UNIVERSITY OF TWENTE.
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Characterisation of hamstring- and anterior cruciate ligament cells

BSc Thesis

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1. Introduction

The anterior cruciate ligament (ACL), located between the femur and the tibia (Figure 1) is responsible for the mechanical stability of the knee joint. However, it is also one of the most susceptible ligaments in the human body, and is often injured while practicing sports like skiing, football, volleyball, hockey, etc. When the ACL is injured, the tissue is either let to heal by itself (in case of small injuries) or surgery is performed (in case of complete rupture). In case of surgery, two different kinds of methods are used in order to replace the ACL: by using an autograft or allograft. When using allograft, tissue from another person or even an animal is used to reconstruct the ACL. The advantage of this method is that no own tendons or ligaments are weakened by using it as donor tissue, while disadvantages are finding a donor, higher risk of inflammations, rejection of the donor tissue by the body or necrosis. Therefore, the use of allografts for ACL replacement is decreasing. In case of autograft, tissue from the same person is used in order to reconstruct the ACL. Examples of ACL reconstruction autografts are the hamstrings or patellar tendon. The hamstring is located posterior to the knee joint.

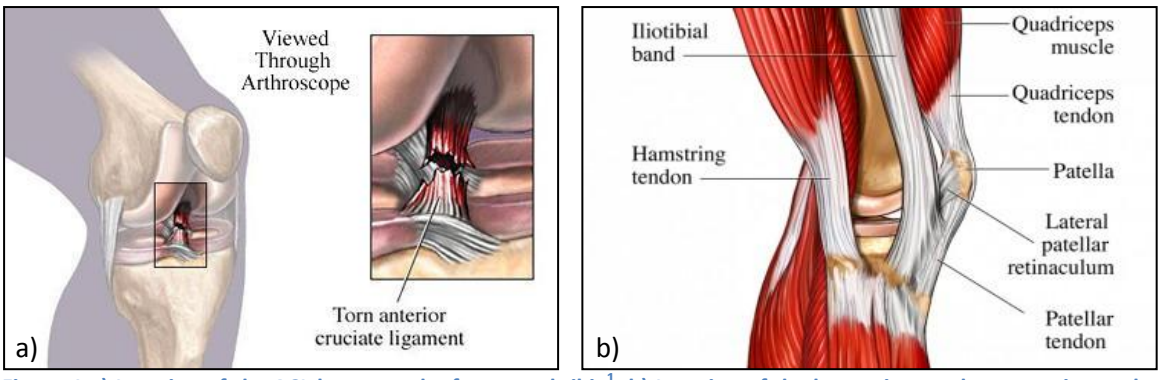


Figure 1 a) Location of the ACL between the femur and tibia[1]. b) Location of the hamstring tendon posterior to the knee joint[2].

In case of autograft, different methods are used. The most common method is by taking a piece of the patellar tendon (located between the patella and tibia), along with a piece of bone to which the tendon is attached, and inserted in the place of the removed ACL[3]. To put the newly achieved tendon into place, a piece of bone from the patella and tibia to which the patellar tendon is attached is removed. A small hole is drilled in the bone, in which the harvested tendon is inserted. The tendon is held in place by bone-to-bone attachment on one side, and by driving a special screw along with the tendon into the bone on the other side, leading to tendon-to-bone attachment[4]. Although this method is referred to as the "Golden Standard", and the patellar tendon can withstand more force than most other autografts (breaking point of 2900 Newton for the patellar tendon compared to 1725 Newton for the ACL[3]), it also has a few disadvantages. By cutting a strip of the patellar tendon, the strength of the tendon decreases leading to instability of the knee. Furthermore, there is an increased risk of patellar fracture, difficulties in achieving full extension of the knee, and long-term pain in the patellar tendon and the patella-femural joint.

Nowadays, the hamstring becomes a more popular autograft in the ACL reconstruction. To put the newly achieved tendon into place, a small hole is drilled into the bone. Then, the harvested tendon is inserted, and held into place by driving a special screw along with the tendon, in the bone[5]. Advantages of this method includes using the same incision for both harvesting the tendon as well as

replacing the ACL, thus reducing damage and risk of complications. The reduction of hamstring strength is way lower than the reduction of the patellar tendon strength in the other method. However, the strength of the hamstring tendon is less than the strength from the ACL (only 1200 Newton3), which has to be taken into account for persons who are involved in sports with high speeds, cutting movements and high impacts.

To understand the suitability of the tendon tissue in ligament reconstruction, a closer look into the composition of the tendon and ligament is essential. If one takes a look at the purpose and components of ligament and tendon, similarities can be found. Even thought~~t~~ the ligament is a bone-to-bone connection, and ~~a~~ the tendon is a muscle-to-bone connection[6], they consist both mainly of collagen 1 and collagen 3 fibers. In both tissues, either the tendon or the ligament, the collagen fibers are bundled together forming a rope-like structure (see Figure 2).

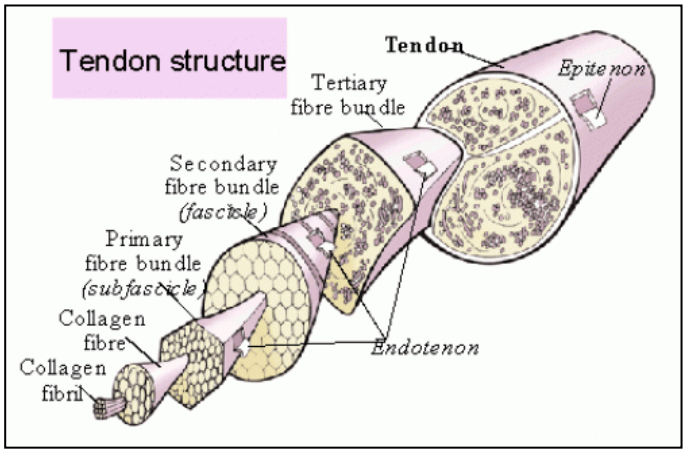


Figure 2 – The composition of a tendon. The composition of ligaments is similar[7].

The main difference between ligament and tendon however, is the way the collagen fibers are alligned[8]. In tendon, the collagen fibers are strictly parallel alligned to each other, while in ligament the fibers are arranged in parallel layers that show sinusoidal waves (see Figure 3).

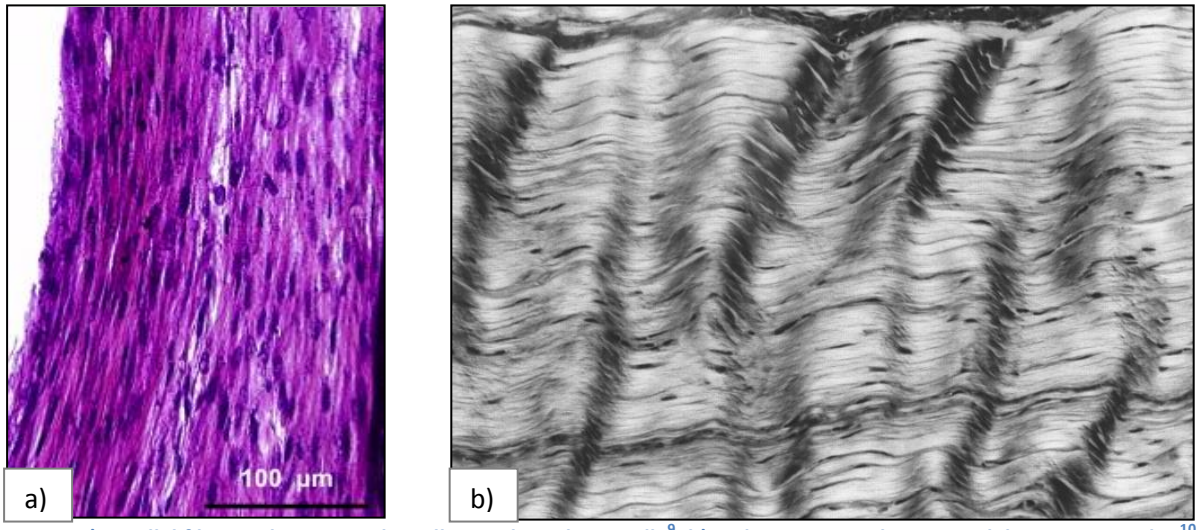


Figure 3 a) Parallel fibers in ligament. The cells are aligned vertically[9]. b) A clear view on the sinusoidal waves in tendon[10].

In both tissues the cells are aligned in between the collagen fibers and in rows along the same axis as the direction of ligament or tendon function[11]. In the ligament tissue itself however, differences in morphology can be seen from the bone to the ligament (see Figure 4 and Figure 5).

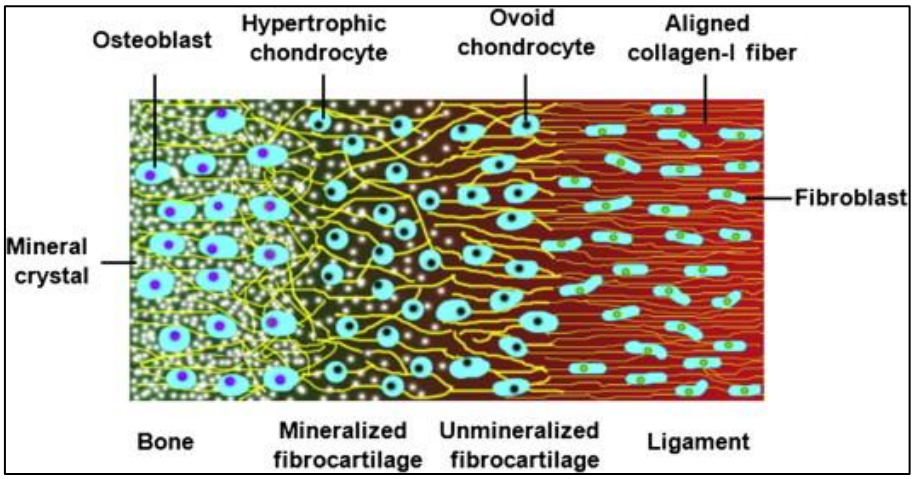


Figure 4 – Schematic view of the differences in morphology of the bone-to-ligament insertion site. Within the insertion, 4 different phases can be distinguished. Starting at the bone, where the cells are rounded and mineralised; mineralised fibrocartilage, containing collagen-producing chondrocytes; unmineralised fibrocartilage, containing more ovoid-shaped chondrocytes; ligament, containing elongated fibroblasts and collagen-fibers.[12]

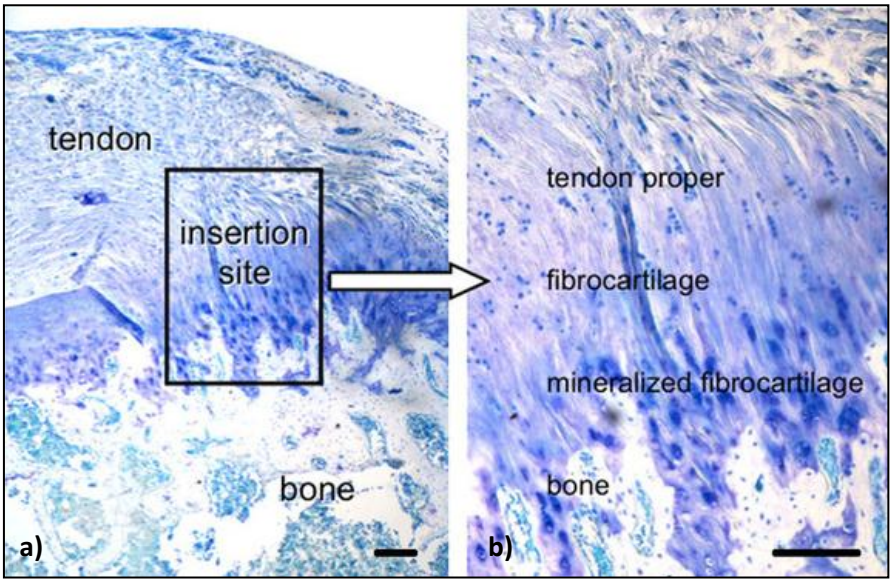


Figure 5 – Microscopic view of the tendon-to-bone insertion site. a) The transition from bone to tendon is clear to see; the elongated tendon cells become more rounded as they are closer to the bone.[13] b) close-up from the insertion site.

Regarding the ACL reconstruction, the surgery itself is well-established. The technique used to reconstruct the ACL is well characterised and the time of surgery is fixed. However, the recovery time of the patient after the surgery is long and the possibility of an after-surgery infection is still high. Therefore, understanding the phenotype of the replaced tissue and of the new inserted tissue, as well as the capability of the new tissue to connect to bone, can lead to a faster healing process of the patient after an ACL reconstruction. Consequently, the aim of this study was to better understand the phenotype of the replaced ACL tissue and of the newly introduced Hamstring tissue. For this, ACL

cells and hamstring cells obtained from human tissue were cultured in vitro in 2D flasks and exposed to different culture conditions. The self-renewal capacity was investigated, as well as the multipotency and multilineage potential, gene expression and cell surface markers of the cells. In this study, the hamstring cells are harvested from the healthy tissue from an injured patient undergoing ACL reconstruction, while the ACL cells are harvested from an injured tissue site.

We investigated the self-renewal capacity of the single seeded cells to investigate their potential to divide, seeded the cells to investigate if they have the ability to mineralise, form fat and form cartilage tissue and to investigate their gene expression and cell surface markers.

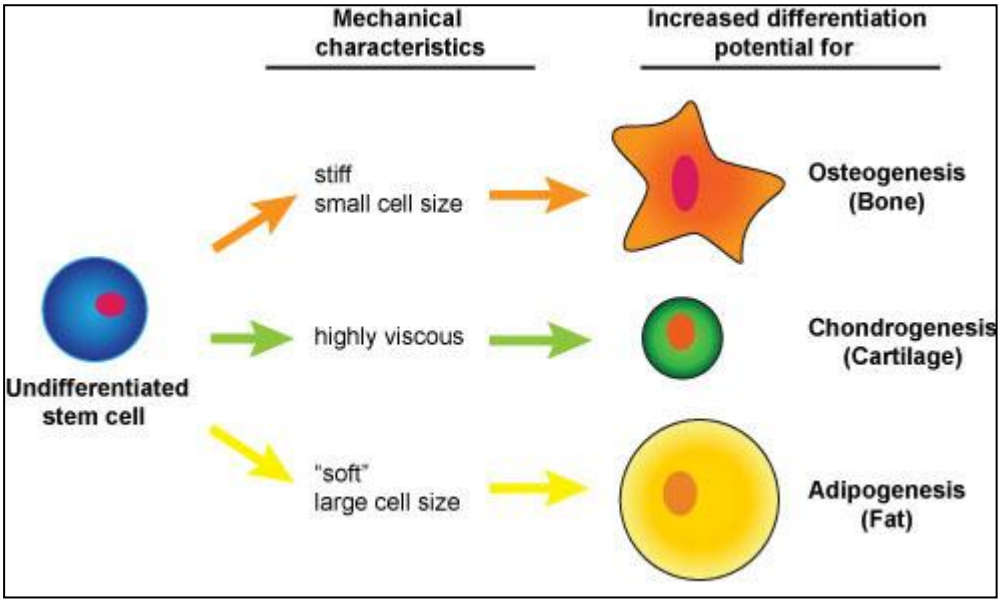


Figure 6 – The differentiation possibilities for multipotent cells. The stem cells have the ability to differentiate to bone, cartilage or fat under different circumstances.[14]

2. Materials and methods

2.1 Seeding and culturing of the hamstring- and ACL-cells

ACL and hamstring cells were harvested from patients undergoing ACL reconstruction. In this study cells from 3 hamstring donors and from 4 ACL donors were used. All donors were female; the tissue from the hamstring donors was healthy tissue while the tissue from the ACL donors was disrupted tissue. An overview from the donors can be found in Table 1.

Tissue	Gender	Age	Cells
H1-2 P2	Female	24	Frozen
H2-1 P1	Female	21	Frozen
H6-4 P1	Female	24	Frozen
ACL D3	Female	70	Frozen
ACL D4	Female	69	Frozen
ACL O3	Female	70	Frozen
ACL O4	Female	69	Frozen

Table 1 – List of donors characterised by gender, age and whether the cells were frozen or not prior experiment.

The obtained cells were harvested either by outgrowing the cells from the tissue (denoted with ACL O) or by collagen digestion of the tissue (denoted with ACL D). The obtained cells were either frozen for later use or further expanded in basic ligament medium, consisting of Dulbecco's Modified Eagle Medium (DMEM) from PAA containing 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin (P/S) and 1% Ascorbic Acid[*]. The cells were cultured in an incubator at 37 degrees Celsius with 5% CO_2 and 95% normal air until full confluency was reached.

When reaching confluency the cells were washed with Phosphate Buffered Saline (PBS) to remove any cell debris and trypsinised for 5 minutes at 37°C, until the cells were detached from the flask. Medium was added to neutralise the effects of the trypsin and after centrifugation the media was exchanged with fresh media. The cells were then used for experimental purpose. For each experimental condition triplicates or higher replicates were used.

2.2 Mineralisation differentiation assay

Hamstring and ACL cells were plated in 6 well plates. A total amount of 50.000 cells were seeded in each well, where 3 wells were used for the control, 3 wells were used for mineralisation in the presence of Dexamethasone (DEX) and 3 wells were used for mineralisation in the presence of Bone Morphogenetic Protein 2 (BMP2). In all 3 conditions the cells were cultured in the presence of basic ligament medium until full confluency was reached. At confluency the control cells were further exposed to basic ligament medium plus 0,01M Bone Gamma-carboxylglutamic Acid-containing Protein (BGP); the mineralisation cells in the presence of DEX were exposed to basic ligament medium plus $10e^{-8}$M DEX, 0,01M BGP and the mineralisation cells in the presence of BMP2 were exposed to basic ligament medium plus 100 ng/mL BMP2, 0,01M BGP. The medium was changed every 3-4 days and the cells were cultured up to 28 days in these conditions.

[*] Detailed information of the different compounds can be found in the "Supporting Information".

For the staining, 2% Alizarin Red at pH 4,2 was used. In order to stain the cells for mineralisation, the cells were washed with PBS and then fixed with 10% formalin for 10-15 minutes. After fixating, the cells were washed with PBS and then were washed twice with demi water. Then the staining was added to the wells for 1-2 minutes. After 1-2 minutes, the staining solution was washed away with distilled water and photos with a photo camera and a bright field microscope were taken.

2.3 Chondrogenesis differentiation assay

Hamstring and ACL were seeded in a 96 well plate with rounded bottom, each well containing 250.000 cells. Three to 5 wells were used as the control and 3 to 5 wells were used for the differentiation. After seeding the cells into the wells, the plate was centrifuged at 300 rcf for 5 minutes so that the cells gather at the bottom of the well. After 1 to 2 days the cells formed a pellet. The medium was changed every 3-4 days. For the control, cells were exposed to basic chondrogenesis medium consisting of basic ligament medium minus FBS, plus 50 mg/mL Insulin, Transferrin and Selenium (ITS) and 40 µg/mL Proline. For the chondrogenic differentiation, cell were exposed to basic chondrogenesis medium plus $10e^{-7}$M DEX and 0,01 µg/mL Transforming Growth Factor Bèta 3 (TGFβ3). The cells were cultured up to 28 days in these conditions.

For the staining, the pellets were dehydrated by embedding them in 70%, 80%, 90% ethanol solution for 30 minutes and 96% ethanol solution for 60 minutes. Eosin staining was performed for 5 minutes to stain the intra- and extracellular proteins. The pellets were then stored at 4°C overnight in butanol.

Next day, the pellets were taken out and put in a 2 mL glass jar. The jar was filled halfway with liquid paraffin at 60°C, and stored in a hot water bath for 2 hours. After that, the paraffin was replaced with fresh paraffin, and the pellets were kept further at 60°C overnight.

Next day, the pellets were taken out of the jar and placed into a base mold, and embedded in fresh liquid paraffin. The paraffin was allowed to cool down on a cool plate for 10 minutes, after which the mold was taken out from the base mold and stored at 4 degrees. The embedded pellets were then sliced into 5 µm thin pieces and placed on glass slides. The slicing was done with a Microm HM 355S.

After drying the pellets for one day on a 40°C hot plate, the pellets were stained with 0,5% Alcian Blue, which stains for certain proteins present in cartilage and counterstained with 0,5% Nuclear Fast Red, which stains for cell nuclei. In order to prepare the pellets for staining, the slides were placed in Xylene twice for two minutes, 100% EtOH twice for one minute, 96% EtOH for one minute, 90% EtOH for one minute, 80% EtOH for one minute, 70% EtOH for one minute and distilled water for 10 minutes. The slides were then placed in Alcian Blue staining for 30 minutes and rinsed with tap water for 2 minutes. The pellets were then counterstained with Nuclear Fast Red for 5 minutes and rinsed with tap water for further 2 minutes. Then the pellets were dehydrated in 96% EtOH twice for two minutes, 100% EtOH twice for two minutes and Xylene twice for two minutes. The cells were then fixed using a mounting medium (Tissue Tek Glas, Centaur Europe).

2.4 Adipogenesis differentiation assay

Hamstring and ACL cells were plated in 24 well plates. A total amount of 25.000 cells were seeded in each well, where 4 wells were used for the control and 4 wells were used for adipogenic conditions. Before reaching confluency the cells were cultured in the presence of basic ligament medium. After confluency was reached, the control cells were exposed further to basic ligament medium, while the cells investigated for adipogenesis were exposed to basic ligament medium plus 0,5M 3-isobutyl-1-methylxanthine (IBMX), 0,2M Indomethacin, $10e^{-6}$M DEX and 10 µg/mL Insulin. The medium was changed every 3-4 days and the cells were cultured up to 21 days in these conditions.

For the staining, 0,3% Oil Red O (Sigma) was used to stain for lipids. In order to stain the cells for adipogenesis, the cells were washed with PBS and then fixed with 10% formalin for 10-15 minutes. After fixation, the cells were washed once with PBS and twice with demi water. 50% Isopropanol was added for 5 minutes to each well followed by staining with Oil Red O solution for another 5 minutes. After that, the excess stain was washed away with distilled water and photos with a bright field microscope were taken.

2.5 Colony forming unit assay

Hamstring and ACL cells were plated in T25 flask. A total amount of 100 cells were seeded in each flask and cultured in basic ligament medium for 14 days, with medium exchange every 5-7 days.

For the staining, 0,5% Crystal Violet has been used. In order to stain the cells for colonies, the cells were washed with PBS and then fixed with 10% formalin for 10-15 minutes. After fixating, the cells were washed once with PBS and twice with demi water. The cells were stained with Crystal Violet for 5 minutes, and excess staining was washed away with distilled water.

2.6 PCR assay

Hamstring and ACL cells were plated in 6 well plates. A total amount of 10.000 cells were seeded in each well. Basic ligament medium was used and the medium was changed every 2-3 days for 7 days. After 7 days, the cells were washed two times with PBS and frozen at -80°C for later RNA isolation and PCR measurements.

RNA was isolated according to the protocol of the NucleoSpin® RNA II kit from Macherey Nagel (Germany). RNA amount and purity was measured with a ND-1000 Spectrophotometer from Thermo Fisher Scientific (US). The same amount of RNA was reverse transcribed into a cDNA. Quantitative real time polymerase chain reaction was performed with the iCycler from BioRad (UK).

Tendon- and ligament cells produce high levels of collagen I and III, therefore collagen I and collagen III were used as target genes. Beta-2 Microglobulin (B2M) was used as a reference gene, since B2M is expressed in every nucleated cell in the human body[15]. A comparison between collagen I and III expression for both hamstring- and ACL donors will be made, as well as a comparison between collagen I and III expression from cells which have outgrown from the tissue and cells which were digested.

2.7 ALP assay

Hamstring and ACL cells were plated in 6 well plates. A total amount of 10.000 cells were seeded in each well, where 3 wells were used for the control, 3 wells were used for mineralisation in the presence of DEX and 3 wells were used for mineralisation in the presence of BMP2. Before reaching confluency the cells were cultured in the presence of basic ligament medium. After confluency was reached, the cells in the control wells were further exposed to basic ligament medium; the cells in the mineralisation wells were exposed either to basic ligament medium plus $10e^{-8}$M DEX or to basic ligament medium plus 100 ng/mL BMP2. The medium was changed every 3-4 days and the cells were cultured up to 7 days in these conditions.

After 7 days fluorescence-activated cell sorting (FACS) was performed to determine if ALP, an early marker for mineralisation was present.

2.8 Fluorescence-activated cell sorting

To characterise the surface markers of the ACL- and hamstring cells and the potential of the cells to form bone, fluorescence-activated cell sorting (FACS) was performed in combination with certain types of antibodies. Markers for stem cells and early mineralisation markers were used to characterise the cells. CD73 PE, CD90 PE, CD105 PE, CD271 PE, CD146 PE, CD166 PE, CD200 PE, CD29 PE, were used as positive markers for stem cells[16,17,18,19,20,21]; while CD34 PE, CD45 PE, CD11bèta PE, CD14 PE, CD19 PE, CD79alpha PE and HLA-DR PE were used as negative markers for stem cells[16]; PE Mouse IgG control and Mouse IgG2a PE were used as isotypes to rule out unspecific binding. ALP antibody binding was used as early mineralisation marker.

Cells from donor H2-2 were investigated for stem cell surface markers. Cells were seeded at a density of 1000 cells/cm^2 in 2x T300 flasks. When confluency was reached the cells were washed with 2 mL Dulbecco's PBS, trypsinised with 5 mL Trypsin, and resuspended in 1,5 mL Block Buffer (BD BioSciences) The block buffer was used to prevent unspecific binding from proteins. After resuspension for each antibody 1,5 mL of cell suspension was pipetted into a glass tube in duplicate. Cells were then centrifuged at 3000 rcf for 2 minutes and resuspended in washing buffer containing the specific antibody. The cells were let one hour for incubation with the antibody. After incubation the cells were washed with 2 mL of Washing Buffer (BD BioSciences) and centrifuged at 3000 rcf for 2 minutes at 4°C. The washing step was repeated 3 times. In the end the cells were ready for FACS. The FACS was done with the FACSCalibur from BD BioSciences; 10.000 cells were counted and plotted.

For the early mineralisation markers, the ALP cells were used. After 7 days of adding the medium, the cells were washed with 2 mL Dulbecco's PBS, trypsinised with 500 µL Trypsin, and resuspended in 1,5 mL Block Buffer. After resuspending, the cells were put in tubes (1 tube per well containing 1,5 mL cell suspension, 3 tubes per differentiation plus 1 tube ISO, containing 500 µL cell suspension per well). The cells were centrifuged at 3000 rcf for 2 minutes, the block buffer and trypsin was taken out, the cells were resuspended, and the anti-ALP B4-78 antibody was added for 1 hour. After 1 hour, 2 mL Washing Buffer was added, the cells were centrifuged at 3000 rcf for 2 minutes at 4 degrees Celsius, the washing buffer plus antibody were taken out, and the goat anti-mouse IgG conjugated with phycoerythrin antibody was added for another 30 minutes. After that, washing buffer was added, the cells were centrifuged at 3000 rcf for 2 minutes at 4 degrees Celsius, the washing buffer plus antibody were taken out, and this step was repeated 3 times in total. After the 3rd time, 300 µL

of washing buffer was added after resuspending the cells, and the samples were ready for the FACS. The FACS was done with the FACSCalibur from BD BioSciences; 10.000 cells were counted and plotted.

3. Results

3.1 Hamstring cells

Mineralisation

After exposing the cells for up to 28 days to mineralisation media containing either DEX or BMP2 and after staining with Alizarin Red no matrix mineralisation of the cells was observed either in H2-1 or H1-2 (Figure 7a, Figure 8a-c and supplementary data A). Figure 7a and Figure 8a-c are representative for H1-2 as well. In contrast, Hamstring H6-4 showed matrix mineralisation in the presence of DEX but not in the presence of BMP2 (Figure 7b and Figure 8e and f). Moreover it is to observe that cells exposed to basic ligament media alone and to basic ligament media plus DEX detach easily from the plate before reaching the 28 days of culture.

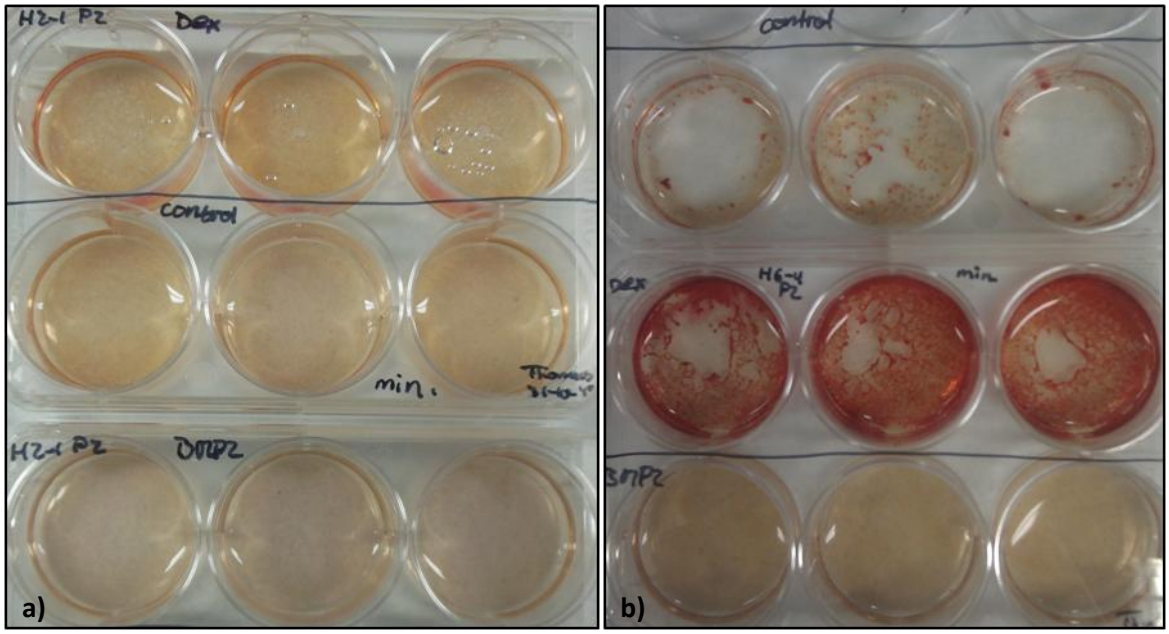


Figure 7 – Alizarin Red staining of H2-1 and H6-4. H2-1 cells a) cells exposed to mineralisation media in the presence of DEX (top), to control mineralisation media (middle) and to mineralisation media in the presence of BMP2 (bottom); and H6-4 cells b) exposed to control mineralisation media(top), to mineralisation media in the presence of DEX (middle) and to mineralisation media in the presence of BMP2 (bottom).

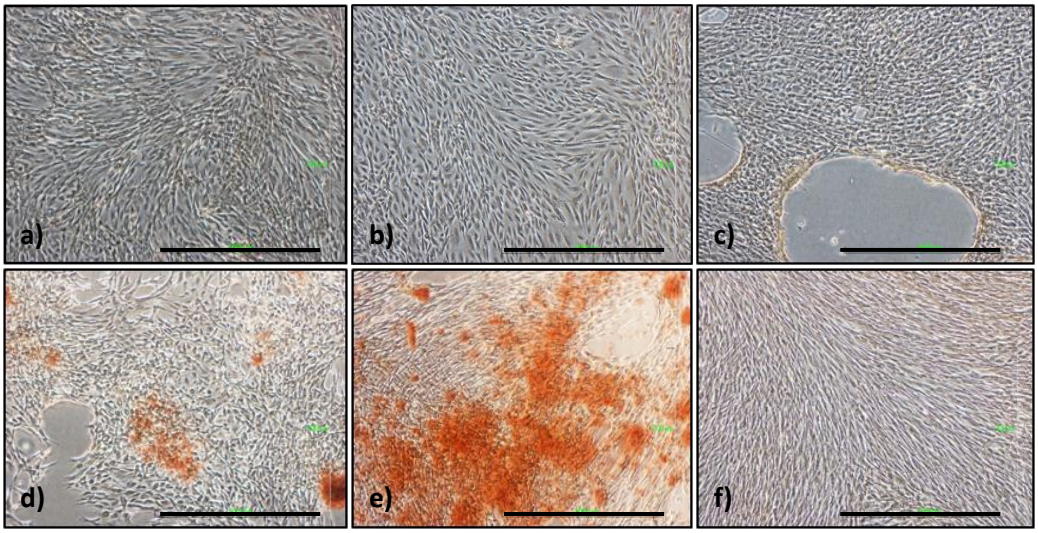


Figure 8 – Bright field microscope images after staining with Alizarin Red for mineralisation of H2-1 and H6-4. H2-1 cells exposed to a) control mineralisation media; b) mineralisation media in the presence of DEX; c) mineralisation media in the presence of BMP2. H6-4 cells exposed to d) control mineralisation media; e) mineralisation media in the presence of DEX; f) mineralisation media in the presence of BMP2. Scale bar: 1000 µm.

Chondrogenesis

After exposing H1-2, H2-1 and H6-4 cells for 28 days to chondrogenic media no cartilage-like tissue was observed in the pellets after staining with Alcian Blue (see Figure 9b and d). Figure 9c and d are representative for H2-1 and H6-4. During media change of H1-2 cells exposed to chondrogenic differentiation media, one of the pellets was accidently destroyed on the 7th day of culture. During the accident part of the cells were aspirated while the rest were heterogeneous distributed on the bottom of the plate. Interestingly, the pellet reassembled itself after 3 days from the accident (see Figure 10a-b). It is also to observe that the size of the reassembled pellet was significantly smaller than the pellets which were not destroyed (see Figure 10b-c). Moreover, the same accident happened in one pellet exposed to basic ligament media but no reassemble potential was observed.

After staining the pellets with Alcian blue no cartilage like tissue was observed in the pellets exposed to chondrogenic differentiation media compared to the pellets exposed to basic ligament media (see Figure 9a-d). Interestingly, the destroyed and reassembled pellet showed cartilage-like structure.

Looking at the morphology of the pellets, it is to observe that the size of the pellet is donor dependent. H1-2 pellets are bigger, containing cells with round nuclei in comparison to H2-1 and H6-4, where the pellets are smaller and the nuclei oval shaped. Also H1-2 pellets show uniform shaped tissue while the H2-1 and H6-4 show more fibrous-like tissue, especially near the edges of the pellets.

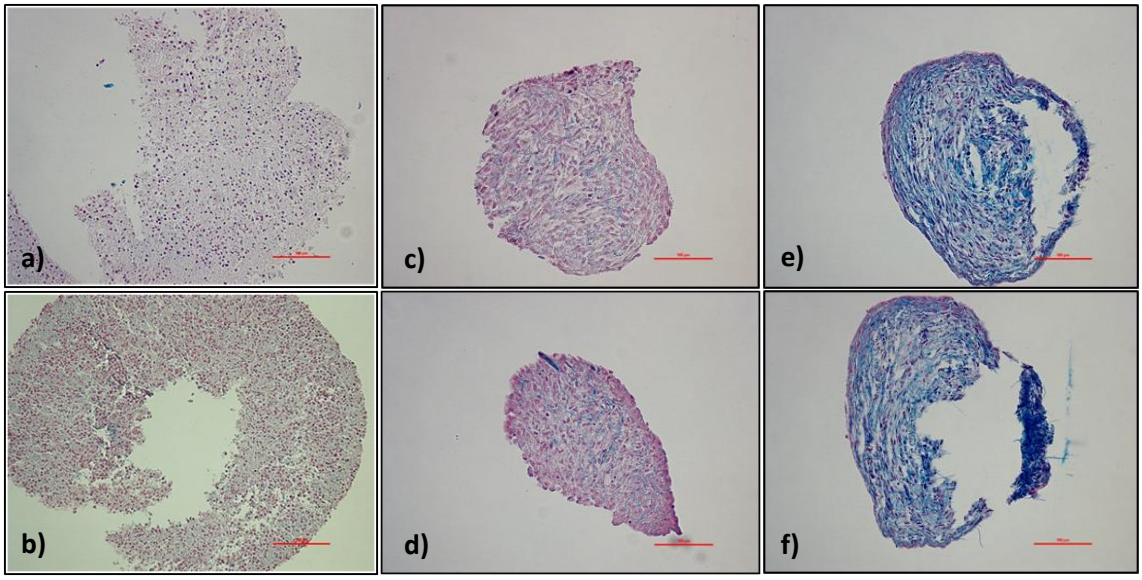


Figure 9 – Alcian Blue based staining of H1-2 and H2-1. H1-2 cells a) cells exposed to control chondrogenic media. b) cells exposed to chondrogenic media. H2-1 cells c) H2cells exposed to control chondrogenic media. d) cells exposed to chondrogenic media. H1-2 destroyed pellet e) f) cells exposed to chondrogenic media. Scale bar: 100 µm.

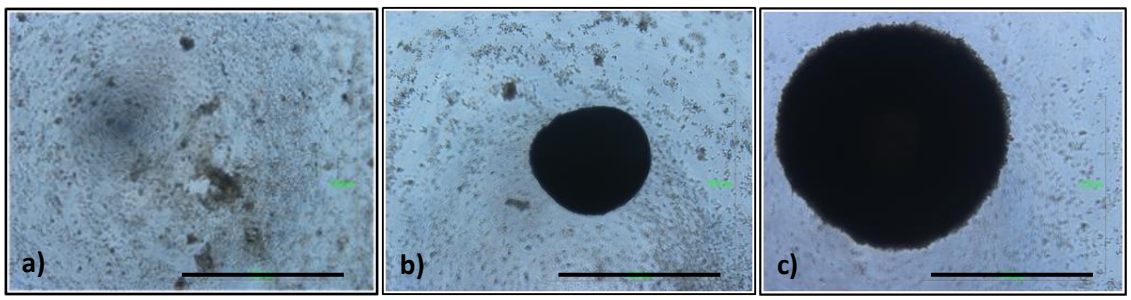


Figure 10 – Donor H1-2 chondrogenic pellets 3 days after two of them were destroyed. a) Destroyed pellet in the control well. b) The regenerated pellet in the differentiation well, smaller than the original pellet. c) An undistorted pellet in a differentiation well, clearly bigger than the regenerated pellet in b). Scale bar: 1000 µm.

Adipogenesis

After exposing the cells to adipogenic media for up to 21 days lipid droplets were observed in all donors. H1-2 and H2-1 (see Figure 11c and d) showed a higher amount of lipid droplets than H6-4 (see Figure 11e and f). Figure 11a-d is representative for both H1-2 and H2-1. The cells in the middle of the well appear to be more round-shaped than the cells near the edge of the well.

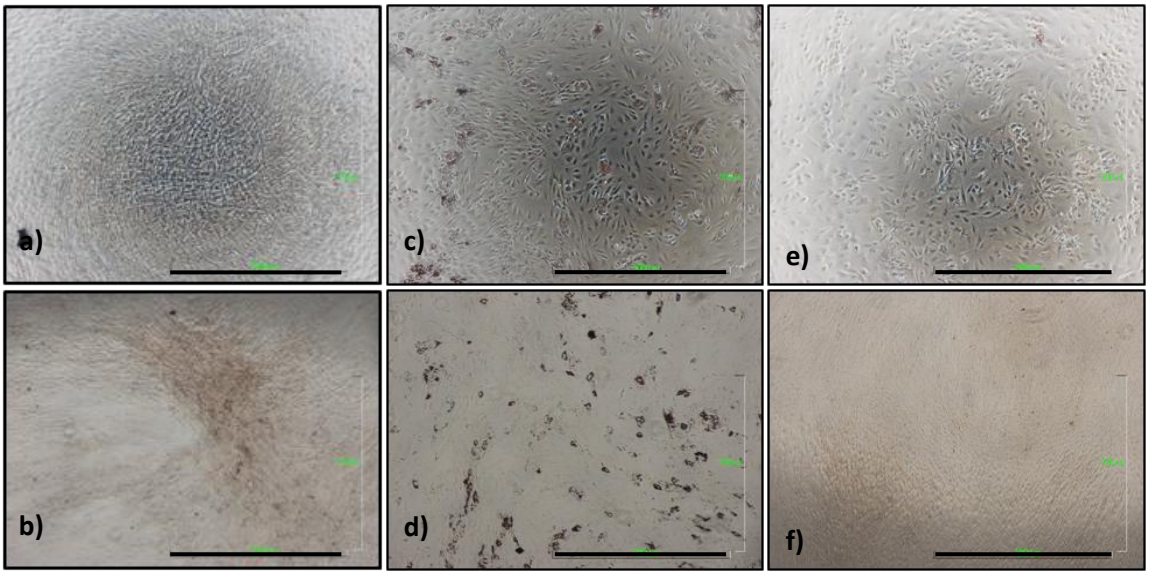


Figure 11 – Bright field microscope image from Oil Red O staining of H2-1 and H6-4. H2-1 cells a) cells exposed to control adipogenic media in the centre of the well. b) cells exposed to basic adipogenic media near the edge of the well. H6-4 cells c) cells exposed to adipogenic media in the centre of the well d) cells exposed to adipogenic media near the edge of the well. H6-4 cells. e) cells exposed to adipogenic media in the centre of the well. f) cells exposed to adipogenic media near the edge of the well. Scale bar: 1000µm.

CFU

After exposing the cells for 7 days to basic ligament media and after staining with Crystal Violet colony formation was observed in both H2-1 and H6-4 (see Figure 12), although more colonies were found in H2-1 than in H6-4. Unfortunately the cells from H1-2 were lost during culturing. The cells were observed to form star-shaped colonies (see Figure 13).

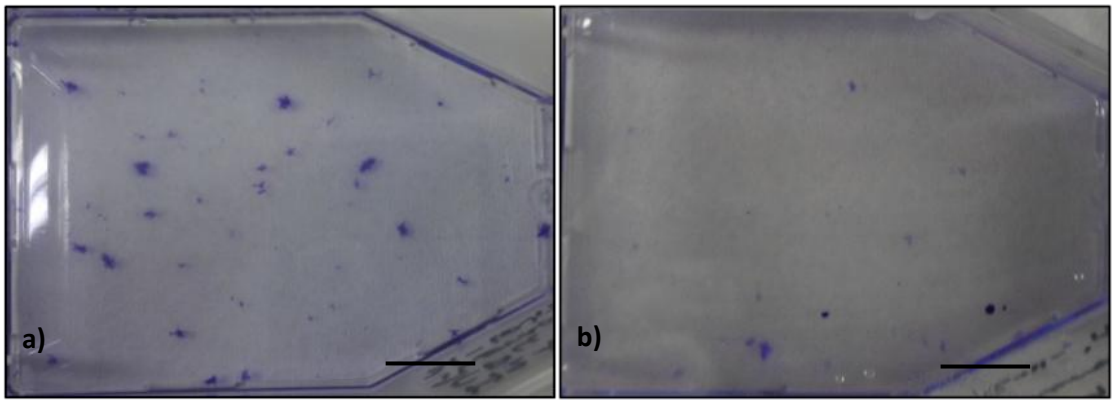


Figure 12 – Crystal Violet staining of H2-1 and H6-4. a) H2-1. b) H6-4. Scale bar: 1000 µm.

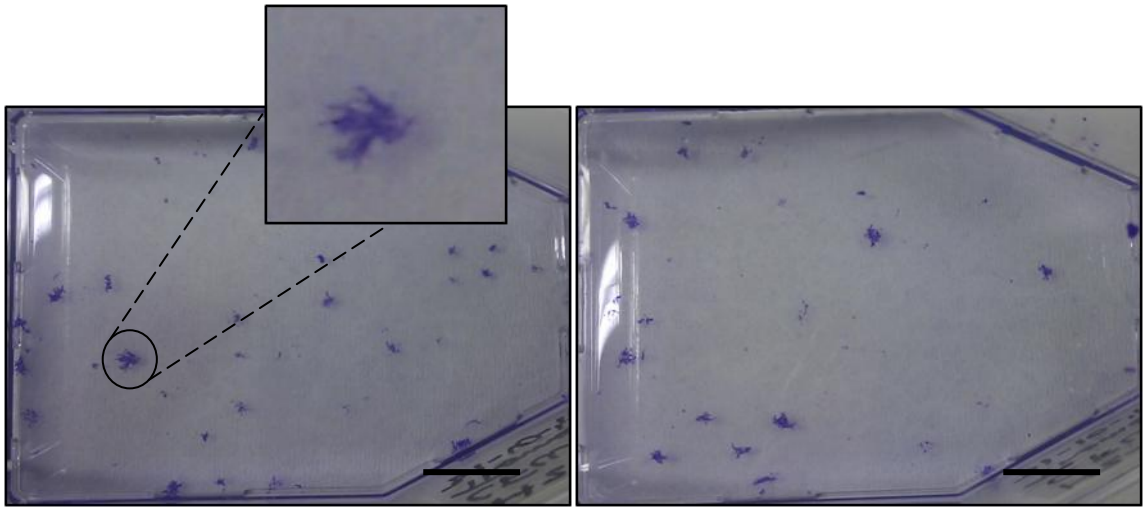


Figure 13 – Crystal Violet staining of H2-1. Donor H2-1 shows clearly visible, star-shaped colonies. Inset: close-up from one of the colonies. The star shape is clearly visible. Scale bar: 500 µm.

FACS

FACS for H2-2 was done in order to see if the hamstring cells contained stem cell surface markers. Cells from H2-2 were used in this procedure. 17 Antibodies were used, which are listed in

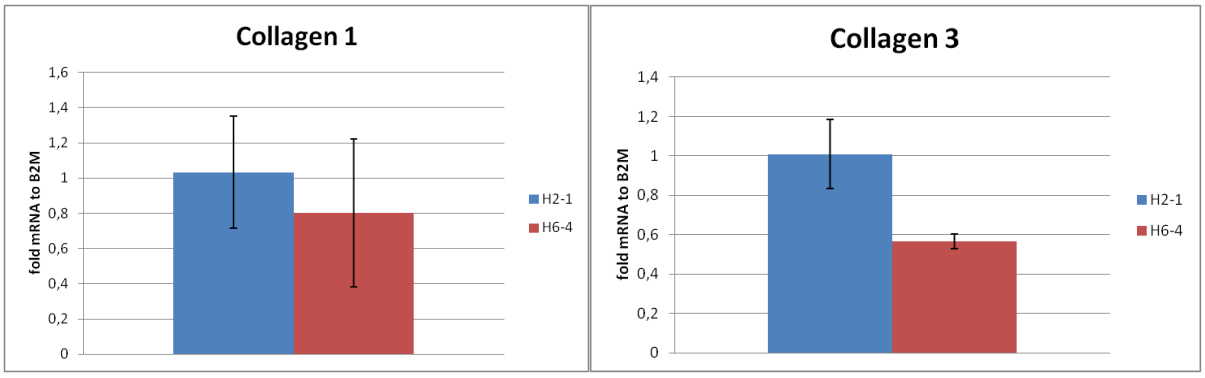
Table 2. The corresponding graphs from the FACS can be found in the supplementary data B.

Positive control	Percentage	Tested	Negative control	Percentage	Tested
CD73 PE	60,28	o	CD34 PE	0,62	-
CD90 PE	96,81	+	CD45 PE	1,57	-
CD105 PE	84,37	o	CD11bèta PE	0,58	-
CD271 PE	0,73	-	CD14 PE	0,24	-
CD146 PE	4,85	-	CD19 PE	0,56	-
CD166 PE	3,53	-	CD79alpha PE	0,53	-
CD200 PE	0,45	-	HLA-DR PE	0,08	-
CD29 PE	0,14	-	Mouse IgG PE	0,86	-
			Mouse IgG2a PE	0,22	-

Table 2 – Antibodies used to test for stem cell markers. Threshold was set at 5%, meaning that everything below 5% is a negative control, and everything above 95% is a positive control. The mean value was taken for the percentages.

PCR

After exposing the cells up to 7 days to basic ligament media the cells were used to test for the presence of collagen type I and III, which are markers for tendon and ligament cells. In Graph 1 the relative expression of H6-4 against H2-1 is given; the cells from H1-2 were unfortunately lost during culturing. H6-4 shows a 1,25-fold decrease for collagen I relative to H2-1 and a 1,75-fold decrease for collagen III relative to H2-1.



Graph 1 – Collagen I and collagen III expression from H2-1 relative to H6-4.

3.2 ACL cells

Mineralisation

After exposing the cells for up to 28 days to mineralisation media containing either DEX or BMP2 and after staining with Alizarin Red no matrix mineralisation of the cells was observed in all four ACL donors (see Figure 14). Figure 14 is representative for all four ACL donors. Detachment of the cells in the DEX-based differentiation well in D3 was visible (see Figure 15).

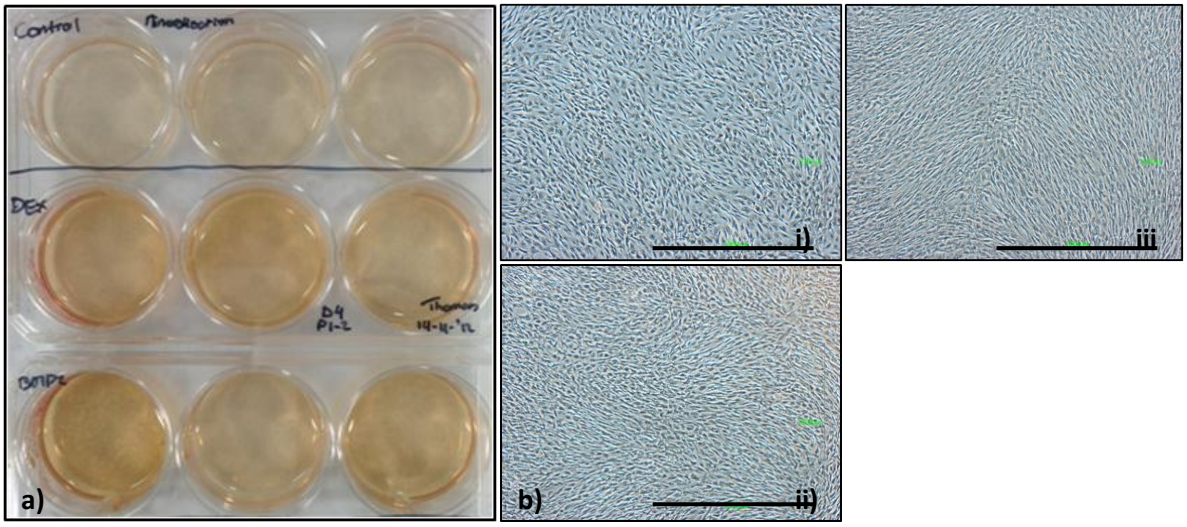


Figure 14 –Alizarin Red staining of D4. a) cells exposed to control mineralisation media (top), to mineralisation media in the presence of DEX (middle), to mineralisation media in the presence of BMP2 (bottom). b) bright field microscope image from the cells exposed to i) control mineralisation media; ii) mineralisation media in the presence of BMP2; iii) mineralisation media in the presence of DEX. Scale bar: 1000 µm.

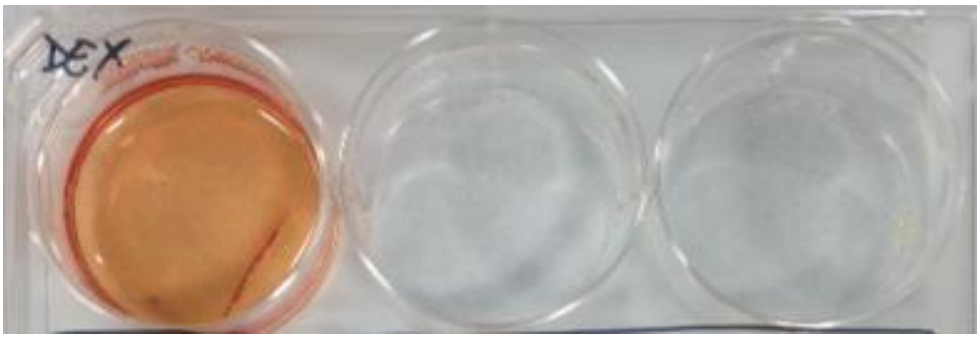


Figure 15 – Detachment of the DEX-based differentiation wells in donor D3. Two out of three wells have completely detached, and the beginning of cell detachment can be seen in the first well in the lower right part.

Chondrogenesis

After exposing O3, O4, D3 and D4 cells for 28 days to chondrogenic media and after staining with Alcian Blue, cartilage-like tissue was observed in the pellets exposed to chondrogenic differentiation media compared to pellets exposed to basic ligament media (see Figure 16). Figure 16a-d is representative for all ACL donors. The nuclei from all donors have shown to be elongated and oval-shaped, and the pellets show fibrous-like tissue near the edges.

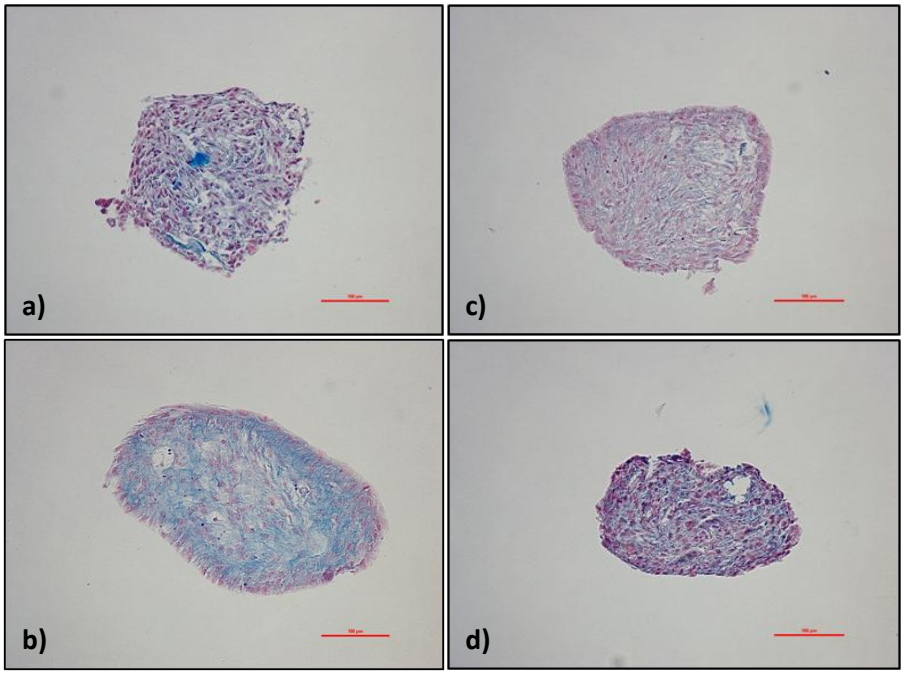


Figure 16 – Alcian Blue based staining of D4 and O3. D4 cells a) cells exposed to control chondrogenic media. b) cells exposed to chondrogenic media. O3 cells c) cells exposed to control chondrogenic media. d) cells exposed to chondrogenic media. Scale bar: 100µm.

Adipogenesis

After exposing the cells to adipogenic media for up to 21 days lipid droplets were observed in all donors. Figure 17 is representative for all four donors. It appeared that the cells were more likely to form lipid droplets near the edge of the well compared to the middle of the well. The cells near the middle of the well appear to be more round-shaped compared the cells near the edge of the well.

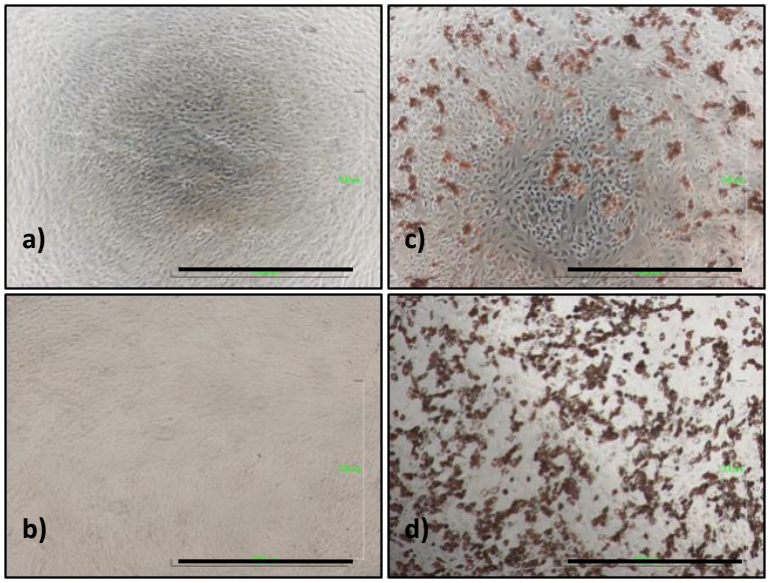


Figure 17 – Bright field microscope image from Oil Red O staining of D3. D3 cells a) cells exposed to control adipogenic media in the middle of the well; b) cells exposed to control adipogenic media near the edge of the well; c) cells exposed to adipogenic media in the middle of the well; d) cells exposed to adipogenic media near the edge of the well. Scale bar: 1000µm.

CFU

After exposing the cells for 7 days to basic ligament media and after staining with Crystal Violet, colony formation was observed in all four ACL donors (see Figure 18) However, ACL D4 has shown to form less colonies than the other donors. The cells were observed to form star-shaped colonies (see Figure 19).

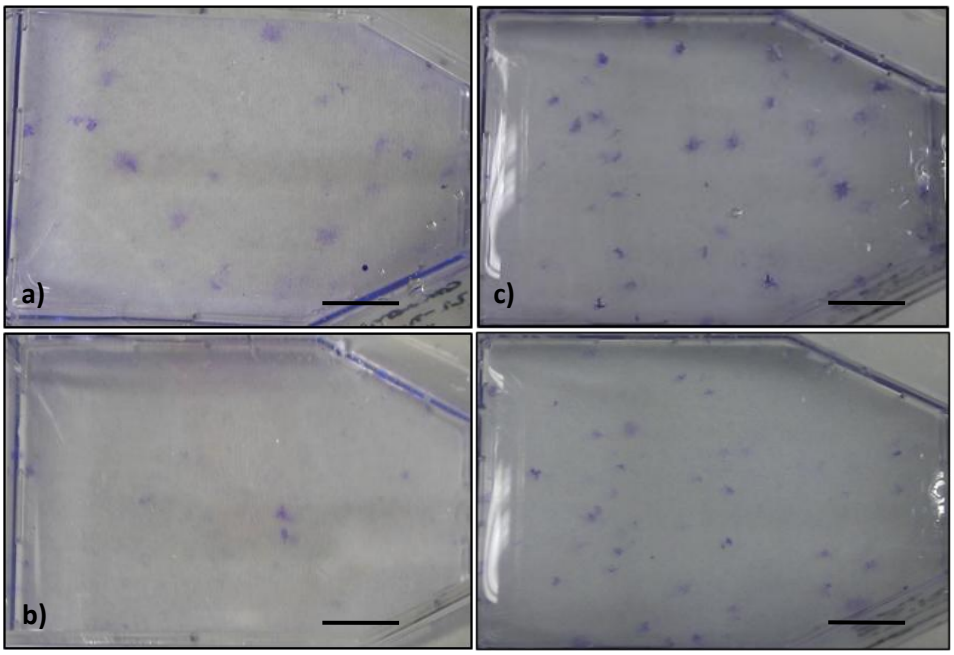


Figure 18 – Crystal Violet staining of ACL a) D3. b) D4. c) O3. d) O4.

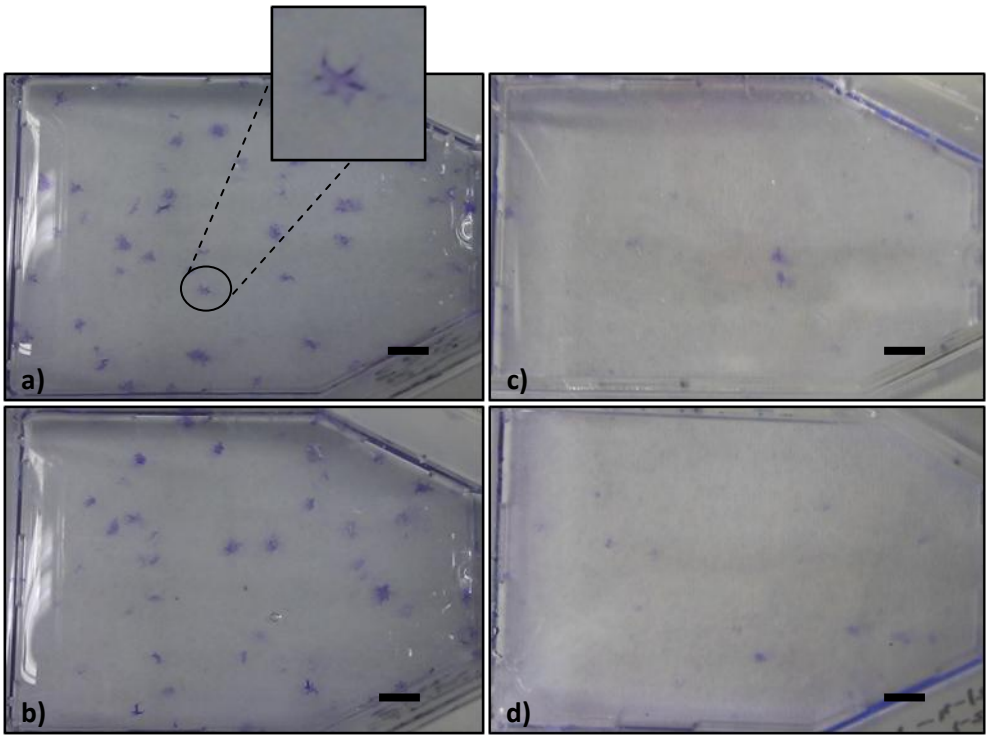


Figure 19 – a) and b) Donor ACL O3 shows clearly visible, star-shaped colonies. Most colonies are smaller than 3 mm. Inset in a): close-up from one of the colonies. The star shape is clearly visible. c) and d) Fewer colonies are formed in donor ACL D4. Scale bar: 500 µm.

FACS

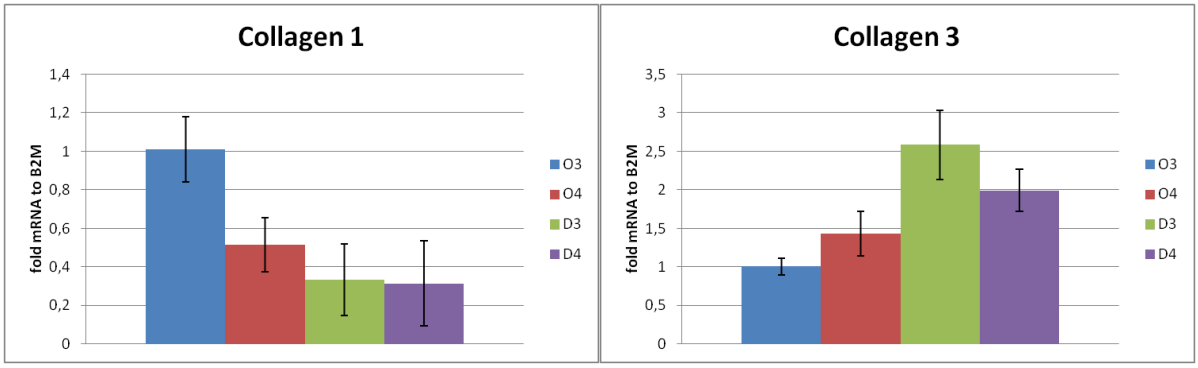
The FACS was done for all ACL donors for Alkaline Phosphatase (ALP), to see if early markers for mineralisation were present. Two antibodies, anti-ALP B4-78 and goat anti-mouse IgG conjugated with phycoerythrin, were used The anti-ALP B4-78 reacts with the ALP-receptor, and the goat anti-mouse IgG conjugated with phycoerythrin attaches to the anti-ALP B4-78 antibody and contains the fluorescent. If the antibodies tested positive on the cells, early mineralisation markers were present. In Table 3 the donors are listed with the outcome of the FACS. An Iso-control was used in order to make sure no unspecific protein binding occurs. The complementary graphs can be found in the supplementary data C.

Donor	Pct.	Tested	Donor	Pct.	Tested
D3 Control[†]	x	x	O3 Control	1,41	-
D3 DEX	1,48	-	O3 DEX	2,71	-
D3 BMP2	1,42	-	O3 BMP2	3,82	-
D4 Control	5,46	o	O4 Control	1,42	-
D4 DEX	25,19[‡]	-	O4 DEX	1,99	-
D4 BMP2	7,91[§]	-	O4 BMP2	3,00	-

Table 3 – Outcome of the FACS in which the cells were tested for ALP-presence. The threshold was set at 5%, meaning that everything below the 5% counts as a negative and everything above the 95% counts as a positive. The mean value was taken for the percentages.

PCR

After exposing the cells up to 7 days to basic ligament media the cells were used to test for the presence of collagen type I and III, which are markers for ligament and tendon cells. The goal of the PCR was to check if there is a significant difference between cells which were outgrown from the tissue and cells which were digested from the tissue and how one donor relates to the other. In Graph 2 the relative expression of ACL O4, D3 and D4 to O3 is shown. For collagen I, ACL O4 shows a 1,95-fold decrease relative to ACL O3, ACL D3 shows a 3,00-fold decrease relative to ACL O3 and ACL D4 shows a 3,19-fold decrease relative to ACL O3. For collagen II, ACL O4 shows a 1,43-fold increase relative to ACL O3, ACL D3 shows a 2,59-fold increase relative to ACL O3 and ACL D4 shows a 1,99-fold increase relative to O3.



Graph 2 – Collagen I and collagen III expression from O3 relative to O4, D3 and D4.

[†] Due to mechanical problems, the D3 Control cells were lost in the process.
[‡] Iso-control gave a value of 38,56; therefore this result is negative.
[§] Iso-control gave a value of 11,76; therefore this result is negative.

4. Discussion

In this study it was investigated whether hamstring- and ACL cells had the potency to differentiate, as well as if ALP- and stem cell markers were present on the cell surface and if differences between collagen I and collagen III expression were present between the donors and between the hamstring- and ACL cells.

Differentiation Assay

Not all the donors show mineralisation potential after staining with Alizarin Red. There was a difference visible between the cells from the fresh tissue and from the tissue from which the cells were frozen first. De Mos et al. has showed that freshly acquired hamstring cells have the potential to form matrix mineralisation in the presence of DEX[22], which is in accordance to our study. The cells of Hamstring H6-4 were not frozen prior experiment and they were the only cells showing mineralisation potential. Therefore, more research has to be done to investigate if the freezing of cells might have an influence on differentiation potential of the cells. Steinert et al. have shown that freshly acquired ACL cells have the potential to form matrix mineralisation[25], while in this study no matrix mineralisation of the ACL cells was observed. Furthermore hamstring cells have shown to detach easily due to overconfluency. Only the cells growing in the presence of DEX were not detaching. This might be due to the fact that cells grow slower in the presence of DEX, therefore reaching overconfluency later, or to the fact that cells in the presence of DEX produce more protein chains which attach to the plastic, and therefore are less likely to detach. In contrast, ACL cells were not detaching; with exception of donor D3, where the cells detached in the presence of DEX. It is yet unknown why this is, this also has yet to be investigated.

Furthermore, no clearly visible cartilage-like tissue was formed in the hamstring cells, while in the ACL cells slight cartilage-like tissue was found after staining with Alcian Blue. Due to the accidental destruction of one of the pellets from both the control and the chondrogenesis wells in donor H1-2, the control pellet was observed not to reform itself, while the chondrogenic pellet was able to reform itself after 3 days (see Figure 10). The regenerated pellet has also shown significantly higher amounts of cartilage-like tissue (see Figure 9). This might be due to the fact that when the pellet was destroyed, only the cells with differentiation potential clustered together, while the cells which didn't have this potential were ruled out in this process because they didn't have this potential (Figure 10a and b). To be sure this is not a onetime coincidence, farther investigation will be done in the near future. The morphology from the pellets differs as the cells itself have shown to be different from donor to donor. As can be seen in Figure 9a and c, the size of the pellet differs even though the amount of seeded cells was the same in the beginning. This might be because the cells in the control pellet were less likely to cluster together, therefore having more space to grow. The nuclei in H1-2 are also more rounded, while the nuclei in H2-1 and H6-4 are more elongated and oval-shaped. This also might be due to the fact that the cells in H1-2 have more space to grow since they don't cluster together as much as H2-1 and H6-4, and therefore are less likely to be deformed due to internal forces between the cells.

All donors have shown to form lipids in the presence of adipogenic differentiation medium, although ACL cells tend to form more lipids than hamstring cells. De Mos et al. have shown that freshly acquired hamstring cells have the potential to form lipids[22], while this research has shown that donor H6-4 forms very few lipids. It might be that this is donor-dependent, and has yet to be investigated.

The cells have also shown to have a different morphology in the centre of the well compared to the cells near the edge of the well. This might be due to the fact that when seeding the cells centrifugal forces caused by movement of the well plates after seeding the cells, pull the cells to the middle of the well, thus causing a higher concentration of cells. Therefore, the cells have less space in the middle of the well, changing their morphology.

CFU Assay

Seeding of single ACL/hamstring cells showed star shaped colonies. The colonies are slightly small in diameter. According to Steinert et al. ACL cells are likely to form round colonies bigger than 3 mm[23]; the ACL/hamstring cells however have shown not to do this in this experiments. The cells are spindle-shaped and fibroblast like, as can be expected from tendon- and ligament cells. Furthermore, no significant difference in the number of colonies was found between the hamstring- and ACL donors; it appears that the number of colonies formed is donor-dependent in both hamstring and ACL donors.

Fluorescence-activated cell sorting

The hamstring donor used for the CD-panel hasn't shown any evidence of the existence of stem cell markers on the cell surface. As can be seen in Table 2, the negative control tested negative for every antibody. However, most of the antibodies in the positive control tested negative as well, indicating that no stem cell markers are present on the cell surface. This is in contrast with G.M. van Buul et al. who state that hamstring cells possess stem cell surface markers[24].

Even though other research has shown that ACL cells contain ALP surface markers[25], no ALP surface markers tested positive in this study. This is in accordance with the results found in the ACL mineralisation differentiation assay.

PCR

We investigated whether there was a difference between collagen I and III gene expression in hamstring cells and in ACL cells. For the hamstring donors, no difference in collagen I expression appears to be present. H6-4 appears to have a lower collagen III expression than H2-1. This might be due to the fact that the cells from H6-4 were not frozen before seeding. For the ACL donors the outgrown cells express a higher level of collagen I, while for collagen III the expression is lower.

5. Acknowledgement

I would like to thank C. Ghebes (daily supervisor) for all the help given at any moment; H. Fernandes (chairperson) for all the help given at any moment; the Tissue Regeneration Department and their members for providing all their materials, equipment and help needed for this study; the University of Twente for making this study possible.

6. Supporting information

DMEM – Basic cell culture medium containing the necessary nutrients.

FBS – Serum containing very few antibodies and more growth factors than normal blood serum.

P/S – Combination of penicillin and streptomycin, used to prevent bacterial contamination.

Ascorbic acid – Basic antioxidant, preventing failure of cell division.

BGP – Osteocalcin, plays a role in the body's metabolic regulation and is pro-osteoblastic, therefore implicated in bone mineralisation.

Dexamethasone – Anti-inflammatory corticoid.

IBMX – Reduces inflammations.

Indomethacin – Anti-inflammatory drug.

Insulin – Peptide hormone, used for the regulation of fat metabolism.

ITS – Insulin, Transferrin and Selenium; insulin is used to promote glucose and amino acid uptake by the cell; transferrin is an iron transport protein which also serves to detoxify the medium from oxygen radicals and peroxidise; selenium is an enzyme cofactor that activates glutathione peroxidise, a player in the detoxification of oxygen radicals.

Proline – Non-essential amino acid.

TGFβ3 – Cell growth factor.

DMSO – Polar aprotic solvent, used to dissolve different organic compounds.

BMP2 – Protein which plays an important role in the formation of bone and cartilage.

PBS – Phosphate buffered saline, containing sodium chloride and -phosphate which helps the cells to maintain a constant pH. Used to wash the cells with to remove nutrients from the medium.

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Supplementary data A

Alizarin Red staining of H1-2. Detachment of the cells is visible in Figure 1a in the dexamethasone-based differentiation wells (top) and in the control wells (bottom).

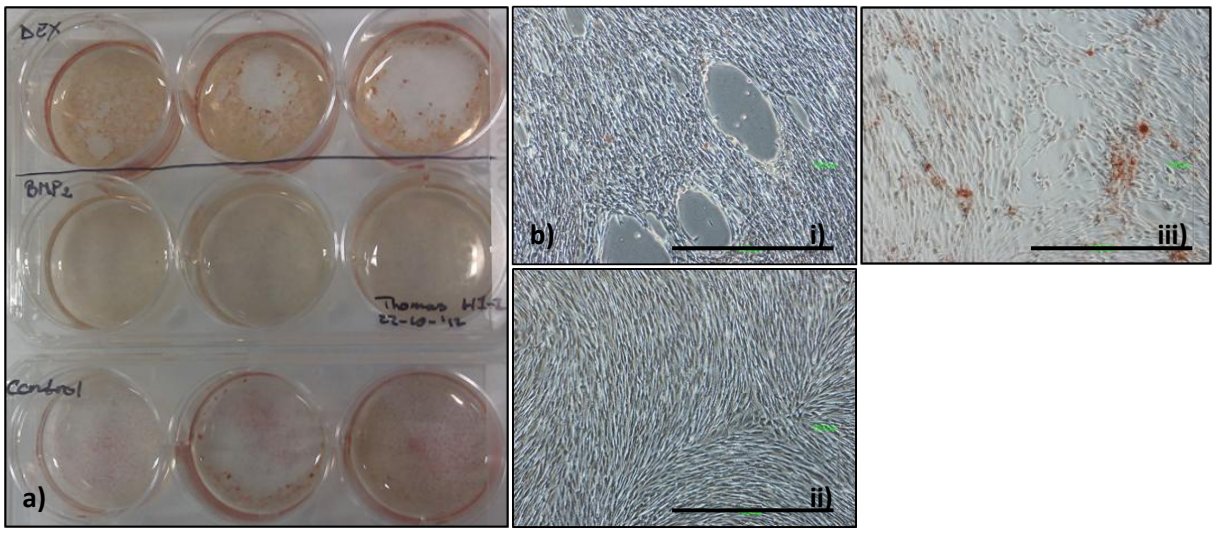
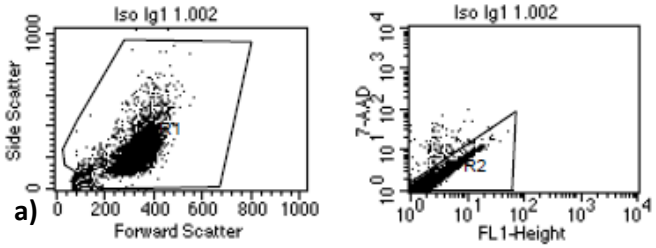
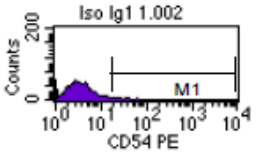


Figure 1 – Alizarin Red staining of H1-2. a) cells exposed to mineralisation media in the presence of DEX (top), to mineralisation media in the presence of BMP2 (middle), to control mineralisation media (bottom). b) bright field microscope image from the cells exposed to i) mineralisation media in the presence of DEX; ii) mineralisation media in the presence of BMP2; iii) control mineralisation media. Scale bar: 1000 µm.

Supplementary data B

a)





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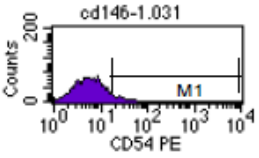

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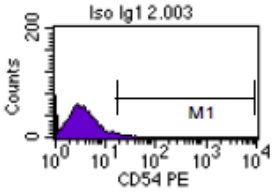

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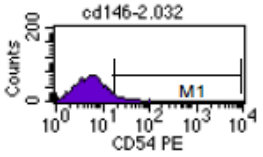

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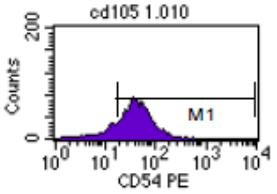

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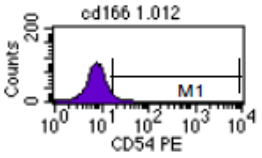

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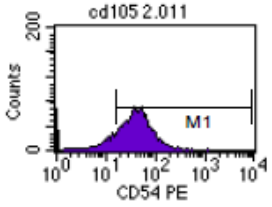

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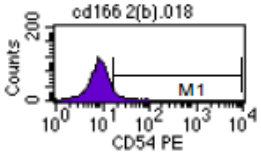

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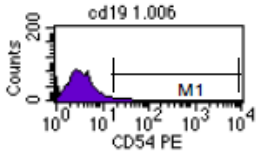

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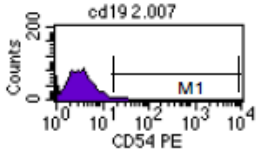

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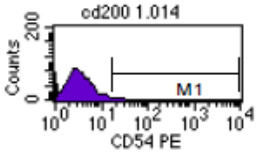

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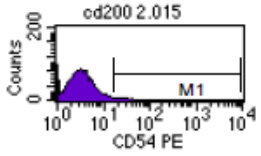
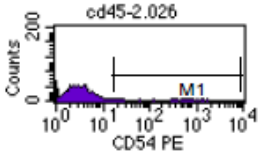
All	100.00
M1	0.28



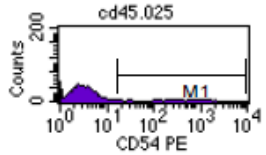
Sample ID: cd200 2
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	0.61



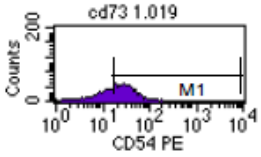
Sample ID: cd45-2
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	1.01



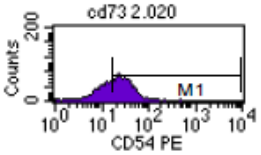
Sample ID: cd45
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	2.12



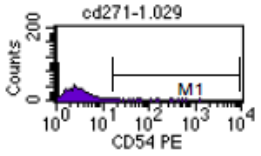
Sample ID: cd73 1
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	62.90



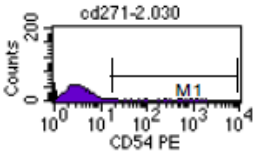
Sample ID: cd73 2
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	57.65



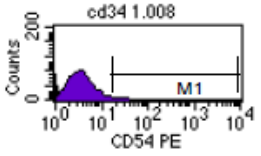
Sample ID: cd271-1
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	0.73



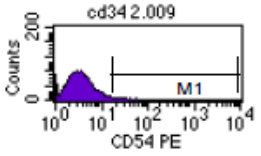
Sample ID: cd271-2
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	0.73



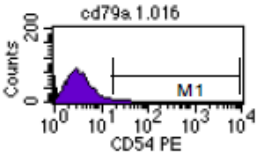
Sample ID: cd34 1
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	0.55



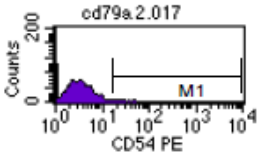
Sample ID: cd34 2
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	0.68



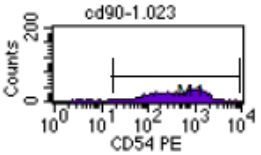
Sample ID: cd79a 1
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	0.55



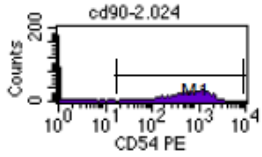
Sample ID: cd79a 2
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	0.51



Sample ID: cd90-1
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	99.59



Sample ID: cd90-2
Acquisition Date: 07-Nov-12

Marker	% Gated
All	100.00
M1	94.02

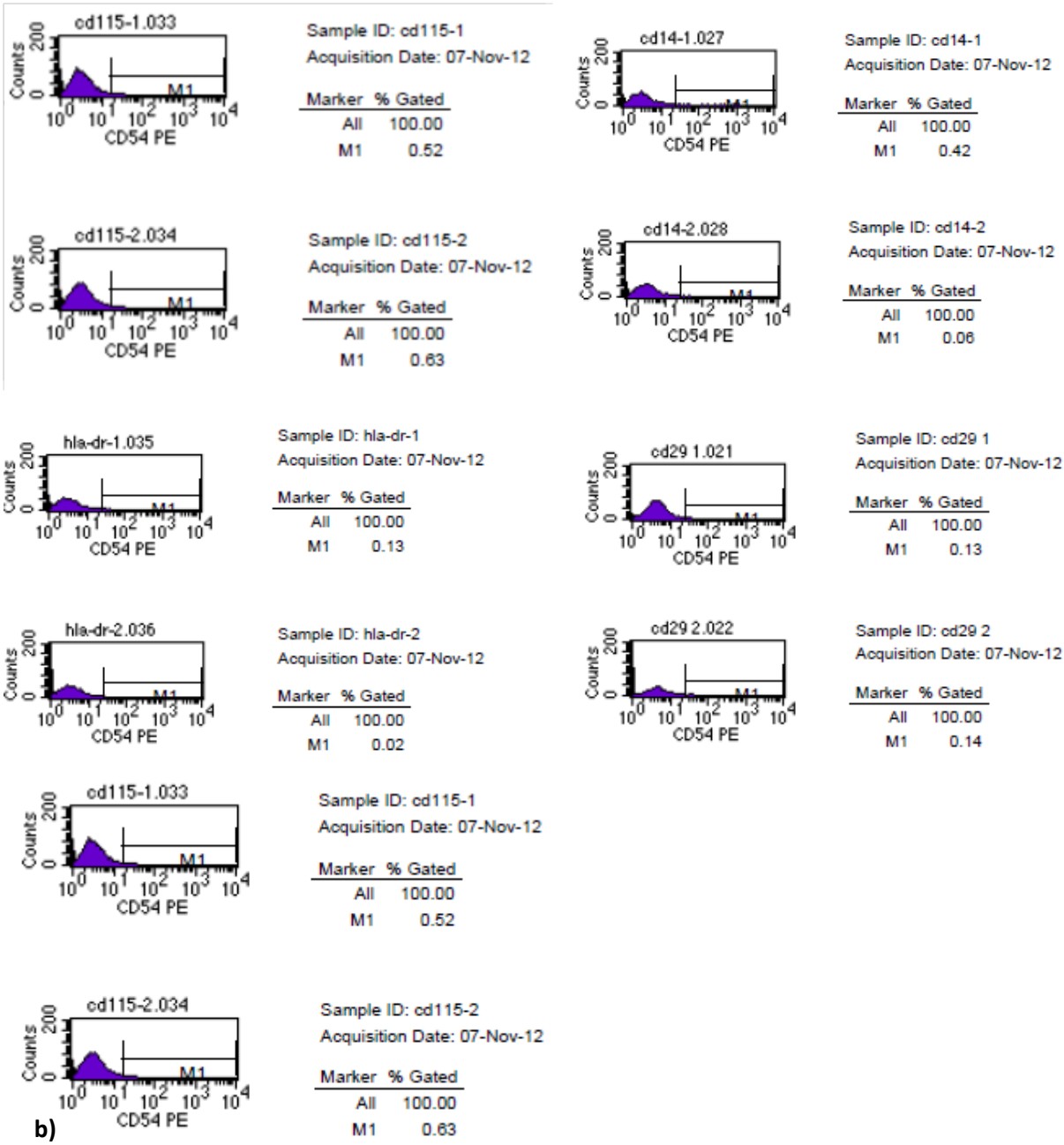


Figure 2 – FACS analysis of H2-2 for stem cell markers. a) Threshold was set to exclude dead cells and cell debris. b) different antibodies tested: M1 is set as threshold for the positive area; name of the antibody is above the graph and the FACS was done in duplicates.

Supplementary data C

Graphs from the ALP FACS antibody experiment. The graphs are ordered in such a way that the wells are in groups of four: the cells treated with the ISO-antibody are in the upper left corner. The numbers with their corresponding donor can be found in

Table 1. Unfortunately, some of the tubes from donor D3 were lost during the experiment (shaded red).

Number	ACL O3	Number	ACL O4	Number	ACL D3	Number	ACL D4
1	C1	13	C1	25	C1	37	C1
2	C2	14	C2	26	C2	38	C2
3	C3	15	C3	27	C3	39	C3
4	C ISO	16	C ISO	28	C ISO	40	C ISO
5	D1	17	D1	29	D1	41	D1
6	D2	18	D2	30	D2	42	D2
7	D3	19	D3	31	D3	43	D3
8	D ISO	20	D ISO	32	D ISO	44	D ISO
9	B1	21	B1	33	B1	45	B1
10	B2	22	B2	34	B2	46	B2
11	B3	23	B3	35	B3	47	B3
12	B ISO	24	B ISO	36	B ISO	48	B ISO

Table 1 – Numbers corresponding with the numbers in the graph. C = control well; D = dexamethasone-based differentiation well; B = BMP2-based differentiation well.

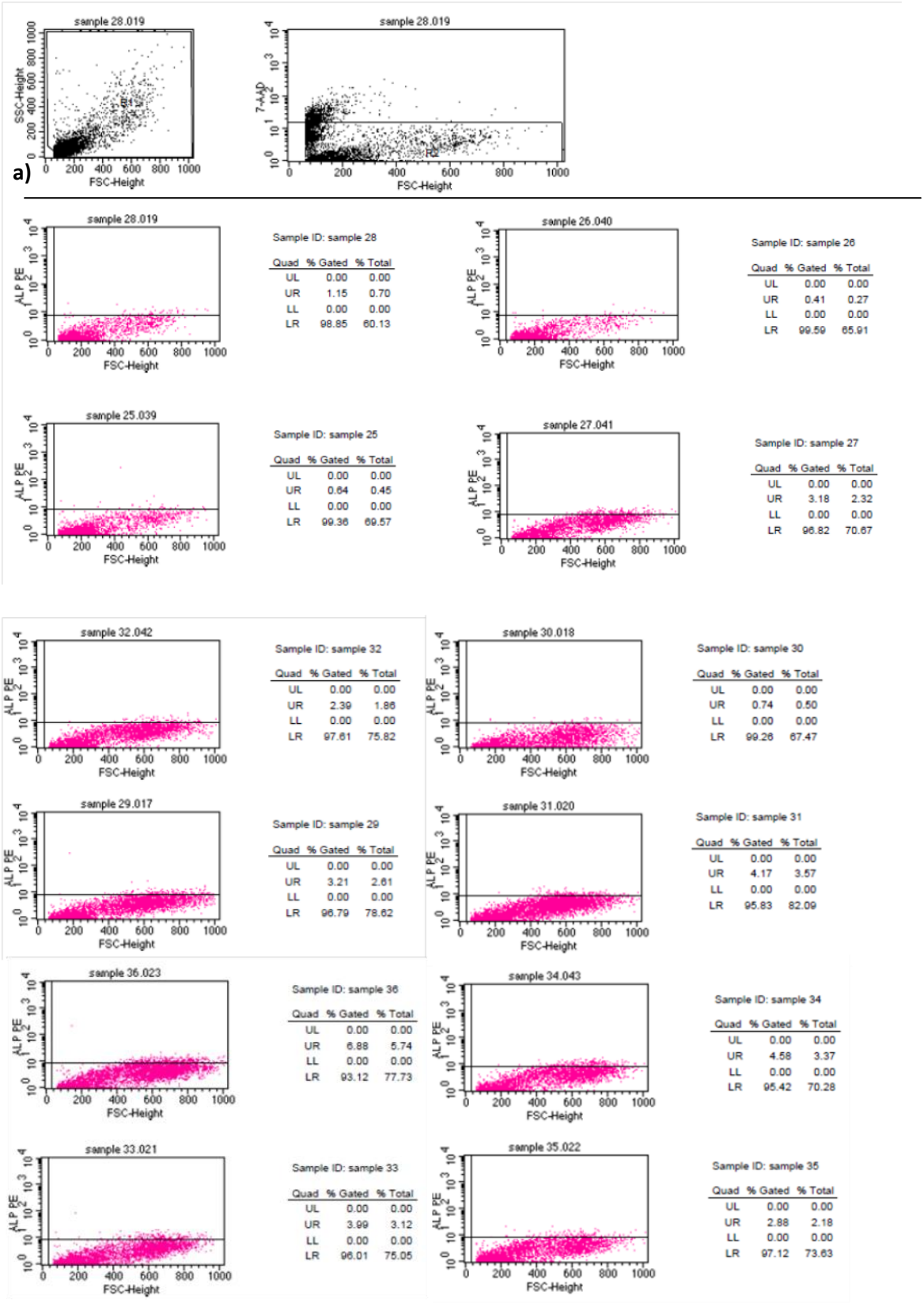


Figure 3 – FACS analysis of O3 for ALP markers. a) From the control well treated with ISO-type antibodies the threshold was set. b) different antibodies were tested: everything below the line is tested negative.

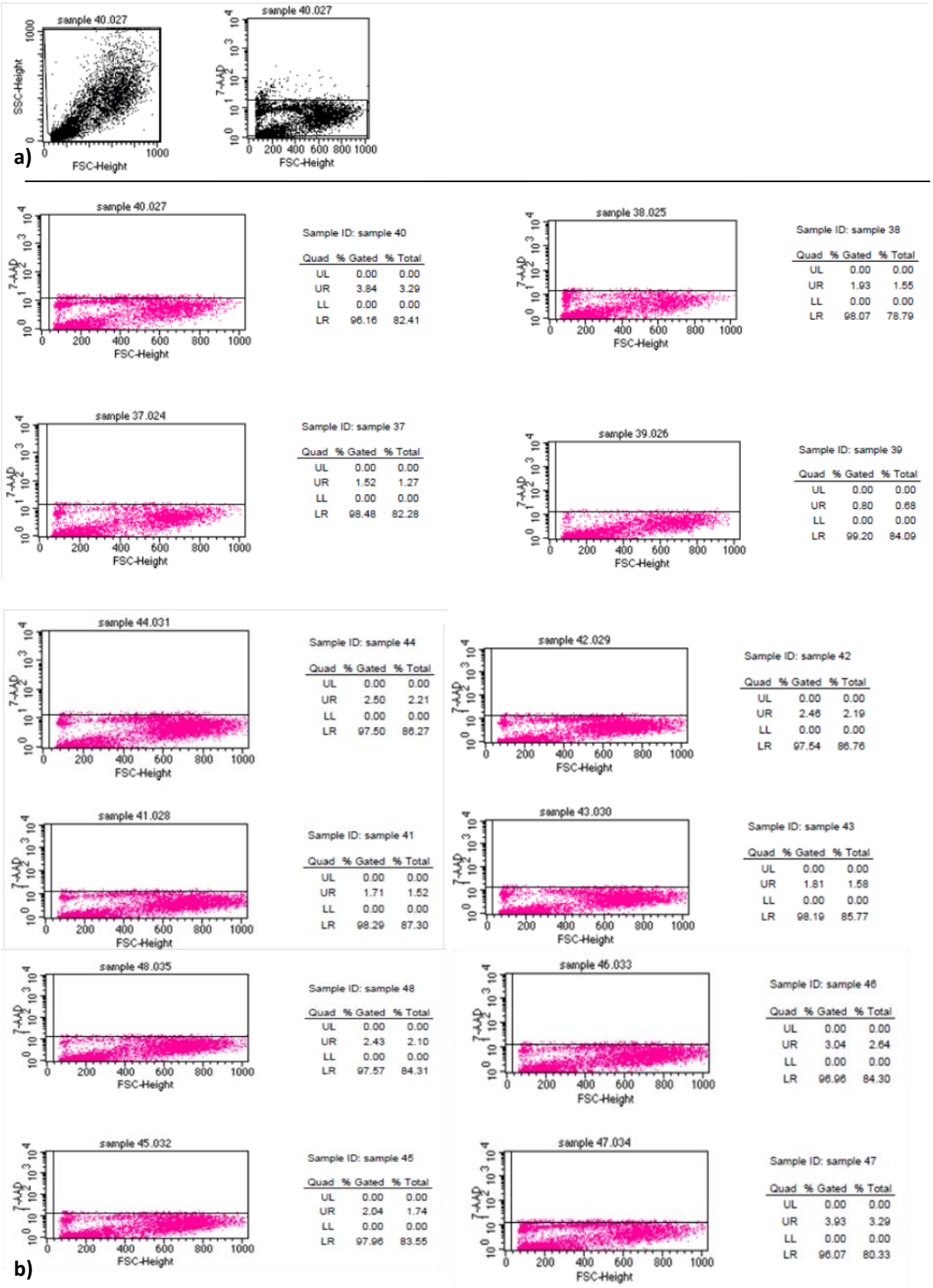


Figure 4 – FACS analysis of O4 for ALP markers. a) From the control well treated with ISO-type antibodies the threshold was set. b) different antibodies were tested: everything below the line is tested negative.

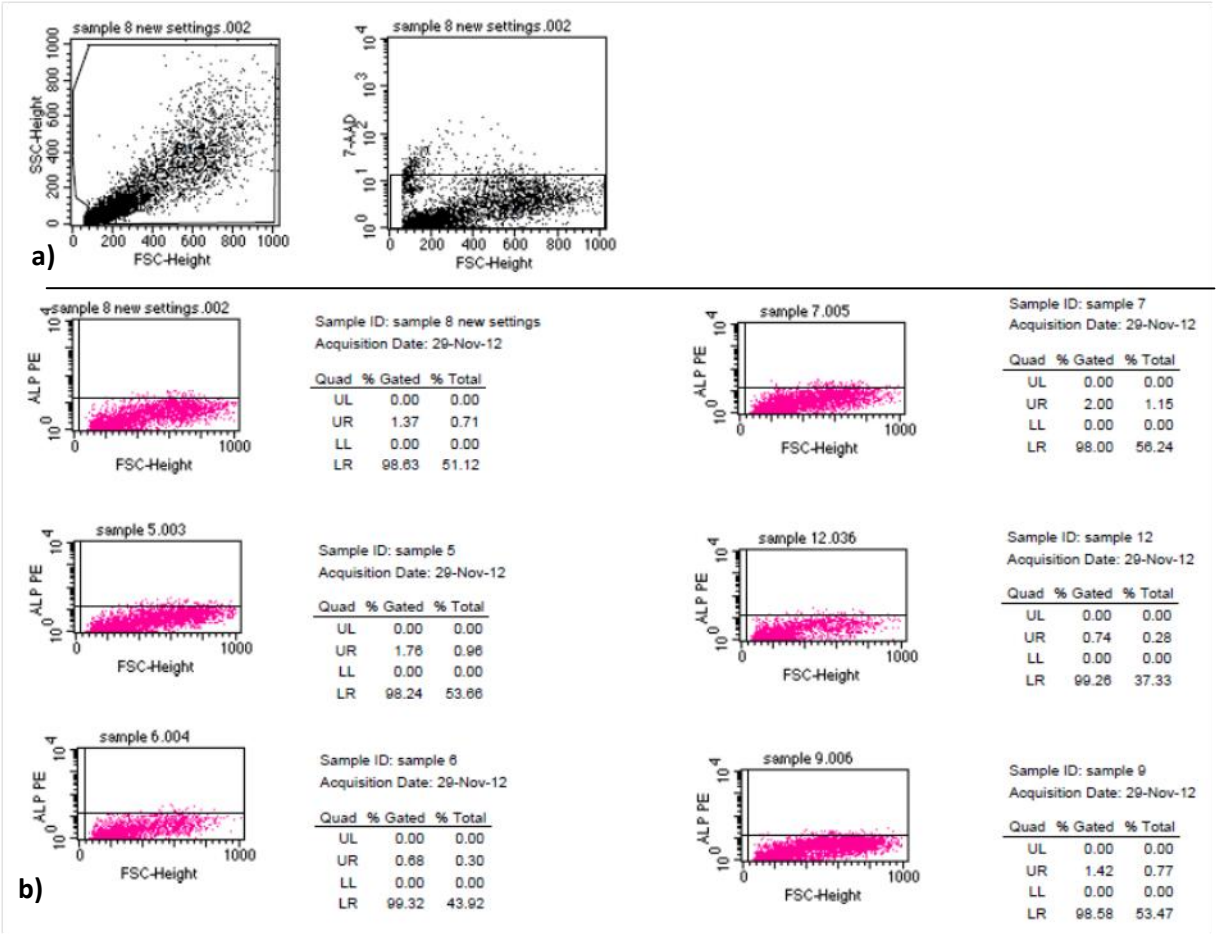


Figure 5 – FACS analysis of D3 for ALP markers. a) From the control well treated with ISO-type antibodies the threshold was set. b) different antibodies were tested: everything below the line is tested negative.

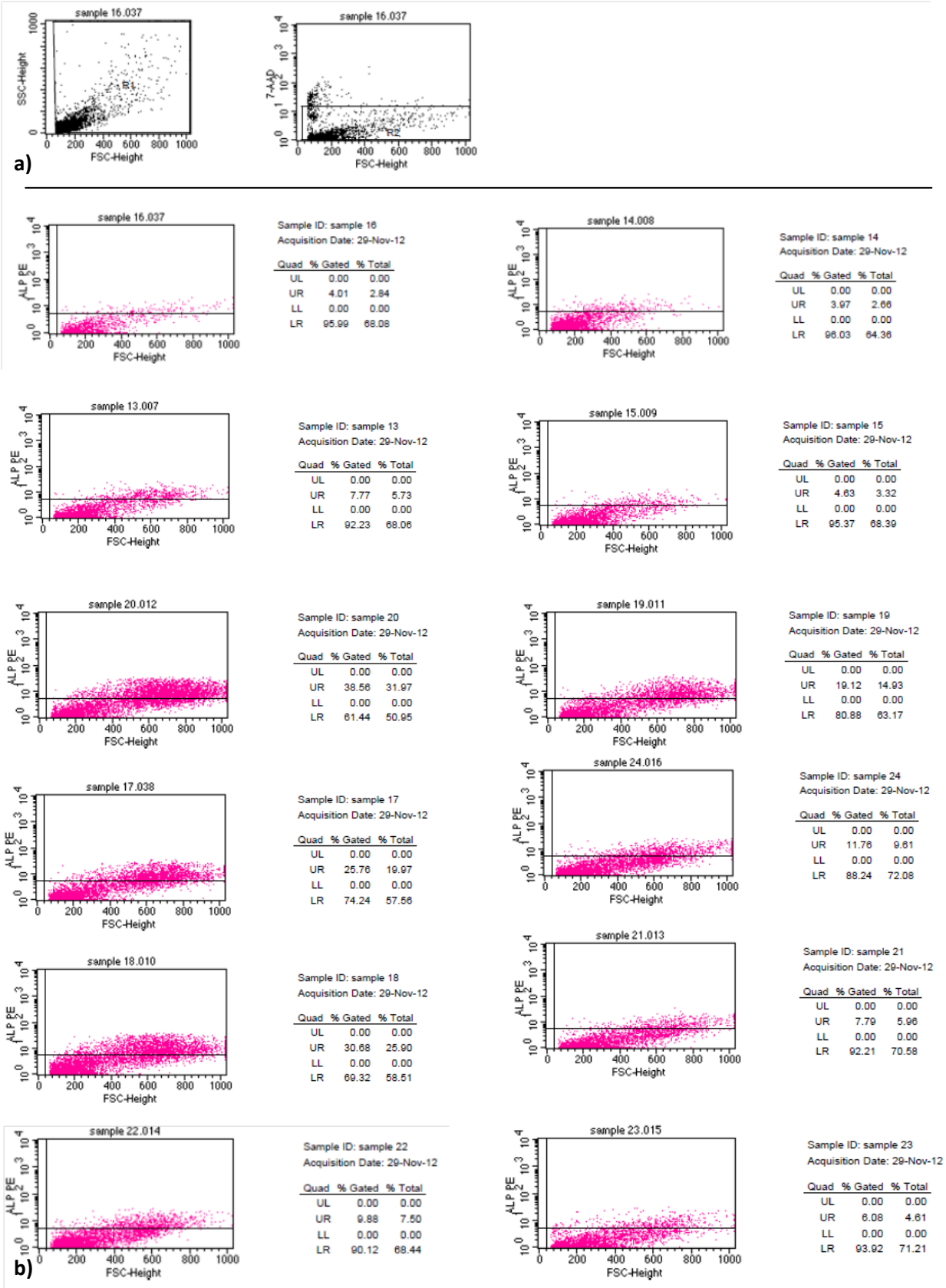


Figure 6 – FACS analysis of D4 for ALP markers. a) From the control well treated with ISO-type antibodies the threshold was set. b) different antibodies were tested: everything below the line is tested negative.