic medium (green) in order to determine whether 3D culture favors osteogenic differentiation compared with 2D culture over the course of 21 days of culture. These two studies were evaluated and a final experiment was performed in which aggregates were cultured for 21 days and treated with expansion and osteogenic medium, and osteogenic medium supplemented with BMP-2 or BMP-6 100ng/ml (purple circles).

The colours corresponding with each experiment are used in the sections of results for reasons of clarity.

Material and Methods

Bone marrow derived stem cells

Bone marrow derived MSCs (BMSCs) of five donors were used during a short term (7 days) and two long term (21 days) experiments. Cell isolation and expanding was performed as described in detail in chapter two. Briefly, MSCs were collected from the humerus of five different donors used in unrelated experiments. Cells were plated in T175 flasks after adding expansion medium (10% FC, α-MEM, 1% penicillin/streptomycin (p/s), 0.1 mM ascorbic acid (Sigma A8960), 10^{-9}M dexamethasone (Sigma D1756)) and FGF (AbD Serotec, PHP105, 1ng/ml) and incubated at 37 °C, 5% CO_2 and 20% O_2. Once they reached a confluency of approximately 60%, BMSCs were cryopreserved at P0 in αMEM containing 10% DMSO and 10% FCS.

Cell media

Prior to the experiment, P0 cells were expanded to P2. Thereafter, cells were seeded with a density of 3,000 cells per cm^2 in monolayers. Cell aggregates were prepared with 200,000 cells per well seeded in an Ultra Low Attachment plate (Corning Incorporated, 07214040). Aggregates were prepared by centrifugation for 5 minutes at 1500 rpm to enable pellet formation.

Experiment A: cells in monolayers and aggregates received expansion medium (α -MEM with 10% FCS and 1% p/s, 10^{-4} M ASAP and 5 µL/mL fungizone (Invitrogen, 15290-018) or osteogenic medium (D-MEM high glucose (Invitrogen, 31966-021), 10% FCS, 1% penicillin/streptomycin, 10mM β-Glycerolphosphate (Sigma, G6376), 0.1 mM ASAP and 5 µL/mL fungizone, 10^{-7}M of dexamethasone) for 7 days. Additionally, BMP-2 and BMP-6 (R&D) were supplemented in a dose of 1, 10, 50 or 100ng/ml for 7 days in monolayers and 10, 50 or 100ng/ml in aggregates. The media for each condition was changed every 3 to 4 days. Additionally, in **experiment B** monolayers and aggregates were cultured for 21 days with expansion and osteogenic medium. Each well received 0.5 ml medium in monolayers and 0.2 ml in aggregates.

Based on the results from the short term (7 days) experiment with BMP-2 and BMP-6 (experiment A) and the long term experiment (21 days) with expansion and osteogenic medium in monolayers and aggregates (experiment B), an additional study was performed with aggregate cultures for 21 days with the addition of BMP-2 and BMP-6 100ng/ml (**experiment C)**. For this purpose, BMSCs of five donors were seeded in a density of 200,000 cells per well and received expansion media the first 24-hours. Thereafter, aggregates were treated with osteogenic medium alone, or osteogenic medium supplemented with BMP-2 or BMP-6 100ng/ml for the period of 21 days.

Alizarin red staining, RNA isolation and qPCR

Alizarin red staining, RNA isolation and qPCR were performed for monolayers as described in detail in chapter two. Briefly, after 4% formalin fixation, cells were washed and stained for 20 minutes with Alizarin red (Sigma A5533-256, pH 4.1-4.3, final concentration 2%) to demonstrate calcium deposits. For each donor two wells were stained. RNA isolation was performed using a RNeasy minikit (Qiagen 74134) according to manufactures protocol, after pooling two wells for each donor. RNA was measured using a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands). An input of 400 ng of RNA was used to make cDNA with iScript™ cDNA Synthesis Kit (Biorad), according to manufactures protocol. Thereafter, qPCR was executed using a BioRad CFX-384 cycler and IQ SYBRGreen SuperMix (BioRad, Veenendaal, The Netherlands). Five reference genes were used; ribosomal protein L8 and ribosomal protein S5 (RPL8 and RPS5) and succinate dehydrogenase complex, subunit A (SDHA), glucoronidase beta (GUSB) and beta 2 microglobulin (B2MG) (primers represented in appendix 1). To determine the amount of osteogenic differentiation, three osteogenic markers, RUNX-2, osteopontin (SPP1) and osteocalcin (BGLAP), were analysed.

Aggregates were tested for the same parameters as monolayers with some small adaptations for the alizarin red staining and the RNA isolation. Aggregates were stained with Alizarin red staining after

they were imbedded in paraffin. Four µm thick sections were deparaffinised and hydrated, the sections were stained with haematoxylin (Vector, H3404) for 10 seconds and washed with tab water. Next, sections were stained with alizarin red staining for two minutes. In aggregates, RNA isolation was performed using a RNeasy microkit (Qiagen, 74004) according to manufactures protocol, after crushing of the pellets using dry ice and liquid nitrogen. Thereafter cDNA, with a RNA input of 50ng/µl, and qPCR was performed as for the monolayers.

Von Kossa staining

Alongside the Alizarin red staining, aggregates were also stained with Von Kossa staining after 7, 14, and 21 days of culturing. A Von Kossa staining is used to give an indication of mineralization through the binding of phosphate (92). Tissue sections were stained with haematoxylin for 10 seconds and washed with tab water. Thereafter, the sections were stained with 5% silver nitrate under Ultra Violet light to establish a Von Kossa staining. After 20 minutes the silver nitrate was removed and 5% sodium thiosulphate was added for 2 minutes.

Alkaline Phosphatase assay and DNA content

Both monolayers and aggregates were evaluated for Alkaline Phosphatase (ALP) activity. ALP is an enzyme that is expressed on the cell membrane and matrix vesicles of bone and calcifying cartilage cells (93). The ALP activity will rise until day 21 in osteoblasts and thereafter declines again (94).

Monolayers were collected after 7, 14, and 21 days of culture in a lysis buffer (1,5M Tris-HCL pH 9,0 + 2% Triton X100 (Sigma-Aldrich) and stored at -20°C until further analysis. Aggregates were crushed using dry ice and liquid nitrogen. The monolayers and crushed aggregates were sonicated in a sonication bath (Sonicor) for 10 minutes to obtain homogenates. From each sample 5 µl was added to a 96-wells plate well. Thereafter, an activity assay buffer (1,5M Tris-HCL pH 9,0 + 1M $ZnCl_2$/1M $MgCL_2$) with 7,5M 4-nitrophenyl-phosphate (pNPP) (Sigma-Aldrich, p7998) was added. The absolute amount of nitrophenyl phosphate formed was determined using a calibration line. The conversion of pNPP to nitrophenyl phosphate was measured after 30 minutes of incubation at 37°C. To stop further conversion of pNPP a stop buffer (1M natriumhydroxide) was added. The amount of conversion was measured using 405nm spectrophotometric analysis. These measurements were then converted to ALP activity with the aid of the calibration line. ALP activity was corrected for DNA content.

The DNA content was measured from the same samples of the ALP activity assay using the Qubit™ dsDNA high sensitivity assay kit (Invitrogen, Q32854) and the Qubit™ 2.0 Fluorometer (Invitrogen, Q32866). High sensitivity standard lines were made, containing 3 µl of lysis buffer, because the lysis buffer interferes with the Qubit. Each sample (3 µl) was measured after adding 197 µl working solution (199 Qubit buffer : 1 Qubit Reagent).

Statistics

Statistical analysis was performed using a cox proportional hazard regression model with a survival analysis in R 3.0.2. CT values, corrected for mean reference genes, were used. The best model was fitted, taken the reference genes, donor, condition, time point and time point:condition interaction

into account. A confidence interval (CI) not containing 1 was considered as significant. Next to the CI, the p-value is represented for those who prefer this.

Data were found to be non-parametric for some culture groups. In those cases statistics was performed using IBM SPSS Statistics 22. First a Kruskal Wallis test was done to determine which parameter was significantly affected by the treatment. Thereafter, the non-parametric Mann-Whitney test was performed to compare all groups with each other. The obtained p-values were then corrected for multiple comparisons using the Benjamini Hochbergs' False Discovery Rate. Multiple comparisons were performed for relevant conditions, i.e. expansion and osteogenic medium treated cells were compared with all other conditions and BMP-2 and BMP-6 were reciprocal analysed for the same dose.

Results

Monolayers and aggregates reached day 7 of culturing (during **experiment A**) successfully for all conditions, including expansion and osteogenic medium, supplementation with BMP-2 and BMP-6 1, 10, 50 and 100 ng/ml in monolayers and 10, 50 and 100 ng/ml in aggregates. Additionally monolayers and aggregates were cultured for 21 days with expansion and osteogenic medium (in **Experiment B**). Unfortunately a few wells of monolayers detached, but enough cells remained to perform analysis. The long term BMP study in aggregates also reached the end of the culture period successfully.

qPCR

Monolayers and aggregates were tested for gene expression of osteogenic markers, RUNX-2, SPP1, and BGLAP. Additionally, noggin, the antagonist of BMP signalling, and Id1, the down-stream target of SMAD 1/5/8 and hence indicator for the activation status of the respective pathway, were examined.

Experiment A: after a 7-day culture period, gene expression profiles of the late osteogenic markers, SPP1 and BGLAP, showed no significant upregulation in both monolayers and aggregates. RUNX-2, Id1 and noggin revealed only moderate changes. Given the relative low number of donors tested in this pilot experiment, the absence of a significant difference does not necessarily exclude a biologic effect. The highest expression in RUNX-2 in monolayer cultures was observed in the conditions with osteogenic medium, supplemented with BMP-2 (no significance) or BMP-6 10 ng/ml (p-value: 0.025) (figure 2). Additionally, BMP-2 50ng/ml (p-value: 0.033) and BMP-6 50ng/ml (p-value: 0.022) and 100ng/ml (p-value: 0.00.014) were significantly upregulated in comparison with expansion in monolayers. Id1 was also highest expressed, in monolayers, in BMP-2 10ng/ml (no significance) and BMP-6 10ng/ml (p-value: 0.019), with the addition of BMP-2 100ng/ml (p-value: 0.017) and BMP-6 50ng/ml (p-value: 0.013) and 100ng/ml (p-value: 0.019) (figure 3). In aggregates the differences in gene expression levels of RUNX-2 were similarly small, with BMP-2 and BMP-6 100ng/ml having the highest expression. Id1 was highest expressed in aggregates in BMP-6 100ng/ml, suggesting that at day 7 the Smad1/5/8 pathway was highest activated in this condition.

Noggin was highest expressed in BMP-6 100ng/ml and thereafter BMP-2 100ng/ml in monolayers after 7 days of culture (figure 4). In aggregates only the lower dose of 10ng/ml was examined and this showed no upregulation in comparison with expansion.

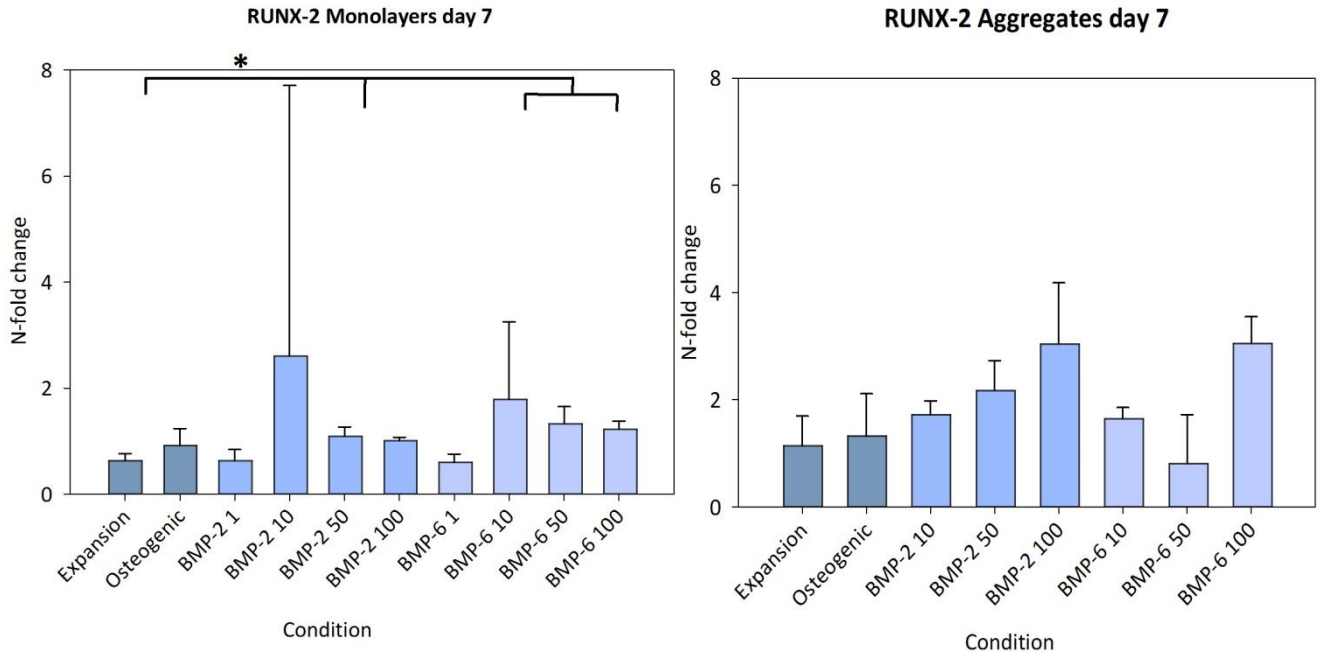

Figure 2. Gene expression profiles of RUNX-2 in monolayers (left) and aggregates (right) after 7 days of culture with expansion medium, osteogenic medium or osteogenic medium, supplemented with BMP-2 or BMP-6 (1, 10, 50 or 100ng/ml). In monolayers in BMP-2 50ng/ml, BMP-6 10, 50 and 100ng/ml is significantly higher in comparison with expansion. *= p<0.05

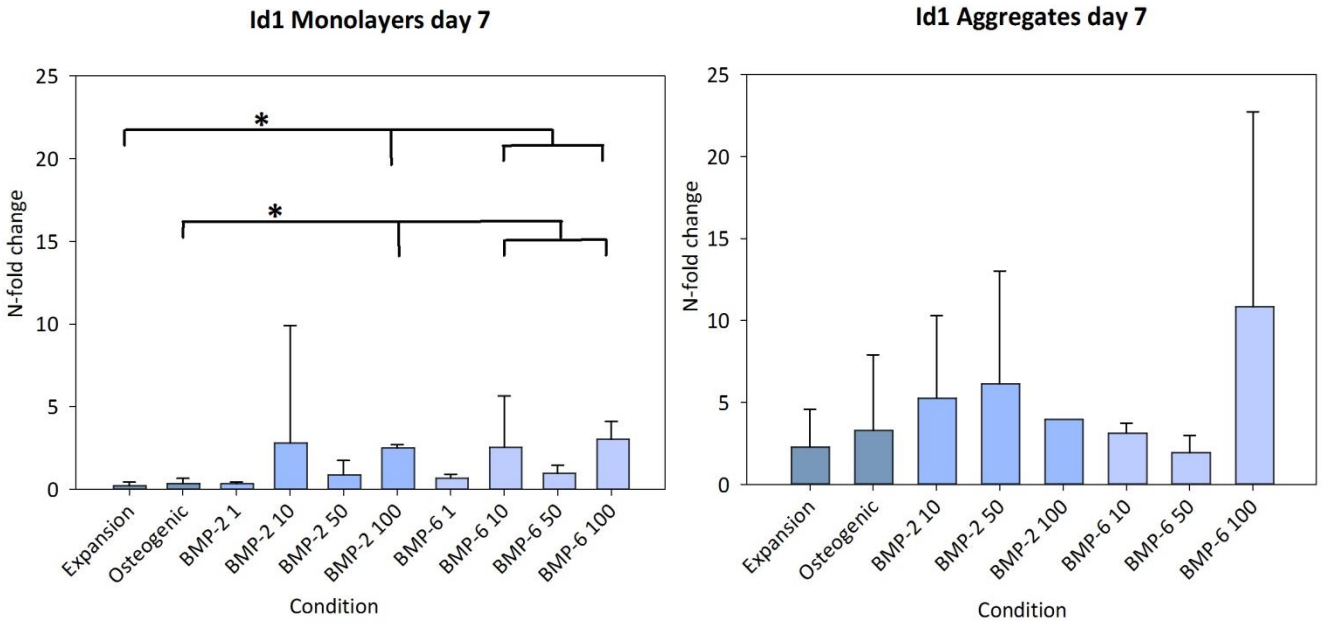

Figure 3. Gene expression profiles of Id1, a target gene indicating SMAD 1/5/8/ activation, in monolayers (left) and aggregates (right) after 7 days of culture with expansion medium, osteogenic medium or osteogenic medium, supplemented with BMP-2 or BMP-6 (1, 10, 50 or 100ng/ml). In monolayers BMP-2 100ng/ml, BMP-6 10, 50 and 100ng/ml is significantly higher in comparison with expansion and osteogenic medium. *= p<0.05

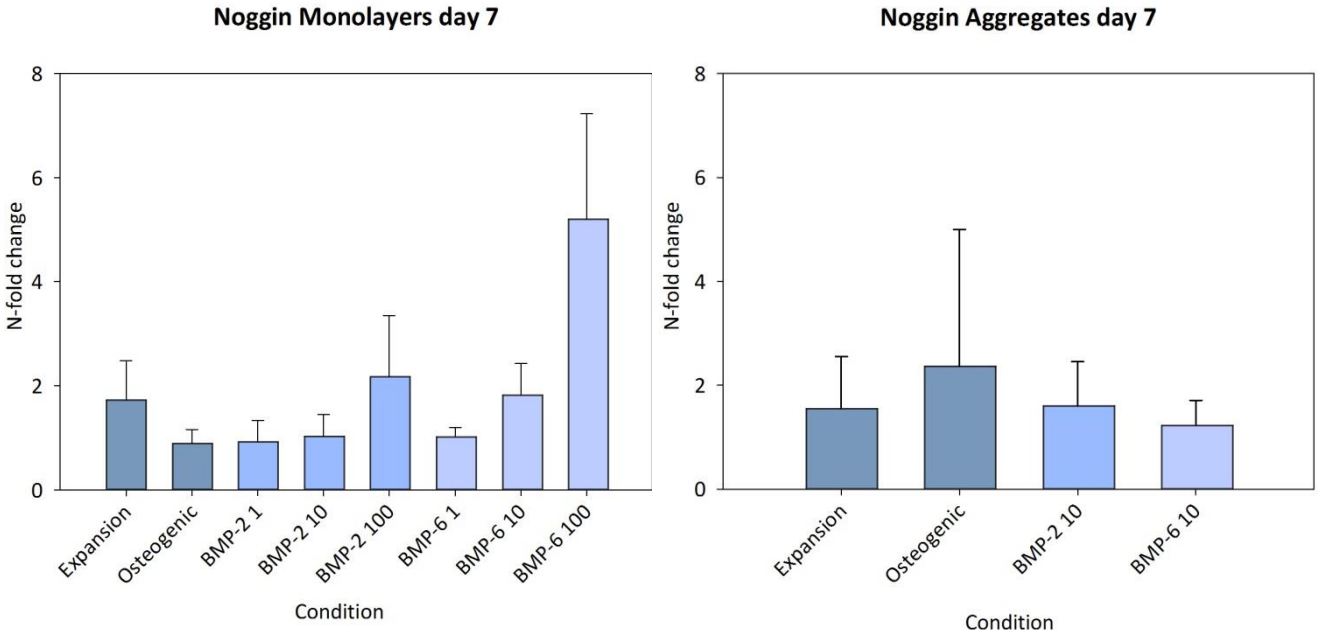

Figure 4. Gene expression profiles of Noggin, the antagonist of BMP signaling, in monolayers (left) and aggregates (right) after 7 days of culture with expansion medium, osteogenic medium or osteogenic medium, supplemented with BMP-2 or BMP-6 (10ng/ml). There were no significant differences between the culture conditions in both culture systems.

During **experiment B**, where monolayers and aggregates of cells received expansion or osteogenic medium, two osteogenic markers that are expressed later during osteogenesis, SPP1 and BLGAP, were evaluated. SPP1 expression of cells receiving osteogenic medium was upregulated at day 7 (p-value: 0.01) in monolayers in comparison with expansion, and thereafter declined (figure 5). The expression in aggregates showed the highest gene expression at day 14, though insignificant due to donors that were not available in SPP1. BGLAP showed a significant upregulation at day 7 (p-value: 0.005) in monolayers in comparison with expansion (figure 6). Additionally, osteogenic medium receiving cells were higher expressed in comparison with expansion at day 14, but no significance was reached, due to low number of available donors. The aggregates showed the highest expression of osteogenic medium receiving cells at day 7 in comparison with day 14 and 21, though the expression of expansion is comparable at this time point.

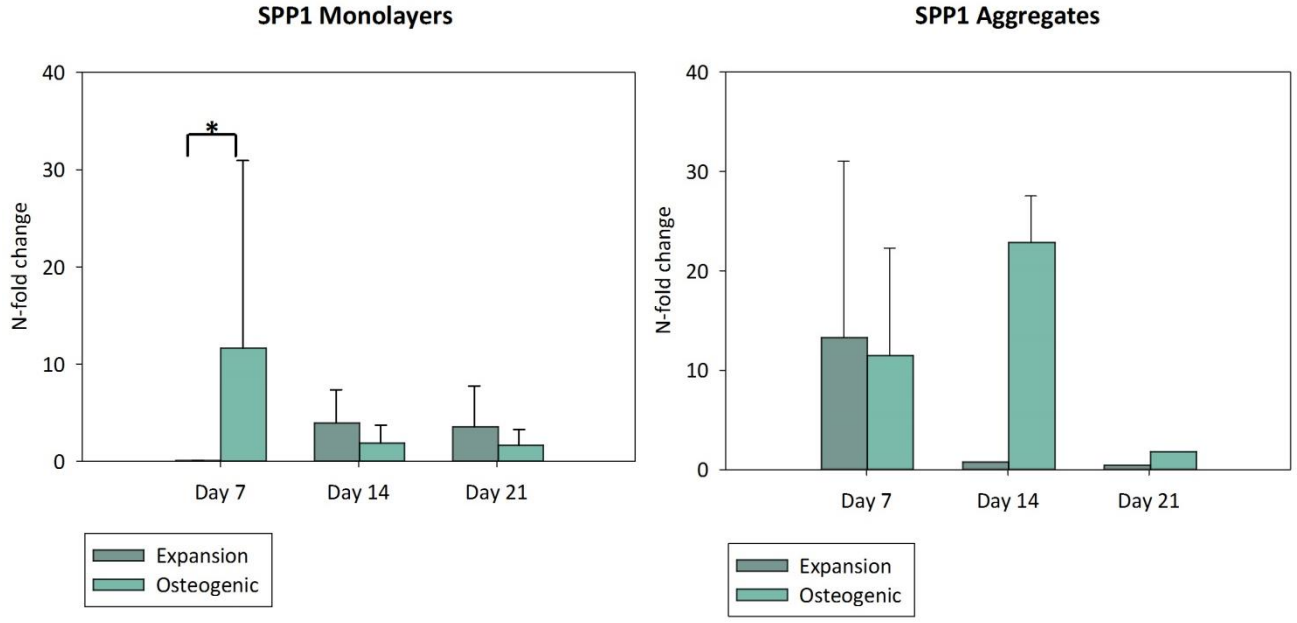

Figure 5. Gene expression profiles of Osteopontin (SPP1), an early marker of osteogenesis, in monolayers (left) and aggregates (right) after 7, 14 and 21 days of culture with expansion medium or osteogenic medium. In monolayers, SPP1 was significantly increased in osteogenic culture conditions compared with expansion at 7 days of culture. *= $p<0.05$

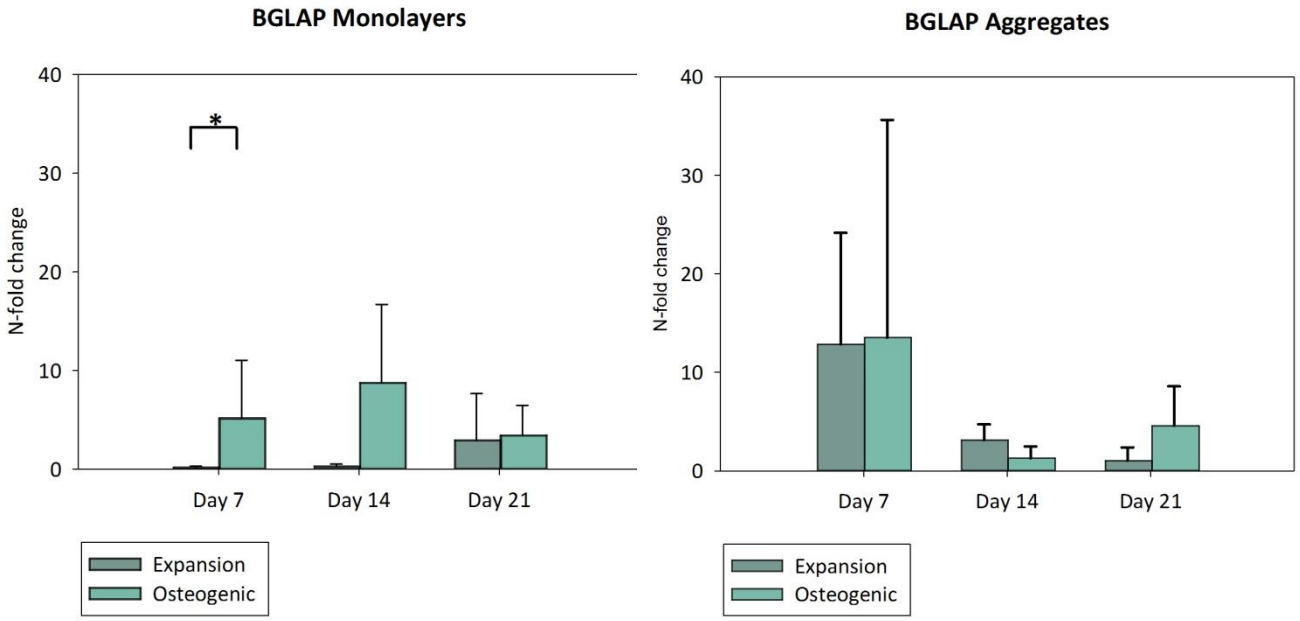

Figure 6. Gene expression profiles of Osteocalcin (BGLAP), a late marker of osteogenesis, in monolayers (left) and aggregates (right) after 7, 14 and 21 days of culture with expansion medium or osteogenic medium. BGLAP was significantly increased in osteogenic culture conditions compared to expansion at day 7 of culture. *= $p<0.05$

Experiment C: in order to determine the additional effect of BMP-2 and BMP-6 supplemented at a concentration 100ng/ml in osteogenic medium the expression of SPP1, BGLAP, Id1 and noggin was determined in aggregates. Notably, all genes revealed a lower expression in this final experiment as compared to experiment B. SPP1 expression was upregulated in all conditions at day 14 in aggregates compared with day 7, but only significantly in BMP-2 and BMP-6 (p-value: 0.04) (figure 7). The highest expression of BGLAP was present at day 14 in cells receiving BMP-2 100ng/ml, though insignificant, most probably due to the fact that only two donors caused this upregulation (figure 8). Aggregates receiving osteogenic medium with or without BMP-6 did not show any BGLAP upregulation at day 7 or 14. Relative gene expression of Id1, was upregulated in the BMP-2 treated aggregates, and in the BMP-6 treated aggregates in lesser extent, at day 7 and 14 (figure 9). Id1 was significantly upregulated in BMP-2 compared to osteogenic at day 7 (p-value: 0.04) and day 14 (p-value: 0.027). Additionally, Id1 was significantly upregulated in BMP-6 compared to osteogenic at day 14 (p-value: 0.02). Similarly, noggin showed a significant upregulation in osteogenic medium supplemented with BMP-2 at day 7 and 14 in comparison with osteogenic (p-value: 0.04) (figure 10). BMP-6 was significantly upregulated compared to osteogenic at day 7 (p-value: 0.053).

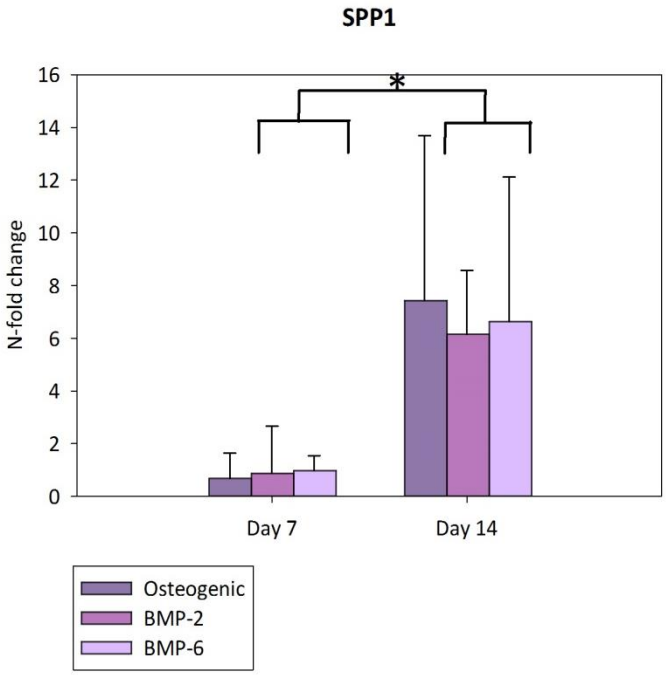

Figure 7. Gene expression profiles of SPP1 in aggregates after 7, and 14 days of culture with osteogenic medium without and with BMP-2 or BMP-6 100ng/ml supplementation. A significance upregulation was present between day 7 and day 14 in BMP-2 and BMP-6. *= p<0.05

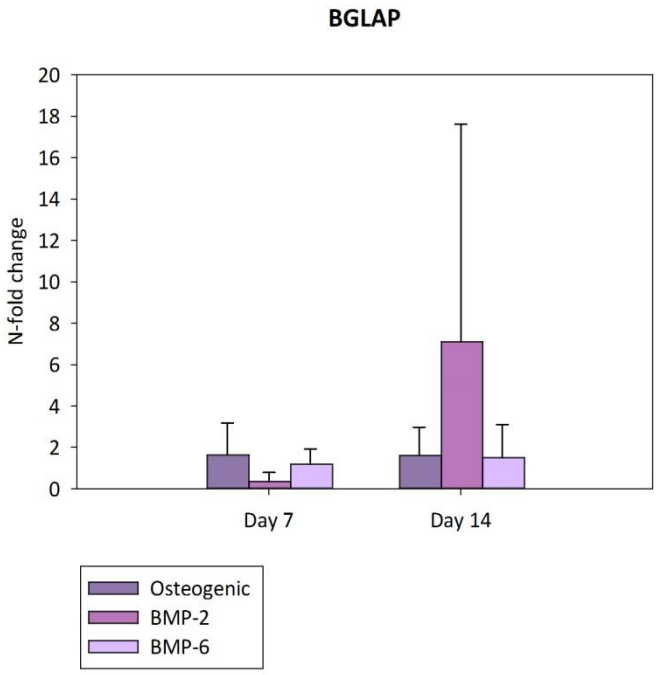

Figure 8. Gene expression profiles of SPP1 in aggregates after 7, and 14 days of culture with osteogenic medium without and with BMP-2 or BMP-6 100ng/ml supplementation.

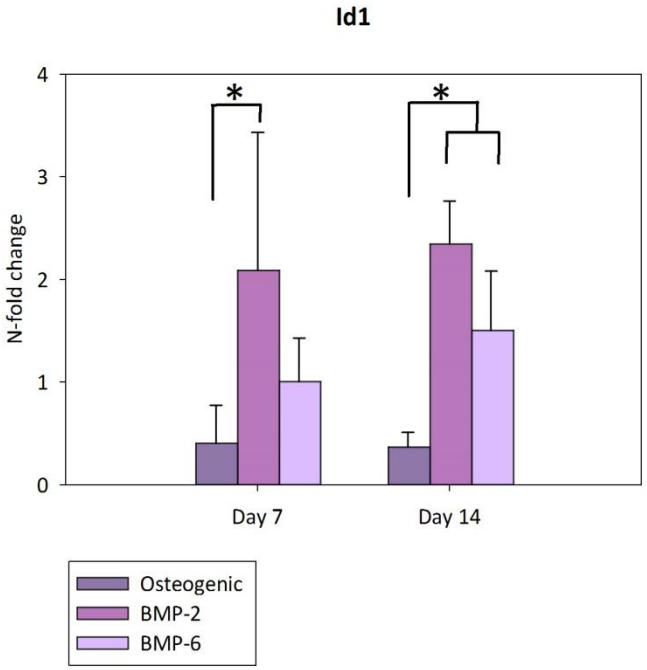

Figure 9. Gene expression profiles of Id1 in aggregates after 7, and 14 days of culture with osteogenic medium without and with BMP-2 or BMP-6 100ng/ml supplementation. BMP-2 was significantly higher expressed in comparison with osteogenic conditions both time points. Additionally, BMP-6 was significantly higher expressed at day 14 in comparison with osteogenic conditions. *= p<0.05

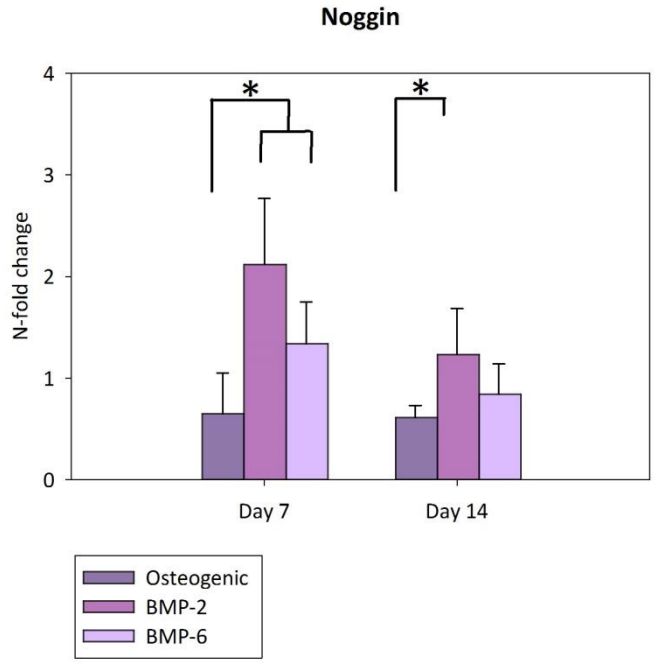

Figure 10. Gene expression profiles of noggin in aggregates after 7, and 14 days of culture with osteogenic medium without and with BMP-2 or BMP-6 100ng/ml supplementation. BMP-2 was significantly higher expressed in comparison with osteogenic conditions both time points. Additionally, BMP-6 was significantly higher expressed at day 7 in comparison with osteogenic conditions. *= p<0.05

ALP

Experiment B: the ALP activity was measured for all conditions and corrected for the DNA content after 1, 2 and 3 weeks of culturing in monolayers and aggregates with expansion or osteogenic medium. The same ALP/DNA ratio was found in expansion and osteogenic medium during the first two weeks of culture in monolayers (figure 11). However, at day 21 the ALP/DNA ratio rises only in osteogenic medium treated cells and is found to be significantly higher in comparison with day 7 (p-value: 0.013). The ALP/DNA ratio in aggregates was almost 10-fold higher in comparison with monolayers allready at day 7, suggesting more ALP activity per cell in aggregates (figure 12). However, treatment of aggregates with osteogenic medium resulted lower ALP/DNA ratio compared to expansion medium. A significant difference for expansion was found at day 7 in comparison with the same condition at day 14 (p-value: 0.041) and day 21 (p-value: 0.003), and for osteogenic at day 7 in comparison with day 21 (p-value: 0.02).

Experiment C: the ALP/DNA ratio was lower in comparison with the aggregates in experiment B, as did the relative gene expression of osteogenic markers (figure 13). The addition of BMP-2 seemed to increase the ALP activity at day 7 in four out of five donors in comparison with osteogenic group and BMP-6, though it was found to be insignificant. At 14 and 21 days of culture the ALP/DNA did not differ between culture conditions.

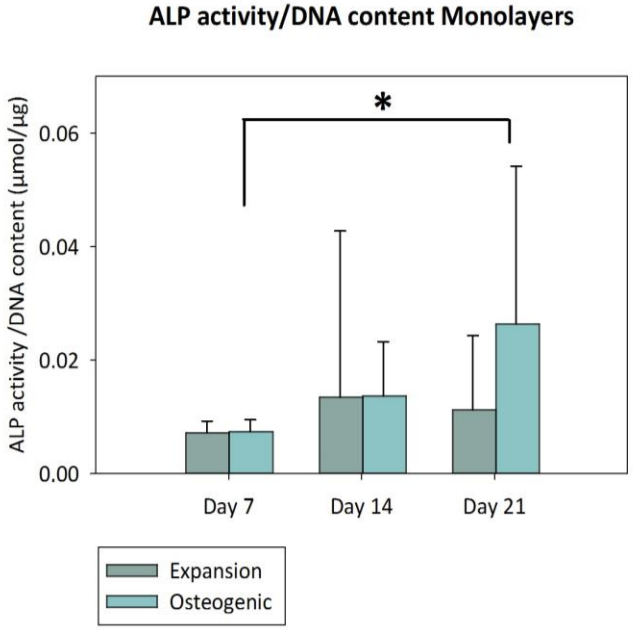

Figure 11. ALP activity corrected for DNA content after treatment with expansion or osteogenic medium in monolayers at day 7, 14 and 21. A significant increase is present from day 7 to day 21 in osteogenic conditions. *= p<0.05

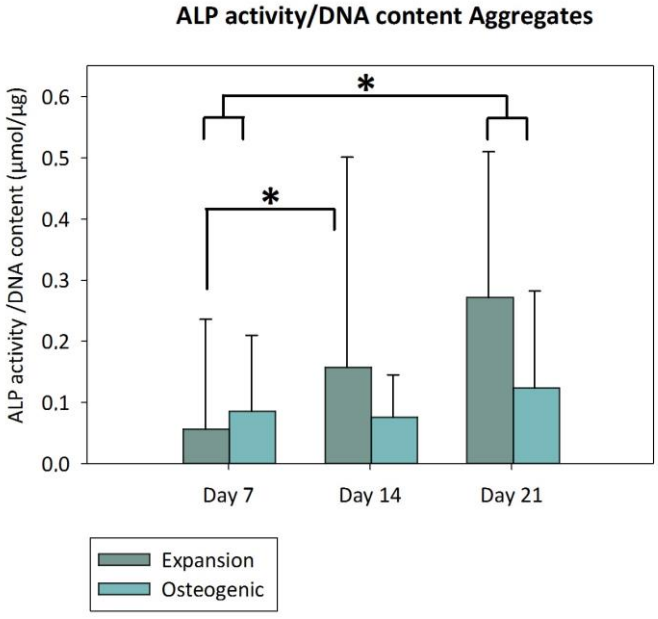

Figure 12. ALP activity corrected for DNA content after treatment with expansion or osteogenic medium in aggregates at day 7, 14 and 21. A significant increase is present from day 7 to day 14 and 21 in expansion. Additionally, osteogenic conditions were significantly increased at day 21 in comparison with day 7. *= p<0.05

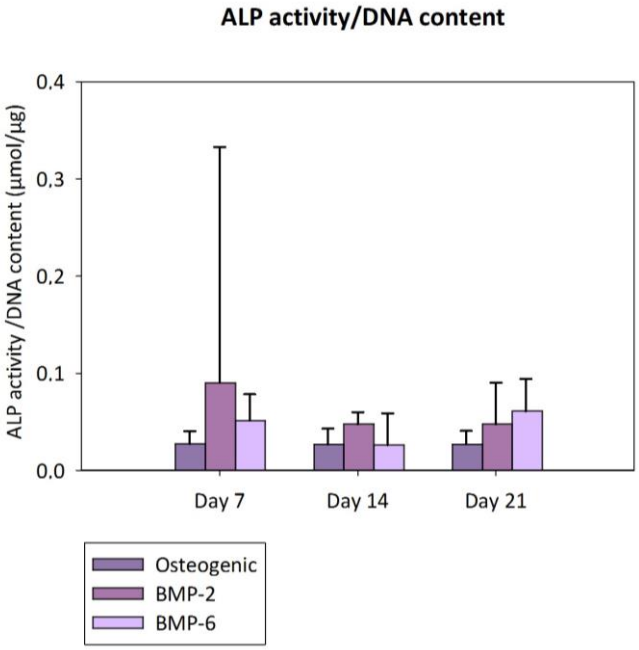

Figure 13. ALP activity corrected for DNA content after treatment with osteogenic medium with or without BMP-2 and BMP-6 100ng/ml supplementation in aggregates at day 7, 14 and 21.

Alizarin red and Von Kossa staining

Experiment A: After 7 days of culturing cells were stained with the Alizarin red staining for both monolayers and aggregates. Expansion did not stain positive in any donor in monolayer culture, as expected. Positive staining in osteogenic medium was present in two out of five donors after 7 days of culturing in monolayers. The supplementation of BMP-2 and BMP-6 at the lowest concentration (1ng/ml) resulted in some noduli formation in two out of three donors cultured in monolayers, while at a concentration of 10ng/ml resulted in nodule formation in three out of five donors. The addition of BMP-2 at 100ng/ml only stained noduli in two out of five donors, whereas the addition of BMP-6 at 100ng/ml resulted in noduli formation staining positive for alizarin red in all donors in a consistent manner (figure 14).

BMSCs cultured in aggregates did not demonstrate positive alizarin red and Von Kossa staining in any of the culture conditions, i.e. expansion medium and osteogenic medium with or without BMP-2 and BMP-6 10ng/ml.

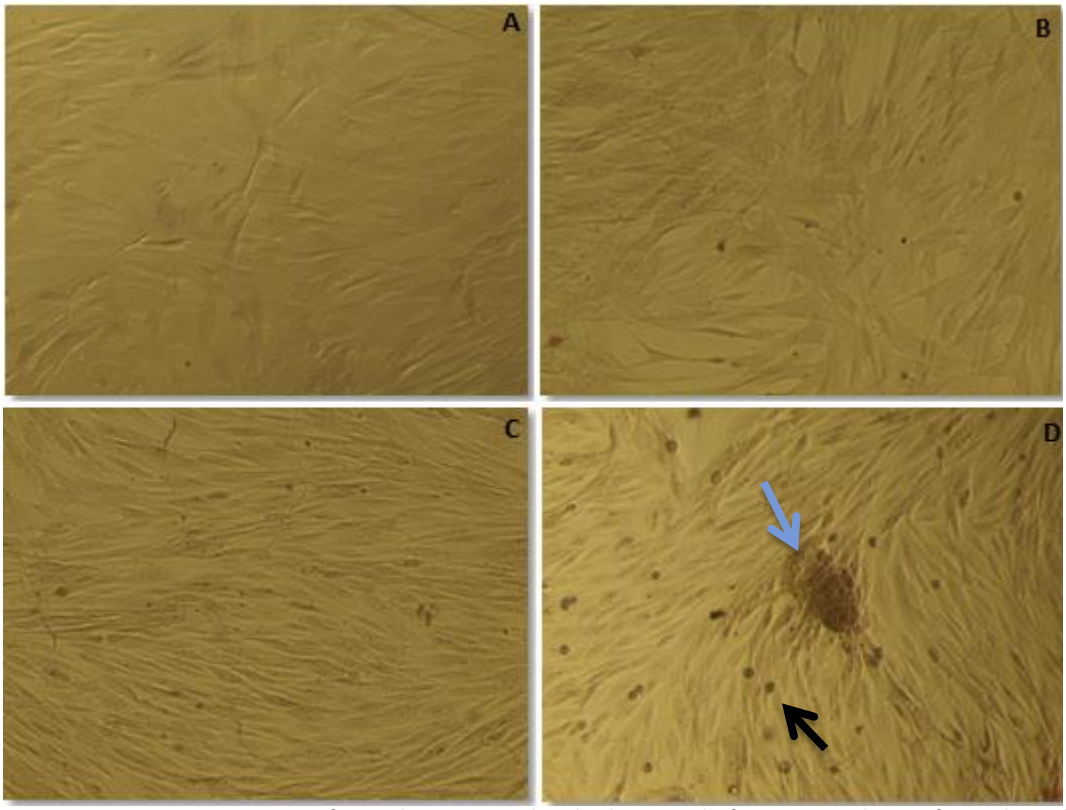

Figure 14. Microscopic pictures of monolayers stained with Alizarin red of one young donor after 7 days of culturing with expansion medium (A), osteogenic medium (B), osteogenic medium with BMP-2 100ng/ml (C) or osteogenic medium with BMP-6 100 ng/ml in which large and small noduli were present. 10x magnification, blue arrow: large noduli, black arrow: small noduli

Experiment B: In the long term study performed with monolayers and aggregates receiving expansion and osteogenic medium, two donors demonstrated alizarin red stained nodule, one at 14 days and one at 21 days of monolayer culture. After 21 days of culture, two donors cultured in aggregates under osteogenic culture conditions stained positive for both alizarin red and von Kossa (figure 15). Unfortunately, the third donor was lost during processing and could not be included in this analysis.

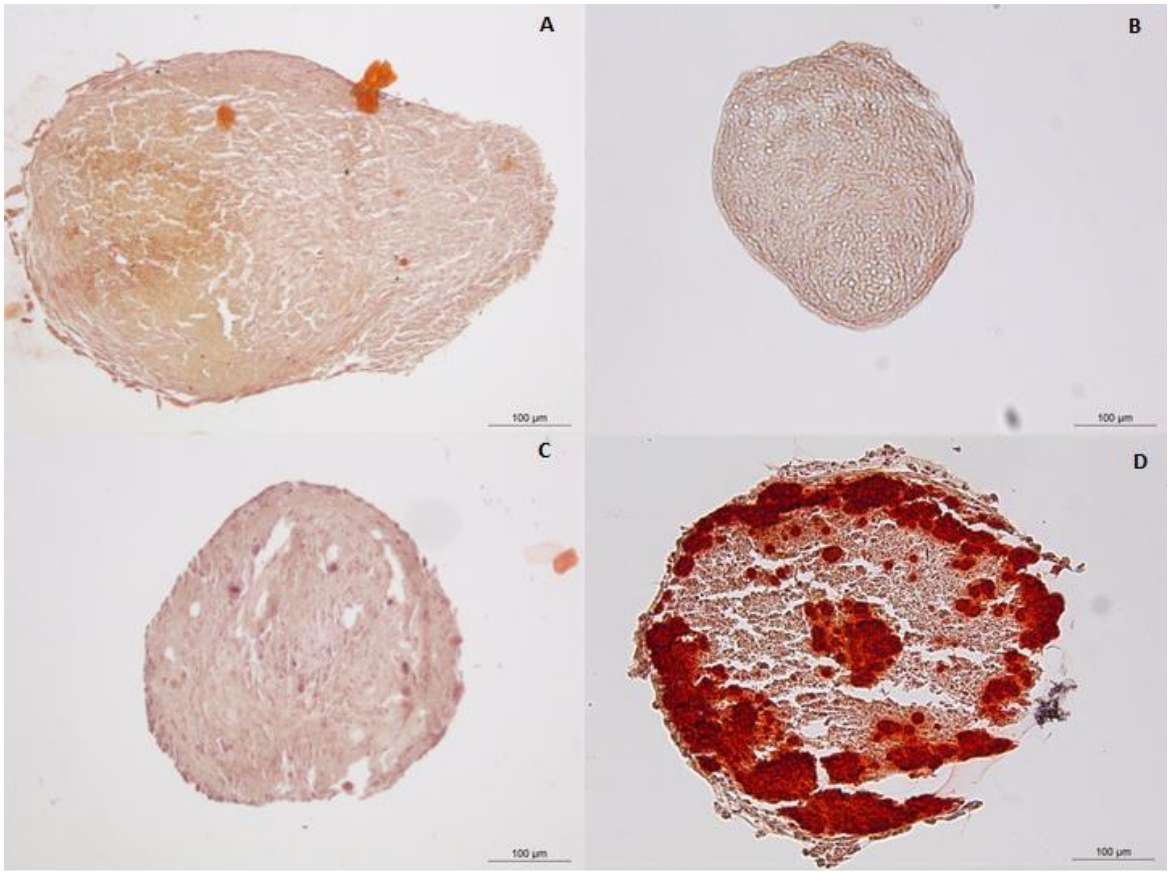

Figure 15. Microscopic images of pellet cultures stained with Alizarin red staining after 14 days (A and C) and 21 days (B and D) of culture with expansion medium (A and B) or osteogenic medium (C and D). 10x magnification

Experiment C: Three out of four donors in the osteogenic group stained slightly for the Alizarin red. Additionally, two donors stained mildly in the von Kossa staining. The addition of BMP-2 to the osteogenic medium demonstrated an additive effect in the Alizarin red and the von Kossa staining in two and four donors, respectively (figure 16). Supplementation with 100ng/ml BMP-6 resulted in more intense staining in comparison with osteogenic medium in three donors for the Alizarin red and the von Kossa staining. It was donor dependent if BMP-2 or BMP-6 had the most additive effect. BMP-2 had a more additive affect in comparison with BMP-6 in one donor for the Alizarin red and two donors in the von Kossa staining. The opposite, with BMP-6 being more stained, was seen in four donors in the Alizarin red staining and one donor for the von Kossa.

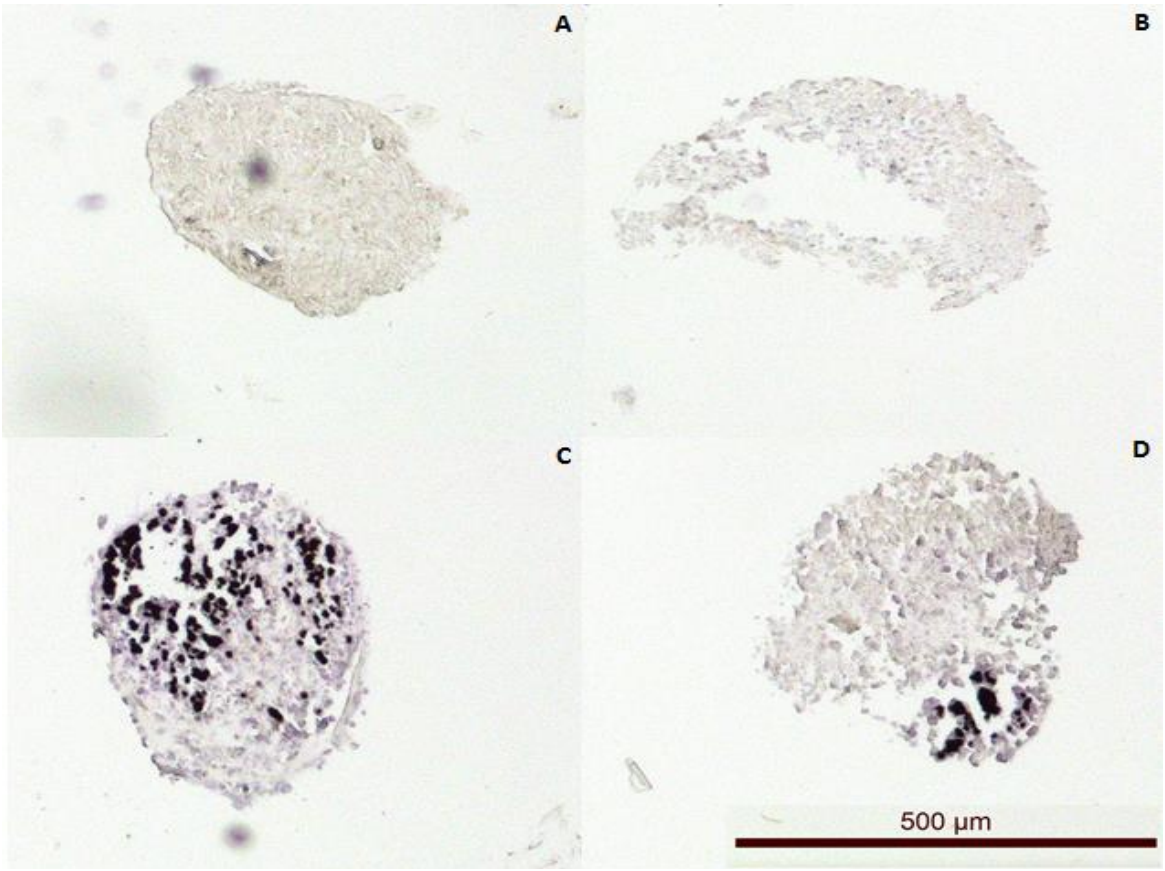

Figure 16. Microscopic images of pellet cultures stained with von Kossa staining after 21 days of culture with expansion medium A, osteogenic medium (B) or osteogenic medium supplemented with BMP-2 (C) or BMP-6 (D).4x magnification, bar 500µm

Discussion

The differential osteogenic potential of BMPs has been the topic of several studies in humans and rats (85) (86) (84). The current study evaluated the differential osteogenic effect of a wide range of BMP-2 and BMP-6 (doses 1-100ng/ml) on canine MSCs. In addition, a 3D culture method was employed to address the technical problems of monolayer culture, i.e. detaching of the cell layer as soon as cells have reached confluency. Three-D culture for osteogenic differentiation of MSCs has been employed recently by others (76) (77) (78). The current study supports those findings based on the Alizarin red and von Kossa staining, qPCR results and ALP activity.

Osteogenic differentiation in monolayers took place after 21 days of culture in two out of three donors, based on positive Alizarin red staining, qPCR expression of BGLAP and the ALP expression. BGLAP was the highest expressed at day 14 and thereafter declined. The ALP activity revealed the highest expression on day 21, as was earlier described in human osteoblasts (94). Additionally, BMP-2 and BMP-6 were evaluated for their osteogenic potential in monolayers and aggregates of canine MSCs at an early time point of osteogenic differentiation, i.e. at 7 days. Monolayers were supplemented with a dose of 1, 10, 50 and 100ng/ml BMP-2 or BMP-6. The best osteogenic

differentiation seemed to be established when monolayers received 100ng/ml BMP-6, with a more consistent result for all donors based on the Alizarin red staining. This is in correspondence with human MSCs, in which BMP-6 has been reported to be more potent in initiating bone formation in a consistent manner in comparison with BMP-2, -4, -7, and -14 (85).

Even in a 3D-culture environment, MSCs underwent osteogenic differentiation when treated with osteogenic medium. Convincing differentiation took place, based on both the Alizarin red and the von Kossa staining and the upregulation of SPP1 at day 14. SPP1 did not only reveal upregulation at day 14, which is in correspondence with literature (67), but additionally it was found that SPP1 was higher expressed in aggregates than in monolayers. ALP activity was already upregulated at day 7 and it is possible that the highest expression occurred before measurements took place. Additionally it was found in aggregates that osteogenic differentiation is stimulated after supplementation of osteogenic medium with 100ng/ml BMP-2 or BMP-6. BMP-2 and BMP-6 100ng/ml led to the highest BMP signalling activity via the Smad1/5/8 pathway, based on the expression of RUNX-2 and Id1. To verify the activation of the Smad pathway, phosphorylation of Smad1/5/8 should be performed. Based on these findings, aggregates seem to have a great potential to differentiate towards the osteogenic lineage and represent a more in vivo like culture method and are therefore a possible solution for detaching monolayers in long term experiments

The differential osteogenic effect of BMP-2 and BMP-6 was evaluated in a long term experiment in the 3D culture model of aggregates. Osteogenic medium, regardless of the supplementation with BMP-2 or BMP-6, induced osteogenic differentiation, indicated by the significant upregulation of SPP1 at day 14 for all culture conditions. BMP-2 was found to be the most potent in initiating osteogenic differentiation in aggregates, based on BGLAP and Id1 gene expression. Additionally both BMP-2 and BMP-6 had the ability to stimulate osteogenic differentiation in aggregates, based on the Alizarin red and von Kossa staining. More intense staining seemed to be present in BMP-6 with Alizarin red staining and in BMP-2 for the von Kossa staining. Alizarin red staining stains calcium, but additionally calcium-binding proteins and proteoglycans. Von Kossa binds only phosphate, which is present in hydroxyapatite in adult bone (92). It is therefore believed that BMP-2 has a greater induction potential in osteogenic differentiation in comparison with BMP-6 in canine MSC aggregates, based on the combination of von Kossa staining, BGLAP and id gene expression.

The difference in osteogenic inducing potential could be explained by differences in receptor expression and noggin. BMP-6 prefers to bind BMP-RII, whereas BMP-2 binds BMP-RIB with the highest affinity (95). In untreated human MSC it was found that BMP-6 had the most osteogenic potential and in agreement with this it was found that the basal expression of BMP-RII was 16-100 times higher in comparison with BMP-RIB (85). To establish if the expression of BMP-RIB is higher expressed in this study in comparison with BMP-RII, qPCR must be performed. Additionally, noggin has shown to antagonise BMP-2, but not BMP-6 and therefore BMP-6 is suggested to be more potent (91). In our study it is not found that BMP-6 had more potential to initiate osteogenesis in comparison with BMP-2. It could be that noggin does not act as an antagonist during osteogenic differentiation in dogs, but enhances it, as has been described in humans (90) (96). The upregulation of noggin in aggregates treated with BMP-2 100ng/ml in experiment B could support this suggestion.

To establish if noggin has a similar affect as in humans, an additional study with down or upregulation of noggin should be performed.

One of the study limitations is the fact that although BMP-2 and BMP-6 seemed to stimulate osteogenesis, less intense Alizarin red staining was observed in aggregates cultured in osteogenic medium in experiment C in comparison with experiment B. Additionally, it was found that gene expression and ALP activity was lower in experiment C compared with experiment B. This unexpected difference could be explained by difference in cell media. During experiment C, aggregates received expansion medium the first 24-hours until a pellet was formed while aggregates in experiment A and B received the osteogenic medium with or without BMP-2 or BMP-6 immediately. In parallel studies concentrating on chondrogenic differentiation it is found that it is crucial to allow pellet formation in the presence of chondrogenic medium rather than allow for pellet formation in the presence of expansion medium and add chondrogenic medium from day 1 and on (unpublished data). Interestingly, if MSCs received expansion medium during first 24-hours, no chondrogenic differentiation occurred. In this study less osteogenic differentiation occurred when pellet formation was allowed to happen in the presence of expansion medium.

Conclusions

Both monolayers and aggregate cultures have the ability to differentiate towards the osteogenic lineage in canine bone marrow derived-MSCs. Osteogenesis is enhanced with BMP-2 and BMP-6 (100ng/ml) supplementation in both culture methods. BMP-2 has the biggest ability to stimulate osteogenic differentiation in comparison with BMP-6, which is in contrast with other animal species. This could be due to other receptor profiling or noggin activity and should be assessed in further experiments.

4 miRNA-2887 and its effect on osteogenic differentiation of canine MSCs

Abstract

microRNAs (miRNAs) are small pieces of RNA of approximately 22-24 nucleotides long and are involved in regulation of genes. Several miRNAs have been reported to be involved in osteogenic differentiation. Whole transcriptome sequencing of the growth plate and primary spongiosum of large, fast growing compared with small, slowly growing dog breed revealed significant local differences including miRNAs. One of these miRNAs, i.e. ENSCAF ENSCAFG00000029043, showed an analogy with the murine miRNA-2887 and was significantly higher expressed both in the growth plate and the primary spongiosum of large vs small breed dogs. One of the predicted target genes of this miRNA is noggin, which is involved in endochondral and intramembranous ossification and antagonises BMP signalling. The aim of this study was to supress noggin through miRNA-2887 and thereby enhance osteogenic differentiation. To optimize transfection in canine mesenchymal stem cells, three different doses of 5, 25, and 50nM miRNA-2887 were tested in expansion and osteogenic medium supplemented with BMP-2 100ng/ml. Surprisingly, two osteogenic markers, RUNX-2 and SPARC, were decreased after successful suppression of noggin with 50nM of miRNA-2887. This suggests that noggin suppression leads to a reduction of osteogenic differentiation, as reported in humans.

Introduction

MicroRNAs (miRNAs) are small RNAs of approximately 22-24 nucleotides long and recently it's been discovered that miRNAs play a bigger role in cell homeostasis than presumed. miRNAs influence gene expression in somatic cells by upregulating or downregulating genes (50). Several miRNAs have been discovered that have an influence on osteogenesis, such as miRNA-29b, miRNA-2861, and miRNA196a (52) (53) (54). Overexpression of these miRNAs causes upregulation of osteogenic genes or downregulation of anti-osteogenic genes and thereby they stimulate osteogenesis. Altogether, this indicates that miRNAs targeting pathways related to osteogenesis could serve as new targets in orthopaedic therapies in stimulating bone formation during fracture healing.

Several studies have been performed to investigate the difference between large, fast growing dog breeds (Great Danes, GD) and small, slow growing dog breeds (Beagles and Miniature Poodles, MP) to determine factors that could stimulate bone formation (9) (10). Elongation of the long bones occurs at the growth plate level through endochondral bone formation. During the process of endochondral bone formation mesenchymal stem cells (MSC's) differentiate into cartilage and subsequently form bone. Within the growth plate, chondrocytes undergo a process of differentiation, starting at the resting phase, followed by proliferative phase and a hypertrophic phase. During the hypertrophic phase, cells will stop producing chondrogenic factors and will turn on the osteogenic genes and the matrix will become mineralized, and this forms the basis for the formation of the primary spongiosum. Tryfonidou *et al*. investigated the differences at the growth plate level between GD and MP and reported that GD have thicker growth plates, with larger proliferation zones (in absolute terms) and larger hypertrophic zones (in absolute and relative terms) (97). Further studies concentrated on the local regulators that define the growth plate phenotype and are inherently accompanied by a higher pace of bone formation. Evaluation of these differences could lead to new factors that are differentially expressed in GDs in comparison with MPs and thereby to new targets that are involved in bone formation (endochondral and intramembranous bone formation). Recently, a whole transcriptome sequencing for the specific growth plate zones of GDs and MPs was performed (Tryfonidou et al., unpublished data). Preliminary analyses of these results gave numerous, significant dissimilarities between GD an MP, including transcripts and miRNAs. (figure 1).

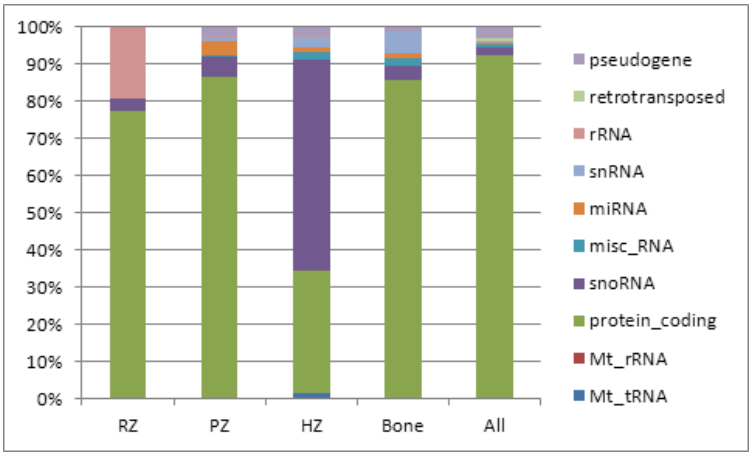

Figure 1. The distribution of different types of RNA in cartilage and bone. RZ: resting zone, PZ: proliferation zone, HZ: hypertrophic zone (Tryfonidou, unpublished data) indicating that next to protein coding transcript, a large proportion of small RNAs are differentially expressed in the growth plate zones

A certain miRNA sequence (CAF ENSCAF ENSCAFG00000029043) was differentially expressed in two consecutive zones of the growth plate: the proliferative and hypertrophic zone, and in the primary spongiosum, i.e., 7-11-fold upregulated in GD compared to MP. Bta-miRNA-2887 showed an analogy for this nucleotide combination (http://www.mirbase.org/). For this miRNA thirty-four predicted gene targets were found, using 'Target Scan Human, release 6.2' (http://www.targetscan.org/) for dogs. At least one of these genes, noggin, plays a significant role in endochondral and intramembranous bone formation.

Noggin is a protein that plays a role in the development of several body tissues, including bone. Noggin reduces BMP activity by antagonising BMP receptors except for BMP-6 (91). Homozygous noggin-overexpressing mice are not viable (98). In heterozygous transgenic mice, noggin overexpression leads to a decrease in trabecular bone volume, osteopenia and impaired osteoblastic functioning in new-borns, and in adult mice inhibited osteoblastic differentiation and bone formation (98) (99). Silencing noggin caused an increase in osteogenic differentiation in mice MC3T3-E1 preosteoblasts (100). Noggin depletion caused higher expression of Smad1/5 in a similar fashion as cells treated with rhBMP-4, i.e. more noduli were found with an Alizarin red staining and a higher expression of BGLAP was present. Furthermore, noggin suppression led to an acceleration of the osteogenic differentiation, which was indicated by an increase in expression of RUNX-2 and SPP1 expression 3 days earlier compared to controls receiving osteogenic medium alone. Additionally, noggin suppression in scaffolds, containing transfected cells, caused an acceleration in fracture healing until week 4 in vivo. After 4 weeks no additional effect of the siRNA was detected, possible due to inactivation of the retrovirus, diminished RNAi activity or the expression of noggin by surrounding cells. In humans however, noggin suppression of human mesenchymal stem cells (MSCs) leads to a decrease of several osteogenic genes, DNA content and protein amount (96) (90). The role of noggin in osteogenesis in the canine species is not yet known.

The aim of this pilot study was to supress noggin expression and thereby augmenting osteogenesis, as seen in rodents by employing miRNA-2887. In order to optimize the transfection, three different doses of miRNA-2887 were tested in MSCs receiving expansion medium or osteogenic medium supplemented with BMP-2. Surprisingly, the highest dosage of 50nM revealed downregulation of osteogenic markers after successfully suppressing noggin gene expression.

Material and Methods

Bone marrow derived stem cells

In this pilot study the bone marrow derived MSCs of one donor was used to test the ability of miRNA-2887. MSCs received expansion medium (10% FC, α-MEM, 1% penicillin/streptomycin (p/s), 0.1 mM ascorbic acid (Sigma A8960), 10-9M dexamethasone (Sigma D1756)) and FGF (AbD Serotec, PHP105, 1ng/ml) and were incubated at 37 °C, 5% CO2 and 20% O2 until 60% confluency was reached. Thereafter the cells were cryopreserved at P0 in αMEM containing 10% DMSO and 10% FCS until further use.

Cell media

BMSCs at P2 were seeded at a density of 5,000 cells per cm^2 in monolayers and transfected once they reached 60% confluency. To establish miRNA transfection, miRNA-2887 (Life Technologies) was added to PBS diluted Lipofectamine™ RNAiMAX (Life Technologies, 13778). The same procedure was performed for a negative control, containing scrambled RNA (Life Technologies, AM17110). Each well received expansion or osteogenic medium with BMP-2 (R&D) (D-MEM high glucose (Invitrogen, 31966-021), 10% FCS, 1% penicillin/streptomycin, 10mM β-Glycerolphosphate (Sigma, G6376), 0.1 mM ASAP and 5 µL/mL fungizone, 10-7M of dexamethasone) and the addition of 5, 25 or 50 nM miRNA-2887, negative miRNA or diluted Lipofectamine™ RNAiMAX alone (table 1). Subsequently three wells received only plain medium. After 48-hours incubation at 37 °C, 5% CO_2 and 20% O_2, cell media was changed with plain expansion or osteogenic medium with BMP-2.

Table 1. study design during the first 48-hours. Thereafter media was changed for plain expansion medium or osteogenic medium supplemented with BMP-2

Expansion medium	+ miRNA-2887 5nM	+ miRNA-2887 5nM	+ miRNA-2887 5nM	+ miRNA negative control	+ Lipofectamine™ RNAiMAX alone
Osteogenic medium with BMP-2					

RNA isolation and qPCR

At day 4 of culturing cells were collected for RNA isolation. RNA isolation was performed using a RNeasy microkit (Qiagen, 74004) according to manufactures protocol. The RNA content was measured with a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands). Maximum RNA input was used to make cDNA with iScript™ cDNA Synthesis Kit (Biorad) and qPCR was performed using a BioRad CFX-384 cycler and IQ SYBRGreen SuperMix (BioRad, Veenendaal, The Netherlands). Five reference genes were selected; ribosomal protein L8 and ribosomal protein S5 (RPL8 and RPS5) and succinate dehydrogenase complex, subunit A (SDHA), glucoronidase beta (GUSB) and beta 2 microglobulin (B2MG) (primers represented in appendix 1). The gene expression of noggin, osteopontin (SPARC) and RUNX-2 were examined. Additionally a second predicted target of miRNA-2887, CUX-1, involved in endochondral ossification was analysed. CUX-1 activates Wnt5a, a member of the Wnt family, which activates the β-catenin independent pathway and causes an increase in chondrogenic differentiation during early stages.

Statistics

This experiment was not subjected to statistics given that it was performed on one donor and served as a pilot study for dose finding for optimization of the transfection process.

Results

qPCR

As expected, treatment of monolayers with osteogenic medium supplemented with 100ng/ml BMP-2 resulted in ~2-fold upregulation of noggin 48 hrs after initiation of the experiment and treatment with BMP-2 resulted in an increase of the Id1 gene expression, a downstream target gene of the Smad1/5/8 signalling pathway that is activated by BMP ligands (figure 4). In the presence of Lipofectamine™ RNAiMAX, BMP-2 treatment resulted in a relatively smaller increment in the expression of noggin (figure 2). In the presence of BMP-2, the expression of noggin did not differ between the scrambled control and miRNA-2887 5nM and 25nM. However, transfection with 50nM miRNA-2887 resulted in almost 4-fold decrease of Noggin in comparison with plain osteogenic medium supplemented with BMP-2. To further corroborate the biological effects of miRNA-2887, another predicted target gene, i.e., CUX-1, was analysed and revealed a similar pattern of expression to noggin (figure 3). In order to determine the effects of miRNA-2887 on osteogenesis, the osteogenic markers SPARC and RUNX-2 were examined for their gene expression. Surprisingly, transfection with 50nM of miRNA-2887 resulted in a decrease in both SPARC and RUNX-2 gene expression compared with all other conditions that received osteogenic medium supplemented with BMP-2 (figure 5-6). Transfection with miRNA-2887 25nM resulted in an upregulation in SPARC without affecting RUNX-2 gene expression in the presence of BMP-2, while the lower dose of miRNA-2887 (5nM) seemed not to affect SPARC expression. Id1 expression, a target gene of the Smad 1/5/8 signalling pathway, remained unchanged in all culture conditions in the presence of BMP-2, except for miRNA 50nM, where Id1 expression was the lowest compared to all other conditions.

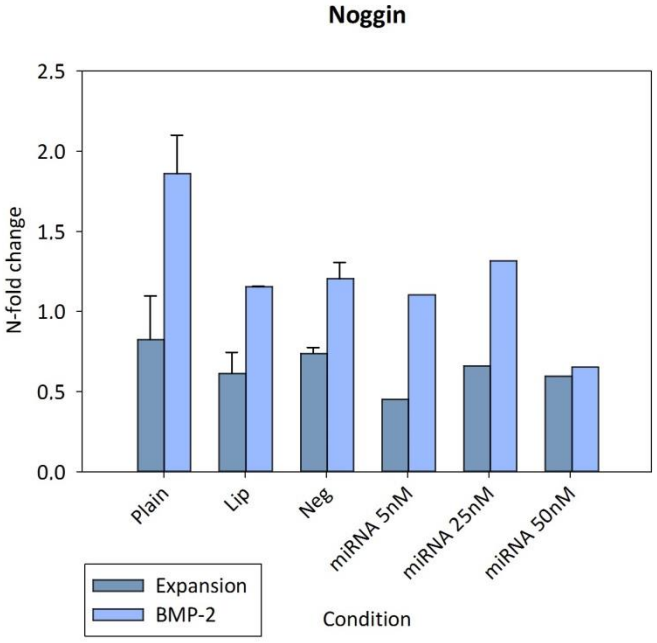

Figure 2. Gene expression profiles of noggin in monolayers after 7 days of culture with expansion medium or osteogenic medium supplemented with BMP-2 (plain), and Lipofectamine™ RNAiMAX (Lip), scrambled negative miRNA (Neg) or miRNA-2887 5, 25 or 50nM

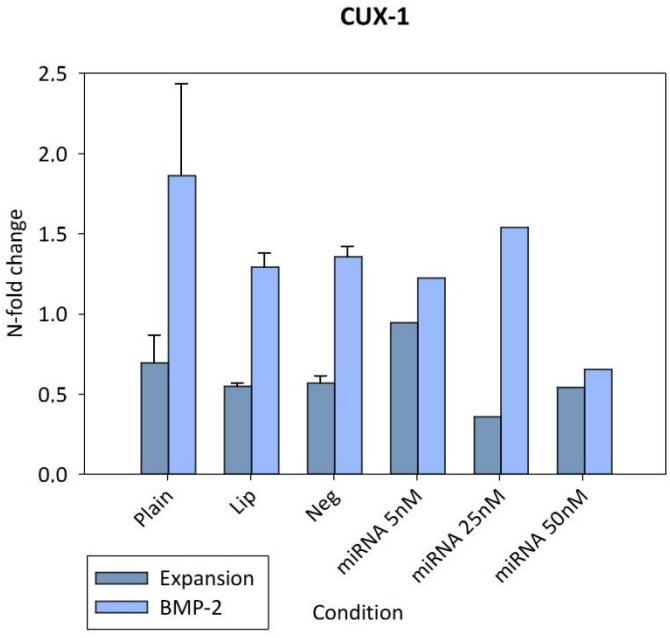

Figure 3. Gene expression profiles of CUX-1 in monolayers after 7 days of culture with expansion medium or osteogenic medium supplemented with BMP-2 (plain), and Lipofectamine™ RNAiMAX (Lip), scrambled negative miRNA (Neg) or miRNA-2887 5, 25 or 50nM

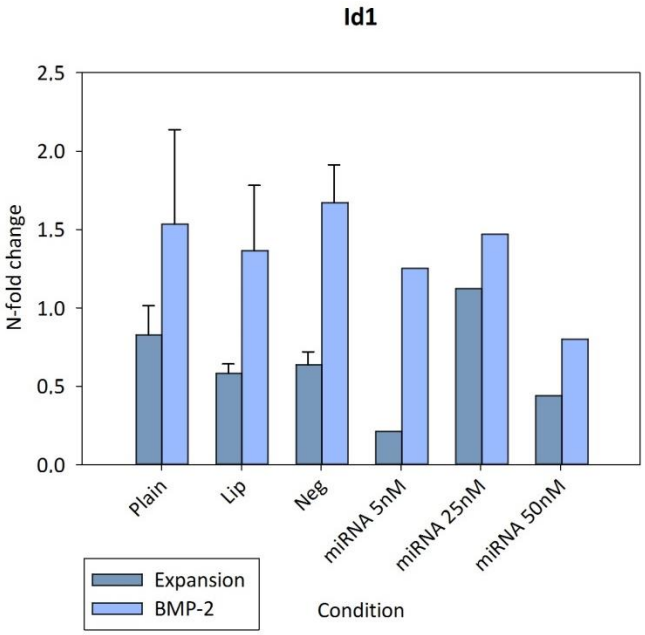

Figure 4. Gene expression profiles of Id1 in monolayers after 7 days of culture with expansion medium or osteogenic medium supplemented with BMP-2 (plain), and Lipofectamine™ RNAiMAX (Lip), scrambled negative miRNA (Neg) or miRNA-2887 5, 25 or 50nM

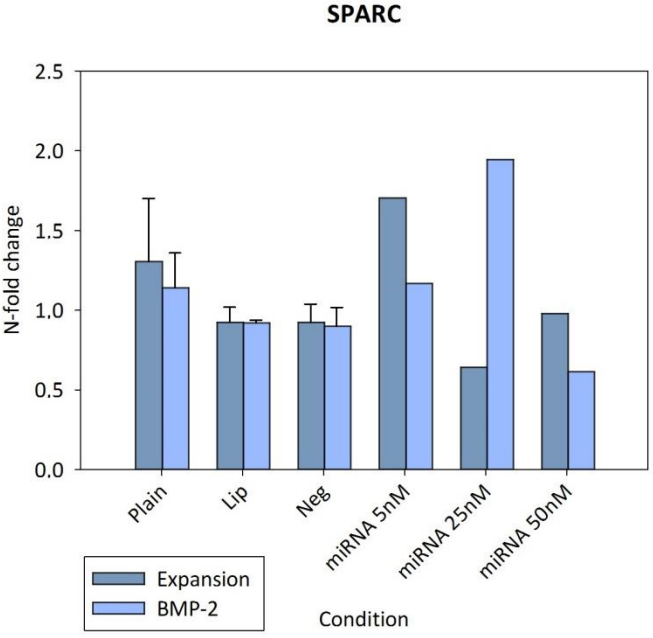

Figure 5. Gene expression profiles of SPARC in monolayers after 7 days of culture with expansion medium or osteogenic medium supplemented with BMP-2 (plain), and Lipofectamine™ RNAiMAX (Lip), scrambled negative miRNA (Neg) or miRNA-2887 5, 25 or 50nM

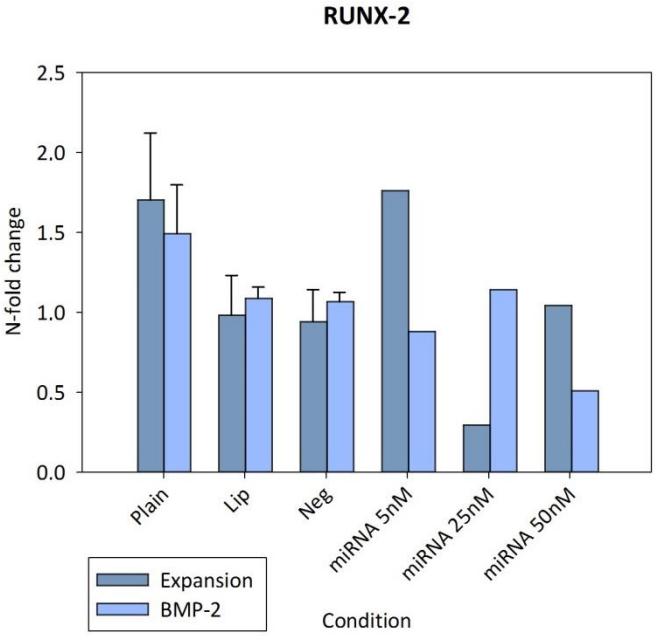

Figure 6. Gene expression profiles of RUNX-2 in monolayers after 7 days of culture with expansion medium or osteogenic medium supplemented with BMP-2 (plain), and Lipofectamine™ RNAiMAX (Lip), scrambled negative miRNA (Neg) or miRNA-2887 5, 25 or 50nM

Discussion

Noggin is a very important protein and involved the development of several body tissues, including bone. Overexpression of noggin is not compatible with life as has been shown in homozygote transgenic mice, while heterozygote transgenic mice reveal decreased trabecular bone volume (98). A reduction of noggin however, stimulates osteogenesis as has been shown in mice MC3T3-E1 preosteoblasts (100)

The same was expected to be found in canine MSCs treated with noggin-downregulating miRNA-2887. However, surprisingly noggin downregulation seemed to negatively affect osteogenesis in canine MSCs. Transfection with 50nM miRNA-2887 in canine MSCs resulted in decreased noggin and CUX-1 gene expression, both genes are predicted targets of miRNA-2887. Additionally, RUNX-2 and SPARC, two early osteogenic markers, were also downregulated in the presence of 50nM miRNA-2887. Altogether, this suggests that osteogenic differentiation is reduced when noggin is downregulated, as reported in human MSCs (90) (96). In order to further underscore the role of noggin in osteogenic differentiation, a long term study with several MSC donors must be performed. Ideally, such an experiment would be designed to perform weekly transfection with miRNA 2887, study early and late markers of osteogenesis throughout the culture period, and use as a main read out parameter the formation of noduli over the course of 21 days of culture. In parallel to that, silencing of noggin by means of siRNA can delineate whether the negative effects of miRNA-2887 are only mediated by noggin or may also target other genes negatively regulating osteogenesis.

Conclusions

Noggin suppression seemed to occur in canine MSCs after transfection with 50nM of miRNA-2887 based on gene expression. Additionally, SPARC, RUNX-2 and Id1 were decreased when cells were treated with 50nM of miRNA-2887. It is therefore tempting to hypothesize that the function of noggin in dogs resembles that of humans instead of rodents. However, more (long term) research must be performed, using more BMSC donors and more read out parameters.

5 General discussion

Regenerative medicine is still challenged by large bone defects that are not able to self-heal, mainly caused by the hypoxic conditions at the fracture site due to the absent/delayed vascular ingrowth. Fracture healing is orchestrated by numerous factors, such as fibroblastic growth factor and platelet-derived growth factor that attract mesenchymal stem cells (MSCs) towards the fracture site, which will then differentiate towards the osteogenic lineage. Several therapeutic strategies were assessed by others, including the use of growth factors, such as BMP-2 and BMP-7, autologous or allogeneic grafts, scaffolds, stem cells or combinations of these (4) (37). MSCs are used in therapies where they are delivered directly at the fracture site and are expected to differentiate to substitute bone (42). Most of the therapies are successful, however complications occur often (infections, rejection, hematoma, pain) and treatments have a high cost. To investigate new therapeutic strategies, MSCs are used during in vitro studies to develop strategies that augment osteogenesis.

The aim of this Honours program project was to establish the role of bone morphogenetic proteins (BMPs) on osteogenesis in canine MSCs and additionally evaluate if downregulation of noggin, an antagonist of the BMP signalling pathway, would enhance osteogenic differentiation. To establish this, culture conditions during osteogenic differentiation were optimizes by fine tuning supplementation with dexamethasone. In the follow up studies this optimized protocol was used to examine the additive effect of BMP-2 and BMP-6. The final study performed included modulation of noggin expression through a novel miRNA during osteogenesis.

Dexamethasone was found to give the best osteogenic differentiation when supplemented during the entire culture period of 21 days in canine MSCs, as opposed to humans where 7 days of supplementation resulted in the best osteogenic differentiation and not 21 days of supplementation. In humans, dexamethasone reduced cell proliferation and viability after 7 days of dexamethasone supplementation (101). We cannot exclude that dexamethasone in canine MSCs has only negative effects on proliferation and viability after day 21. However, this seems highly unlikely given that MSCs proliferate primarily during the early phase of osteogenic differentiation (102). Furthermore, differences between humans and dogs exist in the growth hormone pathway and oestrus cycle that influence bone homeostasis (72). Possible, aryl hydrocarbon receptor (AHR), involved in rapid proliferation, osteoblast differentiation and bone remodelling, through the glucocorticoid receptor, could be different as well, as found in rodents and humans. AHR production is increased in rodents receiving dexamethasone, but in humans dexamethasone reduces AHR transactivation (61) (62).

During this study, complications arose with monolayers: they detached and formed aggregates. After Alizarin red staining of these aggregates, we observed that MSCs had differentiated towards the osteogenic lineage when treated with dexamethasone. Therefore we opted the use of a 3-

Dimensional (3D) culture system during osteogenic differentiation. After 21 days of culture, aggregates were successfully differentiated towards the osteogenic lineage and seemed to have great potential as a replacement for monolayer cultures during long term studies. By coincidence it was found that more osteogenic differentiation was present during the first aggregate-experiments when pellet formation took place in osteogenic medium. During the latter experiments, cells received expansion medium during the first 24-hours for reasons of simplicity of study design. One donor, used in both studies, stained less intense when cells did not receive osteogenic medium during the first 24-hours (figure 1). Additionally, gene expression and ALP activity were found to be lower in aggregates treated with expansion medium the first day and fewer donors differentiate. Altogether, this indicates that the presence of osteogenic medium during the first 24 hrs in which the pellet is formed is important for the induction of optimal osteogenesis. To the author's knowledge there are no reports that have studied this phenomenon.

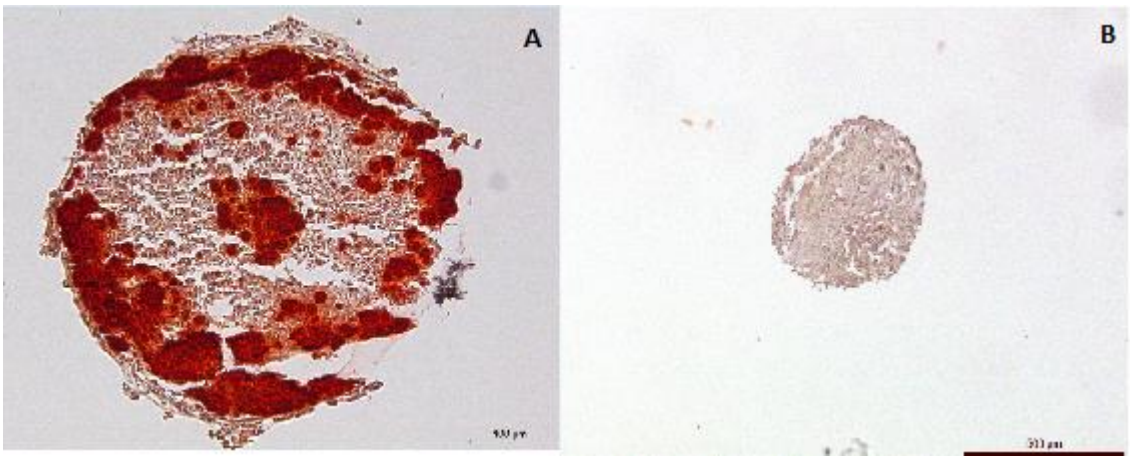

Figure 1. Microscopic images of pellet cultures stained with Alizarin red staining after 21 days culturing with osteogenic medium, with osteogenic medium (A) and expansion medium (B) during pellet formation. A: 10x magnification, B: 4x magnification

BMP-2 and BMP-6 supplementation was assessed in a wide range dose to examine their osteogenic inducing potential. Both in monolayers and aggregates, BMP-2 and BMP-6 100ng/ml had an additive effect on osteogenesis compared with osteogenic medium containing dexamethasone alone. Within contrast to rodents, BMP-2 was more potent in inducing osteogenic differentiation in canine MSCs in comparison with BMP-6 (86) (84). This could be due to BMP receptor expression differences of BMP-RII (preferably bound by BMP-6) or BMP-RIB (preferably bound by BMP-2) or noggin activity (95). Noggin does not antagonise BMP-6 as it does BMP-2, and it is therefore suggested that BMP-6 has a greater potential in initiating osteogenic differentiation as has been shown in mice (91) (86) (84). However, because in this study it was found that BMP-2 was more potent, while having the greatest noggin expression, it is suggested that noggin in canine MSCs acts in the same way as in humans and agonises osteogenic differentiation (90) (96).

In a preliminary study, noggin was found to be suppressed by miRNA-2887, a novel miRNA that is higher expressed in growth plates and primary bone of large, fast growing dog breeds in comparison with small, slow growing breeds. Additionally, another predicted target, CUX-1, involved in endochondral ossification was found to be supressed. Surprisingly, several osteogenic markers were

additionally decreased and therefore it is tempting to hypothesise that noggin enhances osteogenesis, as described also in humans (90) (96).

Future perspectives

To establish if noggin has similar biologic effects in osteogenesis as in humans, noggin suppression with small interference RNA (siRNA) could be examined. In humans this led to a reduction of osteogenic markers, such as RUNX-2 expression and ALP activity, and proliferation was decreased (90). Next to this, miRNA-2887 could further be assessed in more donors and ideally early and late osteogenic markers should be examined during a long term study in which transfection is performed weekly. Alternatively, this long term study could be initiated by using reverse transfection in which cells will be transfected in suspension and thereafter a 3D pellet structure is established and transfection with miRNA-2887 is repeated weekly in the presence of RNAiMax.

As mentioned before, the greater potential of BMP-2 could be explained by BMP receptor expression differences. BMP-6 has a preference for BMP-RII and BMP-2 prefers BMP-RIB and are therefore my main interest. To asses these, BMP receptors should be evaluated for their expression by qPCR in untreated and treated cells to exclude any possible homologous feedback of BMP itself. Next to qPCR, Fluorescence-activated cell sorter analysis (FACS) could be performed for these receptors (103).

Furthermore, given the lack of reports in literature, aggregates should be assessed for the difference in osteogenic potential when they receive expansion medium the first 24-hours instead of osteogenic medium.

Limitations of the study

The osteogenic potential of BMP-2 and BMP-6 supplementation was determined in aggregates. Such culture systems employ smaller volumes of medium (i.e. 200,000 cells in pellet are treated with 200 µl medium, while classically monolayer cultures are performed on 24-well plates due to low density of noduli formation in a 2D environment). BMP-2 and BMP-6 augmented osteogenesis compared with osteogenic medium alone. However, this was based on a small number of donors, due to high costs of BMP-2 and BMP-6. In future experiments additional donors should be used to increase the statistical power.

Appendix 1: Primers

Marker	Gene	Primer sequence	Exon	Amplication size (bp.)	Annealing Temperature (°C)	Accession no.
Osteoblasts	Osteonectin (SPARC)	Forward TCTGTATGAAAGGGATGAGGAC	6	82	64	XM_849889
		Reverse GCTTCTCGTTCTCGTGGA	7			
	Osteopontin (SPP1)	Forward GAATGCTGTGCTGACTGAGG	4	113	66-67	XM_003434024
		Reverse TGGCTATCCACATCGTCTCC	5			
	Osteocalcin (BGLAP)	Forward CTGATGGTCCTTGCCCT	1	116	60-63	XM_547536
		Reverse CTTGGACACGAAGGTTGC	2 and 3			
	RUNX-2	Forward AACGATCTGAGATTTGTGGGC	4	97	64	XM_845779
		Reverse TGTGATAGGTGGCTACTTGGG	4/5			
	Noggin	Forward TGCCGAGCGAGATCAAAGGG	2	99	63-64	
		Reverse AGCCACATCTGTAACTTCCTCCG	3			
	CUX-1	Forward GAACACAAAGTTCAAAGTCTGC	7	108	59	
		Reverse CAATTTCGTCGGCCTTGG	8/9			
	Id1	Forward CTCAACGGCGAGATCAG	1/2	135	59,5	XM_847117.2
		Reverse GAGCACGGGTTCTTCTC				
Housekeeping	RPL8	Forward CCATGAATCCTGTGGAGC	4 and 5	64	55	XM_532360
		Reverse GTAGAGGGTTTGCCGATG	5		3 steps	
	SDHA	Forward GCCTTGGATCTCTTGATGGA	4	92	61	DQ402985
		Reverse TTCTTGGCTCTTATGCGATG	6			
	GUSB	Forward AGACGCTTCCAAGTACCCC	4	103	62	NM_001003191
		Reverse AGGTGTGGTGTAGAGGAGCAC	5			
	B2MG	Forward TCCTCATCCTCCTCGCT	1	85	61,2+63	AB745507
		Reverse TTCTCTGCTGGGTGTCG	2			
	RPS5	Forward TCACTGGTGAGAACCCCCT	2 and 3	141	62,5	XM_533568

Appendix 2: Courses

During my Honours Programme I have had the opportunity to follow several courses and additional education:

- "Molecular methods"
  - Course instructors: Dr. Louis Penning and Dr. Peter Leegwater
  - 2 days, part time (September 17 and September 19)
- "Introductory statistics and RStudio/R"
  - Course instructors: Hans Vernooij and Jan van den Broek
  - 2 days, full time (December 11 and December 12)
- "Modern Methods in Data Analysis"
  - Course instructors: Drs. Cas Kruitwagen and Jan van den Broek
  - 3 weeks, full time (January 6 – January 24)
  - 4 ECTS
- "Presenting in English for PhD researchers"
  - Course instructor: Drs. Margo de Wolf
  - 2 days, full time (April 15 and April 22)
  - 1 ECTS
- "Writing for academic publication in the veterinary and life sciences"
  - Course instructor: Dr. Linda McPhee
  - 10 weeks, part time (3 hours per week), April 17 – June 26
  - 3 ECTs

Attended conventions/seminars:

- Veterinary Science Day (November 2014)
- European Veterinary Voorjaarsdagen 2014 (April 17 – April 19)
  - Presentation Research Award: The effect of dexamethasone on osteogenic differentiation of mesenchymal stem cells
- Monthly RM lunch seminars in UMCU
- Comparative Medicine; The Astonishing Connection Between Human and Animal Health

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