CARTILAGE REPAIR AFTER JOINT DISTRACTION

INTERMITTENT HYDROSTATIC PRESSURE AND ITS INFLUENCE ON MESENCHYMAL STEM CELLS:

AN IN VITRO STUDY

MASTER'S THESIS

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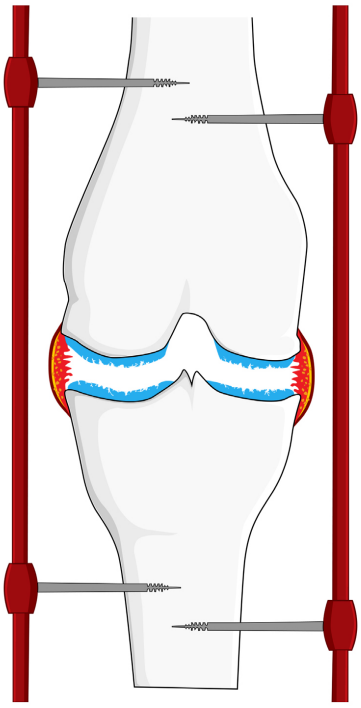

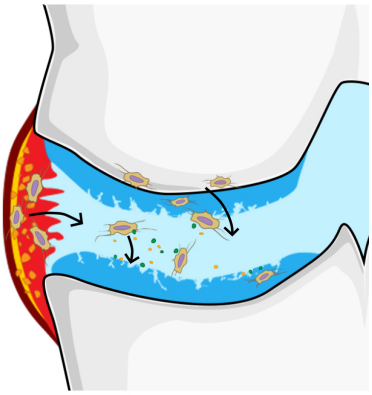

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COLLOQUIUM

Colloquium date: January 29th, 2015
Location: Carré 3022, University of Twente, Enschede
Time: 15.30h

GRADUATION COMMITTEE

Chairman: Prof. M. Karperien
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1. INTRODUCTION

Osteoarthritis (OA) is a rheumatic condition that affects approximately 1.2 million people in the Netherlands (in 2011), from which the largest group (600.000) represents the patients with knee OA[1]. Solely based on demographic changes, the expectation is that the absolute number of patients with OA will increase with almost 40% between 2000 and 2020. This number is expected to rise even further, due to aging and increasing obesity of the population[2-4]. OA is characterized by damaged articular cartilage, changes in the peri-articular bone (formation of osteophytes and subchondral sclerosis) and secondary, joint inflammation[3,5,6]. Treatment usually consists of prevention of excessive joint loading, physical therapy, pain reduction, and inhibition of inflammation[7,8]. In most cases, the extensively damaged joint eventually has to be replaced, in joint replacement surgery. However, this is not a curable treatment, and patients might still suffer from symptoms associated with OA. Additionally, the lifespan of a joint prosthesis is limited to a maximum of about 15 years. Revision or replacement surgery is needed in many cases, with a higher risk of complications, especially in those patients with a relative young age. This makes joint replacement an undesirable treatment mainly for younger people.

The current treatment options for OA exist mainly of conservative options to alleviate pain, inhibit inflammation, joint-preserving surgical options to slow down cartilage degeneration or eventually joint replacement surgery[34]. So far, treatment for osteoarthritis is mainly focussed on joint replacement rather than joint repair. However, evidence has been obtained for a surgical technique called joint distraction that results in a clinical and structural benefit up to 10 years after surgery. Additionally, intrinsic cartilage repair was observed in OA patients eligible for knee joint replacement as a result of knee joint distraction. Knee joint distraction is a surgical procedure in which the two bony ends of the joint are gradually separated to a certain extent and for a certain period of time by use of an external fixation frame. Application of joint distraction has shown to lead to intrinsic cartilage repair in combination with meaningful clinical efficacy for several different joints such as the ankle, hip and the knee[9-11]. As this cartilage repair activity is unique, it is possible for the first time to study the unknown mechanisms required for cartilage repair.

Thus, the exact underlying mechanism of the observed joint regeneration after joint distraction is still (partly) unknown. Looking at the possibilities that joint repair would have for a variety of diseases, the relevance of unravelling this mechanism is very high. If the environment, the (absence of) mechanical stimuli, the trophic factors and the other desired stimuli that are needed to facilitate joint repair can be exactly defined, this could have great implications for the treatment of degenerative joint diseases. Unravelling the MSC secretome under the influence of IHP could potentially lead to further optimisation of joint distraction treatment and to other related treatment strategies for OA. Furthermore, a better understanding of the bio-chemical/mechanical environment stimulating MSCs to initiate cartilage repair leads to optimization of current treatments with MSCs and opens new perspectives for future treatments.

Since the discovery of articular cartilage repair activity after a period of joint distraction, a variety of hypotheses have been formulated, discussed, and some have been proven or rejected. The current hypotheses are:

- There is no direct contact between both articulating surfaces in the joint, also during exercise or use of the joint. This prevents further damage of the articular cartilage, which allows cartilage cells to induce cartilage repair activity.
- Using this method of joint distraction, intermittent joint fluid pressures will be present during use and relaxation of the joint. IHP is a result of movement of the springs that are included in the distraction frame, which allows small axial movements of the knee. This IHP maintains the diffusion of nutrients from the joint (synovial) fluid into the articular cartilage.
- A change in peri-articular bone turnover occurs (osteopenia) because forces that are normally applied on bone will be taken over by the distraction frame. The subchondral bone will become less stiff,

which enables the bone to take over forces acting upon the cartilage. This will promote cartilage repair, also after the distraction period.
- The change in bone turnover results into the release of large quantities of growth factors from the bone. These growth factors are factors that have been shown to play a promoting role in cartilage repair.
- Mesenchymal stem cells (MSCs) originating from either the bone, the synovial tissue/fluid, or the infrapatellar fat pad, exert a trophic effect on the chondrocytes present in articular cartilage, possibly under the influence of the intermittent hydrostatic pressure, which results in a higher production of cartilage matrix components and proliferation of chondrocytes. Denuded bone areas lack presence of resident chondrocytes, which suggests the need for another celltype to initiate cartilage repair (MSCs).

It is conceivable, and proven, that tissues such as cartilage and bone, are influenced by mechanical stimuli. Bone density for example, increases when bone is exposed to higher loads[12]. This is what happens when bone-bone contact is present in joints where the articular cartilage of a joint is severely damaged (subchondral sclerosis). Mechanical stimuli present during joint distraction consist of the intermittent hydrostatic pressure that is present in the knee during joint distraction. The springs that are integrated in the joint distraction frame enable both the femur and tibia to move inside the joint cavity, however they will never be in direct contact with each other. This results in an intermittent hydrostatic pressure inside the joint fluid. This IHP is important for the nutrition of the cartilage that depends upon diffusion. Moreover, IHP is thought to have a, possibly stimulating, effect directly on chondrocytes or indirectly via stimulation of MSCs.

The effect of IHP on cartilage, chondrocytes, and MSCs (table 1), has been extensively studied[13-15]. What is remarkable is that all of the reported results regarding IHP in combination with MSCs are focused on differences in gene expression of genes that are associated with chondrogenic differentiation of MSCs (table 1). Research is focused on chondrogenic genes such as Sox9, Coll2 and aggrecan. This might be because of the dogma that has existed for a long time, since the discovery of multi-potent stem cells. Mesenchymal stem cells were always seen as cells that are able to differentiate into different lineages of cells, mainly into bone, cartilage or fat cells. However, recent results have lead to a shift of this dogma to a more extended function of MSCs. MSCs are able to differentiate *in vitro*, and they are able to produce immune-modulatory molecules, and to produce trophic factors[16-18], which might have a large effect on the role that MSCs play in tissue repair.

As far as is known, no one has studied the effect of IHP on the production of trophic factors: the secretome of MSCs. This research project is a first step in unvravelling the MSC secretome under the influence of IHP, as the MSC secretome might play a significant role in intrinsic cartilage repair observed as a result of joint distraction treatment. Based on the hypothesis that IHP can have a stimulating effect on mesenchymal stem cells or chondrocytes in the intrinsic cartilage repair observed after knee joint distraction treatment, this project aims to further identify and characterize this stimulating effect. Therefore, the first objectives were defined:

- Optimize the previously designed IHP setup, to establish a golden standard for tissue/cell cultures under IHP, improve the ability to monitor culture conditions.
- Identify and characterize the effect of IHP on the secretome of bone-marrow derived hMSCs on a mRNA level
- Evaluate the effect of hMSC (IHP) conditioned medium in explant cultures of osteoarthritic cartilage

Parallel to the first objectives, a protocol will be set up to isolate mesenchymal stem cells from synovial tissue because this is a tissue that is in direct interaction with the damaged articular cartilage of the osteoarthritic joint.

Based on the objectives for this research project, the following questions were formulated. The main question for this research project is:

- *What is the influence of IHP on the trophic role of hMSCs on osteoarthritic cartilage in vitro?*

To answer this question, the following subquestions were defined:

- *What is the effect of IHP on the secretome of hMSCs measured by the mRNA gene expression profile of selected targets?*

- *Can addition of hMSC (IHP) conditioned medium to culture medium in explant cultures have a trophic effect on cartilage matrix turnover in osteoarthritic articular cartilage?*

Study	Cells	Culture conditions	Application of (I)HP	(I)HP conditions	Main findings
Angele et al.[19]	Human BMSCs	Aggregate culture	Custom-built HP chamber	0.55-5.30 MPa, 1 Hz, for 4h/day for 1 or 7 days	Increase in GAG; after 7 days IHP → (92% (day 14), 94.5% (day 28)). Increase in collagen; after 7 days IHP → (10.6% (day 14), 76.8% (day 28))
Elder et al.[20]	Murine embryonic fibroblasts	Monolayer	Custom, temperature-regulated pressure chamber	0.5-5 MPa, 1 Hz, for 3h/day, begin 48h after pellet formation, 3 days.	A 1.9 fold increase in aggregan mRNA expression, and a 2.1 fold increase in collagen II mRNA expression.
Finger et al.[21]	Human BMSCs (2 donors, age 18, 30)	Agarose constructs	Custom HP system	Steady/constant: 7.5 MPa, 14 days. Ramped: 1-7.5 Mpa (increase over 14 days). 1 Hz, 4h/day.	Collagen I: steady → increase at day 4, at day 9-14 decreased to normal. Ramped → 4-fold increase at day 4, day 9 to normal, day 14 further decreased. Sox9: steady → only non-significant. Ramped → increased only at day 9 (5 Mpa). Collagen II and aggrecan mRNA expression remained unchanged.
Li et al.[22]	Rat BMSCs	3D alginate scaffolds	Custom-made pressure system	13-36 kPa, 0.25 Hz, for 1h/day, for 1,3,5,7 days	Increase in col2α1, aggrecan mRNA expression from day 7, further increased at day 10 and 14. Significant increase in Runx2, Ihh, Sox9 mRNA expression. Possibly under influence of the p38 MAPK pathway.
Luo and Seedhom[23]	Ovine BMSCs	Polyester scaffolds	Unknown	0.1 MPa, 0.25 Hz, 30 min/day for 10 days	Increase in total GAG and collagen content after 10 days of PHP.
Meyer et al.[24]	Porcine BMSCs	Agarose constructs, +/- TGFβ3 (10 ng/ml)	Custom bioreactor, water filled pressure vessel	0-10 Mpa, 1 Hz, for 1h/day, 5days/wk. Continuous HP: loaded for 6 wks, begin day 0. Dynamic HP: loading from day 22-42.	Donor 1 → no increase in GAG/collagen production, after exposure to CHP or DHP. Donor 2 → A 1.4 fold increase in GAG production, 1.8 fold increase in collagen production, after CHP. No increases after DHP. Withdrawal of TGFβ3 results in a significant effect on GAG/collagen production.
Miyanishi et al.[25]	Human BMSCs	Pellet culture, +/- TGFβ3 (10 ng/ml)	Water-filled stainless steel pressure vessel	0.1, 1, 10 Mpa, 1 Hz, for 4h/day for 3,7,14 days.	mRNA → SOX9 increased 2.2, 3.3, 2.8 fold, for 0.1, 1, 10 Mpa respectively. Coll2 increased 55.3 fold for 10 Mpa, no increase for 0.1, 1 Mpa. Aggrecan increased 5.6, 7.2, 9.6 fold, for 0.1, 1, 10 Mpa respectively. GAG production increased for 1, 10 Mpa. Collagen

					production (hydroxyprolin) increased due to HP 10 Mpa.
Puetzer et al.[26]	Human ASCs	Agarose constructs	Custom pressure system	7.5 MPa, 1 Hz, 4h/day for 21 days	Sox9, aggrecan, COMP mRNA is upregulated at day 7, but lower compared to controls at day 14. At day 21, expression is decreased to 0.
Steward et al.[27]	Porcine BMSCs	Agarose/fibrin constructs	Custom pressure vessel	10 Mpa, 1 Hz, 4h/day, 5 days/wk, for 3 weeks	Increase in GAG synthesis (1.4 fold) due to HP when cultured in fibrin scaffolds. No increase in collagen synthesis in both fibrin and agarose scaffolds, due to HP. Significant donor variability.
Wagner et al.[28]	Human BMSCs	Collagen I scaffolds/ sponges	Stainless steel pressure vessel in a temperature regulated water bath	1 MPa, 1 Hz, 4h/day for 10 days.	mRNA expression of aggrecan, collagen II, Sox 9 and collagen I increased significantly; 14, 6, 2.5, 15-fold, respectively. Runx2 and TGFβ1 expression did not increase significantly.
Wang et al.[29]	Rat BMSCs	Aggregate culture	Custom-made, computer operated pressure system	10-40 kPa, 0.125, 0.25, 0.5, 1 Hz, 1h/day, 1,3,5,7,10,12,14 days.	Coll2 mRNA expression gradually increases from day 1-14, especially on day 10 and 14. Increased mRNA expression of Ihh was observed at day 1,3,5,7. Study shows that MEK/ERK, p38 MAPK, BMP pathways might be involved.
Zeiter et al.[30]	Bovine BMSCs	Pellet culture	Custom-made pressure vessel, +/- 10 ng/ml TGFβ1, or 50 ng/ml BMP-2	0.5-3 MPa, 1 Hz, 4h/day.	Stimulating effects of TGFβ1 and BMP-2 were found. Loading however, did not significantly influence the mRNA expression of collagen I, II, aggrecan. A small effect on Sox9 expression was observed.

Table 1: Overview of results with MSCs in combination with intermittent hydrostatic pressure

2. IHP SETUP

One of the hypotheses for the observed cartilage regeneration after knee joint distraction is that IHP during knee joint distraction plays a role in the process of cartilage repair, either by stimulating resident chondrocytes or resident hMSCs. To study the effects of IHP on cells and tissue explants *in vitro*, there was a need for a setup that allowed culture of tissue explants and cells under IHP. A basis for this setup was already made in 1986[31], however multiple researchers continuously have optimized this setup[14,32,33].

Briefly, IHP was generated by intermittently compressing the gas phase (5% CO_2 in air) of a closed culture vessel (humidified), which contained the culture well plates and was placed in a 37°C incubator. The maximum pressure applied was 13-15 kPa above atmospheric pressure, which was based on *in vivo* measurements of intra-articular pressure during knee joint distraction treatment. These pressures differ from the pressures that were tested in previous studies (table 1.), but are more representative of the *in vivo* situation during joint distraction. Frequency of pressure application was 0.33 Hz, which was based on the walking pattern of a patient during joint distraction. Control culture well plates were placed in a control vessel, constantly at atmospheric pressure, in the same incubator, also at 5% CO_2 in air. The most recent setup uses a patient ventilating machine (Siemens Servo ventilator 300) to intermittently increase the airflow, therefore pressure, inside the pressure vessel.

To monitor the culture conditions, the previous setup used a combination of sensors. Two intra-vessel temperature sensors were included to measure the temperature inside the pressure and control vessel. A pressure sensor, normally used to measure fluid pressure inside an intravascular infusion system, was connected to the lid of the pressure vessel, to monitor pressure inside the vessel. Furthermore, not all wells of a culture plate were used to culture cells or cartilage explants because of the significant amount of medium evaporation in these wells.

This previous setup was used with success before to culture cartilage explants or cells under IHP. However, upon the start of this study, we observed a need for further optimization and validation of the setup. The first 3-4 months of this internship were used to optimize and validate the IHP setup,

IHP SETUP OPTIMIZATION

PRESSURE SENSORS

Based on the design of the previous IHP setup the assumption was made that the pressure in the control vessel of the system was equal to atmospheric pressure. However, without the presence of a pressure sensor in this vessel, this could not be proved. Additionally, the previous pressure sensor was not placed inside the pressure vessel, which is thought to provide an unreliable estimation of the pressure inside the vessel.

Therefore we modified the system with new pressure sensors (Freescale MPX4200A) inside both vessels of the IHP setup. The choice for these pressure sensors was made on the ability to accurately measure relatively low pressures with a high accuracy. These sensors are able to measure pressures between 20-200 kPa. The main advantage of modifying the culture system with these sensors is that the intra-vessel pressure can now be accurately measured in both the pressure and the control vessel.

To demonstrate that the pressure measured inside the vessel is equal to the pressure that the cells experience; the following formula is used, which is based on Pascal's law.

$$P = P_{air} + \rho g h$$

In this formula; P is the pressure inside a fluid that is in contact with the surrounding air, in Pa. P_{air} is the pressure of the surrounding air, in Pa. ρ is the fluid density (993.36 kg/m^3). g is the gravitational acceleration (9.81 m/s^2), and h is the depth, from the surface of the fluid. Assuming a situation where the measured intra-vessel pressure would be P_{atm} + 15 kPa (P_{atm}: ± 100 kPa), the pressure inside the culture medium in a well of a 24-well plate (max. 1 cm deep) would be:

$$P = 115 \cdot 10^3 + 993.36 \cdot 9.81 \cdot 0.01 = 115097\ Pa = 115.1\ kPa$$

As shown in this example, the difference between the measured pressure and the pressure inside the culture medium is only 0.1 kPa, which is negligible. Therefore, the pressure measured by the pressure sensor can be assumed to be equal to the pressure that the cells experience inside the culture medium.

TEMPERATURE SENSORS

The previous temperature sensors were out-dated and were not compatible with the new sensor interface. Additionally, the resolution of the temperature sensors was low compared to the current temperature sensors. Therefore, the old temperature sensors were replaced with new high-resolution sensors. These TSic™ 501F temperature sensors are able to measure temperatures in a range of -10-60°C, with an accuracy of ± 0.1°C.

SENSOR INTERFACE

The old sensor interface that was used to amplify the signal originating from the previous temperature and pressure sensors was replaced by new hardware based on an Olimexino-328 hardware development board (figure 1). With this new hardware, the new sensors could be successfully read out, without the need to amplify the sensor signal. Additionally, a log board was added that enables the system to log the sensor data to a small mini-SD disk. The old signal amplifier was very out-dated and required a lot of space. Replacing the old hardware significantly increased the usability of the whole IHP setup as well as the accuracy of the measurements.

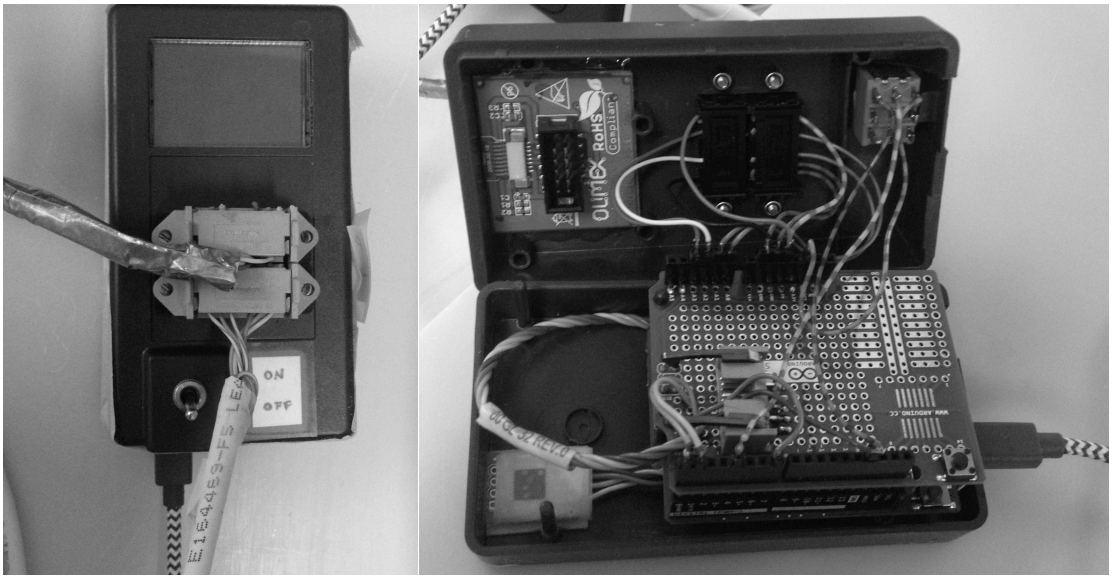

Figure 1: pictures of the new hardware that is used to receive and log the sensor signals

IHP SETUP VALIDATION

Important parameters for cell and tissue explant culture are determined by the culture method that is used. For golden standard cell and tissue explant culture, the culture system should meet the following requirements: temperature = 37 °C, [CO_2]=5%, [O_2]=20%, relative humidity = 95%. To make sure that these parameters are also present in the IHP setup, the pressure and control vessel are placed inside a regular incubator (temperature), connected to a ventilating machine which is able to adjust the CO_2 concentration and the vessels are partly filled with sterile water, to allow evaporation of water inside the vessels to maintain the humidity.

RELATIVE HUMIDITY

Due to the absence of an active humidity regulation inside the system, culture medium evaporates from the well plates, especially inside the pressure vessel and from the wells in the outer rows of the plates. To make sure that the volume of culture medium inside the wells containing cells or tissue stays constant during the culture period, a validation experiment was performed. Different types of Nunc well plates were validated, because these plates fit perfectly inside the vessels, without the need to remove the edges of the plates, as was performed before.

6, 24, and 48 Nunc well plates were used to determine the amount of culture medium volume that evaporates per well. Plates were filled with a constant volume of sterile water (5 ml for 6 well, 1 ml for 24 well, 500 µl for 48 well). Three plates were inserted in each vessel, numbered 1 to 3 to define the location inside the vessel (bottom, middle or top plate). Plates were placed inside the vessels for 48h. The plates were weighed at t=0h, to determine the mass of the plates at the start of the 48h period. After 24h, the plates were weighed again and put back inside the vessels. After 48h the plates were weighed again, and the exact volume of water inside the wells was determined using a pipette. For smaller volumes, a calibrated pipette tip was used.

The highest amount of evaporation is observed in the outer row of wells in a plate (≥10%). This makes the 6 well plates unusable, because of a significant undesired amount of evaporation in each well. The 24 well plates are very well usable inside the culture system, when excluding the outer row of wells and using only the inner 8 wells to culture cells or tissue explants. The same applies to 48 and 96 well plates, which can also be used if the outer row of wells (48 well) or the outer two rows of wells (96 wells) are excluded. The remaining 24 (48 well) or 32 wells can be used for cell or tissue explant culture inside the system. The difference in evaporation between the bottom, middle and top plate is negligible for the inner wells.

CO_2 CONCENTRATION

It is important for cell and tissue explant culture that the concentration of CO_2 inside a culture setup is 5%. To ensure a stable CO_2 concentration, the ventilating machine of the IHP setup is connected to CO_2 supply, which allows adjustment of the CO_2 concentration at the inflow of the IHP setup. To ensure that there is no significant leakage of airflow, or other loss of CO_2, a validation experiment was performed.
To determine the stability of the CO_2 concentration inside both the vessels of the IHP setup, the CO_2 concentrations at the out- and inflow of the ventilating machine were measured for 48h, and compared afterwards. If there would be a significant decrease in CO_2 concentration between the out- and inflow of the system, this could indicate that there is leakage somewhere inside the IHP setup, which results in an undesired variability between the pressure and control vessel.

CO_2 concentrations were measured using a capnograph system (Datex Ohmeda Capnomac Ultima CO2 Monitor), with a sampling tube connected to the IHP setup. Using a combination of tube connectors, it was possible to connect the sampling tube to either the out- or input of the ventilating machine. First, the CO_2 concentration at the outflow of the ventilator machine was measured for 48h, and logged to a file using previously created Simulink software. Second, the CO_2 concentration at the inflow was measured

for 48h and logged. Afterwards, the logged data was imported and analysed in Excel. The results of these measurements showed no significant differences in CO_2 concentration between the in and output of the ventilating machine.

INTERMITTENT HYDROSTATIC PRESSURE (IHP)

The levels of intermittent hydrostatic pressures that are applied to the cells or tissue explants inside the IHP setup are based on pressure measurements in the joint cavity inside the knee and ankle (figure 2) during joint distraction treatment. Intra-articular pressure measurements were performed during joint distraction in knee and ankle, and in human as well as dog joints[14,32]. An interesting finding was that the intra-articular pressures were practically equal for different joints as well as between species. This suggests an important role for the pressure inside a joint capsule in the process of cartilage homeostasis. This finding strengthens the hypothesis that the intra-articular pressures during joint distraction can have an effect on cartilage repair.

According to these measurements, the IHP that is applied by the ventilating machine is set to an IHP between P_{atm} and P_{atm} +/- 14 kPa. To ensure that this IHP was present inside the pressure vessel, and that the pressure inside the control vessel was equal to P_{atm}, a validation experiment using the new pressure sensors and interface was performed.

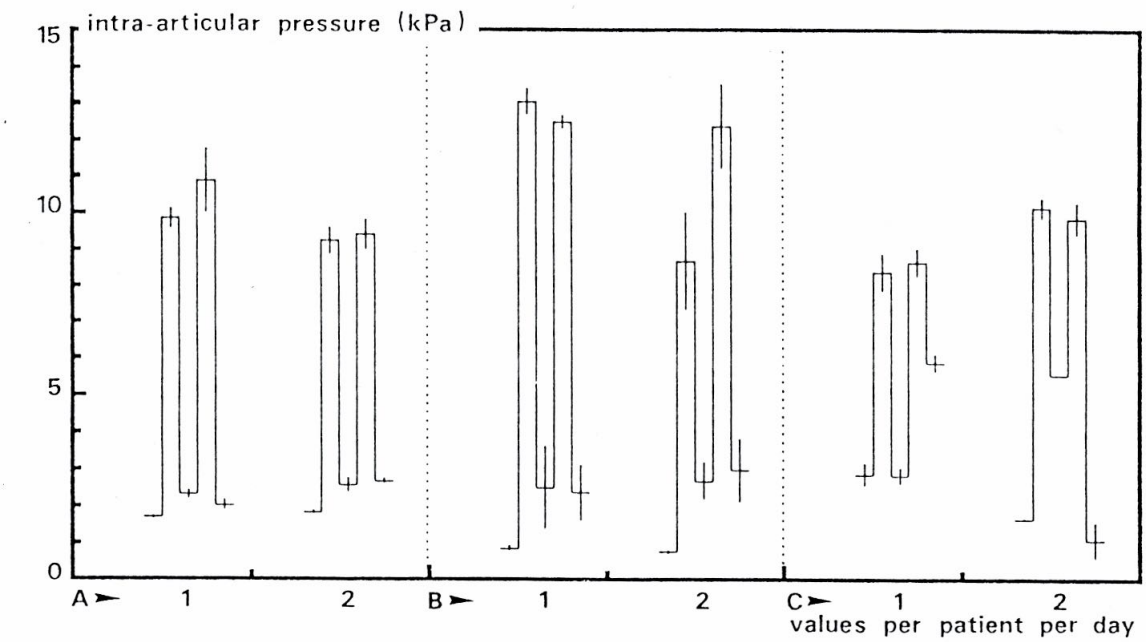

Figure 2: Pressure registration during loading of the treated ankle. Each graph starts with pressure-values measured with the patient lying in bed followed by full weight bearing and relaxation if the treated ankle when standing. Means of one-minute measurements ± SD are shown for three individual patients (A, B and C respectively) for two days (1 and 2)[32].

The pressure was measured using the new sensors inside the pressure and control vessel for 1h. Measurements were logged to a file at a sampling rate of 0.001s. Figure 3 shows the results of a small time period of the total 1h measurements.

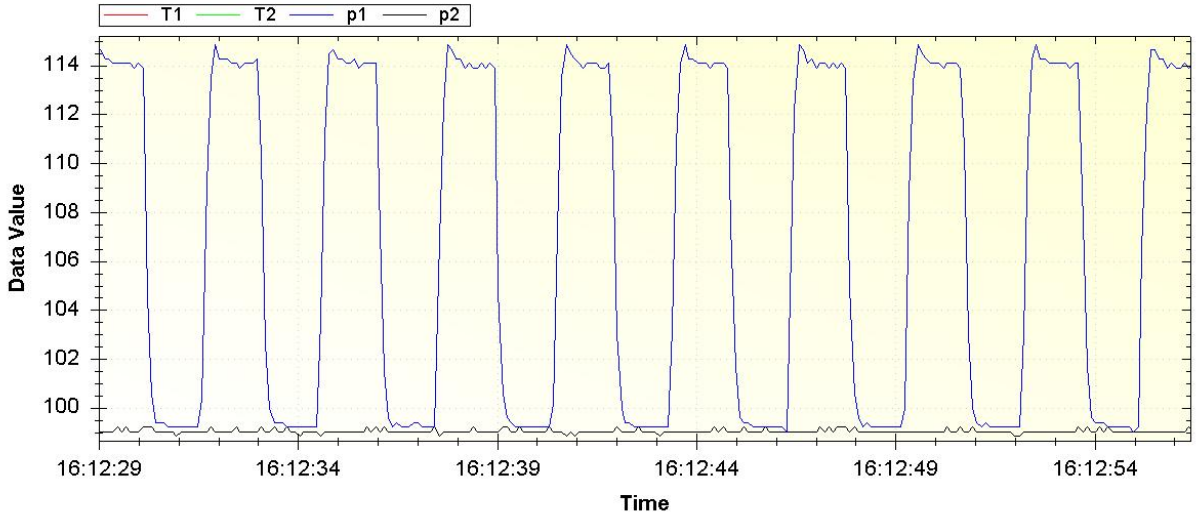

Figure 3: Pressure graph measured using the new pressure sensors and interface

The pressures measured using the new pressure sensors and interface are comparable to previously measured pressures. The pressure that is applied to the pressure vessel varies between P_{atm} and P_{atm} + 14-15 kPa at a frequency of 0.33 Hz and the pressure inside the control vessel is equal to P_{atm}. According to these results, the IHP setup is a good model for the pressures that are present inside the knee and ankle during joint distraction treatment.

3. EXPERIMENT DESIGN

HMSC CHARACTERIZATION

hMSCs originating from bone marrow were obtained in cooperation with MIRA, UTwente (J. Plass, PhD), from varying donors. The available donor characteristics were gender and age of the donor. To characterize the obtained hMSCs based on CD-marker expression, flow cytometry was used. In 2006, the International Society for Cellular Therapy (ISCT) published a position paper on defining minimal criteria for multipotent mesenchymal stromal cells (MSC). In these criteria, a CD-marker expression profile was defined for hMSCs. According to these criteria, hMSCs are CD73, CD90, and CD105 positive, and they lack expression of CD34, CD14 or CD11b, CD79α or CD19, CD45, and HLA-DR.

FLOW CYTOMETRY PROTOCOL

To analyse hMSCs based on CD-marker expression, a Human MSC Analysis Kit (BD Biosciences) was used. Cells were stained with a hMSC positive/non-hMSC cocktail, and a hMSC positive/non-hMSC isotype control cocktail. Stained cells were measured in a BD Biosciences CANTO II flow cytometer.

AUTOFLUORESCENCE

In the first flow cytometry measurements, a lot of autofluorescence was observed for the obtained hMSCs. This was mainly due to the size of the cells, which is relatively large compared to other types of cells. The large amounts of autofluorescence made it impossible to properly determine the CD-marker expression profile of hMSCs. Therefore, it was necessary to optimize the flow cytometer settings for hMSCs.

OPTIMIZATION OF FLOW CYTOMETER SETTINGS

Flow cytometer settings consist mainly of voltage settings for each separate laser, based on the emitted fluorescence of unstained cells. Unstained cells should not give a positive signal for one of the specific filters. Additionally, it is necessary to do a compensation experiment, to compensate for spectral overlap.

A high amount of BM-hMSCs was expanded and cultured to use in the optimization of the flow cytometer settings. These cells were all prepared for flow cytometer analysis without staining the cells. Using the unstained cells, the voltages of each laser were changed, to a level that resulted in a minimal amount of fluorescent signal for each filter. After setting the voltages at the appropriate levels, a compensation experiment was performed, using the CANTO II software. The optimized settings for hMSCs using a BD Biosciences CANTO II flow cytometer are now adopted as a standard protocol in the central flow cytometry facility of the University Medical Centre Utrecht.

SUFFICIENT RNA YIELD

Based on the humidity experiment, 24-, 48-, and 96-well culture plates can be used in the IHP setup. However, if the ultimate goal is to isolate RNA from the cultured cells for mRNA expression analysis using qPCR, the amount of cells per well is important. A sufficient number of cells are needed to obtain enough RNA for further analysis. Therefore, an experiment with a varying amount of cells per well was performed, followed by RNA isolation and quantification of RNA yield. Images by microscope were made to evaluate the level of confluence and adherence of the cells.

A 24 well plate was used for this experiment, because previous experiments with 96-well plates did not results in a sufficient RNA yield. BM-hMSCs ($0.84*10^6$) were thawed (Female donor, 72 yrs old) and seeded at a +/- 5000/cm^2 density in 2 T75 culture flasks. Medium was changed after 3-4 days and hMSCs were trypsinised after 7 days. These hMSCs were seeded and cultured for 48h in a 24-well plate at a varying density of 10.000, 25.000, 40.000, 60.000, 100.000 and 200.000 hMSCs per well in duplo. After 48h, hMSCs were trypsinised using 0.05% Trypsin-EDTA and lysed using β-mercaptoethanol in Qiagen RLT buffer (1:100). Images were made before trypsinisation. RNA was further purified using the

RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and the RNA content was quantified using the Nanodrop 2000 (Thermo Scientific).

No. of hMSCs/well	Total RNA yield (ng)
10.000	246
25.000	384
40.000	671
60.000	679
100.000	927
200.000	834

Table 2: RNA isolation from hMSCs

Due to a pipetting error, a significant amount of hMSCs was accidently removed (for 60-, 100-, 200.000), which has probably resulted in a lower RNA yield as expected for some of the higher cell concentrations (table 2).

RNA YIELD

A choice was made for 40.000 hMSCs/well in a 24-well plate. This results in a sufficient RNA yield. 500 ng is needed for cDNA synthesis, and the cells are not too confluent in the culture plates, because overconfluency would cause significant cell death. Therefore, this amount of cells was chosen for hMSC monolayer culture inside the IHP setup. The average RNA yields for each time point are shown in table 3. These results apply to the RNA of the cells that were used in the mRNA expression analysis, as described in chapter 6.

Time point	RNA concentration (ng/µl)	Total RNA (ng)	260/280 value
t=0h	30.0 ± 11.1	840 ± 310	1.98 ± 0.06
t=6h	28.8 ± 8.0	806 ± 224	1.94 ± 0.12
t=24h	36.9 ± 6.6	1032 ± 186	1.99 ± 0.08
t=48h	31.1 ± 5.6	872 ± 156	2.10 ± 0.09

Table 3: Overview of average ± SD for RNA concentration, total RNA and 260/280 values for each time point.

QPCR MRNA EXPRESSION ANALYSIS

To study the *in vitro* effect of IHP in hMSCs, a choice was made to first determine the mRNA expression levels of a set of cartilage homeostasis related genes using real-time qPCR. At the start, an already available protocol was used. However, eventually the protocol was changed making it less labour-intensive and more compatible with the set of targets that was chosen.

PRIMER DESIGN

First, pre-designed primers were ordered from Life Technologies. A lot of problems were observed using these primers. This was mainly because of inexperience with the technology of qPCR. Eventually, a decision to completely change the primer design, as well as the qPCR protocol was made, based on the experience of a more experienced user of qPCR. The pre-designed primers from Life Technologies resulted in very large product sizes, which significantly complicated qPCR analysis.

Primers were eventually designed using the ProbeFinder from Roche, which is an algorithm that allows the user to choose a gene for a specific organism, from which it generates a set of a forward and reverse primer that covers an intron-spanning region of the gene. It was not possible to generate a primer design from this algorithm for FGF2; therefore a primer design was used from the Harvard PrimerBank. The ProbeFinder delivers primer designs with approximately the same melting temperatures and product

lengths, which allows the user to use the same qPCR cycle settings for multiple genes of interest. Primer sequences are shown in table in chapter 6.

PRIMER VALIDATION

To validate the primers designed using the Roche ProbeFinder, the efficiency of the primers was determined. This is especially important for genes that are low-expressed, to demonstrate that the qPCR results (CT-values) for these genes are reliable.

The primer efficiency can be determined by performing a qPCR run with serial dilutions of a know concentration of cDNA in which the genes of interests are expressed. To determine primer efficiency, qPCR Human Reference Total RNA (Clontech laboratories) is used. This is a mixture of total RNAs from a collection of adult human tissues, chosen to represent a broad range of expressed genes. Both male and female donors are represented. First, cDNA is synthesized from this RNA, according to the cDNA synthesis protocol as described in chapter 4. Serial 5-fold dilutions are made from a stock solution of 10 ng cDNA/µl, representing 10 ng/µl, 2 ng/µl, 0.4 ng/µl, 0.08 ng/µl, 0.016 ng/µl, 0.0032 ng/µl, 0 ng/µl (control). These cDNA dilutions are used in the standard qPCR protocol for the various primers. The primer efficiency is calculated with the following formula:

$$\text{primer efficiency } = \left(10^{\frac{-1}{\text{slope}}} - 1\right) \cdot 100\%$$

The 'slope' in this formula is the slope of the curve that is created with the Ct-values (x-axis) of the different dilutions plotted against the log values of the cDNA concentration (y-axis). For efficient primers, efficiency in a range of 97-103% is accepted.

SELECTION OF CARTILAGE HOMEOSTASIS RELATED GENES

Fibroblast Growth Factor 1 (FGF1)

In 2013, Wu et al[34] have identified FGF1 as an MSC secreted trophic factor whose expression increased in co-cultures with human primary chondrocytes (hPC). Increased FGF1 expression potently stimulated hPCs proliferation.

Fibroblast growth factor 2 (FGF2)

FGF2 was found in several studies as a growth factor that is secreted by adipose tissue-derived MSCs and BM-MSCs[35-39]. FGF2 is known to be a factor that has the potential to simulate proliferation of resident chondrocytes[35].

Fibroblast growth factor 18 (FGF18)

FGF18 is a well known anabolic/hypertrophic growth factor involved in chondrogenesis as well as osteogenesis depending on cell types, in addition to articular cartilage repair[40-43]. FGF18 signals through FGF receptor 3 to promote chondrogenesis. Only one study was found that looked at FGF18 expression in MSCs (from adipose tissue)[44], however it is an interesting target regarding its possible role in cartilage repair[45].

Tissue inhibitor of metalloproteinases 1 (TIMP1)

The protease inhibitor TIMP1 has a protective effect in cartilage degradation[46], due to its inhibitory role against most of the known matrix-metalloproteinases (MMPs). A study by Lozita et al has shown that MSCs inhibit MMPs via secreted TIMPs[47].

Tissue inhibitor of metalloproteinases 2 (TIMP2)

TIMP2 was shown to be secreted by hMSCs, and identified as an anti-fibrotic factor[35]. As noted for TIMP1, MSCs inhibit MMPs via secreted TIMPs[47]. TIMP2 has been shown to interact with MMP2 and

MMP14. Additionally, TIMP2 is regulated in chondrocytes and the basal TIMP-2 levels may be needed for the cartilage ECM integrity[48], which makes it an interesting factor potentially produced by hMSCs.

Transforming growth factor beta 1 (TGFb1)

TGFβ1 stimulates chondrocyte synthetic activity and decreases the catabolic activity of IL1. In vitro, TGFβ1 stimulates chondrogenesis of BM-MSCs[49]. It has also been shown that TGFβ1 is produced by BM MSCs[35,36,50].

Insulin-like growth factor 1 (IGF1)

IGF1 stimulates ECM synthesis (increased proliferation of chondrocytes) and decreases catabolic response in monolayer or explant cultures[49]. IGF1 is also identified as a trophic factor produced by MSCs[35,51].

Hepatocyte growth factor (HGF)

HGF is a well known factor that is produced by hMSCs[35]. HGF was shown to have an effect in cartilage repair through the multiple actions on chondrocytes, including stimulation of cell motility, proliferation and proteoglycan synthesis[52,53].

Hypoxanthine Phosphoribosyltransferase 1 (HPRT1)

HPRT1 is a 25-kDa enzyme that mediates guanine conversion into guanosine monophosphate and hypoxanthine into inosine monophosphate, playing a central function in purine nucleotides generation[54]. In a study by Amable et al. HPRT1 was the most stable reference gene for BM-MSCs, more stable than the conventionally used GAPDH[54]. Therefore, HPRT1 was chosen as reference gene for the qPCR analysis.

4. MRNA EXPRESSION PROFILE OF HMSCS UNDER IHP

INTRODUCTION

hMSCs at a site of injury respond to stimuli that originate from cells in the affected tissue, which describes the trophic role of hMSCs on other cells[16,17]. Depending on the stimuli, hMSCs can exert an anti-inflammatory, anti-apoptotic, anti-fibrotic, or a proliferative effect on resident affected cells[35,55].

The absence of mechanical stresses during knee joint distraction is thought to allow resident chondrocytes and hMSCs originating from various tissues of the knee joint to induce the process of cartilage repair. hMSCs from the subchondral bone, the synovial tissue of surrounding fat tissue can easily migrate into the joint cavity, the site of injury, where they respond to stimuli originating from the diseased knee joint. hMSCs respond to stimuli by secreting injury-specific factors, forming the hMSC secretome. Several groups have used techniques such as genomics and proteomics to identify the secretome of hMSCs under certain conditions[35,36,51,56,57]. An important stimulus that was shown to be present during joint distraction is intemittent hydrostatic pressure (IHP). Previous studies have shown the reponse of hMSCs to IHP, however these studies did not aim to identify the hMSC secretome, but determined the mRNA expression of several targets that indicate chondrogenic differentiation of hMSCs (table 1), at much higher hydrostatic pressures (MPa instead of kPa). hMSCs are thought to play a role in cartilage repair during joint distraction, due to the observed significant decrease in denuded bone area in a short time frame after treatment. The hypothesis is that this repair cannot be solely the result of increased activity of resident chondrocytes. hMSCs are thought to rather play a modulatory, trophic role in tissue repair *in vivo*, than differentiating into chondrocytes[58,59].

This study aims to evaluate mRNA expression in hMSCs under the effect of IHP, for a set of cartilage homeostasis related factors representing a trophic secretome potentially produced by hMSCs. A set of growth factors and metalloproteinase inhibitors was defined, consisting of FGF1, 2, 18, TIMP1, 2, TGF β 1, IGF1, and HGF. The specific role of these targets in cartilage homeostasis was described in chpt. 5.

MATERIALS & METHODS

hMSC preparation

Human MSCs (hMSCs) were obtained from MIRA, University of Twente, Enschede (J. Plass, PhD). The obtained hMSCs were already isolated from heparinized bone marrow aspirates of 5 female donors, aged 75.2 $\pm$ 5 yrs old, using previously described procedures[60]. This isolation procedure of hMSCs from the bone marrow was previously validated based on ISCT characteristics[61]; plastic adherent cells with MSC-specific CD-marker profile and differentiation potential. hMSCs were seeded at a density of +/- 5000 cells/cm^2 and cultured in monolayer in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 100 IU/ml Penicillin, 100 μg/ml streptomycin sulphate, 2 mM L-glutamin, and 0.085 mM L-ascorbic acid. Culture medium was changed every 3-4 days. Cells were trypsinized using trypsin-EDTA (0.05%) and passaged at 90% confluence, until passage two.

hMSC characterization

Passage 2-3 cells were stained for flow cytometry, using a BD Stemflow™ hMSC analysis kit, according to manufacturer's protocol. The kit involved a hMSC positive cocktail of CD73, CD90, and CD105, and a non-hMSC cocktail of CD11b, CD19, CD34, CD45, and HLA-DR. These stains are used to determine the percentage of hMSCs, according to the CD-marker profile, as defined in the 'minimal criteria for hMSCs' previously reported by the International Society for Cellular Therapy[61]. Flow cytometry data was acquired by a FACS Canto II cell analyser (BD Biosciences).

IHP setup: hMSC culture

Subconfluent hMSC monolayer cultures (passage two) were trypsinized, counted manually using a counting chamber and resuspended in culture medium at a concentration of 40.000 hMSCs/ml. Cells were transferred to a 24-well culture plate (Nunc, Thermo Scientific) at a density of 40.000 hMSCs per well (passage three). 24-well culture plates allowed culture of 3 hMSC donors in duplo simultaneously, inside the IHP setup. Cells were allowed to attach to the culture surface overnight. On the condition that the cells were successfully attached, plates were transferred to the IHP setup, and placed in either the pressure or non-pressure vessel. The moment the cells were transferred to the IHP setup was considered t=0h. At this time point, hMSCs from one plate were trypsinized and lysed for RNA isolation. hMSCs were cultured for 48h in the IHP setup [T=37° C, [CO_2]=5%, [O_2]=20%, RH=+/-95%]. At time points t=6h, t=24h, and t=48h, cells were trypsinized and lysed for RNA purification.

mRNA expression analysis

hMSCs were trypsinized and lysed for RNA isolation at several time points. RNA from hMSCs was extracted using beta-mercaptoethanol (14.3M) in RNeasy lysis buffer [1:100] (Qiagen, Venlo, the Netherlands). For a practical reason, lysates were stored for a maximum of two weeks at -80° C before RNA purification. RNA was further purified using the RNeasy Micro Kit (Qiagen), according to manufacturer's protocol. A DNase step was included in this protocol. RNA content was determined for each sample, using the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific). Complementary DNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) with an input of 200-500 ng RNA. Real-time quantitative polymerase chain reactions were performed using Fast Start Universal SYBR Green Master (Roche) based on a previously described protocol. Primer sequences are shown in table 4.

Gene	Nucleotide sequence 5'-3'
FGF1	FWD: ACCAAGTGGATTCTGCTTCC
	REV: CTTGTGGCGCTTTCAAGACT
FGF2	FWD: AGAAGAGCGACCCTCACATCA
	REV: CGGTTAGCACACACTCCTTTG
FGF18	FWD: CGAGGATGGGGACAAGTATG
	REV: CGGACTTGACTACCGAAGGT
TIMP1	FWD: CTGTTGTTGCTGTGGCTGAT
	REV: AACTTGGCCCTGATGACG
TIMP2	FWD: GAAGAGCCTGAACCACAGGT
	REV: CGGGGAGGAGATGTAGCAC
TGFb1	FWD: ACTACTACGCCAAGGAGGTCAC
	REV: TGCTTGAACTTGTCATAGATTTCG
IGF1	FWD: TGTGGAGACAGGGGCTTTTA
	REV: ATCCACGATGCCTGTCTGA
HGF	FWD: GATTGGATCAGGACCATGTGA
	REV: CCATTCTCATTTTATGTTGCTCA
HPRT1	FWD: GCTGACCTGCTGGATTACAT
	REV: CTTGCGACCTTGACCATCT

Table 4: Primer nucleotide sequences of qPCR targets

Data analysis

Flow cytometry data was analysed using FlowJo software (version X, TreeStar, San Carlos, CA, USA). Cells were first gated for PE negativity, followed by gating on the CD105+CD90+PE- population and finally by gating on the CD73+CD90+CD105+PE- population, which represents the hMSC-specific CD-marker profile. For qPCR, all samples were tested in duplo (technical duplicates). Samples with a standard deviation > 0.5 in Ct value for technical duplicates were excluded in the analyses. Ct value quantification was performed for all genes using the same threshold. Relative expression ($2^{-\Delta Ct}$) of the qPCR targets was calculated by normalisation to the HPRT1 reference gene: $\Delta Ct = Ct^{\text{Gene of interest}} - Ct^{\text{Reference gene}}$. HPRT1 was chosen as a reference gene for qPCR in hMSCs based on previous literature[54]. To demonstrate the effect of IHP on mRNA expression in hMSCs compared to the non-IHP situation, the

comparative Ct method was used. Using this method, the fold expression ($2^{-\Delta\Delta Ct}$) for IHP vs non-IHP was calculated, where $\Delta\Delta Ct = \Delta Ct^{IHP} - \Delta Ct^{non\text{-}IHP}$. Graphpad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses and to create graphs. An ANOVA, post-Hoc Tukey, was performed to detect any statistical significant differences ($p \leq 0.05$) in mRNA expression, comparing the fold expression of t=6h, t=24h, t=48h to the baseline expression (t=0h).

RESULTS

hMSC characterization

As described in the ISCT guidelines for defining mesenchymal stem cells[61], one of the criteria is the MSC-specific CD-marker profile of the cells. Shown in table 5, 91.4 ± 4.1% of the BM-MSCs that are used in the experiments exhibit the MSC-specific CD-marker profile. 99.4 ± 0.4% of the cells possess a non-hMSC CD marker expression profile (PE negative), which means that <1% of the cells show a non-hMSC CD-marker profile (PE positive).

Donor ID	PE- (%)	CD105+ CD90+ PE- (%)	CD105+ CD90+ CD73+ PE- (%)
BM-MSC D1	99	94.9	94.8
BM-MSC D2	99	84.7	84.6
BM-MSC D3	99.5	90.9	90.8
BM-MSC D4	99.8	93.8	93.7
BM-MSC D5	99.8	93.2	93
Average	**99.4**	**91.5**	**91.4**
Standard deviation	**0.4**	**4.1**	**4.1**

Table 5: hMSC characterization, % of CD105+CD90+CD73+PE- cells

mRNA expression in hMSCs under the influence of intermittent hydrostatic pressure

To determine if the cartilage-homeostasis related factors respond to stimulation with IHP, mRNA of BM-hMSCs was analysed for gene expression using qPCR. Stimulation of hMSCs with IHP showed an effect on gene expression with a large inter donor variation (as shown in supplementary data – Appendix). Some hMSC donors show very clear trends for up-or downregulation of gene expression, while other donors show the opposite effect.

Looking at the overall (n=5) relative expression data (figure 4), FGF1 expression in the IHP-stimulated hMSCs shows a constant increase over 48h. The non-stimulated hMSCs also show an increased expression after 6h, however the expression stays relatively constant after 6h. FGF2 expression in the IHP-stimulated hMSCs fluctuates over time, but is consistently increased compared to the t=0h situation. The non-stimulated hMSCs show an increase in FGF2 expression up to 24h, which stays constant between 24 and 48h. FGF18 expression in IHP-stimulated hMSCs shows an increase at 6h, which diminishes back to baseline after 6h. FGF18 expression in non-stimulated hMSCs is increased at 6h, but stays constant after 6h, up to 48h. TIMP1 expression decreases over time in IHP-stimulated hMSCs and stays relatively constant after 6h. In non-stimulated hMSCs, TIMP1 expression stays relatively constant, with a small increase after 24h. TIMP2 expression increases over time for both the IHP and non-stimulated hMSCs, however the non-stimulated hMSCs show a significantly higher increase. TGFβ1 expression shows an increase after 6h for both the IHP and non-stimulated hMSCs after which the expression returns to a level equal to t=0h. The expression of IGF1 in IHP-stimulated hMSCs stays constant up to 6h, after which it decreases. IGF1 expression in non-stimulated hMSCs increases over time with a peak at 6h. HGF expression in both IHP and non-stimulated hMSCs increases over time, with a peak at t=6h.

The overall effect (n=5) of IHP on gene expression in hMSCs, when comparing the IHP stimulated and non-stimulated hMSCs, is minimal for FGF1, FGF18, TIMP1, TGFβ1, and HGF. For FGF1, FGF18, TIMP1, and TGFβ1, there seems to be a small increase in fold expression between IHP and non-IHP at t=6h. HGF on the other hand shows more of a small decrease in fold expression at t=6h due to stimulation with IHP (figure 6).

The genes that show a more distinct effect due to stimulation with IHP are TIMP2 and IGF1. TIMP2 expression increases over time (in 48h) for both the IHP stimulated and non-stimulated hMSCs (figure 4). However, the increase in gene expression is significantly higher for the non-stimulated hMSCs; TIMP2 is significantly downregulated due to stimulation with IHP. IGF1 expression stays relatively constant over time (figure 4) for the hMSCs stimulated with IHP, while the non-stimulated hMSCs show a clear increase in expression over time. A trend for downregulation of IGF1 due to stimulation with IHP was observed.

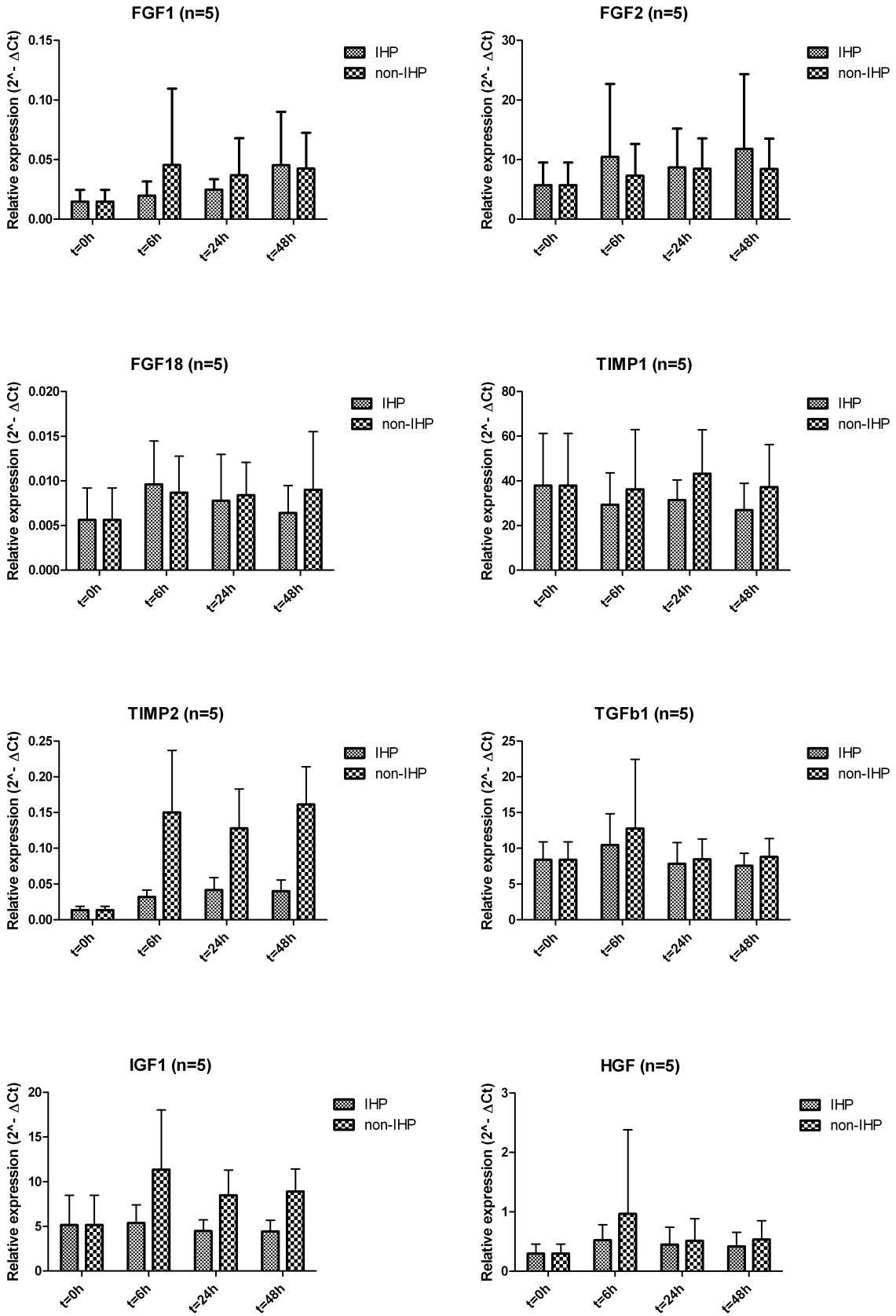

*Figure 4: Relative expression of cartilage-homeostasis related targets on mRNA level in hMSCs under the influence of IHP, compared to non-IHP. * indicates a statistically significant difference (p≤0.05).*

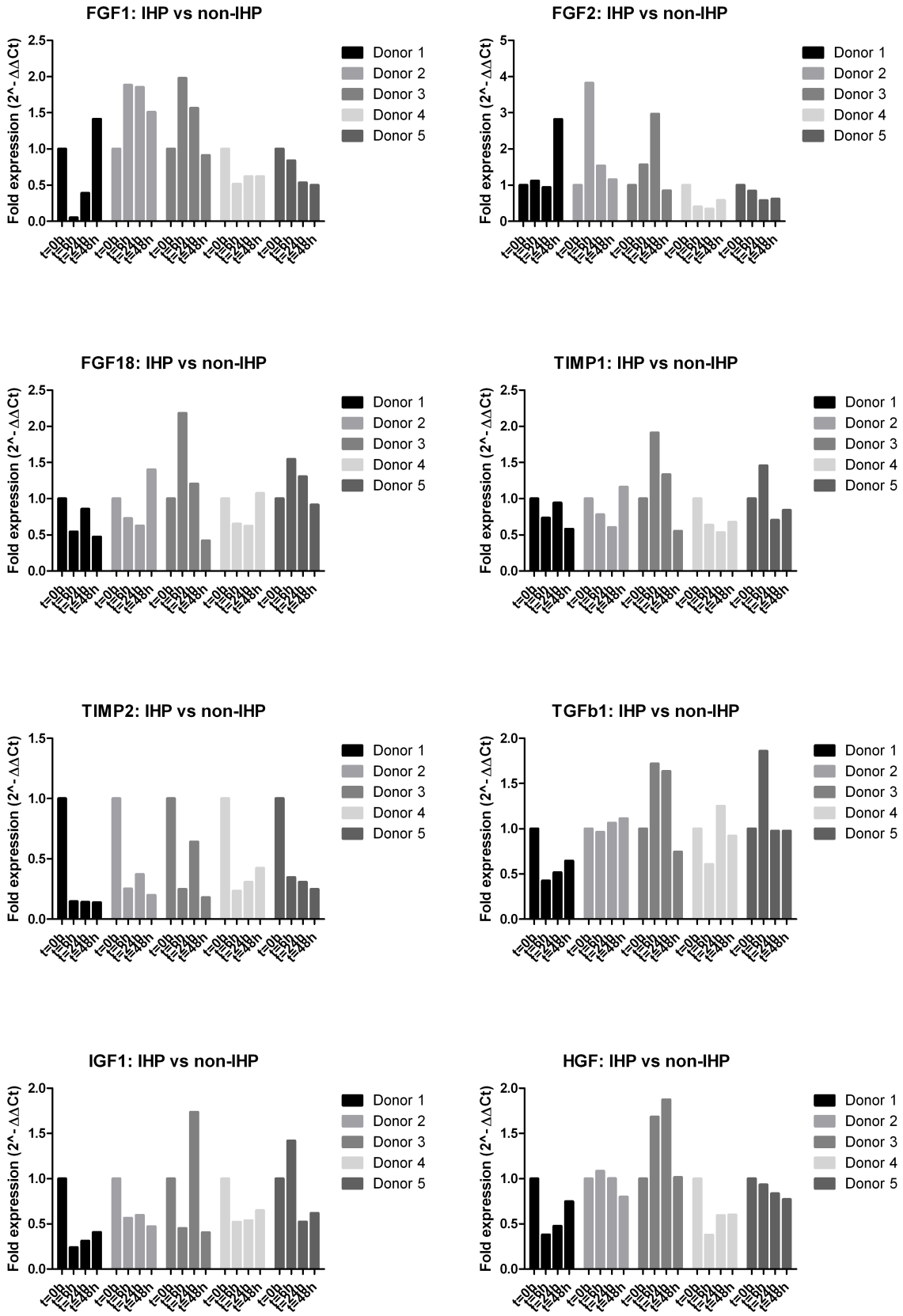

Figure 5: Fold expression of cartilage-homeostasis related targets on mRNA level in hMSCs, comparing the IHP stimulated hMSCs to non-IHP-stimulated hMSCs, using the comparative Ct method.

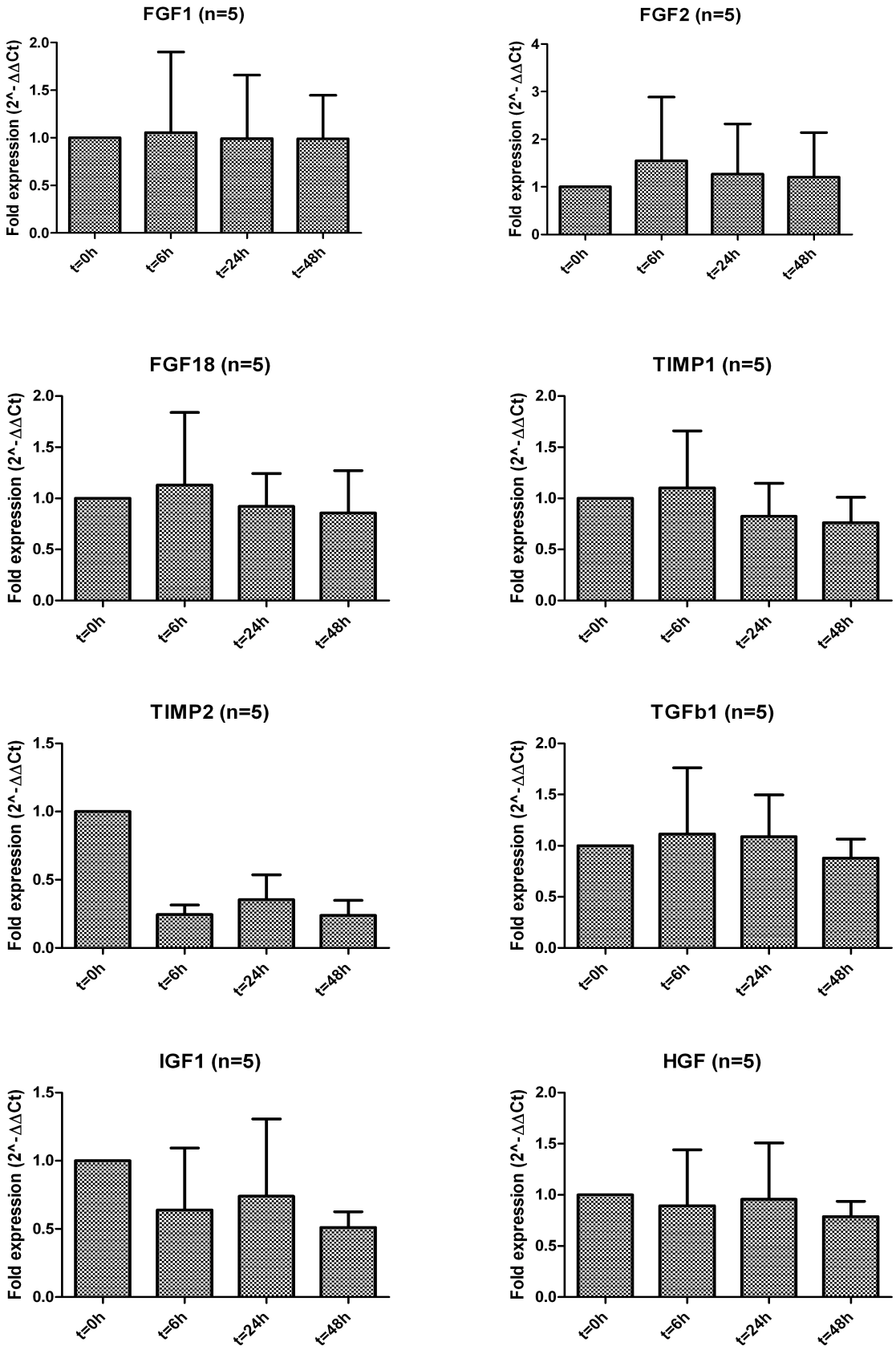

Figure 6: Fold expression of cartilage-homeostasis related targets on mRNA level in hMSCs, comparing the IHP stimulated hMSCs to non-IHP-stimulated hMSCs (averaged for all donors), using the comparative Ct method. Showing the averages for all donors (from figure 5).

DISCUSSION

Osteoarthritis is a disease in which the disturbance of the joint homeostasis plays a significant role[45]. hMSCs have chondrogenic potential *(in vitro),* but can also play a role in immunomodulation and tissue repair by secretion of soluble trophic factors, as they respond to stimuli that are associated with tissue injury. The aim in this study was to evaluate the effect on the hMSC secretome of a stimulus that is present during knee joint distraction as a treatment for knee osteoarthritis. This stimulus is the intermittent hydrostatic pressure that is present during this treatment. As a first step in evaluating the hMSC secretome under the influence of IHP, mRNA expression levels were determined for a set of cartilage-homeostasis related factors in IHP- and non-stimulated hMSCs.

The mRNA expressions that were measured in this study suggest that there is indeed an influence of IHP-stimulation in hMSCs, especially when looking at the individual donors. Overall, it was demonstrated that FGF2 expression shows a trend for upregulation after 6h in IHP-stimulated hMSCs, that TIMP2 shows a significant downregulation over time in hMSCs due to IHP-stimulation, and that IGF1 expression shows a trend for downregulation due to IHP-stimulation. By further increasing the number of hMSC donors, these found effects can possibly be strengthened and other smaller trends can become more evident. FGF2 has the potential to increase proliferation of resident chondrocytes[35]. The observed trend for upregulation of FGF2 expression in IHP-stimulated hMSCs suggests a first step in an early regenerative response upon IHP in hMSCs. TIMP2 is an important factor that is able to inhibit cartilage-degrading metalloproteinases (MMPs). The observed downregulation of TIMP2 in IHP-stimulated hMSCs would not be beneficial in cartilage repair, because it would decrease the inhibitory role of TIMP2. However, a certain level of degradation activity of MMPs might be needed to degrade affected tissue and initiate new cartilage formation. IGF1 has an effect comparable to FGF2; it has the potential to increase proliferation of resident chondrocytes. However, the observed trend for downregulation of IGF1 expression in IHP-stimulated hMSCs would not be beneficial in a diseased joint, because of its inhibitory effect on chondrocyte proliferation. In this study, a first step to evaluate mRNA expression levels for a set of cartilage-homeostasis factors under IHP stimulation was made. In future experiments, it will be interesting to expand the set of targets with catabolic or inflammatory/immunomodulatory factors associated with cartilage-homeostasis. These catabolic targets can be for example MMPs, and immunomodulatory factors can be several interleukins.

In this study, IHP was applied with a maximum pressure of P_{atm}+15 kPa, at a frequency of 0.33 Hz for 48h, constantly. These parameters were chosen based on *in vivo* measurements and as a first step to evaluate the effect of IHP on mRNA expression in hMSCs. However, during joint distraction, IHP will not be present constantly. IHP is an effect of loading and unloading of the joint during treatment, which can be the result of walking. Due to the rigidity and discomfort of the distraction frame, patients will only be loading and unloading the joint for a part of the day. Therefore, in future experiments it will be interesting to evaluate the effect of IHP when applying IHP to the cells only for a set period of time per day, comparable to the time that the joint is loaded and unloaded in patients during treatment. Additionally, it will be interesting to culture hMSCs for a longer time period, to evaluate the mRNA expression for a time period longer than 48h.

By culturing hMSCs in an IHP setup, all the other variables or stimuli, except IHP, that are present in an osteoarthritic knee joint during joint distraction were excluded. This allowed us to study solely the effect of IHP on mRNA expression levels in hMSCs. However, it is known that other injury-specific stimuli can be involved in changes in hMSC secretome. In a diseased joint, hMSCs are exposed to inflammatory factors, cartilage degradation products, and hMSCs can be in direct contact with resident chondrocytes. In order to secrete cartilage-homeostasis related factors, it might be possible that direct contact of hMSCs with cartilage or chondrocytes is necessary. This might also explain why no high expressions of FGF1 were found in this study, compared to the high expressions of FGF1 that were found in co-cultures of primary

chondrocytes and hMSCs[34]. It can also be possible that hMSCs are only able to secrete cartilage-homeostasis related factors when exposed to cartilage degradation products or inflammatory factors. However, it was previously shown that IHP has a stimulating effect on cartilage repair (measured by proteoglycan turnover) in explant cultures of osteoarthritic articular cartilage[32]. Additionally, it was also shown that IHP stimulation results in decreased catabolic activity of mononuclear cells isolated from synovial fluid of osteoarthritis patients. This effect was associated with downregulation of IL1β and TNFα production. It will be interesting to evaluate whether these results can be reproduced using the current, optimized setup. These findings suggested that IHP during joint distraction might be beneficial for osteoarthritic cartilage repair directly, and indirectly via suppression of inflammation[32]. Therefore, this study aimed to study only the effect of IHP in hMSCs, excluding all the other possible stimuli. For future experiments, it is interesting to see whether the presence of osteoarthritic cartilage explants in co-cultures or the addition of inflammatory factors to hMSC cultures have an influence on the IHP-effect that is observed in this study.

CONCLUSION

IHP stimulation has an effect on mRNA expression in hMSCs for a set of cartilage-homeostasis related genes. Due to the heterogeneity of hMSCs and inter-donor variability, no overall effect was observed for FGF1, FGF18, TIMP1, TGFβ1, and HGF. TIMP2 is significantly downregulated in hMSCs due to the effect of IHP, IGF1 show an evident trend towards downregulation, and FGF2 shows a trend towards upregulation after 6h of IHP-stimulation. The upregulation of FGF2 suggests a first step in an early regenerative response upon IHP in hMSCs. However, the downregulation of TIMP2 and IGF1 might be less beneficial in cartilage regeneration, although a certain level of degradation activity might be needed to degrade affected tissue and initiate new cartilage formation. These data suggest that differences in mRNA expression show a large donor variation in general. Therefore, a sufficient number of hMSC donors are needed when evaluating mRNA expression levels in hMSCs. To increase the power of the results of this study, and to decrease the influence of inter-donor variability, these experiments should be repeated for a larger number of hMSC donors.

5. EFFECT OF HMSC (IHP) CONDITIONED MEDIUM ON ARTICULAR OA CARTILAGE

INTRODUCTION

hMSCs are known to secrete factors that can potentially have a proliferating, anti-inflammatory, or anti-apoptotic role[35]. These secreted factors could have a stimulating effect in cartilage repair during knee joint distraction. Another hypothesis is that an intermittent hydrostatic pressure, that is present during joint distraction treatment, could have a stimulating effect on secretion of factors by hMSCs. This study aims to evaluate the effect of hMSC-secreted factors in conditioned medium on proteoglycan turnover in OA cartilage explants.

MATERIALS & METHODS

Production of hMSC (IHP) conditioned medium

hMSCs were obtained, prepared and cultured (as described before in chpt. 6) for 48h inside the IHP setup [T=37° C, [CO_2]=5%, [O_2]=20%, RH=+/-95%], in either the pressure or the non-pressure vessel. At time points t=0h, t=6h, t=24h, and t=48h, supernatants were collected, immediately frozen in liquid nitrogen, and stored at -80° C. Supernatants from donor 1-5 (chpt. 6) were used as hMSC (IHP) conditioned medium.

Osteoarthritic articular cartilage preparation

Human osteoarthritic articular cartilage was obtained after knee replacement surgery from knee condyles within maximum 4-6 hours after surgery and transported in phosphate buffered saline (PBS). Cartilage was obtained from female donors (n=3) with a known history of knee osteoarthritis, aged 63 ± 11 yrs. Slices of cartilage were cut aseptically from the articular surface, excluding the underlying bone. To ensure full thickness cartilage cubes, the outer, thinner regions of the slices were removed. Subsequently, the slices were cut into square pieces (±4-6 mm^2) and the cubes were allowed to stabilize for a minimum of 30 minutes in Dulbecco's modified Eagle's medium (DMEM). This step is included to allow any cartilage handling-related cytokine release to be released. After stabilization, excess DMEM is removed by shortly placing the cubes on sterile filter papers, before weighing the cubes aseptically. Cubes in a range of 5-15 mg were cultured individually in 200 µl of cartilage culture medium; DMEM supplemented with 10% heat inactivated pooled human male AB^+ serum, 100 IU/ml Penicillin, 100 µg/ml streptomycin sulphate, 2 mM L-glutamin, and 0.085 mM L-ascorbic acid.

Ex vivo cartilage explant culture in hMSC-conditioned medium

Osteoarthritic cartilage tissue was cultured for 4 days in cartilage culture medium, or in a 1:1 mixture of 100 µl cartilage culture medium supplemented with 100 µl hMSC-supernatant. The supernatants from the hMSCs cultured under IHP as well as the hMSCs cultured without IHP were used. An extra control condition was added by culturing cubes in a 1:1 mixture of cartilage medium and hMSC culture medium (foetal calf serum (FCS) instead of male AB^+ serum) that was cultured for 48h in the IHP setup. Each condition was applied to 8 cartilage cubes. Conditioned medium from hMSC donor 1 and 2 was tested on cartilage from cartilage donor 1. Conditioned medium from hMSC donor 3 was tested on cartilage donor 2, and conditioned medium from hMSC donor 4 and 5 was tested on cartilage donor 3. After 4 days, culture was stopped to determine cartilage matrix turnover (proteoglycan synthesis, release).

Analysis of cartilage matrix turnover

After 4 days of culture, medium was collected per cartilage cube, and stored at -20 ° C for further analysis. Culture medium supplemented with 5 µCi of $Na^{35}SO_4$ per cube was added. Sulphate incorporation rate was determined, as a measure of the proteoglycan synthesis rate, during the last 4

hours of the 4-day culture period, as described previously[14]. After 4 hours labelling, radioactive labelling medium was removed and the cartilage explants were rinsed with PBS and stored at -20 ° C. Cartilage tissue samples were digested in papain buffer as described before[14].

$^{35}SO_4^{2-}$ radioactivity of the samples was measured by liquid scintillation analysis. Proteoglycan synthesis rate was normalized to the weight of the cubes and expressed in $nmol/(h \cdot g)$.

Release of proteoglycans as a measure of cartilage matrix degradation was measured in an Alcian Blue assay, as described before[32]. Glycosaminoglycans (GAGs) from the medium obtained after 4 days of culture, before adding the radioactive labelling medium, were stained and precipitated with Alcian Blue dye solution with a chondroitin sulphate (Sigma-Aldrich) solution as a standard. Quantities of GAGs were measured spectrophotometrically. Proteoglycan release was measured, normalized to the weight of the cartilage cubes, and expressed in mg GAGs per g of cartilage tissue.

Data analyses

Microsoft Excel 2010 was used to process the data from the liquid scintillation and the spectrophotometric measurements. Graphpad Prism 6 (GraphPad Software, San Diego, CA, USA) was used to generate graphs and to detect any statistically significant differences in proteoglycan turnover between the cartilage cubes cultured in conditioned medium and the controls. A one-way ANOVA with a post-hoc Tukey test was used to detect any statistical differences ($p \leq 0.05$).

RESULTS

To determine the effect of hMSC (IHP) conditioned medium on proteoglycan turnover in OA cartilage, proteoglycan release and synthesis were measured after 4 days of OA cartilage explant culture in hMSC (IHP) conditioned medium. Overall, no significant differences were observed in proteoglycan release due to stimulation with hMSC (IHP) conditioned medium, only minimal trends were observed. Regarding total proteoglycan synthesis, conditioned medium from some hMSC donors had an effect, however this effect was not consistent for all cartilage donors.

Practically no significant differences in proteoglycan release were observed for the three cartilage donors due to stimulation with hMSC (IHP) conditioned medium. The only significant difference was observed between the 48h medium and the hMSC-IHP conditioned medium from donor 3, which showed a higher proteoglycan release in cartilage donor 2. Overall, there seems to be a trend towards a slightly higher proteoglycan release for the cartilage explants cultured in conditioned medium in general, compared to fresh medium. Additionally, there is a trend towards a higher proteoglycan release in cartilage explants cultured in hMSC-IHP conditioned medium, compared to the explants cultured in hMSC-non-IHP conditioned medium.

More evident differences were observed in proteoglycan synthesis. Overall, there is a trend towards a lower proteoglycan synthesis for the cartilage explants cultured in conditioned medium in general, compared to fresh medium, except for cartilage donor 2 (B). Furthermore, the effects of hMSC-IHP conditioned medium compared to hMSC-non-IHP conditioned medium are cartilage donor dependent. In cartilage donor 1, hMSC donor 1 shows no difference in proteoglycan synthesis between IHP and non-IHP conditioned medium. However, hMSC donor 2 shows a trend towards a lower proteoglycan synthesis for the IHP-conditioned medium compared to the non-IHP conditioned medium. In cartilage donor 2, there is a trend towards a higher proteoglycan synthesis for the IHP-conditioned medium compared to the non-IHP conditioned medium. In cartilage donor 3, both hMSC donors show a higher proteoglycan synthesis for the IHP-conditioned medium compared to the non-IHP conditioned medium. This difference is statistically significant ($p<0.05$) for hMSC donor 5. The overall effect of culturing OA cartilage explants in either IHP or non-IHP conditioned medium, measured by proteoglycan synthesis seems to be very cartilage- and hMSC donor dependent.

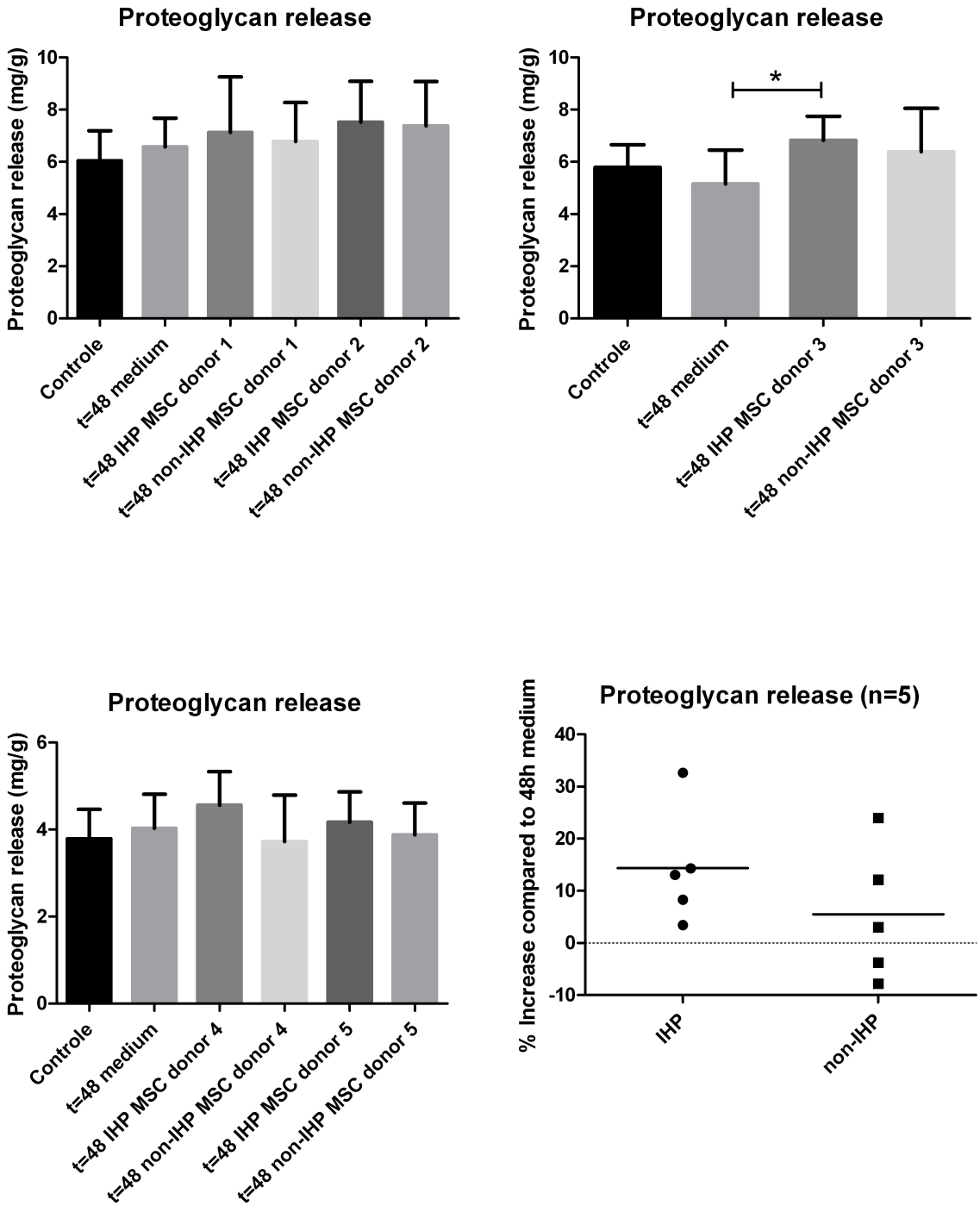

Figure 7: Proteoglycan release measured by Alcian Blue assay for three experiments with different OA cartilage donors.

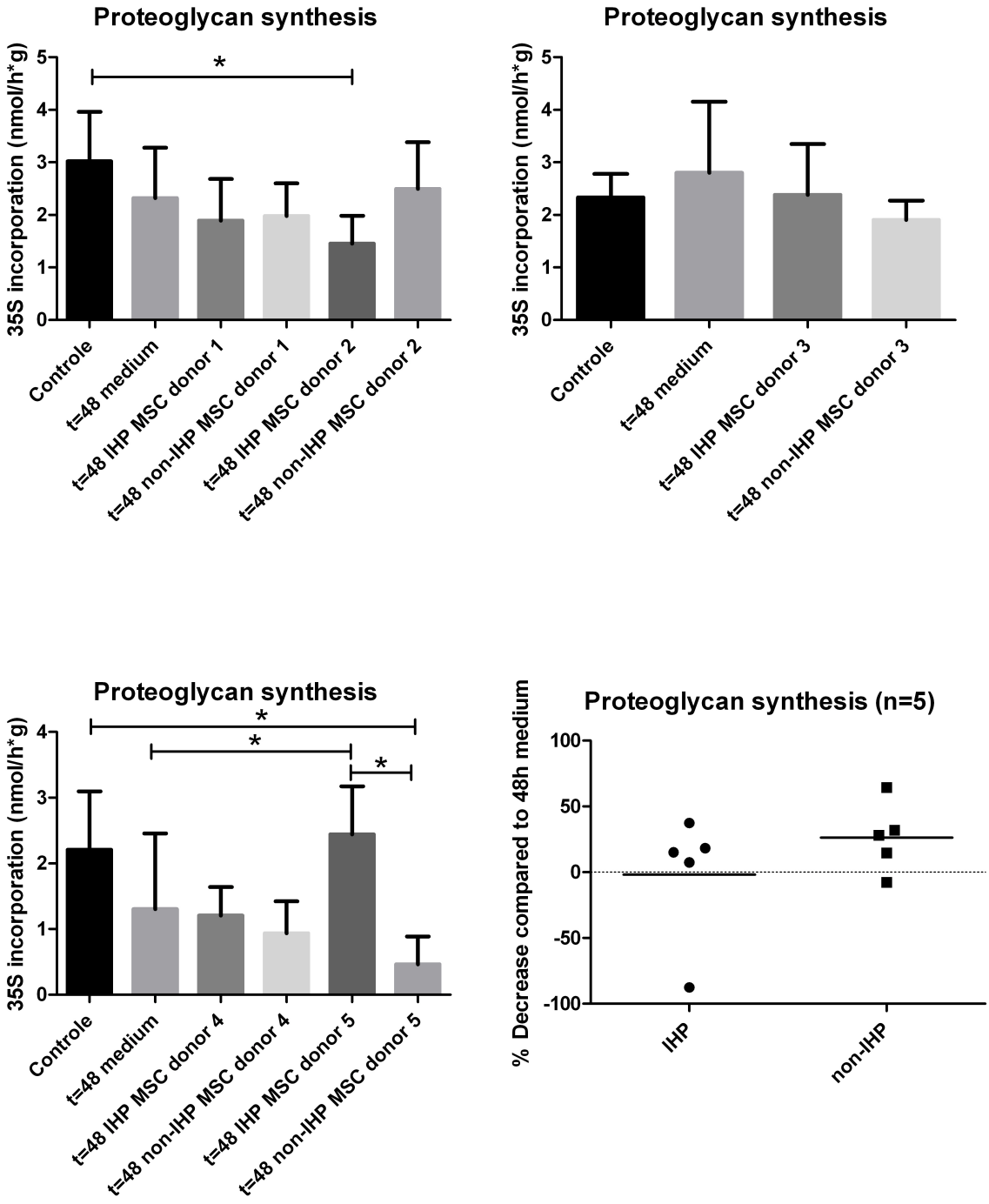

Figure 8: Proteoglycan synthesis measured by $^{35}SO_4$ incorporation for three experiments with different OA cartilage donors.

DISCUSSION

The hypothesis was that hMSCs secrete trophic factors, either under the influence of IHP, which could have a stimulating effect in cartilage repair. To test this hypothesis, culture medium in which IHP and non-IHP stimulated hMSCs were cultured was collected after 48h of culture in the IHP setup. This conditioned culture medium was used in OA cartilage explant cultures to evaluate the effect of this conditioned medium on proteoglycan turnover in OA cartilage.

Culturing OA cartilage explants in hMSC-conditioned medium did not result in a significant difference in proteoglycan release between hMSC-IHP and non-IHP conditioned medium. This is a positive finding, as it implies that there is no increased degradation of extracellular matrix due to the factors secreted by hMSCs under the influence of IHP. However, a trend was observed towards a higher proteoglycan release for all cartilage explants cultured in conditioned medium, compared to explants cultured in fresh medium. An explanation for this finding could be that the hMSC-conditioned medium is depleted from essential nutrients during 48h of culture in the IHP setup. Changing the proportion of fresh medium in the mix with hMSC-conditioned medium (1:1 in these experiments) could resolve this issue. Another explanation for this finding could be the difference in serum that was used for hMSC and cartilage cultures. Foetal calf serum was used in hMSC cultures in contrary to cartilage explants that were cultured in human serum.

OA cartilage explants cultured in hMSC-conditioned medium showed an inter-donor variation regarding the proteoglycan synthesis in the cultured explants. In two of the cartilage donors, hMSC conditioned medium did not have a significant effect on proteoglycan synthesis. One of the cartilage donors showed a lower proteoglycan synthesis for the explants cultured in hMSC-IHP conditioned medium compared to the non-IHP conditioned medium, while the other cartilage donor showed a higher synthesis for the IHP-conditioned medium compared to the non-IHP medium. In the third cartilage donor, one of the hMSC donors showed a statistically significant positive effect on proteoglycan synthesis of the IHP-conditioned medium compared to the non-IHP medium. Overall, no distinct consistent effect of the hMSC-IHP conditioned medium was observed, compared to the hMSC-non-IHP conditioned medium. First, to improve the results of these experiments, conditioned medium from these hMSC donors should be tested on a higher number of cartilage donors. Second, hMSC conditioned medium after 48h of culture was used in these experiments, instead of after 6 hours, the time point at which the most significant differences on a mRNA expression level were observed. Finally, it is advisable to perform these experiments with a higher number of hMSC donors, as large inter donor variations were observed when evaluating the effect of IHP in hMSCs on a mRNA level.

CONCLUSION

Culturing OA cartilage explants in hMSC-IHP or non-IHP conditioned medium did not have a significant effect on proteoglycan release, which implies that there is no effect on degradation of extracellular matrix components. On the other hand, no consistent effect was found in proteoglycan synthesis, only one hMSC donor was able to increase the proteoglycan synthesis in OA articular cartilage explants under the influence of IHP, compared to the non-IHP situation. Additional experiments with a higher number of cartilage and hMSC donors are needed to truly evaluate the effect of factors secreted by IHP-stimulated hMSCs on proteoglycan turnover in OA cartilage.

6. ISOLATION OF (OA) SYNOVIAL MEMBRANE HMSCS

INTRODUCTION

Human mesenchymal stem cells have been isolated from adipose and synovial tissue[62], synovial fluid[63], peripheral blood, skeletal muscle, umbilical cord blood, placenta, and bone marrow[64]. Their usefulness in different disease processes may depend on their source. Therefore, synovial tissue is an interesting source of hMSCs, regarding the observed cartilage repair due to joint distraction treatment, because this tissue surrounds the degenerated articular cartilage. In this study, a first step was made to isolate hMSCs from osteoarthritic synovial tissue. A basic isolation protocol, based on the adherent capacity of hMSCs was used and the isolated cells were characterized using flow cytometry.

MATERIALS & METHODS

Tissue preparation

Human synovial membrane tissue was obtained after knee replacement surgery from knee joints within maximum 4-6 hours after surgery. Synovial tissue resection was performed during surgery as accurately as possible by the orthopaedic surgeon and transported in sterile phosphate buffered saline (PBS). Tissue was obtained from female donors (n=2) with a known history of knee osteoarthritis, aged 64.5± 14.8 yrs. Excessive fat tissue was removed aseptically, isolating the synovial tissue. Synovial tissue was minced in a sterile petri dish in pieces (±2x2x2 mm) small enough to enter a glass pipette. Culture medium [DMEM, 10% FCS, 100 IU/ml Penicillin, 100 μg/ml streptomycin sulphate, 2 mM L-glutamin, and 0.085 mM L-ascorbic acid] was added to the petri dish and the tissue suspension was transferred to a T75 culture flask (Nunc, ThermoScientific).

Synovial tissue and cell culture

Synovial tissue was cultured for a week to allow the synovial cells to migrate from the tissue and adhere to the culture plastic. Medium was changed every 1-2 days depending on the level of decolourization of the medium. After a week, tissue suspension was discarded and culture of the plastic adherent cells was continued in fresh medium. Cells were cultured and passaged when confluent (as described before), up to maximum passage 1-2, medium was changed every 3-4 days.

Cell characterization

Passage 1-2 cells were stained for flow cytometry, using a BD Stemflow™ hMSC analysis kit, according to manufacturer's protocol. The kit involved a hMSC positive cocktail of CD73, CD90, and CD105, and a non-hMSC cocktail of CD11b, CD19, CD34, CD45, and HLA-DR. These stains are used to determine the percentage of hMSCs, according to the CD-marker profile, as defined in the 'minimal criteria for hMSCs' previously reported by the International Society for Cellular Therapy. Flow cytometry data was acquired by a FACS Canto II cell analyser (BD Biosciences). Data was analysed using FlowJo software (version X, TreeStar, San Carlos, CA, USA).

RESULTS

Adherent cells were successfully isolated from synovial tissue, using a basic isolation protocol. According to table 6, 93.9% of the isolated cells from synovium donor 1 possessed the hMSC-specific CD-marker profile. Additionally, less than 2% of the cells were PE positive (non-hMSC). According to the ISCT criteria[61] for defining mesenchymal stem cells, the cells can almost completely be considered to be hMSCs. The cells are plastic adherent, the cells form colony-forming units (figure 9), and less than 2% of the cells are PE positive (non-hMSC). The criterion of ≥95% of CD105+ CD90+ CD73+ PE- cells was not completely met (93.9%) however; the measured percentage is very close to 95%.

Donor ID	PE- (%)	CD105+ CD90+ PE- (%)	CD105+ CD90+ CD73+ PE- (%)
Synovial cells D1	98.2	94.4	93.9
Synovial cells D2	86.9	60.5	60.4

Table 6: hMSC characterization, % of CD105+CD90+CD73+PE- cells

The isolated cells from synovium donor 2 did not meet the ISCT criteria for defining mesenchymal stem cells. The cells were plastic adherent and formed colony forming units, however more than 2% of the cells were PE-positive (non-hMSC) and only 60.4% of the cells possessed the hMSC-specific CD-marker profile.

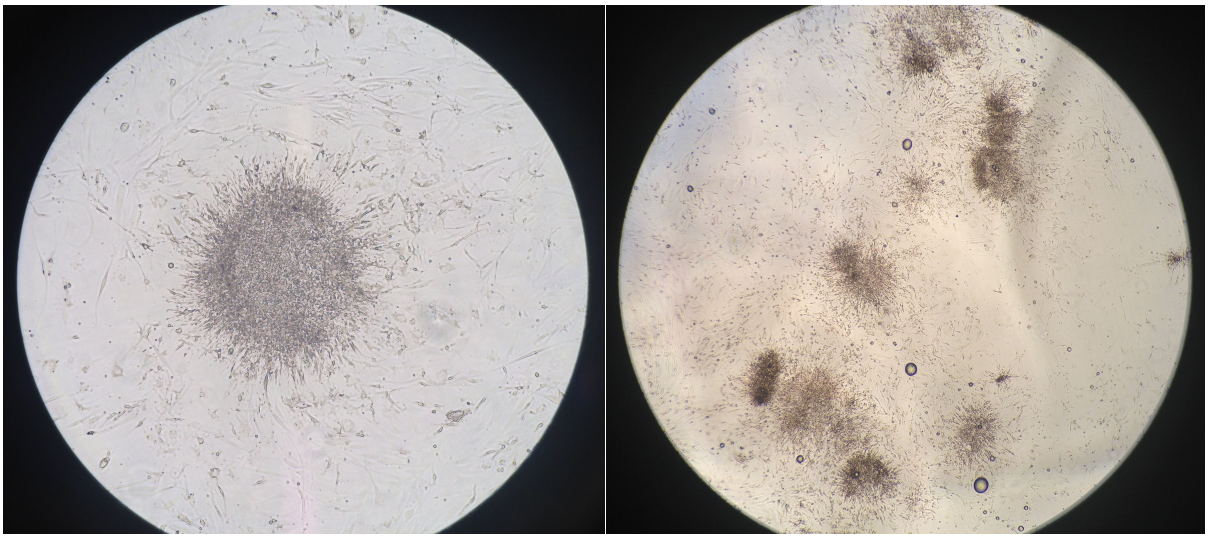

Figure 9: Microscopic images of adhered cells originating from synovial tissue. hMSC-specific colony forming units are clearly visible.

DISCUSSION

The aim of these experiments was to study the feasibility of a basic isolation protocol for hMSCs from synovial tissue, with the ultimate goal to compare hMSCs from different origins (bone marrow and synovial tissue) under the influence of IHP. The results of these experiments show that it was possible, at least for one donor, to isolate hMSC-like cells from synovial tissue. The cells isolated from donor 1 meet most of the characteristics for defining hMSCs. To completely proof that the isolated cells from donor 1 are indeed hMSCs, a differentiation experiment should be performed. The last criterion for hMSCs is their capacity to be differentiated into the adipogenic, chondrogenic and osteogenic lineage *in vitro*.

The cells isolated from synovial tissue from donor 2 didn't meet the criteria for defining hMSCs. A significant portion of the cells was PE-positive (non-hMSC). The PE-positive cells showed expression of CD45, CD34, CD14 or CD11b, CD79a, CD19 or HLA class II. These CD-markers are specific for cells with a hematopoietic origin. One of the first steps of this protocol was to separate the synovial tissue from excessive tissue. One of the explanations for the portion of PE positive cells could be that the synovial tissue was not properly separated from excessive tissue. It could be that some well-vascularized tissue, including blood, was cultured together with the synovial tissue. As a result, hematopoietic cells could have migrated from this tissue, or from blood, and have adhered on the plastic culture surface. This finding is a strong argument for very accurate separation of synovial tissue from the excessive tissue.

Recently, more sophisticated protocols have been developed for isolating hMSCs from synovial tissue[65]. A future step would be to apply these protocols to isolate hMSCs from synovial tissue. This procedure is faster because of the application of enzymatic digestion. Additionally, more extended characterization methods, with an expanded panel of CD-markers, have been developed[66]. A new, more sophisticated protocol for isolation of hMSCs from synovial tissue, based on literature, is included in the appendix.

CONCLUSION

In agreement with previous literature, we have shown that it is possible to isolate hMSC-like cells (based on CD-marker profile) from synovial tissue using a basic isolation protocol. A very important step in this protocol, and in future protocols is to accurately separate the synovial tissue from the excessive tissue. A new protocol for (enzymatic) isolation of hMSCs from synovial tissue was created based on literature, however due to limited time in this project, this protocol was not yet applied. The cells isolated from donor 1 can be used to compare BM-hMSCs with synovial hMSCs under the influence of IHP, on a mRNA level.

7. GENERAL DISCUSSION

Application of joint distraction has shown intrinsic cartilage repair in combination with meaningful clinical efficacy for several different joints such as the ankle, hip and the knee in patients with osteoarthritis[9-11]. This observed cartilage repair activity is unique, but the exact underlying mechanism of cartilage repair due to joint distraction is still unknown. Looking at the possibilities that joint cartilage repair would have for a variety of degenerative diseases, the relevance of unravelling this mechanism is very high. If the environment, the (absence of) mechanical stimuli, the trophic factors and the other desired stimuli that are needed to facilitate cartilage repair can be exactly defined, this could have great implications for the treatment of degenerative joint diseases.

Osteoarthritis is a disease in which the disturbance of the joint homeostasis plays a significant role[45]. hMSCs have chondrogenic potential *(in vitro),* but can also play a role in immunomodulation and tissue repair by secretion of soluble trophic factors, as they respond to stimuli that are associated with tissue injury[17]. The aim in this study was to evaluate the effect of IHP on the hMSC secretome, IHP being a stimulus that is present during knee joint distraction. As a first step in evaluating the hMSC secretome under the influence of IHP, we have optimized and validated the previous IHP culture system, established standardized protocols for hMSC culture under IHP, flow cytometry, RNA isolation and qPCR, and mRNA expression levels were determined for a set of cartilage-homeostasis related factors in IHP- and non-IHP stimulated hMSCs from multiple donors. Additionally, a first step was made to isolate hMSCs from osteoarthritic synovial tissue, to enable future experiments for comparison of hMSCs from different origins.

The current, optimized experimental setup consists of closed culture vessels in which the temperature, CO_2 concentration, O_2 concentration and airflow (pressure) can be accurately regulated and monitored. IHP is applied by intermittingly increasing the airflow inside the pressure vessel, thereby exerting IHP on the cultured hMSCs. The main question for this research project was whether IHP has an effect on the trophic role of hMSCs on cartilage *in vitro*. This setup allows us to study solely the effect of IHP on cells or tissue explants, excluding other variables that are present inside an osteoarthritic knee joint.
Using this setup, mRNA expression levels in hMSCs were determined in monolayer cultures of bone marrow derived hMSCs. In this case, the setup was an appropriate model to study the effects of IHP on mRNA expression in BM-hMSCs, however this setup didn't directly allow us to study the effects of IHP-stimulated hMSCs on cartilage. Additionally, the relevance of the BM-hMSCs as a source of trophic factors in relation to cartilage repair can be questioned, as it is anticipated that their direct interaction with cartilage is limited due their location. Nevertheless, for pragmatic reasons the first experiments were done with these cells.. Future experiments should focus on hMSCs derived from a source more related to the osteoarthritic knee, for example from osteoarthritic synovial tissue. This increases the relevance of measuring mRNA expression levels in hMSCs under the influence of IHP, regarding the observed cartilage repair.

It was previously shown that IHP has a stimulating effect on cartilage repair (measured by proteoglycan turnover) in explant cultures of osteoarthritic articular cartilage[32]. Additionally, it was also shown that IHP stimulation results in decreased catabolic activity of mononuclear cells isolated from synovial fluid of osteoarthritis patients. This effect was associated with downregulation of IL1β and TNFα production. These findings suggested that IHP during joint distraction might be beneficial for osteoarthritic cartilage repair directly, and indirectly via suppression of inflammation[32]. Moreover, this implies that solely IHP can be a stimulus that can exert a stimulating effect on cells, tissue or even MSCs, which might be beneficial for cartilage repair. It will be interesting to study the direct effect of IHP on cartilage using the current IHP setup, to further validate the current IHP setup. These studies measured other parameters than gene expression. As shown in table one, several groups have studied the effects of IHP on gene expression levels in MSCs, however with a main focus on genes that are characteristic for chondrogenic

differentiation of MSCs (collagen, TGFb3, Sox9, Runx2). In comparison to our setup, MSCs were in most studies cultured in hydrogel constructs or scaffolds. Pressure was usually applied by increasing the fluid or air pressure in a custom-built pressure chamber, comparable to our setup. The applied pressures in these studies were higher in comparison to our setup (MPa rather than kPa). Pressures in a range of MPa are not representative for the situation *in vivo.* The pressures that were applied using our setup, were based on *in vivo* measurements of intra-articular pressures during joint distraction treatment[32] (Figure 2). Therefore, this setup is a clinically relevant model for the application of IHP to cells or tissue explants; the applied pressures resemble the *in vivo* situation during treatment. The main difference between previous studies regarding IHP and gene expression in MSCs with our project is that previous studies focussed on the ability of IHP to induce chondrogenic differentiation of MSCs, while our project focussed on the expression of trophic factors due to application of IHP.

In a review by Elder et al., the role of (intermittent) hydrostatic pressure (HP) in tissue engineering, the chondroprotective effects of (I)HP, MSC differentiation and effects of high (I)HPs were evaluated. According to these results, IHP stimulation (IHP: 5-10 MPa) of chondrocytes has a significant beneficial effect on proteoglycan, aggrecan and collagen synthesis. Additionally, stimulation of MSCs *in vitro* with (I)HP has shown to induce chondrogenic differentiation characterized by increased GAG and collagen production and increased collagen, sox9, aggrecan mRNA expression levels. These genes might serve as an appropriate positive control to validate the application of IHP using the current IHP setup. This would allow us to more certainly conclude that our current IHP setup indeed is an appropriate model for the application of pressure to cells or tissue explants. However, it would be interesting to see if these upregulations seen in other studies are also present when applying lower magnitude IHP levels (15 kPa) in our setup. Finally, when applying very high levels of IHP (30-50 MPa), IHP has a detrimental effect on chondrocytes and generally results in a stress response and decreased metabolic activity[13]. This could also serve as a positive control for the application of IHP. However, such high pressures cannot be applied using the current IHP setup and need rigorous technical adaptations.

One of the important hypotheses for the observed intrinsic cartilage repair due to knee joint distraction is that the cartilage repair cannot be solely the result of increased activity of resident chondrocytes. It was shown that the denuded bone area of the most affected compartment of the knee decreased from 22% at baseline to 5% at 1-year follow-up after knee joint distraction[67,68]. The very low density of resident chondrocytes in denuded bone areas suggests that there are other cells needed to facilitate cartilage repair. hMSCs are suggested to be an important candidate for this repair activity, by playing a trophic and/or immunomodulatory role. An argument which strengthens this hypothesis, is shown by Wu et al; hMSCs are thought to rather play a modulatory, trophic role in cartilage tissue repair *in vivo*, than differentiating into chondrocytes[58,59]. Moreover, hMSCs have been isolated from a variety of joint-related tissues, including the infra-patellar fat pad and synovial tissue[62], bone marrow[64], and the synovial fluid[63]. Higher numbers of synovial fluid MSCs, possibly derived from dislodged synovial fragments, were found in early-OA synovial fluids[63]. These synovial cells have been suggested to contribute to spontaneous cartilage repair[69]. Additionally, the absence of mechanical stress would possibly be very beneficial for synovial derived MSCs and their contribution to cartilage repair during joint distraction. However, this role for synovial MSCs in cartilage repair due to joint distraction has not been shown yet, mainly due to the fact that it is still very difficult to determine the origin of isolated MSCs. A synovial-MSC specific CD-marker profile still has to be found, however first steps have been made[63,70].Testing the hypotheses of the presence of synovial derived MSCs in the synovial fluid facilitating cartilage repair during joint distraction treatment is current subject of study within the department.

Overall, there are strong hypotheses for the mechanisms behind the intrinsic cartilage repair that is observed after knee joint distraction treatment in patients with osteoarthritis. First, the absence of mechanical stress during joint distraction prevents further wear and tear of the joint cartilage. The

absence of mechanical stress is also thought to allow synovial fluid MSCs, originating from either the synovial tissue, or another joint related tissue, to initiate cartilage repair, probably by playing a trophic and/or immunomodulatory role, especially in denuded bone areas. Third, the intermittent hydrostatic pressure that is present in the synovial fluid during joint distraction 1) stimulates the diffusion of nutrients into the residual articular cartilage and 2) can possibly act as a stimulus for MSCs that induces the secretion of (trophic) cartilage-homeostasis related factors. During this research project, we have optimized and validated an IHP culture setup, which can now be used to study the influence of IHP in cells or tissue explants; first steps have already been made during this project. This research project enables subsequent researchers to further study the role of IHP and the (trophic) role of hMSCs in the process of cartilage regeneration.

8. FUTURE PERSPECTIVES

In this study, the previous IHP setup was optimised, to establish a golden standard for tissue/cell culture under IHP, hereby creating an *in vitro* model for culture of hMSCs and/or articular cartilage explants under the influence of IHP, which is observed during joint distraction treatment.

- IHP stimulation definitely shows an effect in BM-hMSCs on an mRNA level, for a set of cartilage-homeostasis related factors. With this optimised *in vitro* model that was established during this internship, experiments should be repeated with a larger number of hMSC donors, to increase the power and consistency of the results.
- This study is a first step in defining the hMSC secretome (on a mRNA level) under the influence of IHP. In future experiments, the set of genes should be expanded, to more extensively study the effects of IHP on the hMSC secretome. µ-Array gene expression analyses might be an interesting option. However, because of the large inter-donor variation, a large number of hMSC donors would have to be included in these analyses.
- It is known that hMSCs respond to injury-specific stimuli by secreting trophic, proliferative, or immune-modulatory factors. It will be interesting to study the gene expression profile of hMSCs under IHP in co-cultures with osteoarthritic cartilage. This might unravel the mechanism that leads to changes in the hMSC secretome under the influence of IHP.
- hMSCs are normally *(in vivo)* situated in the 3D structure of a tissue. In this study, hMSCs were cultured in monolayer, which does not perfectly represent the *in vivo* situation. A next step would be to culture hMSCs in a 3D structure, which can be a 3D scaffold based on for example agarose. This would better mimic the situation *in vivo.*
- In future experiments, it will be very interesting to study the effects of IHP on hMSC from different origins. Synovial hMSCs were successfully isolated in this study. The next step is to apply more sophisticated isolation protocols for isolation of synovial hMSCs. Additionally, it will be interesting to use more extended characterization methods to be able to discriminate between the hMSCs from various origins.

Overall, this internship has resulted in an established (optimised) *in vitro* model to study the effect of an intermittent hydrostatic pressure in hMSCs. By doing this, a first step was made to unravel one of the mechanisms behind the intrinsic cartilage repair that is observed after knee joint distraction treatment. The optimised culture setup allows us to study several hypotheses for the mechanisms behind cartilage repair. Future experiments should aim at further identification of the hMSC secretome under the influence of IHP either on a gene expression or protein secretion level. The potential effect of IHP has already been shown for osteoarthritic cartilage and inflammatory cells, and this study suggests that IHP also has an influence on hMSCs. However, the effect of IHP on hMSCs should be further unravelled. Culture of hMSCs in 3D scaffolds/constructs, co-cultures of hMSCs and osteoarthritic cartilage, genomics and proteomics can be very interesting techniques that could be used to establish this goal and finally confirm or reject the hypotheses regarding IHP and its effect on hMSCs.

Unravelling the MSC secretome under the influence of IHP could potentially lead to further optimisation of joint distraction treatment and to other related treatment strategies for OA. Furthermore, a better understanding of the bio-chemical/mechanical environment stimulating MSCs to initiate cartilage repair leads to optimization of current extrinsic treatments with MSCs and opens new perspectives for future treatments.

9. CLINICAL STUDIES – PATIENT FOLLOW-UP

Parallel to my work in the laboratory, where I studied the effects of intermittent hydrostatic pressure in human mesenchymal stem cells, I have participated in clinical studies where I saw patients that returned to the hospital for follow-up visits after surgical treatment for knee osteoarthritis. I had the possibility to participate in clinical studies in the Rheumatology department, which, in my opinion, has contributed significantly to the development of my clinical competences. To increase the experience in clinical research, and as recommended by my supervisors, I followed a one-day course in WMO (wet medisch-wetenschappelijk onderzoek met mensen) and good clinical practice (GCP), for which I gained a certificate (Appendix) After completing the WMO/GCP course I was also allowed to discuss and sign the informed consent, if there were any changes with the version that was signed before surgery. During this M3 internship, I saw ± 35 patients that participated in one of three studies.

All three clinical studies aim to evaluate the clinical and/or structural benefit of knee joint distraction treatment in patients with osteoarthritis. The first clinical study aimed to evaluate the effectiveness of the treatment and to study the underlying mechanisms that result in the observed structural and clinical benefit. Patients were invited to return to the hospital at multiple time points after joint distraction surgery, up to 10 years after surgery. Depending on the time point after surgery, patients were asked to fill in a questionnaire about the daily functioning and pain of the patient (WOMAC), blood and urine was collected for biomarker analyses, and a standardised X-ray image and an MRI (Eckstein protocol) was made. Additionally, patients visited the Rheumatology department, where I did a short anamnesis in which any changes in the clinical status of the patient were discussed. During these visits, blood and urine was collected and patients were asked to fill in the questionnaires. The second and third studies are comparative studies in which two different treatments for knee osteoarthritis were compared with the knee joint distraction treatment. One study compares the clinical and structural benefits observed after knee joint distraction with the results that were observed after high tibial osteotomy (HTO) treatment. The other study compares the results of joint distraction and total knee prosthesis (TKP) treatment. Depending on the time point after surgery patients were also asked to fill in questionnaires; KOOS, VAS, COSTS, Sf-36, and EQ-5D. During patient visits, health status of the patients was discussed, blood and urine was collected for biomarker analysis and optionally, X-rays or MRI's were discussed. In this study, dGEMRIC MRI scans with Magnograv© contrast were made for the patients treated with knee joint distraction or HTO (after the plate was removed).

Thus, my participation in the clinical studies consisted of patient visits, discussion of health status of the patients (related to surgery), discussing X-rays, communicating with research nurses/clinical researchers/radiology technicians, and with the laboratory technicians. Clinical activities included intravascular infusion of MRI contrast and blood collection by venepuncture. By seeing a relatively large number of patients I got much more comfortable in patient/clinician contact over the course of my internship. I experienced that participation in a clinical study also means collaboration in a clinical study. Over the course of this internship I became more aware of all the parties that collaborate in a clinical study, and while participating in these studies I felt very involved and I felt a strong sense of responsibility for my work.

I have found the combination of my laboratory, more fundamental research, with the activities in the clinical studies very interesting. This was mainly because of the fact that my laboratory research was fundamentally based on the results that were observed after knee joint distraction in patients with osteoarthritis. By seeing a relatively large number of patients treated with joint distraction, I became aware of the heterogeneity of the clinical and structural benefits of this treatment. This observation lead to a large number of questions, which was specifically interesting in my laboratory research, where I was also confronted with large inter donor variations.

10. PERSONAL DEVELOPMENT (POP)

At the start of my M3 internship, I set myself personal goals aimed at acquiring competencies as a clinical physician, as well as more research specific competencies. These goals have originated from experience obtained during previous internships and feedback from supervisors that I obtained in these internships. My aim for this year was to obtain a variety of skills in both clinical and more fundamental research, to prepare myself for a future PhD project, as a graduated technical physician in reconstructive medicine. Looking back at this year at the Rheumatology department, I felt very involved in the whole research group, and I especially appreciated the supervision of my direct supervisor, Simon.

My objectives in clinical experience for this year were mainly focussed at clinical research. I wanted to gain more experience in physician-patient conversations, physical examination, clinical skills such as venepunctures and intravascular infusions, and I wanted to try to gain more knowledge about what it's like to work in a research group, which is directly connected to the clinic. As described before, I have participated in clinical studies, which has taught me a lot about which parties are involved and the specific roles they have in the clinical studies. In these activities I have also gained more experience in venepunctures and intravascular infusions, because these were activities essential for the follow-up of joint distraction patients. Physician-patient conversation and physical examination (minimally) were also a part of the patient follow-up. I think I have improved my skills in communicating with patients, due to the number of patients I've seen. Over time, I felt more and more confident and competent in physician-patient conversations. Additionally, I gained more general knowledge in the field of rheumatology and osteoarthritis by visiting the weekly clinical research meetings. In these meetings, researchers, either physicians, medical biologists, or epidemiologists discussed the progress in their research. This also contributed to the experience I gained about working in a research group that is closely connected to the clinic.

On the other hand, I also wanted to increase my experience as a more fundamental researcher. My first objective was to gain more experience in setting up, executing laboratory experiments and interpreting/analysing the results. A logic second objective was to gain more experience in scientific writing and presentation of results. Additionally, I wanted to acquire more practical skills in laboratory techniques such as cell culture, flow cytometry, RNA isolation, qPCR, and laboratory animal science, for which I followed the laboratory animal science course (LAS) at the Radboud University in Nijmegen. In this internship research project I have optimized and validated a previous culture setup, set up an experiment protocol to study gene expression in hMSCs cultured in the IHP setup, and I've analysed the results. I have described and discussed the results in this thesis, and I've presented some of my preliminary results in the Monday discussion of progress meeting, with all the researchers in the Rheumatology department as the audience. I think that I still have a lot to learn when it comes to setting up and executing experiments and presenting the results, however the experience in this project has taught me on which parts I can still improve. During the first months of this project, I spent a substantial amount of time optimizing and validating the culture setup, which could have been performed faster to my opinion. I think that I can improve my long-term planning, which can be beneficial for a long-term project like this.
Regarding practical skills in laboratory research, I think I have learned a lot. I've learned to optimize a protocol for flow cytometry, I have gained more experience in cell cultures, and I've gained experience in RNA isolation and qPCR by setting up a qPCR analysis specific for the hMSCs that were cultured for this project. In future research, I'd like to gain more experience in other laboratory techniques such as ELISA's, Western Blots, immunohistochemistry, and techniques in tissue engineering.

Through 360-degree feedback, I've received feedback that was very positive and feedback that was more constructive. The constructive feedback I received was that I could position myself more prominent, and that I could be more explicit in presenting my ambitions and myself. Another point of feedback was that I could sometimes take more self-control in my project. These points are not new to me, as I've

received as feedback in previous internships. If I look at the way I presented myself in previous internships, compared to this internship, I think I've made a lot of progress. Participating in clinical studies has especially been beneficial for my progress in the way I present myself, to my opinion. Regarding my ambitions, I have had multiple conversations with my medical supervisor, prof. Floris Lafeber. We discussed the optional possibilities for a PhD project in the Rheumatology department, but we also discussed doing a PhD in general. He has given me good advice about writing job application letters and job interviews. A point, in which I still want to make progress, is the way I visit discussion of progress, or clinical research meetings. I'd like to participate more in these meetings by asking relevant questions. Questions come across my mind during meetings, however I should feel less restrained in asking these questions. Overall, I think I've made progress during this graduation year. The fact that I've really felt part of a clinical research group, with my specific responsibilities, has made it easier for me to gain more progress on the topics of feedback I've received.

APPENDIX

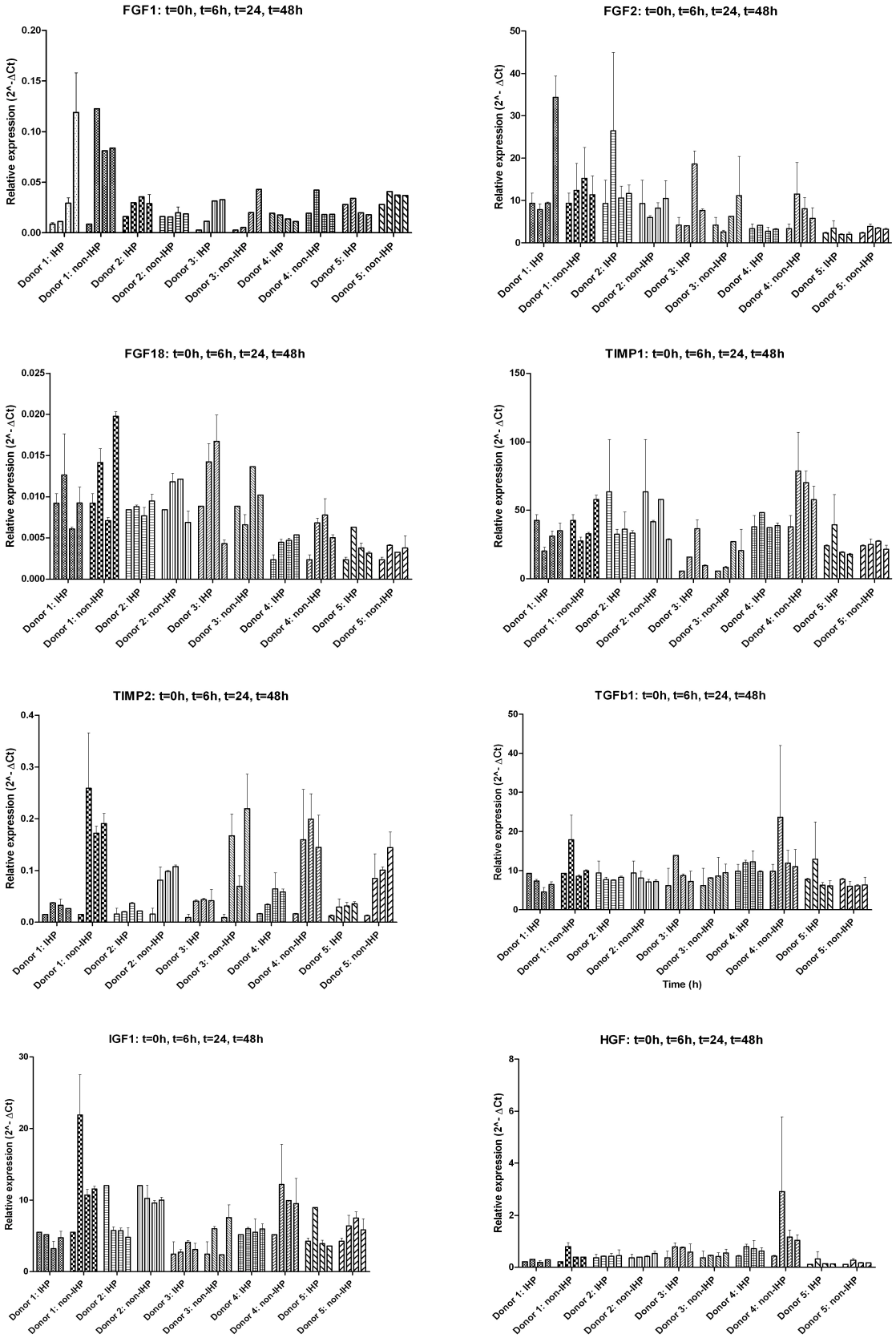

Figure 10: Relative expression data for all hMSC donors

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