The effect of a novel miRNA on the expression of osteogenic differentiation related genes.

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Contents

Introduction

In the orthopaedic area, large bone defects represent a great challenge. They arise after removal of tumours such as osteosarcoma, chondrosarcoma or a squamous cell carcinoma. But also healthy people and dogs can have large bone defects in the form of complicated fractures. Osteosarcomas are primary bone tumours which have a high metastatic rate. In 2007 in the United States, over 10.000 dogs and 1.000 humans were diagnosed with osteosarcoma.(Withrow, Vail 2007) They mostly arise in the long bones, but can also be found in the flat bones (Selvarajah, Kirpensteijn 2010). To treat osteosarcomas, surgery has to be performed together with chemotherapy. Limp amputation is a first-line procedure which prolongs the survival time of the dog and brings pain relief(Selvarajah, Kirpensteijn 2010). Limb-sparing surgery is also possible. The tumour will be removed and the surrounding bone will be stabilized via a prosthesis or with a cortical allograft. But the surgery site often gets infected or the implant gets rejected by the body(Szewczyk, Lechowski & Zabielska 2015).

Chondrosarcomas are the second most common primary bone tumour in dogs. They account for approximately 5-10% of all primary bone tumours in dogs.(Withrow, Vail 2007) They are often found in the axial skeleton such as the ribs and the facial bone, but they can also be found in the long bones or at extra skeletal sites. Treatment consists of surgery (either amputation or limb sparing) with radiotherapy. (Farese et al. 2009)

A squamous cell carcinoma(SCC) is the second most common oral tumour in dogs. Of all metastatic tumours, 28% are SCCs.(Brønden, Eriksen & Kristensen 2009) They can arise from the gingiva, tongue or tonsils. Metastasis can be found in the lungs or in the regional lymph nodes.(Baines, Shales & White 2013) The treatment is either mandibulectomy or maxillectomy with radiation therapy.(Kühnel, Kessler 2014)

In these three tumours, large bone defects are created to ensure removal of the tumorous site. The size of this defect often exceeds the maximum regeneration capacity of the body. (Dimitriou et al. 2012) The same problem is seen in complicated fractures, where the body is unable to regenerate the amount of bone needed to ensure a stable fracture site. Therefore new regenerative strategies are needed. At this moment bone morphogenic protein (BMP) treatments are already clinically available. Long bone fractures, abnormal vertebrae motion, spinal deformities or facial defects can be treated with BMP nowadays, but these treatments are very expensive. Human recombinant BMP-2(hrBMP-2) is oftently used.(Lo et al. 2012) The most important problem is that hrBMP-2 has a half time of only 6,7 minutes and therefore a carrier is needed to ensure continuous deposit of hrBMP-2 at the fracture site. These carriers can either be natural polymers, synthetic polymers, natural ceramics, synthetic ceramics or composites of these three groups. Each of these carriers has its own characteristics making it more or less suitable for the injury. (Lo et al. 2012) The carrier is then left near the site of interest to gradually deposit the BMP and after a long period of time, new bone is formed(Lo et al. 2012, Terbish et al. 2015). Some other downsides to treatment with BMPs are that ectopic bone formation can occur (Fu 2013) and the carriers can cause soft tissue swelling, which causes pain for the patients.(Smucker 2006, Pountos et al. 2014) There also seems to be a correlation between treatment with BMPs and cancer(Pountos et al. 2014). Therefore, there is still a need for cheaper, safer and more stable treatments.

Physiological fracture healing

Fracture healing is a complex process ultimately leading to optimal skeletal repair and restoration of skeletal function. During this process, a great amount of physiological processes take place in the compact bone, the periosteum, the bone marrow and the soft tissues surrounding the fractured bone (*figure 1a*). The compact bone's main function is to bear the wait of the body and to protect organs. It is composed of thin lamellae which are circularly arranged around a canal, the haversian cannel. Via these canals blood vessels and nerves can enter the bone. The periosteum is a tough fibrous membrane that surrounds the outer part of bones, except for the joint surface in long bones. It has a layer of cells which are capable of bone formation during development, but as well during the healing of a fracture. The medullary cavity of the bone is filled with bone marrow (*figure 1c*). There are two types of bone marrow, red and yellow. The red bone marrow is highly vascularized and plays an important role in the genesis of blood cells. The yellow marrow is red marrow which is infiltrated with fat and therefore the hemopoetic properties of this marrow are dormant.(Dyce, Sack & Wensing 2010) To initiate and continue the process of osteogenesis, different types of growth factors, cytokines, minerals and hormones are needed, which are orchestrated in a complicated way to ensure bone formation.

In general, two types of bone healing can be identified. The first is the primary or direct healing pattern. This occurs when a rigid internal fixation can be maintained around the fracture and when the fragments are close aligned. In this type of fracture healing, only the Harvesian system and the lamellar bone have to be reformed. To do so, osteoclasts will be formed out of osteoprogenitor cells, which will be provided by the vascular endothelial and the perivascular mesenchymal cells. During this process no callus will be formed, meaning there is no periosteal response. (Dimitriou, Tsiridis & Giannoudis 2005, Panteli et al. 2015)

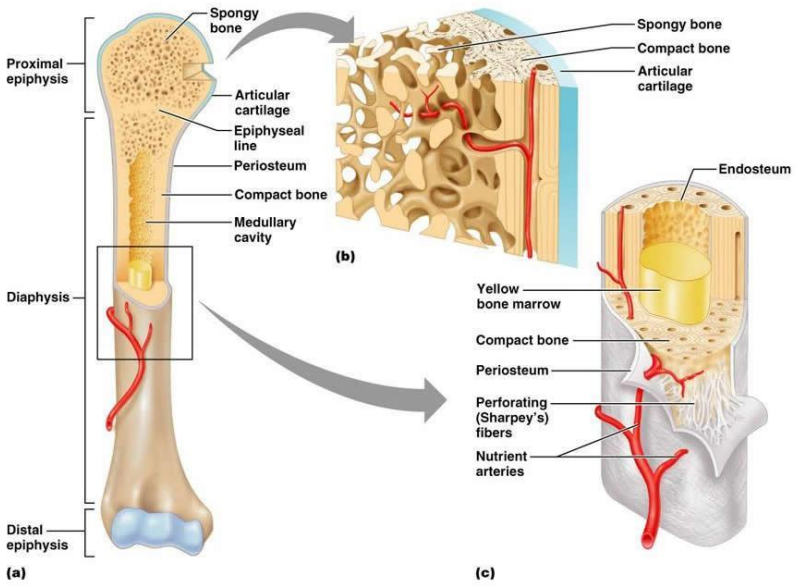

Figure 1: Anatomy of bone. Picture taken from http://www.imagekb.com/endosteum, accessed at 30-07-2015

The majority of bone fractures are healed via the secondary or indirect healing pattern. This type of healing relies on the formation of fibrocartilaginous callus. During this process, five stages can be seen. The first of these stages is inflammation. The fragmented bone bleeds from the damaged ends, which will soon turn into a clot between the fragments (*figure 2*). This clot consists of cells from bone marrow and peripheral and intramedullary blood. The surrounding soft tissue will show signs of acute inflammation. Tumor necrosis factor-alpha (TNF-alpha), Interleukin-1(IL-1) and IL-6 will be secreted and recruit inflammatory cells such as macrophages and neutrophils.(McKibbin 1978,

Marsell, Einhorn 2011) During the following stage, granulation tissue will be formed and replace the haematoma in a structure called callus.

In the third phase, prostaglandins, cytokines and other proteins contribute to cellular migration, proliferation and differentiation of mesenchymal stem cells(MSCs). MSCs are cells who have remained in an undifferentiated state and have a great potential to self-renewal, meaning that they can preserve their undifferentiated state. They can also differentiate into various cell types such as osteoblasts, chondrocytes, myocytes and adipocytes.(Pittenger et al. 1999, Kolf, Cho & Tuan 2007)These adult stem cells are easy to isolate, culture and manipulate in vitro, thus they conform a potential source for cell therapy. Interestingly, they can be found throughout the entire body, but adipose tissue, the umbilical cord and bone marrow are the tissues with the higher content in MSCs. (Pittenger et al. 1999, Kolf, Cho & Tuan 2007)

The MSCs differentiate into chondrocytes with a rich extracellular matrix (ECM), forming hyaline cartilage. The ECM of this type of cartilage mainly consists of type two collagen fibrils (ColII), chondroitin sulfate, proteoglycans and water. Chondroitin sulfate is a glycosaminoglycan(GAG) and is attached to proteoglycans(PG) such as aggrecan.(Palukuru, McGoverin & Pleshko 2014, Danielson, Knudson 2015). The formed structure, called soft callus, will stabilize the fracture.(Panteli et al. 2015, McKibbin 1978)

Within this tissue bone formation occurs via endochondral or intramembranous ossification which is the fourth step of fracture healing. (Marsell, Einhorn 2011) Endochondral bone formation occurs between the two bone fragments and periosteal to the fracture site (*figure 2*). This type of bone formation mostly occurs in the long bones. (Dimitriou, Tsiridis & Giannoudis 2005, Marsell, Einhorn 2011). The calcification of hypertrophic chondrocytes mineralizes the callus that was formed between the fragments. These cells are removed by chondroclasts and new MSCs are recruited and differentiated into an osteogenic cell lineage, forming a woven bone.

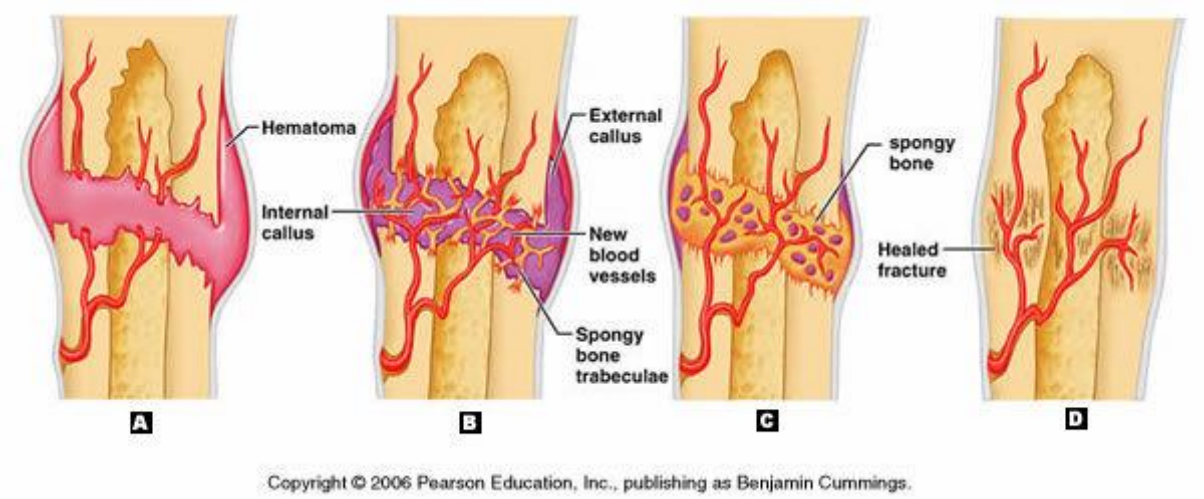

Figure 2: Endochondral bone formation. Picture taken from http://wps.aw.com/bc_marieb_happlace_7_oa/42/10965/2807221.cw/, accessed at 30-07-2015

The intramembranous ossification skips the forming of cartilage and directly forms bone via osteoblasts. This ossification is further away from the fracture site compared to the endochondral ossification.(Dimitriou, Tsiridis & Giannoudis 2005, Panteli et al. 2015) This type of bone formation mostly occurs during the formation of the flat bones of the skull. (Percival, Richtsmeier 2013)

The final step in fracture healing is the remodelling of the formed bone. During this phase, osteoblasts and osteoclasts are highly active. They resorb the bone and deposit it at an alternative place in the bone to ensure maximum strength of the bone. This phase could take up to several years.(Panteli et al. 2015)

BMP's

BMPs are important factors in osteogenesis. As stated before, BMP-2 and BMP-7 are already being used in the treatment of large bone defects.(Terbish et al. 2015, Tasli et al. 2014) BMPs are part of the transforming growth factor-β (TGF-β) superfamily and have a widely spread cellular function. They bind to BMP receptors (BMPr) on the cell membrane of their target cells. The receptor then phosphorylates Smad 1, 5 and 8. Together with Smad 4 they form the Smad complex which will translocate into the nucleus where it can activate gene transcription (*figure 3*).(Matthews 2005, Chen, Zhao & Mundy 2004) Some of the main targets of BMP's are the ID proteins 1 to 4. They regulate transcription by sequestering E proteins. The ID proteins themself cannot bind to DNA, but the E proteins can regulate transcription of DNA. By stopping the E proteins, ID stops DNA transcription. (Ling, Kang & Sun 2014)

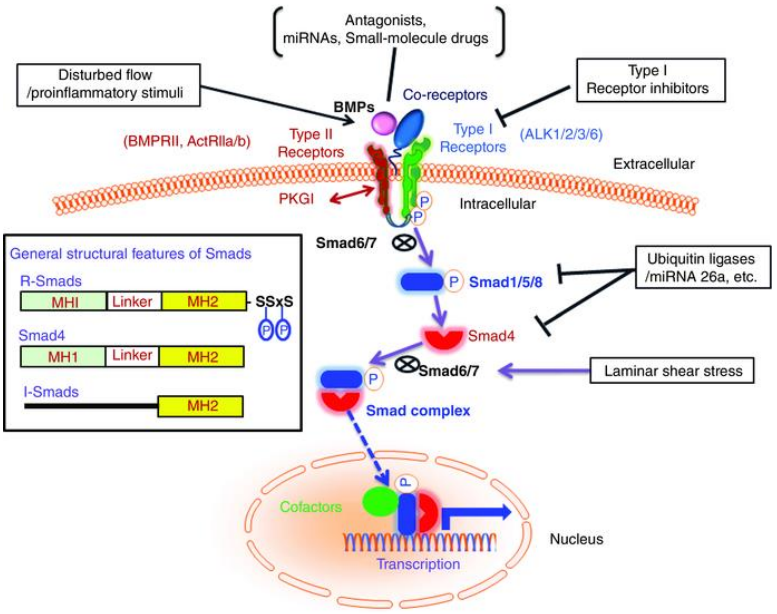

Figure 3: BMP signaling pathway. Picture taken from the article of Luo et al. [25]

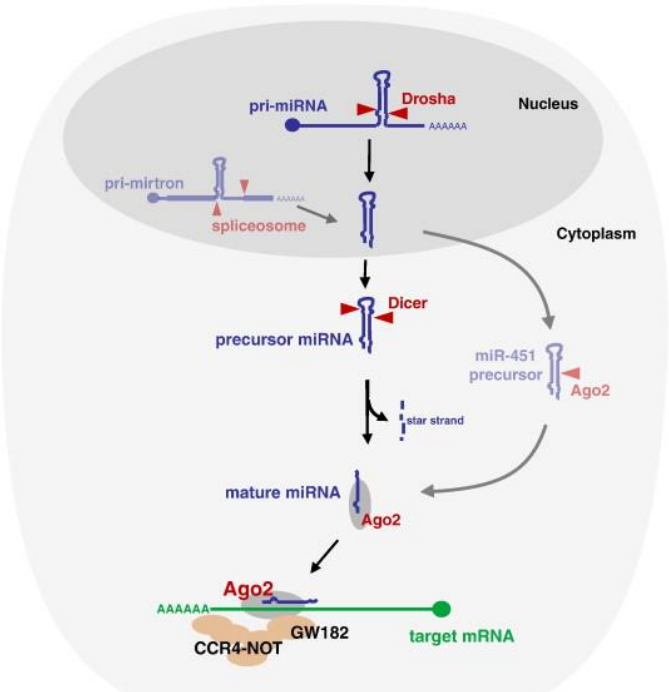

Figure 4: Scheme of the miRNA synthesis. Picture taken from the article of Hammond et al. [27]

miRNA

According to earlier research, some microRNAs(miRNAs) are important factors in bone formation. The research of Li et al showed that suppression of miRNA 216a shows a significant suppression of osteogenic differentiation in human adipose derived MSCs(Li et al. 2015a). Suppression of miRNA 375 on the other hand shows a significant increase in osteogenic

differentiation, therefore suggesting a negative role for this miRNA on osteogenic differentiation(DU et al. 2015). miRNA 145 also shows a negative effect on osteogenic differentiation by targeting the transcription factor *Osterix*(Jia et al. 2013). The world literature shows many more examples on how miRNA can influence the osteogenic differentiation.

miRNA are small noncoding pieces of RNA which consist of 20-22 nucleotides. At the 5'-end of the miRNA, nucleotides 2 to 8 are the so called *seed region*. This *seed region* is complementary to messenger RNA(mRNA). Because of the small size of this region, the complementary base pairing can be possible with several mRNA, therefore making several mRNA the target of one miRNA.(Fang et al. 2015)

In the nucleus pri-miRNA is formed by RNA polymerase II. This pri-miRNA consists of a nucleotide chain which has folded at some points and therefore creating a hairpin like piece (*figure 4*). The pri-miRNA is cleaved by Drosha and Dicer proteins. Drosha cuts the hairpin like piece of the pri-miRNA. This is then transported out of the nucleus. Dicer cleaves the loop of the precursor miRNA, leaving a duplex of miRNA. One of the strands, the star strand, is degraded. The other one is loaded onto the RNA-induced silencing complex(RISC)which contains an argonaute 2(ago 2) protein. This protein can bind to complementay mRNA. This way it will initiate degradation of the mRNA via two main pathways.(Fang et al. 2015, Hammond 2015)

The first pathway cleaves the mRNA via the endonuclease activity of Ago2, but only if the mRNA is complementary to the miRNA sequence in the central region of the miRNA (nucleotide 9-11). The second pathway occurs more often. The mRNA is complementary to the miRNA, but not in the central region of the miRNA. Via a series of reactions, the poly(A) tail of the mRNA will be removed, which will ensure degradation of the mRNA target.(Hammond 2015)

For biologists and other scientists, defining the targets of a specific miRNA is important. Not only to understand the biological role of the miRNA, but also to determine the efficacy of a miRNA mimic or inhibitor. Since the miRNA only has a short sequence to bind with the mRNA, a large amount of predicted targets can be found. By developed algorithms, a substantial amount of false negative targets can be filtered out.(Hammond 2015)

Pilot results

A novel miRNA has been identified during a study where large (Great Dane) and small breed dogs (Miniature Poodle) were compared to each other on a transcriptional level. The growth plate tissue of large breed dogs showed an increased amount of this novel miRNA when compared to small breed dogs.

In a pilot experiment, canine MSCs undergoing osteogenic differentiation were transfected with this miRNA or with a control miRNA (mock). This mock miRNA does not have a biological effect. Via quantitative PCR(qPCR) the gene expressions of osteogenic genes were measured. The preliminary results reveal that transfection with this miRNA in the presence of BMP-6 positively regulated osteogenic differentiation compared to the mock miRNA.

Another goal of the pilot study was to determine what was the best concentration for the miRNA. The miRNA was used in two different concentrations, 12,5nM and 25nM. The 12,5nM has proven to be the most effective, therefore for future studies, the 12,5nM will be used.

Furthermore was decided during this pilot study to work with BMP-6 instead of BMP-2 to induce the osteogenic differentiation. Earlier research from our group and others has shown that BMP-6 has the same or even a better effect at inducing the osteogenic differentiation when compared to BMP-2. (Bozkurt et al. 2014, Açil et al. 2014)

Research goal

The purpose of this study is to evaluate whether the novel miRNA has a positive effect on osteogenic differentiation in canine MSCs. Dogs are considered to be a superior model for the translational research into human than rats. Dogs are, the same as humans, higher mammals and therefore their physiology has great resemblances.(de Bakker et al. 2013) By evaluating the expression of eight osteogenic genes, we will evaluate whether the miRNA has an effect on osteogenic differentiation. These gene markers are:

- Alkaline phosphatase (*ALP*) can be formed in all cells of the body. The ALP formed in hepatocytes and bone has a long duration of action. It is one of the first genes expressed in the process of calcification and it is therefore likely that at least one of its functions is during the early mineralization process of bone.(Ram et al. 2015) (role of alkaline phosphatase in mineralization—E.E. Golub)
- Osteocalcin, also known as bone gamma carboxyglutamic(gla) acid protein (*BGLAP*) is formed by osteoblasts. The gla domain of this protein has a function in binding calcium to hydroxyapatite, which is the mineral component of bone. It plays a role in bone resorption and in bone mineralization.(Ram et al. 2015)
- Collagen type I replaces the collagen type II which was formed during the third stage of secondary bone formation. Collagen 1 is the main component of the ECM of bone. (Palukuru, McGoverin & Pleshko 2014)
- Inhibitor of DNA binding protein 1 (*ID1*) is a downstream target of the BMP pathway. In a BMP rich environment, ID1 will be upregulated.(Valdimarsdottir et al. 2002) It plays a role in the differentiation of MSCs.(Yokota 2001)
- Noggin is a BMP-2, 4 and 7 antagonist. It binds them and therefore ensures they cannot bind to the BMPr. Smad 6 binds to the BMPr type 1. It prevents Smad 1/5/8 to be phosphorylated and therefore activated. (Rifas 2007)
- Runt-related transcription factor 2 (*RUNX 2*) is critical for osteoblast differentiation. It binds specific DNA sequences and can therefore regulate transcription of several genes. This way RUNX 2 controls osteoblast development from MSCs.(Schroeder, Jensen & Westendorf 2005)
- Osteonectin, also known as secreted protein acidic and rich in cysteine (*SPARC*), is a calcium binding glycoprotein which is secreted by all sorts of cells. It consists of three domains, of which one can bind calcium ions to hydroxyapatite. (Yan, Sage 1999) It has a function in the cell-matrix interaction during remodeling of the bone tissues and is associated with mineralization of the bone. (Ram et al. 2015, Yan, Sage 1999)
- Osteopontin, also known as bone sialoprotein I (BS1) or secreted phosphoprotein 1(SPP1), is an attachment protein. It links cells to the bone minerals via a hydroxyapatite binding region in the protein. (Kazanecki, Uzwiak & Denhardt 2007) Beside its role in the cell-matrix interaction, it also plays a role in the resorption and remodeling of bone. (Ram et al. 2015)

Materials and methods

Preliminary experiment

The patient samples are bone marrow derived MSCs that are being biobanked by the division of orthopaedics at a regular basis. Four donors have been studied in a follow up experiment. The cells have been expanded and differentiated following protocol. They are plated in 48-wells plates and divided into three groups, giving a different medium in each:

- Expansion medium *(α-MEM (Invitrogen, 22561-021) with 10% FCS (PAA Cell Culture Company), 1% penicillin/streptomycin(p/s), 10^{-4} M ASAP and 5 µL/mL fungizone (Invitrogen, 15290-180))*
- Osteogenic medium *(D-MEM high glucose (Invitrogen, 31966-021), 10% FCS, 1% p/s, 10mM β-Glycerolphosphate (Sigma, G6376), 0,1 mM ASAP, 10^{-7}M dexamethasone and 5 µL/mL fungizone)*
- Osteogenic medium + BMP-6 *(the medium as described above and 250 ng/mL BMP-6 (R&D))*

The cells in these three groups are again divided into two or three groups per medium. These three groups receive different treatments. The first group is transfected with the novel miRNA, the second group is transfected with a mock miRNA and the third group remains untreated. This group is hereafter called the negative control group.

At day 7 and day 14 of this experiment, the cells were harvested and stored properly for further analysis.

mRNA isolation

Total mRNA extraction of the materials collected in the follow up experiment was performed with a RNeasy® mini kit (*Qiagen*) for the monolayers and a RNeasy® micro kit (*Qiagen*) for the pellets. Manufacturer's protocol is followed. To ensure no ribonucleases (RNase) could interfere with the outcomes, RNase free water, tips and pipets were used. To ensure limited DNA interference, desoxyribonuclease (DNase) was used. This was added to the protocol according to manufacturer's protocol. After isolation of the mRNA, 1 µl of each sample was placed in the nanodrop® to determine the amount of RNA.

cDNA synthesis

The cDNA was synthesized by using the iScript™ cDNA Synthesis Kit (*BIO RAD*) according to manufacturer's protocol. The same amount of mRNA(60ng) was used in each reaction. A maximum amount of 15µl mRNA solution is added to 5µl supermix of the cDNA Synthesis Kit, making a total amount of 20µl. The mixture was incubated 5 minutes at 25˚C, 30 minutes at 42˚C and finally another 5 minutes at 85˚C. Thereafter the samples were kept at -20˚C.

qPCR

The cDNA samples were diluted with 180µl miliQ to make a 1:10 dilution. A standard dilution series was made and pipetted in the 384-well qPCR plate. The cDNA samples were further diluted by taking 44µl of cDNA 1:10 solution and adding an extra 172µl of miliQ. The dilution of the cDNA was then 1:50. This is the dilution needed to run the samples in the PCR. The mastermix was made with 782µl supermix, 6,3 µl fw primer (100µM), 6,3µl rv primer (100µM) and 144µl miliQ. Primers with their characteristics are shown in appendix 1.

6µl of the mastermix and 4µl of each sample were pipetted in each well of the 384wells plate. The plate was placed in the BIORAD 384FSX.

Statistics

To test for normality, a kolmogorov-smirnof and shapiro-Wilk test are performed. If the data was normally distributed a general linear model used in SPSS (*IBM version 22*). In a general linear model there can be determined whether the means of two groups show a statistical difference. A post-hoc LSD test was used to correct for multiple testing. When the data was not normally distributed, a cox proportional hazard model was made in R studio. Data is shown as the mean + the standard deviation.

Results

MSCs from 4 different donors (445, 405, 545 and 608) were seeded in monolayers during 14 days in three different conditions: expansion, osteogenic and osteogenic with BMP-6(BMP-6 group). Interestingly, in the first two groups, many of the monolayers turned into pellets. This happened at different time points for each different well. Moreover, it seemed to be a tendency in the BMP-6 group to keep the monolayers stable for a longer period of time. As a result, at day 14 almost all wells from the expansion and osteogenic groups were pellets, compared with only two wells in the BMP-6 group.

Osteogenic differentiation

To ensure that osteogenic differentiation has been initiated, the MSCs which only received one of the medium types and not the mock or the miRNA, the negative controls, were compared with each other (*figure 5*).

Almost all measured osteogenic related genes were upregulated in the BMP-6 group. Concretely, the transcription of *ALP, BGLAP* and the master osteogenic regulator *RUNX2* showed a significant upregulation for the BMP-6 group when compared with the expansion group. Moreover, the first two together with ID1, a downstream target of the BMP pathway, and SPP1 were also found to be more expressed when compared with the osteogenic group, suggesting a more potent osteogenic differentiation by the addition of BMP6.

In the osteogenic group, *ALP* and *SPARC* showed a significant upregulation when compared with the expansion group. For *RUNX2* the same tendency was shown although not significant (*p-value = 0,060*). Curiously, and contrary to what in the BMP6 group happened, SPP1 was significantly downregulated highlighting a different transcriptional behavior between the two treatments.

Collagen1, which is the most abundant collagen type in bone, showed a significantly lower expression in the BMP-6 group when compared with the expansion and osteogenic group. Finally, Noggin, the BMP-2/4/7 inhibitor, showed no significant differences.

These results suggested that the osteogenic differentiation has at least been initiated and maybe even further progressed.

Mock versus miRNA

Having strong suspects that osteogenic differentiation has occurred in the MSCs, we further analyzed in deep the effects of the miRNA in the osteogenic process. By comparing the expression levels of the mock with those of the miRNA, the effect of the miRNA can be assessed.

ALP (*figure 6*) showed no significant increases in transcription in the osteogenic group by the addition of the miRNA at day 7, but a trend can be seen at both time points (day 7 and 14), where all the miRNA groups seem to have a higher expression when compared to the mock. In the BMP-6 group, no differences can be found between the mock and the miRNA.

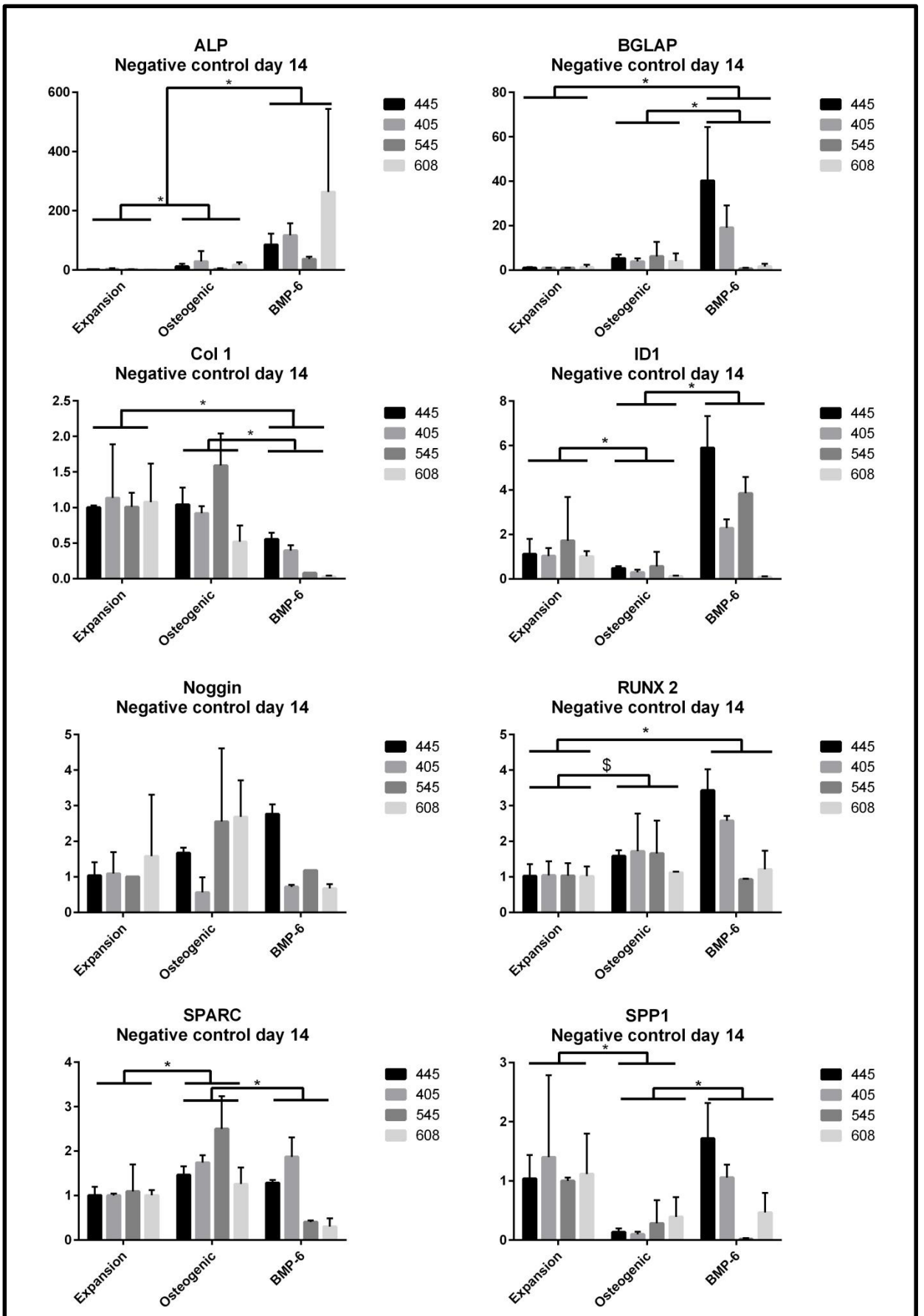

Figure 5: The mean n-fold changes of different osteogenic differentiation related genes. The expansion group was taken as reference point and therefore as 1. An asterix (*) indicates a significant difference with a *p*-value lower than 0,05. A dollar sign indicates a borderline insignificant *p-value* between 0,08 and 0,05.

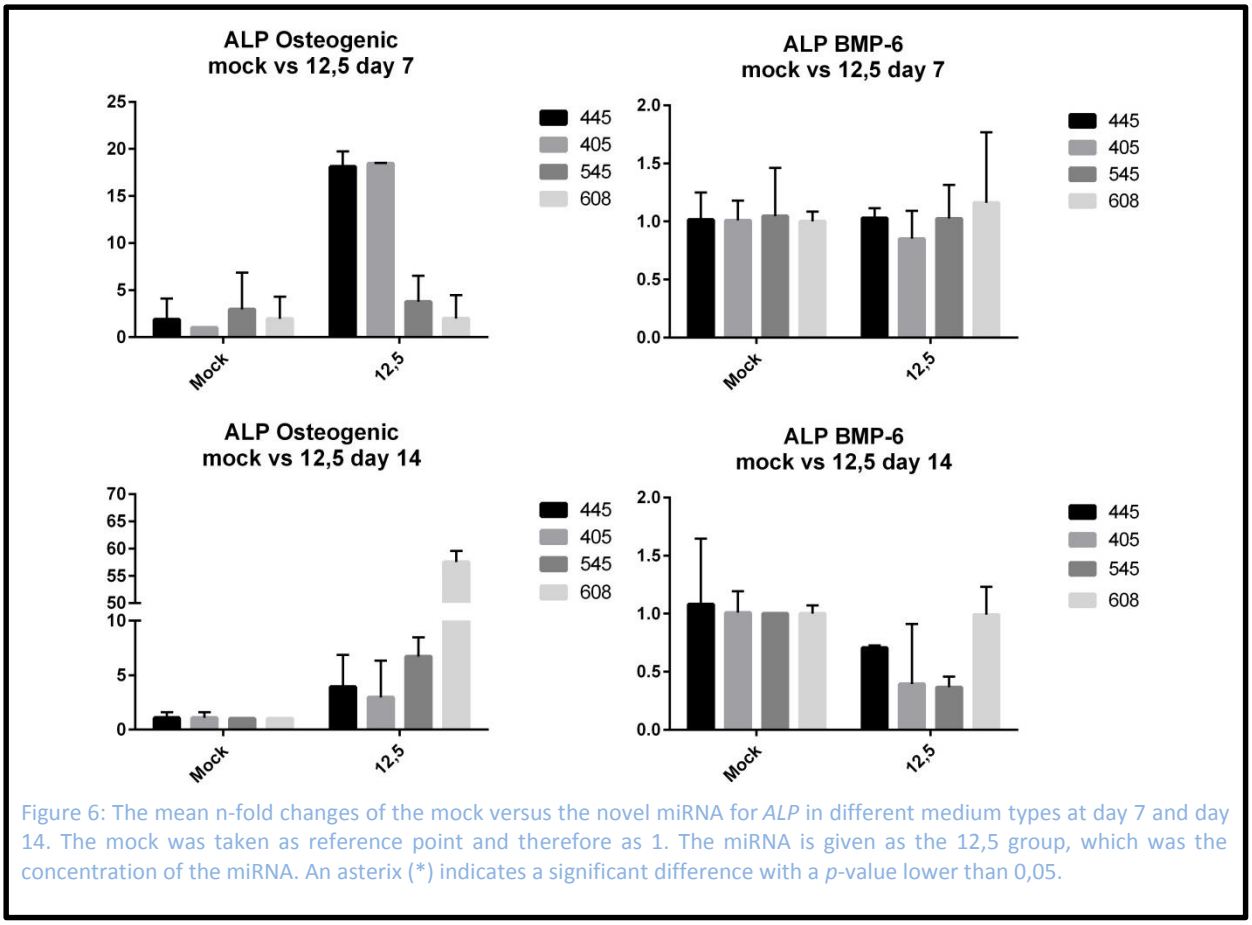

Figure 6: The mean n-fold changes of the mock versus the novel miRNA for *ALP* in different medium types at day 7 and day 14. The mock was taken as reference point and therefore as 1. The miRNA is given as the 12,5 group, which was the concentration of the miRNA. An asterix (*) indicates a significant difference with a *p*-value lower than 0,05.

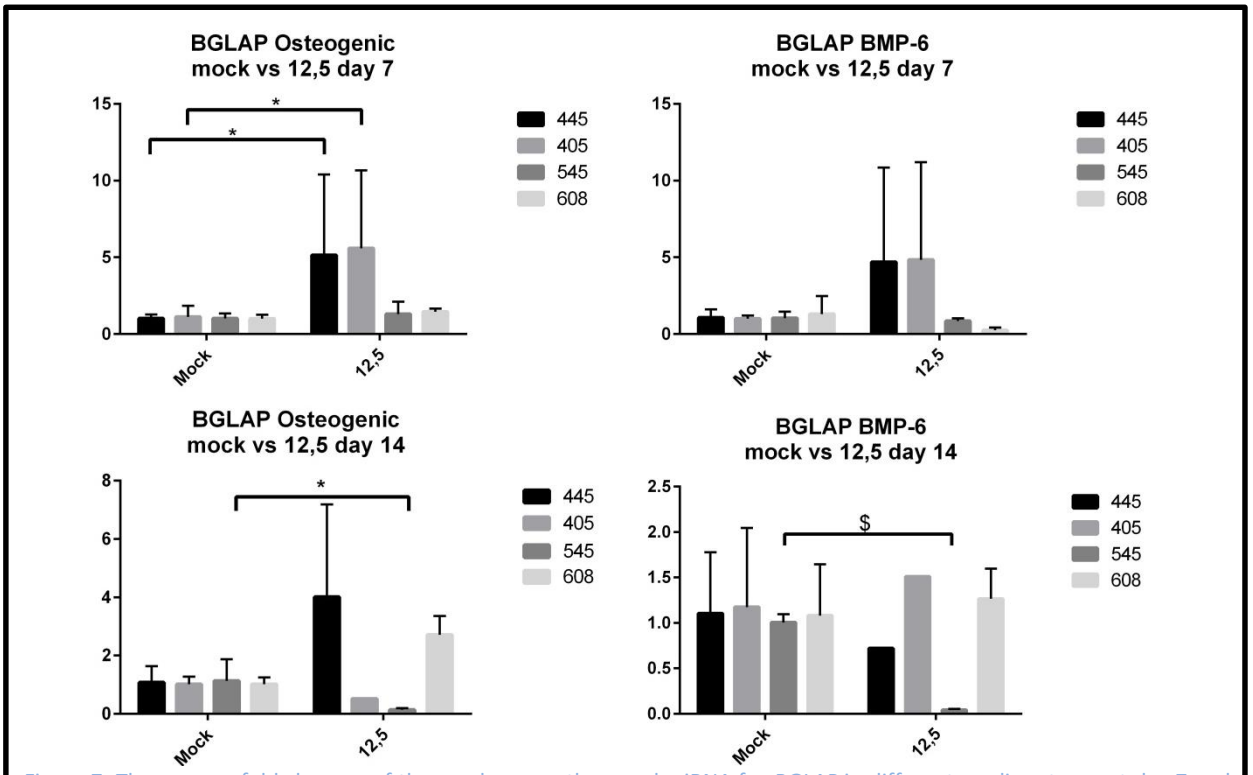

Figure 7: The mean n-fold changes of the mock versus the novel miRNA for *BGLAP* in different medium types at day 7 and day 14. The mock was taken as reference point and therefore as 1. The miRNA is given as the 12,5 group, which was the concentration of the miRNA. An asterix (*) indicates a significant difference with a *p*-value lower than 0,05. A dollar sign ($) indicates a borderline insignificant difference with a *p*-value between 0,08 and 0,05.

At day 7 in the osteogenic group, two donors showed a significant increase in expression for BGLAP (*figure 7*). This trend is also visible for the BMP-6 group, although not significantly different. At day 14, these differences disappeared.

Collagen 1 showed at day 7 in both the osteogenic and the BMP-6 group significant decreases of the miRNA expression compared to the mock (*figure 8*). At day 14 these differences are no longer significant, but the decreasing trend is still visible in three out of four donors for both groups. (Even, donor 545 still shows a significant decrease in the osteogenic group).

At day 7 in the osteogenic group, *SPARC* (*figure 9*) showed a borderline significant decreased expression (*p-value = 0,078*) between the mock and the miRNA for one donor and a significant decrease for another. Together, 3 out of 4 donors seemed to downregulate SPARC as a response to the miRNA. At day 14, these differences were no longer visible. In the BMP-6 medium, only donor 545 showed a significant decrease at day 7..

Finally, SPP1 showed no significant differences in transcription at day 7, behalf a downregulation for donor 545 in the BMP-6 group (*figure 10*). But there seems to be a tendency towards an increase of expression in the osteogenic group. Indeed, for this group at day 14 , *SPP1* showed an upregulation in the miRNA group when compared with the mock. This is not present in the BMP-6 group.

No significant changes or trends were found for *Noggin, RUNX2* and *ID1* (data not shown).

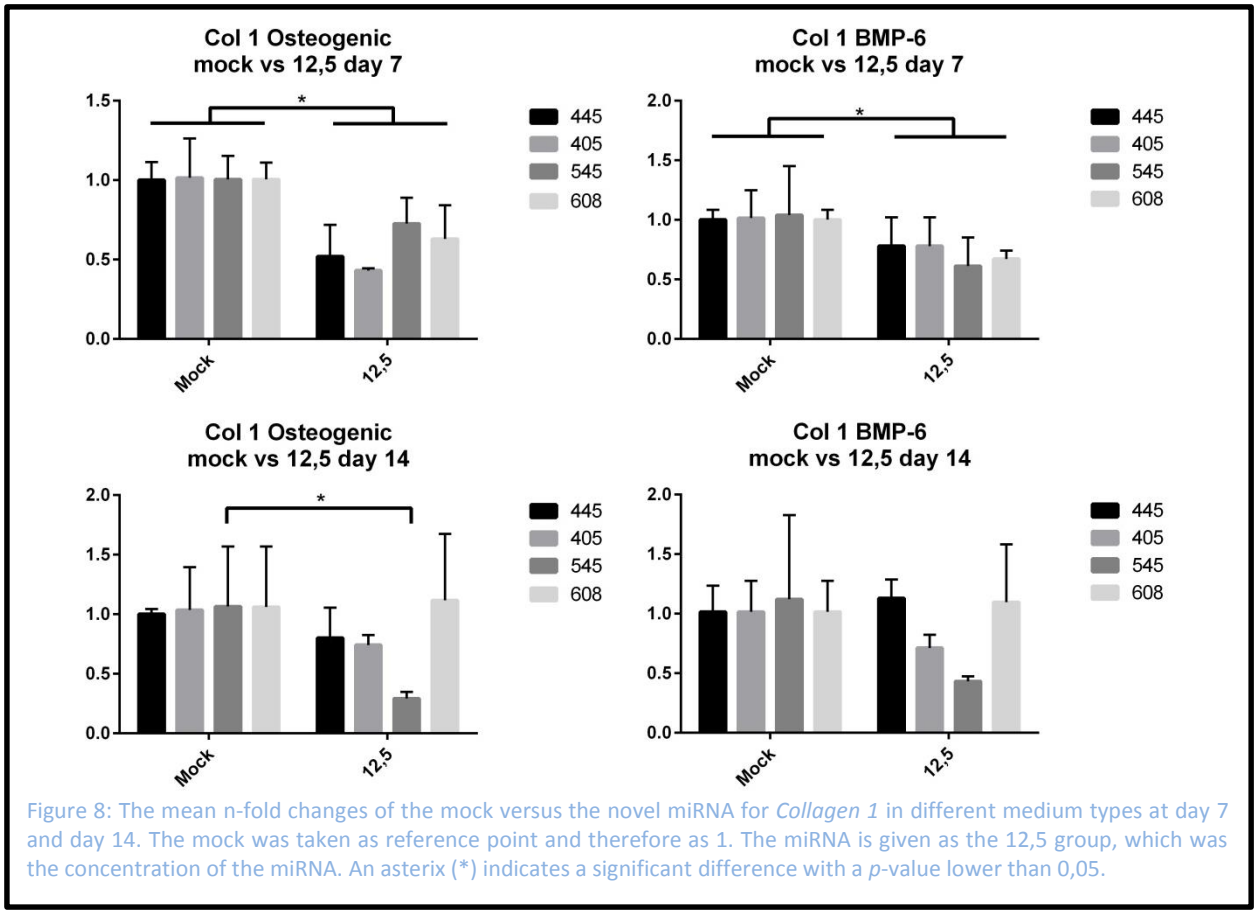

Figure 8: The mean n-fold changes of the mock versus the novel miRNA for *Collagen 1* in different medium types at day 7 and day 14. The mock was taken as reference point and therefore as 1. The miRNA is given as the 12,5 group, which was the concentration of the miRNA. An asterix (*) indicates a significant difference with a *p*-value lower than 0,05.

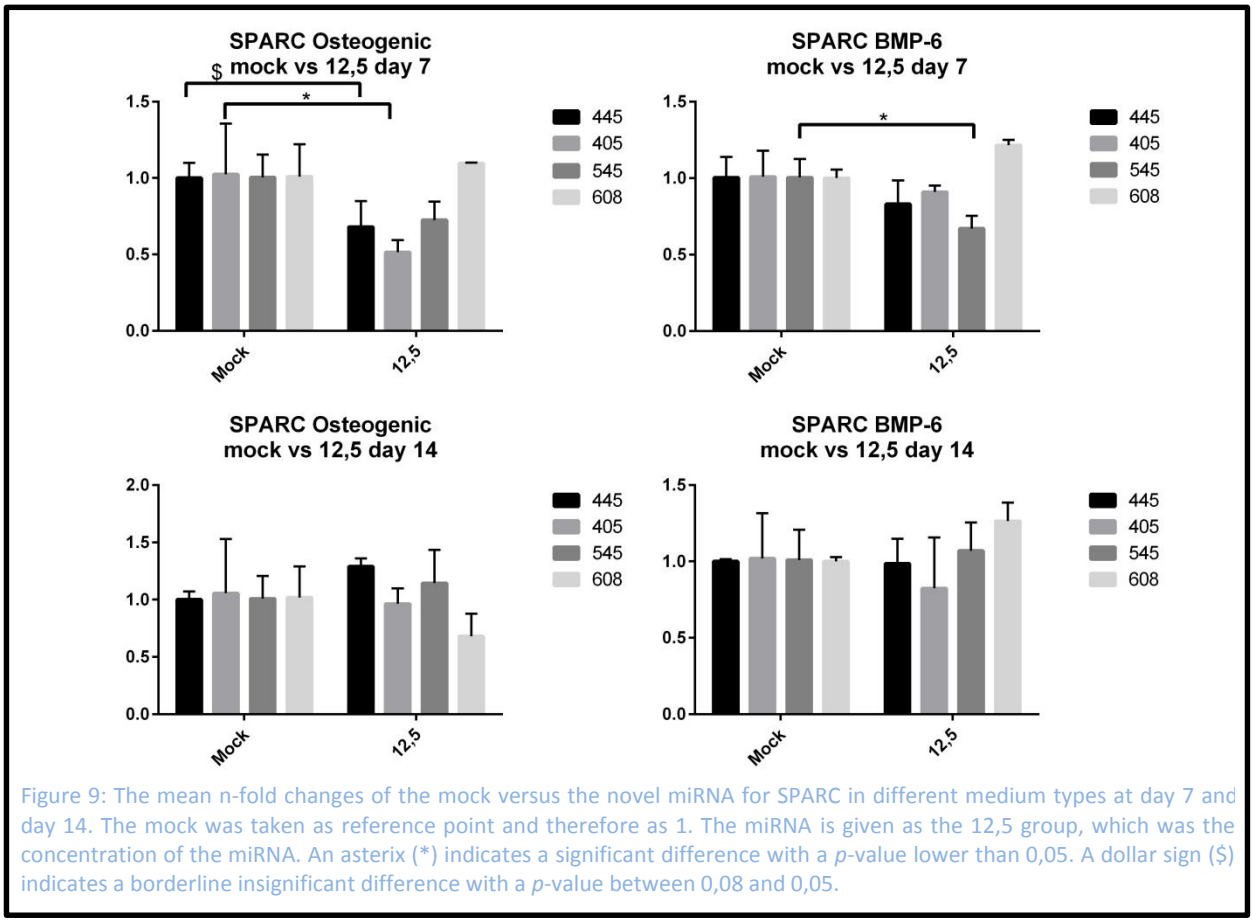

Figure 9: The mean n-fold changes of the mock versus the novel miRNA for SPARC in different medium types at day 7 and day 14. The mock was taken as reference point and therefore as 1. The miRNA is given as the 12,5 group, which was the concentration of the miRNA. An asterix (*) indicates a significant difference with a *p*-value lower than 0,05. A dollar sign ($) indicates a borderline insignificant difference with a *p*-value between 0,08 and 0,05.

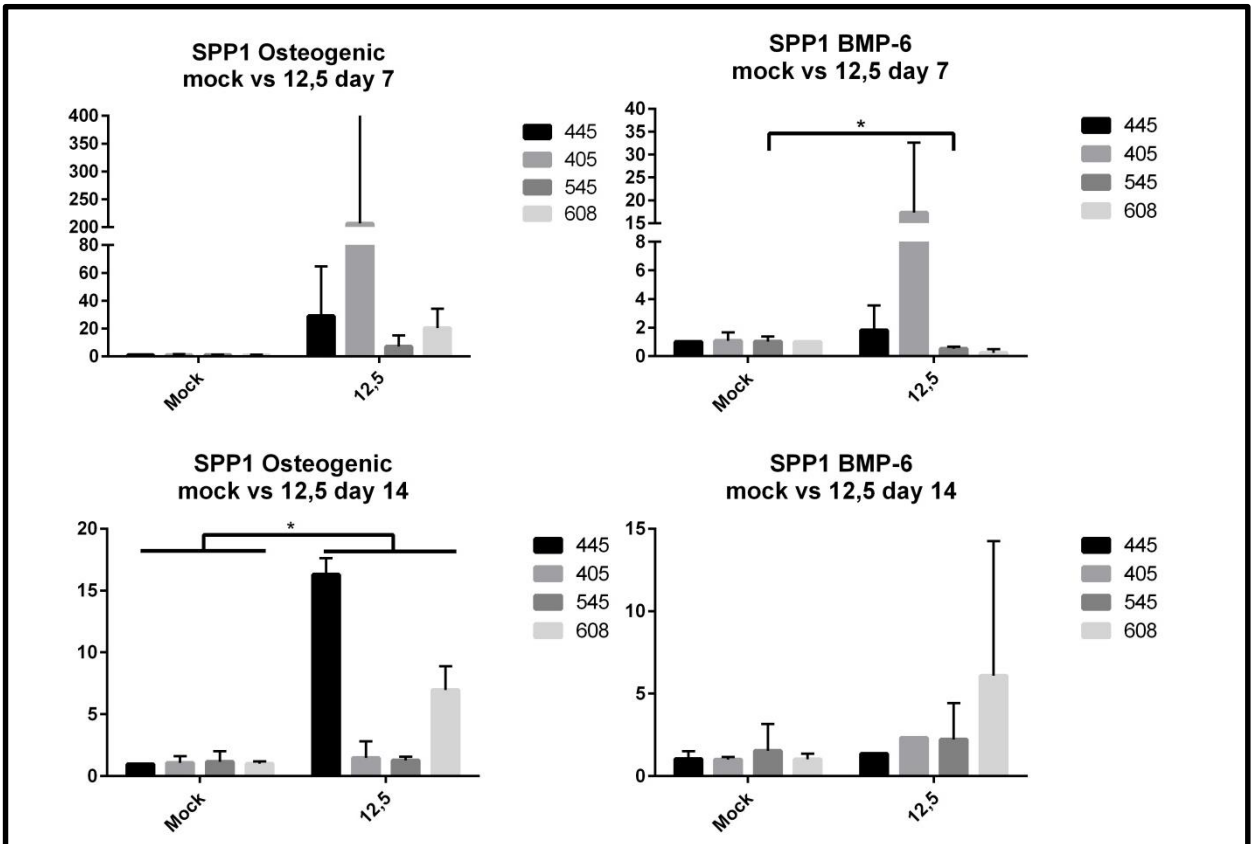

Figure 10: The mean n-fold changes of the mock versus the novel miRNA for SPP1 in different medium types at day 7 and day 14. The mock was taken as reference point and therefore as 1. The miRNA is given as the 12,5 group, which was the concentration of the miRNA. An asterix (*) indicates a significant difference with a *p*-value lower than 0,05.

Expansion

Unexpectedly, several different expression features were found for the the miRNA treatment in the expansion group when compared with the mock in almost all the tested genes.

At day 7 (*figure 11*), *ALP* seemed to show a trend for upregulation in two donors of the miRNA group when compared with the mock. Moreover, *BGLAP* showed a significant upregulation in two donors. *Collagen 1* and *SPARC* showed a significant downregulation of the miRNA group, whilst *SPP1* showed a significant upregulation. In the other hand, *ID1* showed a borderline significant upregulation (*p-value = 0,057*), while *Noggin* showed a significant upregulation of one donor and a borderline significant upregulation of another donor (*p-value = 0,068*). Finally, *RUNX2* showed no significancies or trends (data not shown).

At day 14 (*figure 12*), *ALP* showed a trend in two donors to be downregulated in the miRNA group. Curiously, *BGLAP* and *SPP1* were significantly downregulated, whilst *Collagen 1* was upregulated. *ID1*, *Noggin*, *RUNX2* and *SPARC* showed no significancies or trends (data not shown).

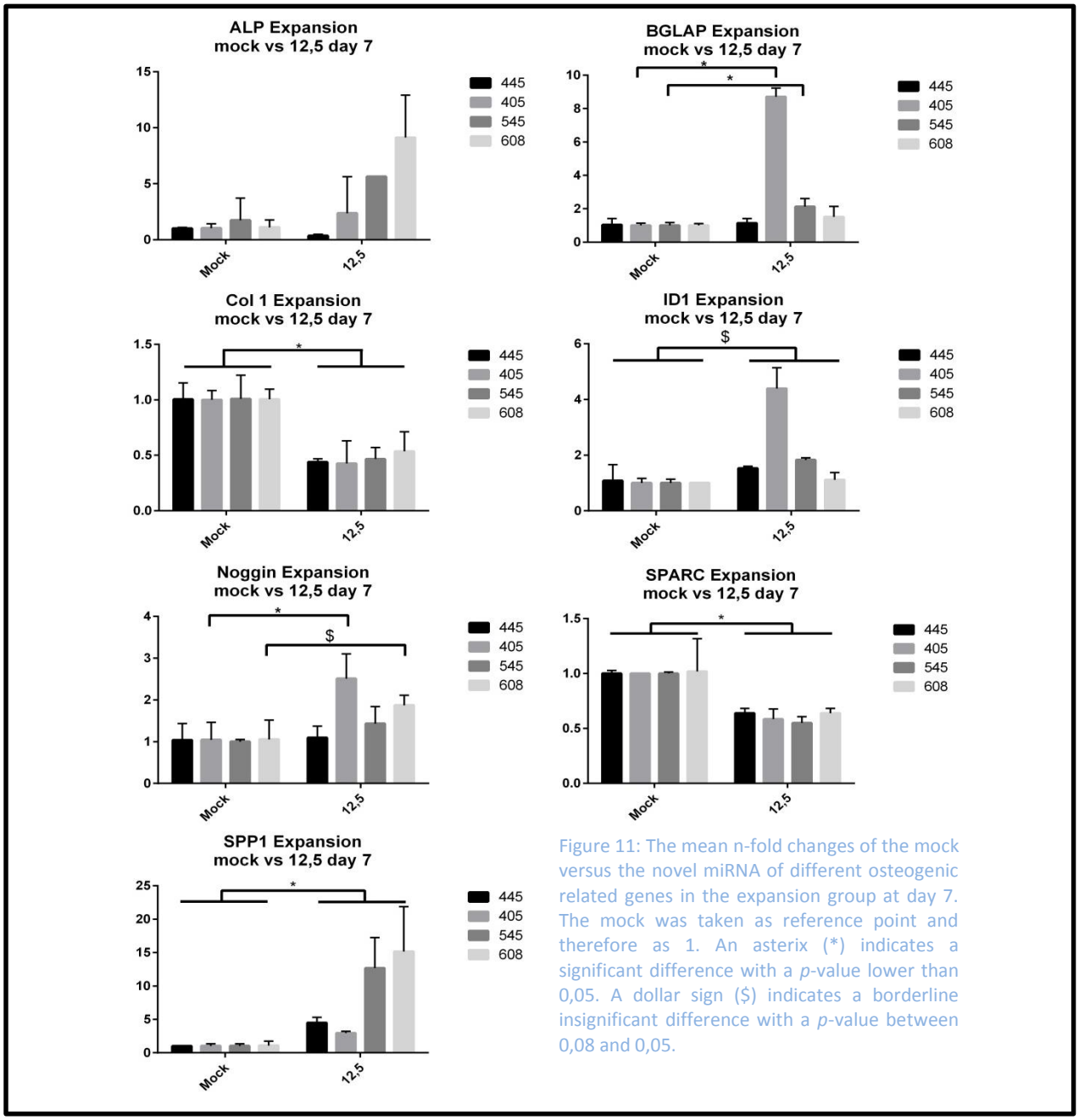

Figure 11: The mean n-fold changes of the mock versus the novel miRNA of different osteogenic related genes in the expansion group at day 7. The mock was taken as reference point and therefore as 1. An asterix (*) indicates a significant difference with a *p*-value lower than 0,05. A dollar sign ($) indicates a borderline insignificant difference with a *p*-value between 0,08 and 0,05.

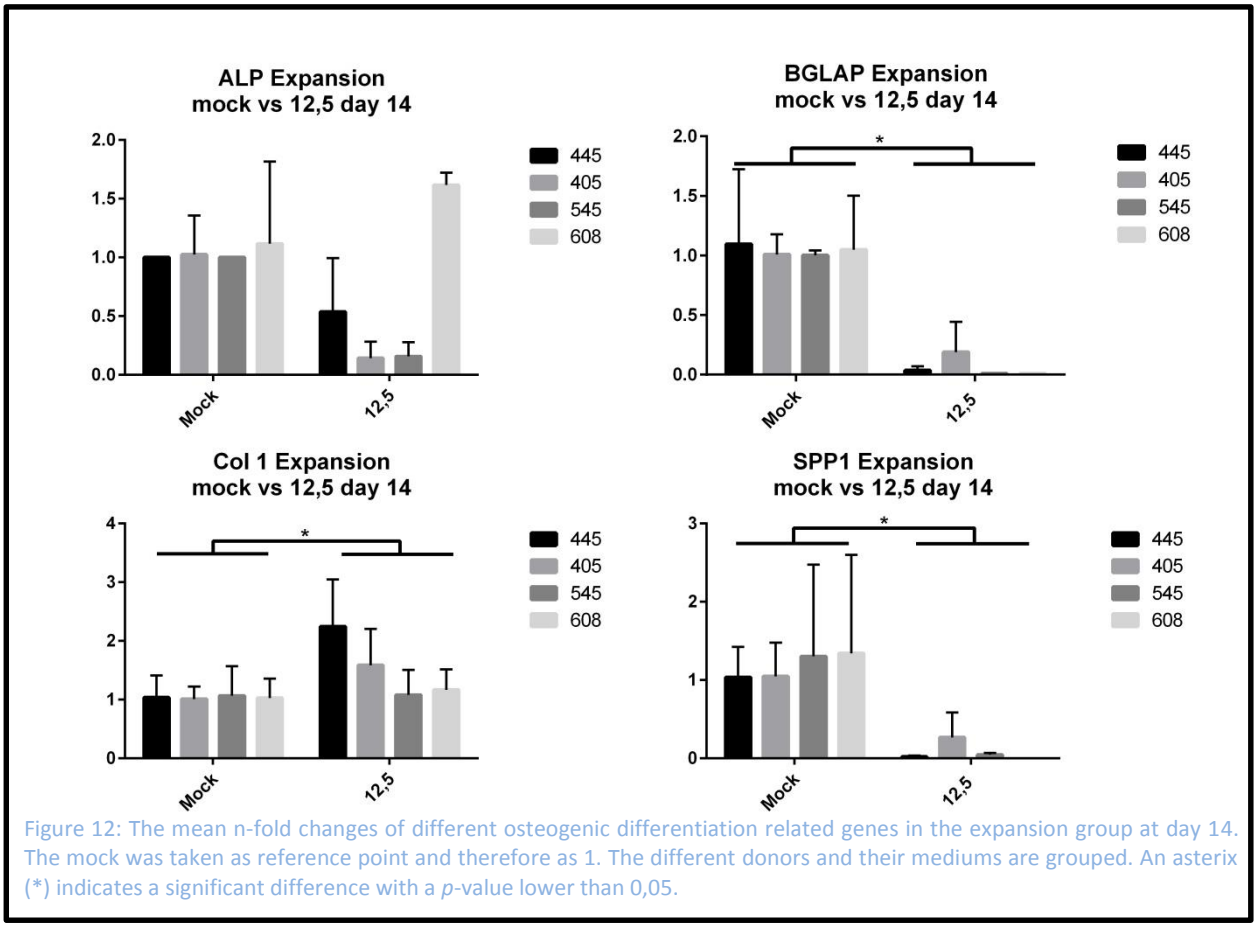

Figure 12: The mean n-fold changes of different osteogenic differentiation related genes in the expansion group at day 14. The mock was taken as reference point and therefore as 1. The different donors and their mediums are grouped. An asterix (*) indicates a significant difference with a *p*-value lower than 0,05.

Discussion

The main goal of this study was to evaluate whether the novel miRNA has a positive effect on the osteogenic differentiation of MSCs. Via analyzing the PCR outcomes of eight osteogenic differentiation related genes, it can be inferred whether the miRNA has a positive or negative effect on the osteogenic differentiation of the MSCs. At first, it is needed to ensure that osteogenic differentiation has occurred in the MSCs. After that, the comparisons between the mock and the miRNA can be made.

A first unexpected difficulty appeared soon because during different time points the monolayers detached from the plate, folded within them and turned into pellets. Hence, it is not possible to state that all wells got the same treatment and followed the same differentiation course. In the wells which still contained monolayers, by definition, all cells have been transfected with the mock or the novel miRNA homogeneously, but that cannot be assured for the pellets. They have acquired a three dimensional conformation and therefore a stimulation gradient from the outer cell layers towards the inner ones was established. It is therefore impossible to say whether the cells in the middle of the pellet got transfected with the same amount of miRNA compared to the cells on the border of the pellet or whether it even reached the cells in the middle. Even though the forming of the pellets was taken in consideration as a random effect in the statistics, it would still be better for further research to seed the cells directly in pellets, thus eliminating definitely this variable in the analysis.

The folding of the monolayers was not something expected to happen, but it might have had a positive effect on osteogenic differentiation. Although we seeded the cells as a monolayer, maybe it is the natural trend of the cells to detach from the plate and fold into pellets.It can be suggested therefore that folding into pellets is helping the cells in their osteogenic differentiation. The fact that this has not occurred in the BMP-6 group, might be because BMP-6 is already helping the cells differentiate and therefore the formation of the pellets is not needed immediately.

Osteogenic differentiation

By comparing the negative controls of each medium type, we were able to state that osteogenic differentiation indeed had occurred in the MSCs. *ALP*, *BGLAP*, *Collagen 1* and *RUNX2* showed significant differences in the BMP-6 group when compared to the expansion group. Furthermore, *ALP*, *ID1*, *SPARC* and *SPP1* showed a significant difference and *RUNX2* a borderline significant difference when the osteogenic group was compared with the expansion group, therefore suggesting that in the osteogenic group the osteogenic differentiation has taken place as well. *ALP*, *BGLAP*, *Collagen 1*, *ID1*, *SPARC* and *SPP1* also showed significant differences in the BMP-6 group when compared with the osteogenic group, suggesting an additive effect of the BMP-6 group on the osteogenic differentiation.

The fact that *collagen 1* showed a downregulation at day 14 (*Figure 5*) in the BMP-6 group might be because it has its peak of expression somewhere in between the time points we have measured. Normally, *collagen 1* is highly expressed in the ECM at day 15 after osteogenic differentiation (Collin et al. 1992) with its peak of gene expression around day 21.(Eslaminejad, Taghiyar 2010)(Li et al. 2015b) When treated with BMP-6(50 ng/ml), a significant increase in collagen 1 was seen at day 10 of the treatment when compared with a non-treated control group. At day 20, these significant differences were no longer visible. When BMP-6 was added in a concentration of 100 ng/ml, no significant differences were found between the control- and the treated group (Bozkurt et al. 2014).

The experiment of Açil et al shows an upregulation of *collagen 1* at day 10 and day 18 in human MSCs after adding an increasing amount of BMP-6 during the time of the experiment. (Açil et al. 2014) It might be possible that between day 10 and day 18, *collagen 1* expression is downregulated again. Therefore in this experiment a downregulation of *collagen 1* is found at day 7 and at day 14.

SPARC and *SPP1* seem to have a different effect solely related to the medium types (*Figure 5*). These genes both express a protein which is involved in the cell-matrix interactions and the remodeling phase of the bone formation(Ram et al. 2015, Yan, Sage 1999, Kazanecki, Uzwiak & Denhardt 2007), meaning they would probably be expressed around the same time during osteogenic differentiation. But our results show that where *SPARC* is being upregulated by the osteogenic group, *SPP1* is being downregulated. This suggests that *SPARC* expression is needed at the measured time points, but *SPP1* is not. They therefore do have a different expression throughout osteogenic differentiation.

Another explanation for a downregulation in general, is that the miRNA not only has an enhancing effect on upregulations, but it can also have an enhancing effect on downregulations. In this way, the downregulations which already occur during differentiation are enhanced as well.

SPARC and *SPP1* also showed no differences between the expansion and BMP-6 group (*Figure 5*). Taken in account that *SPARC* has a higher and *SPP1* a lower expression in the osteogenic group, this suggests that BMP-6 has a negative effect on the expression of *SPARC*, but a positive effect on the expression of *SPP1*.

Mock vs miRNA

Earlier research in our group has shown that that the transfection of the miRNA's via lipofectamine has a negative effect on the expression of the osteogenic proteins. Therefore the novel miRNA has to be compared to the mock instead of the negative control, to see the actual effect of the miRNA.

We can state that the miRNA has an effect on *collagen 1*. It shows a significant decreased expression in the miRNA group at day 7 in both the osteogenic and the BMP-6 mediums, being still mostly visible at day 14(*Figure 8*). The fact that some of the donors do not show an increase or decrease at day 14, might be because of the donor variation. It is not given that the donors are all at the same point in their osteogenic differentiation and therefore they can have a different expression of the genes.

Furthermore, a tendency can be seen in *ALP* at day 7 and day 14 in the osteogenic group (*Figure 6*). At day 7, two donors seem to have a higher expression when compared to the mock, whilst at day 14 all four donors seem to have an increased expression of *ALP*. The fact that these differences are not significant might be due to the high donor variation. In an experiment with more donors, these upregulations would probably be significant.

The expression of *SPARC* at day 7 in the osteogenic group, seemed to be downregulated in three donors in the group treated with the miRNA. This is interesting, because when looking at the negative controls (*figure 5*), *SPARC* was upregulated in the osteogenic group at day 14, but BMP-6 downregulated the expression of *SPARC*. In the osteogenic group at day 7(*figure 9*), the same trend is visible for the miRNA for three donors, therefore suggesting that the miRNA has the same effect as BMP-6 regarding these gene transcriptions.

SPP1 showed a downregulation in the osteogenic group in the negative controls, but with the added effect of the miRNA, it showed an increase of expression at day 14 in the osteogenic group (*figure 10*). At day 7 the same tendency can be seen. This suggests again that the miRNA has the same effect as BMP-6 in the osteogenic group, since the BMP-6 group in the negative controls was significantly upregulated when compared to the osteogenic group (*figure 5*).

The fact that we do not see a clear effect of the miRNA in the BMP-6 groups in any of the genes, except for *collagen 1*, might be because the cells in the BMP-6 groups already produce the maximum amount of gene which can be made in the cells. *Figure 5* shows an upregulation in the BMP-6 group for *ALP*, *BGLAP*, *ID1, RUNX2* and *SPP1* when compared with the expansion group and/or the osteogenic group. The osteogenic group has a lower expression of these genes than the BMP-6 group. We therefore know that the cells in the osteogenic group can still increase the expression of these genes. On those grounds it might be that these cells can still react to the miRNA.

What could be possible as well is that the cells in the BMP-6 group do react to the miRNA, but they already have such a high expression that therefore the extra expression through the miRNA could be of no significant importance. Thus, the effect of the miRNA in the osteogenic group is relatively larger, because of the lower expression it has at the starting point (*figure 5)*.

Donor 545 has been proved in other studies from the group to be less responsive to BMP-6. This was confirmed by the fact that in the negative control group, it showed a lower expression of osteogenic markers than the rest. The reason of its inclusion in the study was to see whether the miRNA can have an effect on a reluctant donor. Unfortunately, while the other donors showed a tendency towards a positive response to the miRNA, the resistant transcriptional behavior was maintained within the miRNA transfected cells and, as a consequence, the data from this donor have negatively influenced the statistics, increasing the dispersion. An increase of biological replicates is desirable to properly assess the effect of the miRNA, because the tendencies or even the non-significant expression patterns could turn into a significant transcription feature.

It is important to mention that the statistical comparison per donor between the mock and novel miRNA in the different medium types was performed according to the same statistical analysis as has been noted earlier, with only two biological replicates in each medium. It gives a low statistical power thus all the trends and significances from these individual comparisons should be taken cautiously and were just stated to demonstrate the inter-individual variation.

Expansion

The expansion medium is a medium which does not induce any differentiation of the MSCs. It expresses genes without the influences of differentiation, so therefore the effect of the miRNA on the expression of these eight genes is clearly visible.

Concretely, at day 7 (*figure 11*) the miRNA addition has a general positive effect on the undifferentiated cells. *ALP* showed a trend towards an increase in two donors. *BGLAP*, *ID1*, *Noggin* and *SPP1* show either as a group or in a few donors a significant increase in expression of the genes, while *collagen 1* and *SPARC* show a significant decrease. This means that the miRNA has an influence in the expression of these genes. Some are positively upregulated and some are downregulated, but the influence of the miRNA is unmistakable.

For *Collagen 1* the groups treated with the miRNA showed a significant decrease in expression. which is the same pattern in the expansion group at day 7 as it does in the osteogenic and BMP-6 groups at day 7(figures 8 and 11). *SPARC* shows the same trend in the osteogenic group at day 7(*figures 9 and 11*) when compared with the expansion group at day 7 as well. In the osteogenic group three out of four donors seem to be downregulated, while in the expansion group, the entire miRNA group is significantly downregulated. *SPP1* also showed similarities between the expansion and osteogenic groups at day 7 (*figures 10 and 11*). In the osteogenic group only a trend is visible towards an increase of expression in the miRNA group, but the expansion group showed a significant increase. What can be concluded from this is that the miRNA seems to show a similar effect on the gene expression MSCs undergoing osteogenic differentiation as on MSCs which are not undergoing this differentiation. This indicates that the miRNA might have a great influence in the early phases of osteogenic differentiation.

At day 14 in the expansion medium the miRNA downregulated *BGLAP* and *SPP1* significantly when compared with the mock (*figure 12*). For *ALP* a trend was visible in two out of four donors towards a downregulation, but no significances were found. The fact that these genes are downregulated suggests that the expression of these genes is at this point in time no longer needed. *Collagen 1* is on the other hand upregulated when compared with the mock. So it can be concluded that the miRNA influences the expression of these genes at day 14 as well.

What is also interesting is the expression of *ALP* in the expansion medium at day 14. It shows the same decreasing trend as *ALP* in the BMP-6 medium at day 14(*Figures 6 and 12*). Even though these differences were not significant, the same three donors showed a downregulation of *ALP* in the miRNA group when compared to the mock. But other than this, no similarities were found between the osteogenic and BMP-6 groups.

To summarize, based on this transcriptional behavior, it is tempting to think that with longer incubation periods (up to 28 days or longer) the addition of the miRNA to the expansion medium could induce the osteogenic differentiation *in vitro*. If this extreme is confirmed, a new treatment strategy for bone defects could be opened in the way of direct delivery of the novel miRNA into the damaged zone.

Future studies

For future studies it is best to start by culturing the cells in pellets instead of monolayers. In that way, a biological bias in the experiment can be overcome. Culturing the cells in pellets instead of monolayers also comes with other advantages, because pellets are a 3D structure, which has a higher resemblance to tissues in vivo. Therefore the effect of the miRNA has a higher chance of being reproducible in vivo.

Furthermore, a longer period of time, at least 28 days, with more time points to measure the gene expressions and more donors to decrease the influence of the inter-individual differences would be of importance in future studies. Gene expression can be followed in time during the whole osteogenic differentiation so all peaks and lows of the different gene expressions can be found. The expansion group has also proven to be of great interest. Future experiments should prove whether or not osteogenic differentiation has occurred in the expansion medium after a culturing the cells for a longer period of time while adding the miRNA.

Finally, a collagen 1 staining should be performed to see whether *collagen 1* has been produced and at what amount. In this experiment, *collagen 1* expression has always been downregulated, so it is not possible to state for certain that collagen 1 has been produced.

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