UC DAVIS
UNIVERSITY OF CALIFORNIA
MEDICAL CENTER

fhs Fachhochschule Salzburg University of Applied Sciences

# *Development of novel laboratory-based techniques to analyze the mode of action of placental mesenchymal stem cells (PMSCs) protecting neurons in the treatment of myelomeningocele (MMC).*

## BACHELOR THESIS 2


Student Stefanie Emrich, 1410434006

Supervisor Gertie Janneke Oostingh, PhD

Supervision at the UC Davis Laboratory Research II: Aijun Wang, PhD


Salzburg, 6th of June 2017

## Declaration in lieu of an oath

I hereby declare in lieu of an oath, that the following bachelor thesis was written independently and without external help. In addition, no sources, other than those that are indicated and cited, were used. I also confirm that I identified the cited passages as such.

This thesis has never been presented to any other committee before, whether in Austria nor in a foreign country and was not published prior.

06/06/2017

Date

Signature

**Contents**

## 1. List of abbreviations

| | |
|---|---|
| APC: | Allophycocyanin |
| BDNF: | Brain Derived Neurotrophic Factor |
| DEPC: | Diethylpyrocarbonate |
| dNTP: | deoxynucleotide triphosphate |
| DTT: | Dithiothreitol |
| EGF: | Epidermal Growth Factor |
| ELISA: | Enzyme Linked Immuno-Sorbent Assay |
| FBS: | Fetal Bovine Serum |
| FGFb: | Fibroblast Growth Factor basic |
| FITC: | Fluorescein Isothiocyanate |
| HGF: | Hepatocyte Growth Factor |
| GFP: | Green Fluorescent Protein |
| GvHD: | Graft-versus-Host-Disease |
| MMC: | Myelomeningocele |
| MSC: | Mesenchymal Stromal Cell |
| NIR: | Near Infrared |
| O.D.: | Optical Density |
| P: | Passage |
| PBS: | Phosphate Buffered Saline |
| PCR: | Polymerase Chain Reaction |
| PDGF: | Platelet-Derived Growth Factor |
| PE: | Phycoerythrin |
| PMSC: | Placental Mesenchymal Stem Cell |
| RFU: | Relative Fluorescence Unit |
| RT: | Reverse Transcriptase |
| sFRP-3: | secreted Frizzled-Related Protein 3 |
| TGFβ: | Transforming Growth Factor-β |
| T75: | 75 $cm^2$ cell culture flask |
| T150: | 150 $cm^2$ cell culture flask |
| VEGF: | Vascular Endothelial Growth Factor |

## 2. Abstract/ Zusammenfassung

### 2.1 Abstract

Myelomeningocele (MMC) is a congenital birth defect which is caused by incomplete neural tube closure during fetal development. It causes paralysis, musculoskeletal deformities, incontinence and cognitive disabilities. The application of placental mesenchymal stem cells (PMSCs) during the surgical defect repair is hypothesized to significantly improve the paralysis. This is due to the broad range of properties of PMSCs. In this bachelor thesis, laboratory based technologies were developed and used to characterize and analyze ovine PMSCs to further the knowledge on the mechanisms of action for later autologous stem cell treatment in the ovine model. The analysis by flow cytometry has been shown to be a powerful method, but it depends on the availability of species-specific antigens and antibodies. In addition, enzyme-linked immunosorbent assay (ELISA) and cytokine array were proven to be effective techniques. The limiting factor in the scope of this study was the rare availability of commercial sheep reactive reagents. Compared to ELISA, cytokine array and flow cytometry, polymerase chain reaction (PCR) was species independent and an excellent method for investigating ovine PMSCs. Every experiment delivered valuable results, the array experiment pointed to two new cytokines which have previously not been investigated within this field. The data gained showed that the use of novel laboratory-based methods will support the further analysis of PMSCs. This will aid the development of therapies, needed to improve paralysis in children affected by MMC.

### 2.2 Zusammenfassung

Myelomeningozele (MMC) ist ein angeborener Geburtsfehler, der durch unvollständigen Neuralrohrverschluss während der fetalen Entwicklung verursacht wird. Dies führt zu Lähmungen, muskuloskeletalen Deformitäten, Inkontinenz und kognitiver Behinderung. Es wird vermutet, dass die Applikation von plazentalen mesenchymalen Stammzellen (PMSCs) während des chirurgischen Defektverschlusses die neurologische Funktion signifikant verbessert. Dies liegt an den komplexen Eigenschaften von PMSCs. In dieser Bachelorarbeit wurden laborbasierte Technologien entwickelt und angewendet, um PMSCs von Schafen zu charakterisieren und zu analysieren. Für die zukünftige autologe Stammzelltransplantation im Schafmodell sollte das Wissen über die Wirkungsmechanismen der Zellen weiterentwickelt werden. Die Analyse der Durchflusszytometrie hat sich als leistungsstarke Methode erwiesen, hängt aber von der Verfügbarkeit von Spezies-spezifischen Antigenen und Antikörpern ab. Wirksame Techniken sind Enzym-Immunosorbent-Assays (ELISAs) und Zytokin-Arrays, ebenfalls mit dem limitierenden Faktor der eingeschränkten Verfügbarkeit von Schafreaktionsreagenzien. Das Array-Experiment deutete auf zwei neue Zytokine hin, die

bisher nicht in diesem Bereich untersucht wurden. Die Polymerase-Kettenreaktion (PCR) war speziesunabhängig und eine hervorragende Methode. Jedes Experiment lieferte wertvolle Ergebnisse und die gewonnenen Daten zeigten, dass der Einsatz neuartiger Labor basierter Tests die weitere Analyse von PMSCs unterstützen wird. Dies wird die Entwicklung von Therapien unterstützen um die Lähmungen von MMC betroffenen Kindern zu verbessern.

## 3. Introduction

Myelomeningocele (MMC) is a congenital birth defect which is caused by incomplete neural tube closure during fetal development. It is a kind of Spina bifida, which is defined as defective fusion of posterior spinal bony elements in the open dysraphism. (Özek et al., 2008) In the US, four children are born with this defect every day. It causes paralysis, musculoskeletal deformities, incontinence and cognitive disabilities. The Management of Myelomeningocele Study (MOMS) was a clinical trial that showed that *in utero* surgical defect repair could diminish the disabilities, but still only 42% of the affected children were able to walk without assistance at 30 months of age. (Wang et al, 2015). Within the proposed project, the **aim** was to develop several different novel laboratory-based techniques for the generation and analysis of placental mesenchymal stem cells (PMSCs). These techniques will aid the ongoing research within this field and guide potential future therapies. It is **hypothesized** that improved methods can be established, which will aid in the application of PMSCs during the surgical defect repair and thereby significantly improve the paralysis. This is due to the broad range of properties of PMSCs.

### 3.1 Description of MMC-associated disease

Spina bifida is a spinal cord malformation. This is a congenital anomaly of the nervous system which causes a defect of the closure of the neural tube during fetal development. The defect occurs during gastrulation (2 to 3 weeks of gestation), primary neurulation (3 to 4 weeks of gestation) and secondary neurulation (5 to 6 weeks of gestation). Spinal malformations are divided into open spinal dysraphisms and closed dysraphisms. The first category is characterized by an exposure of nervous tissue through a skin defect. The second one is characterized by a complete skin coverage of the beneath lying anomaly. Open malformations occur due to a defective closure of the primary neural tube in fetal development. This leads to the persistence of a non-neurulated placode segment. The open spinal dysraphisms include spina bifida, with MMC and other rarer forms like myelocele. In open malformations, there is a leakage of cerebrospinal fluid in the amniotic cavity. This can trigger a cascade of events and may lead to a Chiari II malformation. Closed malformations may cause much milder forms of illness because there is no loss of cerebrospinal fluid. Spina bifida has a higher prevalence in Caucasians and Hispanics than in Asians and African Americans. Most cases can be detected via ultrasound. (Özek et al., 2008)

MMC is the most frequent form of spina bifida. Typical for MMC is the extrusion of the spinal cord into a sac filled with cerebrospinal fluid. At birth, damage to the spinal cord and peripheral nerves is usually observed. Paralysis, bowel and bladder dysfunctions are further effects of MMC. These lead to irreversible disabilities despite early postnatal surgical repair. Most of the

children born with MMC also suffer from an Arnold-Chiari ll malformation. This includes hindbrain herniation and brain stem anomalies. A hindbrain herniation is a downward displacement of the medulla, fourth ventricle, and cerebellum into the spinal canal. The Arnold-Chiari II malformation has effects of motor and cranial-nerve, as well as cognitive functions. It is often characterized by hydrocephalus, which is treated by diverting the cerebrospinal fluid into the peritoneal cavity. A shunt is placed surgical and needs lifelong monitoring to prevent infection or failure. Folic acid helps to avoid MMC and is applied to most pregnant women. Nevertheless there are 3.4 cases per 10 000 live births in the US. The death rate is about 10%. (Adzick et al., 2012)

In America, a lot of nutrition is supplemented with folic acid. In Austria, women are often prescribed prenatal vitamins including folic acid to prevent spina bifida. The problem is, that neural tube closure completes at the 28$^{th}$ embryonic day (Pritchard & Allowy, 1999). Therefore, supplementing is often too late for many pregnant women and supplementation before gestation is therefore recommended.

### 3.2 Treatment of MMC at the example of the MOMS trial

Early clinical trials have confirmed that prenatal treatment of MMC has benefits for the affected child. In particular, an improvement in hindbrain herniation after treatment has been known for almost 20 years. (Sutton, 1999) The first *in utero* repair of spina bifida in humans was performed in 1997. Six years later, more than 200 fetuses received prenatal surgery for spina bifida. For fetal surgery, a laparotomy and a hysterotomy of the pregnant women is performed. The surgeon makes an incision along the arachnoid mater and skin junction around the MMC. The neural placode is visualized and then covered by closing the dura mater. The MOMS Trial, published in 2012, showed that the need for cerebrospinal fluid shunt at the age of 12 month was 98% for children who had a postnatal surgery. However, in the prenatal surgery group, only 68% of the children needed that treatment. 64% of infants from the prenatal surgery group showed a hindbrain herniation compared to 96% of the postnatal surgery group. The prenatal surgery group also had lower rates of brain-stem kinking and abnormal fourth-ventricle location. In the rates of epidermoid cysts identification, there was no significance between the groups. For delayed spinal cord tethering, the infants treated after birth had to undergo more procedures. (Adzick et al., 2012)

The study by Adzick et al. (2012) also showed that the risk of preterm birth in the prenatal surgery group was higher than in the postnatal surgery group. The first group of infants was born at an average gestational age of 34.1 weeks and 13% were born before 30 weeks of gestation. In the postnatal surgery group, the average gestational age at the time of birth was

37.3 weeks and no birth before 30 weeks of gestation occurred. Two perinatal deaths occurred in each group. The affected neonates in the postnatal surgery group died because of severe symptoms of Chiari II malformations. In the prenatal surgery group, a stillbirth at 26 weeks of gestation and one neonatal death because of prematurity (23 weeks of gestation) was recorded. At the age of 30 month, the groups were compared again and the functional and anatomical status of the children was significantly worse in the postnatal surgery group. In the prenatal surgery group, 42% of the infants were able to ambulate, twice as much as in the postnatal surgery group (21%). The prenatal surgery group had a higher risk for preterm delivery, although the outcomes of this group were significantly better than the postnatal surgery group. (Adzick et al., 2012)

### 3.3 PMSCs in stem cell therapy

In the early 1980s, isolation of mouse embryonic stem cells was first performed. This was a huge step in the field of stem cell biology and opened many new possibilities for therapeutic interventions in humans. (Harding et al., 2013) Nowadays, numerous cell types have been explored for cell transplantation applications all over the world. Cellular transplantation therapy has already shown a high potential for regenerative medicine and tissue repair. In preclinical studies, many beneficial applications of stem cell therapies have been reported. One type of cells that has been a focus of investigation are the mesenchymal stromal cell (MSC) type, these were initially discovered in the bone marrow. Obtaining bone marrow MSCs requires an invasive procedure and the yield of cells by this technique is generally low. For this reason, other sources of MSCs were sought. The placenta and other gestational tissues (placental membranes, umbilical cord and the amniotic fluid) were recognized to contain a source of cells suitable for regenerative medicine. The placenta is a rich source of stem cells that display strong immunosuppressive properties, making the potential for their use in cell therapy very promising. (Parolini et al., 2008)

The placental tissue is considered extra-embryonic due to its developmental origin within the trophectoderm layer of the fetal blastocyst and subsequent development outside the fetus. In addition, the placenta is typically discarded after birth and tissues can be easily obtained. MSCs can be isolated from all regions of placental tissue, including those which are of fetal origin. In other words, the placenta contains fetal tissue that can be used to isolate cells for autologous cell therapy for the fetus *in utero.* The characteristics of these PMSCs show promising therapeutic benefits for numerous different pathological conditions. Furthermore, the tissue can be obtained non-invasively which enhanced PMSCs potential for use in several types of stem cell therapy. (Meierhenry et al., 2015)

Former research proved that PMSCs are neuroprotective, improve wound healing and have immunomodulatory properties (Wang et al., 2015). The cells can be isolate either from discarded placenta or from chorionic villus sampling, which can be performed in the first trimester between the 9th and 12th week of gestation. During this procedure, a small mass of the chorionic villi is aspirated by a needle (Thomas, 1998). The procedure is either made through the lower abdomen or trans-cervical depending on different factors, such as location of the placenta, maternal weight or preference of the operator (Stergiotou et al., 2016). For a stem cell therapy, the prenatal period is believed to be the ideal time for treatment, because the fetal immune system is naïve and naturally receptive to stem cell mediated remodeling (Wang et al., 2015).

### 3.3.1 Stem cell therapy in the lamb model

Wang et al. proved in 2015 in a fetal lamb model that PMSCs secrete high level of the human brain-derived neurotrophic factor (BDNF) and hepatocyte growth factor (HGF). These factors are known to be neuroprotective. This was demonstrated using ELISA in which the concentrations of secreted cytokines and growth factors were measured and quantified. In the fetal lamb model, a surgically created MMC defect was induced in 12 lambs. At an average gestational age of 77.3 days, a spinal column defect of approx. 3 cm was created by laparotomy and hysterotomy of the pregnant sheep. The defect of the fetal lamb was created by removing the skin, paraspinal muscles, lamina of six lumbar vertebrae and dura over the spinal cord (see figure 1A and B). At a mean gestational age of 103.5 days (range, 97 to 107 days) a second operation for repairing the defect was performed (see figure 1 C to G). In this study, there were 6 lambs treated with human PMSCs on a delivery vehicle during *in utero* repair and 6 lambs which underwent no PMSC treatment. In the treatment group, 6 randomly chosen lambs received 1 mL of collagen gel seed with 2.5 million Green-Fluorescent-Protein (GFP)-tagged PMSCs as a direct application on the spinal cord. The no-treatment group received the delivery vehicle without PMSCs as a negative control. In all surgeries, any fibrinous exudate present on the spinal cord was removed. Before the defect was closed, a commercially available extracellular matrix patch was placed on it to keep the collagen in place. All 12 lambs survived to term at the average gestational age of 145.7 days. (Wang et al., 2015)

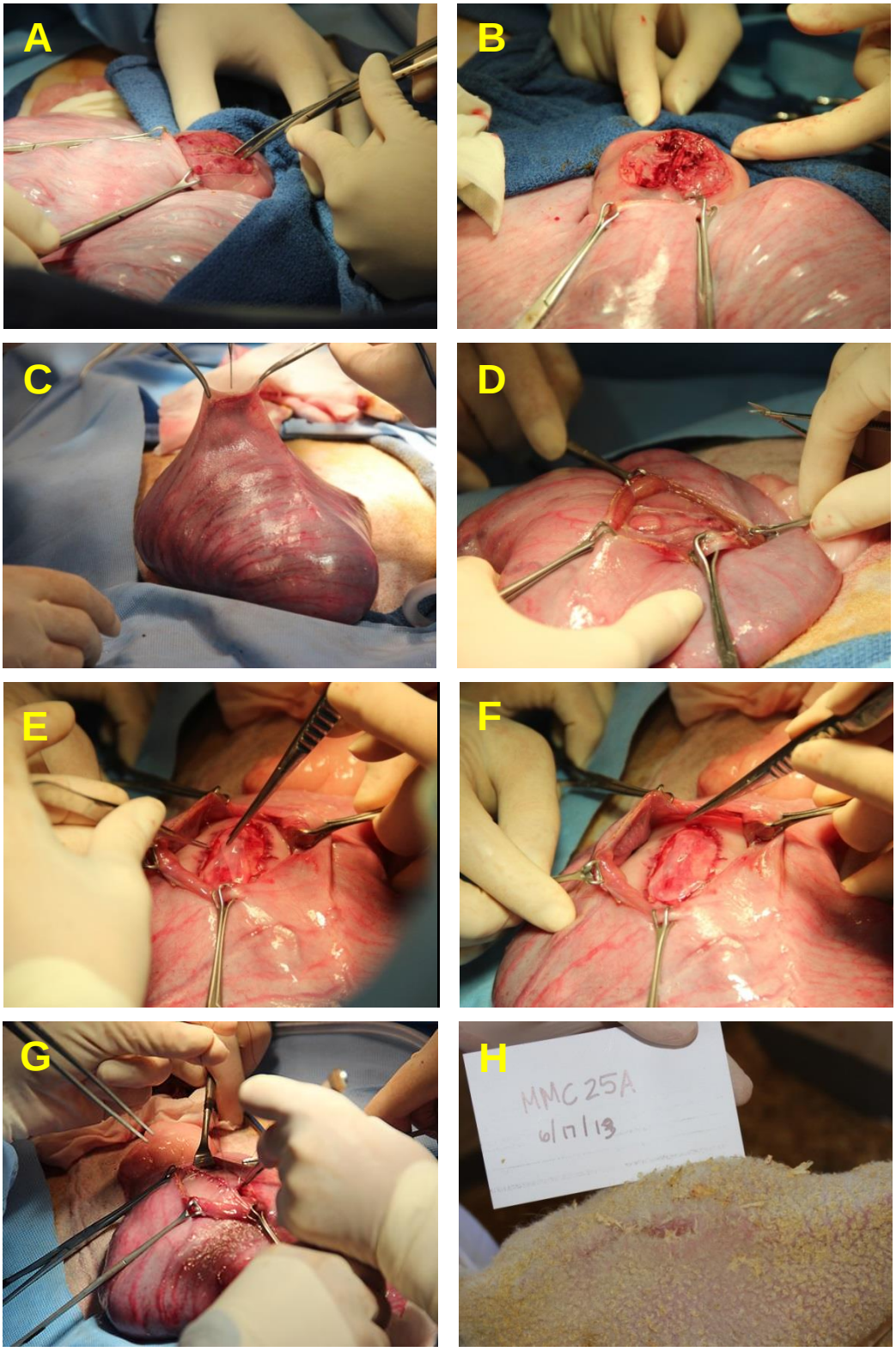



**Figure 1: Surgically created MMC defect induced in a lamb.** A: Exposed ovine uterus with removed skin during defect creation. B: Skin, paraspinal muscles, lamina of six lumbar vertebrae and dura over the spinal cord was removed. C: The ovine uterus at defect repair surgery is exposed. D: Open spinal cord defect. E: Delivery vehicle with PMSCs in collagen is placed on the defect. F: Repaired defect with PMSCs before closure. G: After repair, the uterus was closed. H: The spinal cord defect at the time of birth. (Permission obtained from C. Pivetti, taken at the UC Davis in 2016)

Within the first 24 hours after birth, the motor function was scored using the sheep locomotor rating (SLR) scale. In figure 1H, the outward appearance of the spinal cord defect after birth is shown. The SLR scale measures motor function in 7 categories. Every category has a scale from 0 to 15 where 15 is the highest score associated with a completely normal motor function. A score from 10 to 14 describes a mild motor deficit, from 5 to 9 a moderate deficit and from 0 to 4 a high deficit. As a positive control, three lambs not receiving a surgical procedure were classified by the SLR scale, and all scored 15. In the study, 67% of the PMSC-treated lambs were able to ambulate. Whereas the untreated control group, none of the lambs could ambulate. The PMSC treated lambs had significantly higher SLR scores compared to the untreated group. Two of the PMSC treated lambs reached a SLR score of 15. The others reached a score of 13, 10, 8 and 5. In the untreated group the highest score were 8 and 6, the other four sheep were severely restricted (scores ranged 2-4). (Wang et al., 2015)

After the SLR analysis, all lambs were euthanized and the spinal cord was dissected for further tests. The tissue was immunohistochemically stained and the tissue was then observed by bright field. In the histopathologic analysis of the spinal cord, the number of large neurons of the treated lambs was significantly higher than that of the untreated ones. Therefore, the application of human PMSCs for *in utero* surgical repair improved neurologic function and preserved spinal cord neuron density in the ovine model of MMC. (Wang et al., 2015) Thus, PMSCs have a high potential to be therapeutically beneficial in enhancing the current standard of *in utero* MMC treatment. (Wang et al., 2015)

### 3.3.2 Essential characteristics of PMSCs

In 2006, a position paper was published by the International Society for Cellular Therapy in which multipotent MSCs were defined. To be classified as MSC a cell must meet different criteria which are listed in table 1.

The ability to differentiate into chondroblasts, osteoblasts and adipocytes under standard conditions must be shown *in vitro*, which means that the cells are cultured in a lineage-specific differentiation medium for a set duration. The differentiation is demonstrated by different stainings. For example, adipocytes can be stained with Oil Red, chondroblasts can be stained with Alcian Blue and the osteoblasts can be stained with Alizarin Red. The expression of the surface antigens CD73, CD90 and CD105 must be present in ≥ 95% of the cell population. These markers or antigens are proteins on the outside of the cell and are used to identify and distinguish different cell populations. The expression of the markers is analyzed by flow cytometry. The method is explained in chapter 3.4.1.

**Table 1: Minimal set of standard criteria for MSCs**. CD = Cluster of Differentiation

| |
|---|
| Cells show the potential of tri-lineage differentiation, which means they can differentiate into chondroblasts, osteoblasts and adipocytes |
| The cells adhere to plastic in the standard culture environment using tissue culture flasks |
| The cells express the following surface markers:<br>• CD73<br>• CD90<br>• CD105 |
| The cells do not express the following surface markers:<br>• CD11b/CD14 (typical for blood cells like monocytes or macrophages)<br>• CD34 (marker for hematopoietic progenitor cells and endothelial cells)<br>• CD45 (marker for leukocytes)<br>• CD79α/CD19 (B cell marker with ability to adhere to MSCs in culture conditions)<br>• HLA/DR markers (expressed on interferon gamma stimulated MSCs only) |

(Dominici et al., 2006)

Depending on the tissue from which MSCs are isolated, they show different capabilities of plasticity, division rate and secretion. For example, hepatocyte growth factor (a cytokine which induces angiogenesis and other biological functions) is presented in higher concentration in PMSCs than in MSCs. (Meierhenry et al., 2015) In the course of *in vitro* expansion, PMSCs withstand higher passage numbers compared to bone marrow driven MSCs. In addition, the seeding density is lower in PMSC-culture. Therefore, the growth rate of PMSCs has been shown to be higher than bone marrow derived MSCs. (Barlow et al., 2008) MSCs isolated from pre-term placenta tissue have certain characteristics of multipotent cells, such as long telomeres and expression of markers such as Oct-4. Oct-4 stands for octamer binding factor, a pluripotent stem cell specific transcription factor. Cells expressing Oct-4 have shown the capacity to differentiate into adipogenic, myogenic, osteogenic, endothelial, hepatic and neuronal cells. Furthermore, they have never shown to form tumors *in vivo*. (Miki T et al., 2005)

Recent studies suggest that MSCs have a high potential to aid in the healing of damaged tissue. The chemokines and cytokines produced by these cells inhibit inflammation and promote vascularization. The secreted exosomes by MSCs have shown cytoprotective abilities. PMSCs can secrete many cytokines involved in wound healing, such as IL-6, IL-8, Plasma-Derived Growth Factor (PDGF) and Transforming Growth Factor (TGF)-β2 (Meierhenry et al., 2015). Interleukins are proteins which are secreted by various cells during physiological processes (Gressner and Arndt, 2013). PDGF is a dimeric glycoprotein produced by platelets during platelet activation. It is also secreted by various normal cells,

such as activated macrophages, endothelial cells or smooth muscle cells and plays a major role in wound healing. TGFβ 2 is also a protein and plays a major role in the differentiation between apoptosis and immune regulation (Nanadakumar et al., 2016).

Treatments using cell and organ transplantation are limited by the development of graft-versus-host-disease (GvHD). The immunomodulatory capability of PMSCs may overcome this problem, since the cells are able to inhibit T-cell and other responses. MSCs from bone marrow showed a decrease in GvHD after transplantation in prior studies. The unique ability of the immunomodulatory potential of PMSCs is illustrated by the ability to maintain tolerance toward the fetomaternal placental tissue Therefore, PMSCs might be suitable for allogeneic as well as autologous stem cell therapies. (Meierhenry et al., 2015)

### 3.4 Laboratory based methods for analyzing PMSCs

Several different methods are used for the characterization and analysis of PMSCs. ELISA is used to measure and quantify immunomodulatory and chemotactic cytokines. (Wang et al., 2015) For quantitative and qualitative characterization of cells and exosomes, flow cytometry is a powerful method (Witwer et al., 2013). To investigate and characterize the PMSCs in this Bachelor thesis the main method of choice was flow cytometry. Due to the rare availability of sheep reactive antibodies and a lack of results in flow cytometry, PCR was performed to determine genes encoding surface markers specific for PMSCs. Additionally, ELISA and cytokine array testing for secretory factors of PMSCs, as mentioned in chapter 3.3.1, were performed.

#### 3.4.1 Flow cytometry

Flow cytometry is used to analyze cells and other small particles. Scattered light as well as fluorescence emission of labeled antibodies bound to the cells/ particles or intracellular dyes can be detected. Flow cytometry was introduced about 65 years ago and detected the size of cells for hematological analysis. In 1965, Max Fulwyler described the first fluorescent activated cell sorter (FACS). This instrument charges cells and separates them into different collection tubes. In human medical centers, flow cytometers were invented in the 1980s and were used to define different lymphocytes. Nowadays, flow cytometers are able to analyze 14 parameters simultaneously. Flow cytometry is based on the analysis of single cells or particles. The benefit of flow cytometry is the capacity to analyze different properties of cells including chromosomes, proteins and also nucleic acids. (Wilkerson, 2012)

The main modules of a flow cytometer are the fluidic transport, the optical system, the detection and the translation components. To obtain single cell or particle detection, the cells/ particles in the suspensions are separated using sheath fluid, often with PBS as buffer solution. The sample cell suspension is injected through a pressurized airline with greater pressure than the sheath flow and is thereby transported passed the detecting point. The cells are in the center of the sheath flow and pass the core center of the flow cytometer one by one. This fluidic system is called hydrodynamic focusing. (Adan et al., 2017)

Detection in flow cytometer occurs with a laser hitting the cell. The laser produces photons that strike a cell and result in the detection of two different light scatter grams. The forward scatter (FSC) is detected in the same axis as the laser beam and represents the volume of the cells. Therefore, the FSC provides information about the size of the detected cell or particle. The side scatter (SSC) is the second detected scatter, mostly at an angle of 90°. The SSC provides information about the intern structure of a cell such as the granularity and the nucleus. At the same angle as the SSC, the fluorescent light emitted by fluorescently labeled antibodies or intracellularly stained cells or particles is detected. Light signals from detectors (photomultiplier and photodiodes) are converted to electrons, which amounts proportionally to the amount of light signal. The emitted light by fluorescence probes gets detected by optical lenses, mirrors and filters to separate the specified wavelengths. After conversion of the light signal to an electrical current, an analog signal is generated. An analog to digital converter changes the signal for further computer processing. The digital data is displayed as plots or histograms. (Adan et al., 2017)

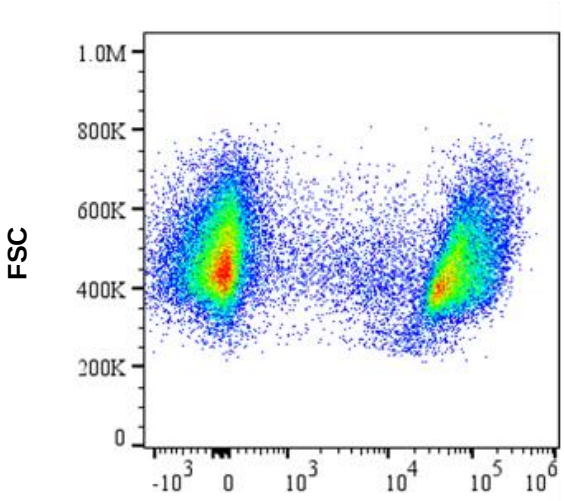
In the scope of stem cell research, investigating PMSCs by flow cytometry using immunophenotyping has proven that PMSCs have a similar profile when compared to other MCSs. The PMCS express well established surface markers and lack markers typical for hematopoetic and endothelial cells. (Lankford et al., 2015) In the scope of this bachelor thesis, flow cytometric analysis of the PMCSs was performed. For this, the following aspects were taken into consideration.

Fluorochromes attached to antibodies specific receptors are used to highlight these cell surface receptors. (Adan et al., 2017) There are different laser configurations for the excitation and these can be used for different fluorochromes. A typical laser used in flow cytometry is an argon laser with an excitation wave length of 488 nm. The emission wavelength of a fluorochrome is longer than the excitation wavelength. For example, an often used fluorochrome called Fluorescein Isothiocyanate (FITC) gets excited by the wavelength of 400 - 550 nm and the absorption maximum is near 490 nm. At this wavelength, most

photons are absorbed and the fluorescence has the highest intensity. After light absorption, the maximum emission wavelength is about 495-520 nm. Thus, the absorption and emission spectra are overlapping. (Adan et al., 2017) If more fluorochromes are used in one sample, overlapping emission spectra are present. To correct the data, a process is performed called compensation. For example, if FITC and phycoerythrin (PE) are used for one sample, the fluorescein emitted light will pass through the PE filter at the same wavelength. Spectral overlapping is corrected by subtracting a fraction of signal released by FITC from PE or the other way around. For this purpose, the fluorochromes have to be measured alone. The percentage overlap between the emission spectra is calculated. Based on this information, the compensation is applied on the dataset. (Adan et al., 2017) For the flow cytometric analysis applied in this bachelor thesis a compensation using beads (fluorochromes connected to beads) protocol was set up as described in chapter 4.2.1. The advantage of using beads instead of using cells stained with just one fluorochrome labeled antibody per sample is that the beads are cheaper and no valuable cells are needed for compensation. (Adan et al., 2017)

The sensitivity of the flow cytometry analysis depends on many different factors. One is the specificity of the antibody used. If the antibody amount in the suspension is too high, unspecific binding occurs. In addition, cell surface Fc receptors may bind some antibodies, depending on the species the antibodies are derived from. Aggregates are sometimes formed during storage, but these can be resuspended by centrifugation. (McCarthy & Macey, 2001) For both, an isotype control antibody should be included to determine eventual Fc-mediated or unspecific binding. The isotype controls used in this thesis were fluorochrome-conjugated antibodies without Fab region which are not supposed to bind and should therefore not provide a signal. If signals get detected when using the isotype control, the data can be adjusted via software to ensure precise analysis.

Another problem associated with unspecific binding is the presence of dead cells. Dead cells aggregate and show greater non-specific binding compared to viable cells. (McCarthy & Macey, 2001) In addition, dead cells or cells in apoptosis often express a high auto fluorescence, which can interfere with the staining. In this thesis, a Near Infrared (NIR) live/dead fixed cell sample was measured to gate for dead cells. This procedure is provided in chapter 4.2.1. For this analysis, a procedure was set up to create a population of half live and half dead cells. Due to a difference in FSC and SSC, the dead cells were plotted as a separate population in the flow analysis and can thus be excluded (figure 2). This gate was applied to all samples to exclude dead cells from viable cells in the flow cytometric analysis. This process increases the accuracy of the flow cytometry analysis.




**NIR Viability Figure 2: The NIR Live Dead Control imaged with FlowJo.**

### 3.4.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a technique in which very small amounts of DNA sequences are copied many times to allow their detection. The technique allows the amplification of specific sequences from a small amount of starting material. For a PCR, the template DNA, primers, free nucleotides and polymerase have to be provided. In a first step, the double-stranded DNA is denatured by heat. The DNA becomes single-stranded and can be used as template for the DNA polymerase. Thereafter, the primer, which is an oligonucleotide, binds to a specific region of the template. After the primer has annealed to the template DNA, the polymerase binds at the free 3'OH end to add free bases to the template DNA to the bound primer and thereby copies the starting material. These three steps are called one cycle. For additional copies, the process of denaturation, annealing and chain elongation is repeated. Usually, 30 to 35 cycles are performed to enhance the material for analysis, whereby the PCR product increases exponentially after every cycle. This method is very efficient; 30 cycles give rise to over $10^9$ replicates. (Palmer MS, 1995)

The PCR was first performed by Kary B. Mullis in 1986. Initially, the DNA polymerase was added after every cycle, because it was not heat-stable. Nowadays, polymerase only has to be added at the beginning, since the thermally stable Taq-polymerase is used. Taq means *Thermus aquaticus* which is a thermophilic bacterium. The PCR process is performed in a thermal cycler, in which the number of cycles, the temperature and the time can be programmed. (Nordheim and Knippers, 2015)

Compared to DNA, RNA is a less stable biological material. Degradation during storage and transport is a technical challenge. Also cross contamination is a significant problem when using

RNA as template for PCR. That is why RNA is often converted into copy DNA (cDNA) for PCR application (Kizaki, 2016). The RNA is converted by reverse transcription. Reverse transcriptase is a RNA dependent DNA polymerase and uses RNA as a template for cDNA synthesis. (Kück, 2005)

### 3.4.3 ELISA and cytokine array

ELISA is a method based on the specific antibody-antigen reaction and is usually performed in microtiter plates. In the first incubation step, antigens or antibodies are coated to the surface of the wells. Thereafter, the antigens or antibodies from the samples of interest are added and allowed to bind specifically. Thereafter, a detection antibody is added. Free antigens or antibodies have to be removed in washing steps to prevent unspecific signals, which would lead to measurement errors. The detection antibody is normally labelled with a marker such as horse-radish peroxidase. Therefore, a substrate will be added the wells, which initiates an enzyme reaction. The product of the reaction is measured spectrophotometrically. A special type of ELISA test used in this thesis is a competitive ELISA. Labeled antigens compete with antigens from the analyte. Both bind to limited number of antibody sites. If the sample has a high concentration of antigen, less of the enzyme labeled antibodies bind to the limited capture antibodies and the optical signals are low. By using this method, the optical signal is inversely proportional to the concentration of the analyte. (Hallbach, 2011)

The cytokine array is also based on an antibody-antigen reaction, and it has several advantages over ELISAs. Cytokine arrays can detect different cytokines simultaneously in one sample, because different antibodies are coated onto a sample slide. The costs for the experiments and the amount of sample needed can thereby be reduced. The optical signals can be measured by different means. (Fung, 2004)

### 3.4.4 Cell culture

Cell culture technique was introduced approximately 100 years ago and was used to explore most basic questions in developmental biology. Ross Harrison used cell culture in 1907 to observed nerve fibers in embryo tissues from frogs. In the late 1970s, the use of cell culture techniques spread and researchers demonstrated that different cells needed different medium for supporting their viability and function *in vitro.* In cell culture, the behavior of a cell under different circumstances can be observed. Its stability and the ability to react to different external influences can be investigated. The type of cell culture can be “primary” which means the cell stem from an organ or tissue slice or “cell lines”. Cell lines mostly originate from tumors (like

the well-known HeLa cell line) or from cells that are transformed *in vitro.* But there are also cell lines from normal tissues with a limited life span. (Mather & Roberts, 1998)

For culturing cells such as the PMSCs used in the present study, a culture medium is needed in which the cells grow and divide. The basal medium contains water, glucose, amino acids, vitamins and a physiologically balance pH buffered saline solution. To this basal medium, growth factors, hormones or proteins can be added In addition, serum is used as supplement in the cell culture medium. The standard universal growth supplement for medium is fetal bovine serum (FBS). In culture of most primary cells, antibiotics such as streptomycin and penicillin are used to prevent cells from getting contaminated with bacteria. Primary cells can also get contaminated with fungi and mycoplasma. (Pamies et al., 2016) Thawed cells in culture are incubated at 37 °C and supplied with $CO_2$ (normally 5%) because the conditions *in vitro* should be as close as possible to their *in vivo* environment. Also the pH, the $O_2$ and the osmotic pressure are factors which can be controlled in cell culture. (Harrison & Rae, 1997)

To maintain high cell viability, culture conditions must be fixed and controlled. Cells should not stay longer outside the incubator as needed. For passaging and counting the adherent cells are detached by enzymes. To track the relative age of cells in culture, the split ratio and the number of times a culture was passaged should be recorded. Most cells divide at least once in each passage. Cells from higher passages still replicate but are more likely to develop genetic abnormalities. Cells can be stored for later investigations by cryopreservation. Before cells get frozen, they are diluted in a cryoprotectant solution. Recommended cooling rates range between 0.3 – 5 °C per minute in the cryopreserving process. After primary cooling, cells are stored overnight in a -80 °C freezer before they get transferred to liquid nitrogen tanks. (Pamies et al., 2016)

For cell culture, significant parameters are cell counts, viability and the ratio of alive and dead cells. To count the cells, they get stained and are usually counted in the Neubauer hemocytometer. The cell suspension can be stained with 0.5% trypan blue (1:1 ratio). Under the microscope, the dead cells are stained blue, whereas the viable cells stay unstained. For accurate counting in the hematocytometer, the cell suspension must contain between $10^5$ and $10^6$ cells/ mL. This complies with 5 to 50 cells/ square. Counting more than 50 to 100 cells/ square is not feasible. By diluting the suspension, counting can be reduced. (Storhas, 2013)

The project described in this thesis was performed in the scope of a Marshall Plan Scholarship. The project was part of a large study on *in utero* transplantation of placental mesenchymal stem cells (PMSCs) to treat spina bifida. Basic investigations on PMSCs by flow cytometry, PCR, ELISA and cytokine array were performed in this thesis. The aim of this bachelor project was to develop different laboratory-based techniques to investigate PMSCs and their mechanism of action, in order to allow a further improvement of the treatment for MMC. This aim lead to the following research question: **Which laboratory based technologies can be used to further our knowledge on the mechanisms of action provided by PMSCs and how can these be used to treat MMC in humans?** The preliminary hypothesis is, that the use of novel lab-based methods will aid the further analysis of PMSCs used to improve paralysis in children affected by MMC**.**

For this purpose, autologous transplantation (cells are from the person who is treated) is the ideal treatment. The xenogeneic transplantation (donor is another species) method, which was published for the lamb model (see chapter 3.2), could reduce the beneficial factors of PMSCs. Xenogeneic transplantations are well known to cause severe immune responses. To suppress this effect, the host animal can be treated with immunosuppressive substances, but the process complicates the interpretation of the result. (Harding et al., 2013) Cells taken from the chorionic villus for autologous stem cell therapy are preferable to allogeneic (donor is same species as host), because the patient's own cells are likely to cause no immune response. (Lankford et al., 2015) Also, allogeneic transplantation is known to cause GvHD in humans. Thus, autologous transplantation is proposed to be the ideal method for the treatment of MMC. Therefore, planned experiments will use placental sheep tissue for autologous transplantation in the lamb model. The characterization of the PMSCs, as listed in chapter 3.3.2, was performed by flow cytometry. For these investigations, sheep placental tissue was collected and cultured. The cells were antibody-labeled and analyzed by flow cytometry. In addition, PCR was performed to further analyze the PMSCs. PMSCs secrete a variety of paracrine factors capable of angiogenesis, neurogenesis and neuroprotection. These cells could therefore be responsible for the observed loco motor improvements *in vivo*. To determine cell secreting factors, ELISAs and a cytokine array were performed with the cell culture medium of the cells. The investigations of PMSCs are very promising for future trials to improve paralysis in children who are affected by MMC. **Therefore, novel laboratory-based techniques to analyze the mechanism of action of PMSCs should be further explored.**

## 4. Materials and methods

To investigate sheep cells, different batches of cells (MMC 29A, MMC 96A1 and MMC 96B1) were thawed and cultured. After expanding the cells, flow cytometry was set up and the cells were analyzed. Cells from the cultures were lysed and the target RNA was amplified by PCR after the generation of cDNA from the RNA. In addition, cell culture medium was collected for ELISA and cytokine array analysis of the secretory factors.

### 4.1 Culturing ovine PMSCs (MMC 29A, MMC 96A1, MMC 96B1)

The cell line MMC 29 P3 was thawed on 2/21/17. This cell line was passaged twice (2/27/17 and 3/2/2017) to gain 9 $10^6$ cells for flow cytometry. The cells were harvest at P5. MMC 96A1 and MMC 96B1 were thawed on 3/10/2017 for further expansion and testing. The procedures were the same except for the size of the flasks used. The volumes were minimized to half of the amount for T75 flasks (75 cm² cell culture flask). All reagents and materials used are listed in table 2.

**Table 2: Reagents and materials used for cell culture.**

| **Reagents** | **Vendor** | **Lot** Nr. | **Exp. Date** |
|---|---|---|---|
| DMEM High Glucose (L-glutamine) | HyClone | AB10134659 | 09/2017 |
| FBS | HyClone | AB10156964 | 02/2020 |
| DPBS | HyClone | SH30028.02 | 09/2018 |
| Penicillin/ Streptomycin (penstrep) | Gibco | 151a336 | 09/2017 |
| Recombinant Human FGFb | Advent Bio | AB1752323111 | 07/2017 |
| Recombinant Human EGF | Advent Bio | - | 07/2017 |
| Dimethylsulfoxide (DMSO) | Sigma | SHBF8746V | - |
| StemPro Adipocyte Differentiation | Gibco | 1837104 | 10/2017 |
| StemPro Osteocyte Differentiation | Gibco | 1832381 | 10/2017 |
| Trypan Blue | HyClone | 1835251 | 10/2019 |
| TrypLE Select | HyClone | 1799536 | 09/2018 |
| Vacuum filter flask | Thermo Scientific | 1186337 | 10/2021 |
| Filter Unit (0.22 µm) | Millex GP | R6JA81487 | 07/2019 |

#### 4.1.1 Medium for cell culturing

For culturing MMC 29A, MMC 96A1 and MMC 96B1, Medium D5+GF was used. The cell culture medium was prepared as follows: 25 mL FBS, 5 mL penstrep, 20 ng FGFb aliquoted in sterile $H_2O$ (40 µL), and 20 ng EGF aliquoted in sterile $H_2O$ (10 µL) were added to 470 mL DMEM High Glucose (containing L-glutamine), which resulted in a total volume of 500 mL

complete cell culture medium. All reagents were mixed and filtered using a 500 mL vacuum filter flask. The medium was prepared once or twice a week to feed and passage the cells.

For cryopreservation, freezing medium with 10% DMSO in FBS was prepared. The freezing medium was prepared as follows: 2.5 mL DMSO was added to 22.5 mL FBS resulting in 25 mL freeze medium (10% DMSO in FBS). The reagents were mixed and then filtered into a conical tube.

### 4.1.2 Thawing PMSCs

To expand the cells for flow cytometry experiments, the MMC 29A PMSC at explant passage 3 (P3) with a density of $1x10^6$ cells/ vial frozen on 3/21/2016 were thawed as follows on the 2/21/17. The vial was taken out of the liquid nitrogen tank and placed in a water bath (37 °C) for 1 minute. Thereafter, the freezing medium (1 mL) containing cells, was diluted in 9 mL D5+GF medium. The cell suspension was centrifuged (1,500 rpm for 7 minutes), the supernatant was discarded and the cell pellet was resuspended in 1 mL D5+GF medium. A volume of 20 µl cell suspension was mixed with 20 µl Trypan Blue reagent for counting the cells by hemocytometer. Two grids were filled with 10 µl suspension and four large squares were counted per grid. Unstained (viable) and stained (dead) cells were counted separately. The remaining cells were placed on ice during this procedure. A volume of 29 mL D5+GF medium was added to the cell suspension and the complete solution was transferred to a T150 flask (150 $cm^2$ cell culture flask). Cells were incubated at 37 °C and 5% $CO_2$ and high humidity.

### 4.1.3 Changing the medium

The medium of the MMC 29A PMSCs was changed every second day to provide the optimal environment for the cells to divide and to maintain the cell viability. For this, D5+GF medium was aliquoted (30 mL) and placed in a 37 °C water bath for 5 minutes. The confluence and condition of the cells (blebbing, swimming, attaching to culture flask) was controlled microscopically. The cell culture medium was discarded and 30 mL fresh D5+GF medium was added per T150 flask. The cells were placed in the incubator at 37 °C and 5% $CO_2$.

### 4.1.4 Passaging the PMSCs

Passaging the MMC 29A PMSC, passage 3 (P3) thawed on the 2/21/17 was performed by discarding the medium first and washing the cells by pipetting 15 mL PBS in the T150 flask. The PBS was discarded before the cells were detached by adding 6 mL of TrypLE Select to the flask, followed by incubation for 4 min at 37 °C. DMEM (18 mL) was added to inhibit TrypLE Select. The cells were detached from the bottom of the flasks by repetitive pipetting and then

the cell suspension was taken out and transferred to a centrifuge tube. The cell suspension was centrifuged at 1,500 rpm for 7 minutes. The supernatant was removed using a vacuum line. The pellet was resuspended in 12 mL D5+GF medium, stained and counted as described in chapter 4.2.1. Viability and cells/mL were calculated to seed $1.2*10^6$ cells/T150 flask. D5+GF was added to a total amount of 30 mL and placed in the incubator (37 °C, 5% $CO_2$).

The remaining cells were centrifuged at 1,500 rpm for 7 minutes and resuspended in 1 mL freezing medium. The vial was stored in an ethanol storage container at -80 °C overnight. The following day the vial was transferred to liquid nitrogen.

### 4.1.5 Preparing 60 mm dishes for RNA isolation

In order to gain RNA for PCR analysis, MMC 29A, MMC 96A1 and MMC 96B1 were cultured from P4 thawed on 3/10/2017. For this purpose, 84.000 cells of each cell line were plated in one 60 mm dish each. A volume of 5 mL medium D5+GF was added. Cells were placed in the incubator (7 °C, 5% $CO_2$). On the 3/20/2017, the cells were 80 - 90% confluent and ready for RNA isolation (see chapter 4.3.1).

### 4.1.6 Preparation of 24-well plates for testing cell secretion

For ELISA testing, MMC 29A P5, MMC 96A1 P4 and MMC 96B1 P4 were cultured in a 24-well plate. The cells were plated in three wells per cell line for triplicate ELISA testing. Per well 190,000 cells were plated, amounting to 100,000 cells per $cm^2$ in a total amount of 500 µL medium D5+GF. Cells were placed in the incubator (37 °C, 5% $CO_2$). After 24 hours, three times 0.5 mL medium of each cell line was collected in a 1 tube (1.5 mL per cell line). The samples were centrifuged for 10 minutes at 3,500 rpm and stored in -80 °C until ELISA analysis.

### 4.1.7 Preparation of 24-well plates for differentiation

For cell differentiation, MMC 29A P5 and MMC 96A1 P4 were cultured in a 24-well plate. The cells were plated on 3/28/2017 with two triplicates for each cell line. One triplicate was cultured in differentiation inducing culture medium. The other one was cultured in D5+GF as a negative control. Per well, 50,000 cells were plated and 1 mL medium. Cells were placed in the incubator (37 °C, 5% $CO_2$) for 24 days. Medium was changed 3 times a week on Monday, Wednesday and Friday.

### 4.2 Flow cytometry analysis

For flow cytometry, MMC 29A cells were collected, stained and measured using an Attune NxT Life Technology flow cytometer. In the following analysis, CD44 was tested instead of CD105 for the first flow cytometry analysis because of an unavailability of reagents. CD44 is known as a transmembrane glycoprotein which is found in a broad variety of tissues. The CD44 marker is also expressed during development and therefore detectable in embryonic tissue. (Sneath & Mangham, 1998) McCarty et al., (2009) showed that CD44 was highly expressed by flow cytometry in their ovine trial. Another stem cell marker, CD29, was also positive in the ovine analysis of McCarty et al (2009). CD31 is an endothelial marker and tested negative in prior ovine tests. (McCarty et al., 2009)

#### 4.2.1 Setting up flow cytometry

**Table 3: Reagents and materials used for the flow cytometry analysis.**

| Reagents | Vendor | Lot | Exp. Date |
|---|---|---|---|
| FBS | HyClone | AB10156964 | 02/2020 |
| PBS | HyClone | AB10156968 | 09/2018 |
| DMEM High Glucose | HyClone | 8H30243.01 | 10/2017 |
| D5+GF medium | | see 4.1 and 4.1.1 | see 4.1 and 4.1.1 |
| Formalin | Protocol | 360772 | 03/2020 |
| Accutase | - | - | - |
| Filter Unit (0.22 µm) | Millex GP | R6JA81487 | 07/2019 |
| Tube with Cell-Strainer Cap | Falcon | 18116020 | - |

All reagents and materials needed for the flow cytometric experiments are listed in table 3. To prepare the washing buffer, 0.25 mL FBS was added to 49.75 mL PBS. To produce staining buffer, 1 mL FBS was added to 49 mL PBS. For the fixation buffer, 5 mL 10% Formalin was added to 15 mL PBS to produce 2.5% formalin in PBS (20 mL), 0.2 mL FBS was added to 19.8 mL PBS to produce 1% FBS in PBS. Formalin in PBS (2.5%) and FBS (1%) in PBS were combined 1:2. After the reagent-preparation, all reagents were syringe filtered through 0.2 µm filters.

For the Compbead control procedure, 1 drop of negative bead solution was added to a 1.5 mL microcentrifuge tube for each of the three fluorochromes used. One drop of mouse IgG (pos) bead solution was added to each of the 1.5 mL microcentrifuge tubes in part 1. The antibody was added directly to the positive/negative bead combination at the staining concentration. Only one antibody was added per tube and stained at room temperature for 30 minutes.

Washing buffer (500 µL) was added to the tubes before the beads were pelleted by centrifugation at 3,500 rpm for 5 minutes. The washing buffer was discarded and the compbeads were resuspended in 500 µL fixation buffer. In this buffer, the samples could be stored for up to one week at 5 °C.

The NIR dead cell staining protocol was performed as follows. The cells were detached using TrypLE (3 mL for a T75 flask) for 4 minutes. DMEM (9 mL) was added and the suspension was centrifuged at 1,500 rpm for 7 minutes before the pellet was resuspended in 2 mL DMEM + 10% FBS. The suspension was transferred into two 15 mL conical tubes, 1 mL per tube. One tube was placed in a water bath at 56 °C for 1 hour to create the dead cell population. The other tube was placed on ice. After this incubation step, the suspension from the water bath was cooled down on ice for 10 minutes, 6 mL PBS was added to both tubes before centrifugation at 1,500 rpm for 7 minutes. The supernatant was aspirated and cells were resuspended in 100 µl PBS, 0.2 µL NIR Dead Stain Reagent was added to the suspension and incubated for 30 minutes. The staining buffer (300 µl) was added to the cell suspension and cells were centrifuged at 1,500 rpm for 8 minutes. The supernatant was aspirated again and the pellet was resuspended in 300 µL of 10% formalin for 30 minutes to fix the cells. Washing buffer was added (400 µl) and the suspension was centrifuged again (1,500 rpm for 8 minutes). The supernatant became discarded and the cells were resuspended in 500 µl fixation buffer and kept overnight at 5 °C.

The antibody-staining procedure was performed as follows. Cells were washed with sterile PBS (15 mL per flask). Thereafter, the cells were detached with 6 mL accutase per T150 flask (incubation at 37 °C for 5 minutes). The suspension was diluted by adding 18 mL DMEM and centrifuged at 1,500 rpm for 7 minutes. The cell counting procedure was performed as explained in chapter 4.1.2. For the unstained control, $1x10^6$ cells were removed immediately. The remaining cells were pelleted and resuspended in 10% formalin. After incubation for 30 minutes on ice, the cells were pelleted again and resuspended in fixation buffer. For each sample, $1x10^6$ cells were fractioned into 50 mL conical tube. Cells were pelleted again and resuspended in 100 µL sterile PBS per $1x10^6$ cells. NIR Dead Cell Stain Reagent was added (0.2 µl per $1x10^6$ cells) to the conical tube and incubated on ice for 30 minutes. For each $1x10^6$ cells, 1 mL staining buffer was added to the tubes containing the samples before the cells were pelleted and resuspended in 100 µL staining buffer per $1x10^6$ cells; than cells were fractioned into 1.5 mL micro centrifuge tubes with 100 µL each. Appropriate amounts (see table 4 and 5) of antibodies were added to the suspensions, followed by an incubation for 30 minutes whereby the samples were protected from light. Washing buffer (300 µl) was added and the samples were centrifuged at 1,500 rpm for 10 minutes. The pellets were resuspended in 300

µL 10% formalin in PBS for 30 minutes. After cell fixation, cells were centrifuged again at 1,800 rpm for 10 minutes and resuspended in 500 µL fixation buffer overnight. Immediately before running the samples on the flow cytometer, the cell samples were passed through sterile 70 µm filter caps into flow cytometry tubes. Stained samples were kept on ice and protected from light until use. Flow cytometry was performed on Attune NxT life technologies Fisher Scientific flow cytometer. The flow cytometer was proper calibrated and all samples were measured continuously within one hour.

**Table 4: Overview over the samples analyzed for the first flow cytometry experiment 3/3/2017**

| Sample | Ab | Fluorochrome | Vendor | Catalog | Isotype | Reactivity | Conc (mg/mL) | Volume (µL) |
|---|---|---|---|---|---|---|---|---|
| **1** | CD44 | FITC | Bio-Rad | MCA2219F | Ms IgG1 | Sheep | 0.1 | 10 |
| | CD45 | RPE | Bio-Rad | MCA2220PE | Ms IgG1 | Sheep | 0.1 | 10 |
| **2** | CD31 | FITC | Bio-Rad | MCA1097F | Ms IgG2a | Sheep | 0.1 | 10 |
| | CD14 | PE | abcam | ab186689 | Ms IgG2a | Sheep | - | 10 |
| **3** | CD90 | Unconj. | Ab Online | ABIN728038 | Rb Poly | Sheep | 1 | 1 |
| **4** | CD73 | PE | B.D. | 561014 | Ms IgG1, κ | Hu | 0.00625 | 20 |
| | CD29 | APC | B.D. | 561794 | Ms IgG1, κ | Hu | 0.05 | 20 |
| **CompBead controls** | | | | | | | | |
| **5** | CD44 | FITC | Bio-Rad | MCA2219F | Ms IgG1 | Sheep | 0.1 | 10 |
| **6** | CD45 | RPE | Bio-Rad | MCA2220PE | Ms IgG1 | Sheep | 0.1 | 10 |
| **7** | CD29 | APC | B.D. | 561794 | Ms IgG1, κ | Hu | 0.05 | 20 |
| **Isotype controls** | | | | | | | | |
| **8** | PE - Ms IgG1 | | B.D. | 555749 | Ms IgG1 | N/A | 0.05 | 20 |
| | FITC - Ms IgG1 | | B.D. | 555649 | Ms IgG1 | N/A | 0.05 | 20 |
| | APC - Ms IgG1 κ | | B.D. | 550854 | Ms IgG1, κ | N/A | 0.0125 | 80 |
| **9** | PE - Ms IgG2a | | abcam | ab91363 | Ms IgG2a | N/A | 0.025 | 20 |
| | FITC - Ms IgG2a | | Bio-Rad | MCA929F | Ms IgG2a | N/A | 0.1 | 10 |
| **10** | Rabbit IgG Poly | | Invitrogen | 026102 | Rb IgG1 | N/A | 0.2 | 5 |
| **Other control samples** | | | | | | | | |
| **11** | Near IR Live/Dead Fixed cell sample | | | | N/A | N/A | | 0,2 |
| **12** | Unstained Sample | | | | N/A | N/A | | N/A |

The second flow cytometry was performed on 3/9/2017 according to the scheme depicted in table 4. CD90 was expected to be positive on ovine cells. Secondary antibody staining was performed using different antibodies. The cells used to perform flow cytometry were the MMC29A P4 thawed on 3/9/2017.

**Table 5: Overview over the samples analyzed for the second flow cytometry experiment 3/3/2017**

| Sample | Ab | Fluorochrome | Vendor | Catalog | Isotype | Reactivity | Conc (mg/mL) | Volume (µL) |
|---|---|---|---|---|---|---|---|---|
| | **Secondary stain with anti-rabbit AF488** | | | | | | | |
| **1** | CD90 | Unconj. | Ab Online | ABIN728038 | Rb Poly | Sheep | 10 | 1 |
| | **Secondary stain with anti-rabbit AF546** | | | | | | | |
| **2** | CD90 | Unconj. | Ab Online | ABIN728038 | Rb Poly | Sheep | 10 | 1 |
| **CompBead Controls** | | | | | | | | |
| **3** | CD44 | FITC | Bio-Rad | MCA2219F | Ms IgG1 | Sheep | 0.1 | 10 |
| **4** | CD45 | RPE | Bio-Rad | MCA2220PE | Ms IgG1 | Sheep | 0.1 | 10 |
| **5** | CD29 | APC | B.D. | 561794 | Ms IgG1, κ | Hu | 0.05 | 20 |
| **Isotype Controls** | | | | | | | | |
| | **Secondary stain with anti-rabbit AF488** | | | | | | | |
| **6** | Rabbit IgG Poly | | Invitrogen | 026102 | Rb IgG1 | N/A | 0.2 | 50 |
| | **Secondary stain with anti-rabbit AF546** | | | | | | | |
| **7** | Rabbit IgG Poly | | Invitrogen | 026102 | Rb IgG1 | N/A | 0.2 | 50 |
| **Other control samples:** | | | | | | | | |
| **8** | Unstained Sample | | | | N/A | N/A | | N/A |

### 4.3 PCR

A PCR was set up on 4/5/2017 from frozen RNA isolations according to the protocol described below.

#### 4.3.1 RNA isolation

For PCR analysis RNA isolation was performed using the Qiagen RNeasy Plus mini Kit (Lot:154014614). The reagents and materials needed are listed in table 6.

**Table 6: Reagents and materials used for RNA isolation.**

| Reagents | Lot | Columns/Tubes | Lot |
|---|---|---|---|
| RLT buffer | 154012635 | RNeasy Mini Spin Column | 15401228 |
| RW1 buffer | 151054002 | gDNA Eliminator Column | 154012285 |
| RPE buffer | 154010956 | Collection Tube 1.5 mL | 154012588 |
| RNase-free water | 154012129 | Collection Tube 2.0 mL | 154012103 |
| 70% EtOH in DEPC | Nalgene P/N AM9915G L/N 160704 | QIA shredder Mini Spin Column | 11876917 |
| β-mercaptoethanol | 210007534 | | |

To prepare the lysis buffer, 6 µL β-mercaptoethanol was added to 600 µL RLT buffer resulting in 606 µL lysis buffer. This is an appropriate amount for one 60 mm dish. To extract RNA from

cells, the medium was removed using a sterile tip and the cells were lysed by adding 606 µL lysis buffer to the 60 mm dish. The tip was used to scrape the cells off the dish. Immediately thereafter, the lysed cells were collected and centrifuged in the QIAshredder column at 13,000 rpm for 30 seconds. The flow through was transferred to the genomic shredder column and centrifuged at 10,000 rpm for 30 seconds. An equal amount of 70% ethanol in DEPC (~600 µL) was added and mixed by gently pipetting up and down. Then, 700 µL suspension was transferred to the RNeasy column and centrifuged at 10,000 rpm for 15 seconds. The step was repeated with the rest of the ethanol-RNA suspension. The buffer RW1 was added (700 µL) and centrifuged at 10,000 rpm for 15 seconds. The flow through was discarded and 700 µL buffer RPE was added and centrifuged at 10,000 rpm for 15 seconds. Another 700 µL buffer RPE was added and centrifuged at 10,000 rpm for 2 minutes. The column was transferred to a clean 2 mL collection tube and centrifuged at 13,000 rpm for 1 minute. The column was transferred to a clean 1.5 mL collection tube and 30 µL RNase-free water was added directly to the center of the column. After one minute, the column was centrifuged at 10,000 rpm for 1 minute. The RNA was then in the RNase free water in the collection tube and was stored at –80 °C until the PCR was performed.

### 4.3.2 cDNA synthesis

For the PCR analysis, a reverse transcriptase-reaction was performed and cDNA was used to perform a PCR. The reagents used are listed in table 7.

**Table 7: Reagents and materials used for cDNA synthesis.**

| Reagents | Vendor | Lot. | Exp. |
|---|---|---|---|
| RNA | - | - | - |
| DEPC treated water | Ambion | 1612018 | - |
| 10 mM dNTP mix | Invitrogen | Mat.: 1005631 | - |
| Random Hexamer | aliquoted | - | - |
| 100 mM DTT (=dithiothreitol) | Invitrogen | 1842667 | - |
| RNase OUT | Invitrogen | 1835412 | 11/2019 |
| 5X FS buffer | Invitrogen | 1835412 | - |
| Superscript II | Invitrogen | 1775933 | 08/2017 |

Preparation of premix 1 was performed as follows. For each PCR tube, 1 µL Random Hexamer was added to 1 µL dNTP. This resulted in 2 µL per sample. For the preparation of premix 2 (-RT), without reverse transcriptase as a negative control, 2 µL DTT, 1 µL RNase OUT (=RNase inhibitor), 4 µL 5X FS buffer and 1 µL DEPC treated water were added together, which resulted in a total amount of 2 µL premix 2 -RT per PCR tube. The preparation of premix 3 +RT (contains reverse transcriptase) was done in the same way as premix 2 with the difference that 1 µL

Superscript II (=reverse transcriptase) was added. This resulted in a total amount of 2 µL premix 3 (+RT) per PCR tube.

To measure the RNA concentration, the Thermo Scientific Nanodrop 2000 was used and 1 µg RNA in 10 µL DEPC treated water was used for the reverse transcriptase reaction. For MMC29, a concentration of 1.09 µg/µL was calculated by the Nanodrop 2000. This resulted in the following calculation: $\frac{1\ \mu g}{1.09\ \mu g/\ \mu L}$ = 0.92 µL RNA extraction, 1 µg = 0.92 µL RNA extraction. To this volume, 9.08 µL DEPC treated water was added to gain an amount of 10 µL. The other RNA samples were prepared in an equal manner, using their respective RNA concentrations (MMC96 A1 0.4703 µg/ µL, MMC96 B1 0.3722 µg/ µL).

For the reverse transcriptase reaction, 10 µL (1 µg) RNA in DEPC treated water and 2 µL premix 1 was added to RNase free PCR tubes. The suspension was heated in the thermocycler for 5 minutes at 65 °C. Samples were chilled on ice immediately thereafter for 1 minute, then they were centrifuged for 5 seconds. Premix 2 (-RT) was added (8 µL) to each negative control, premix 3 (+RT) was added (8 µL) to the each sample and all were mixed by gently pipetting up and down. The thermal cycler program was set up at 42 °C for two hours and 70 °C for 10 minutes. Then, the cDNA samples were stored at -80 °C.

### 4.3.3 PCR protocol

For amplification of the cDNA, the Bioneer Accupower TaqPCR PreMix was used (Lot: 141320111J, Exp 10.2016). The used reagents and materials are listed in table 8.

The primers were supplied at concentrations between 18.0 and 26.0 nmol. In a first step, a stock of 100 µM was created. For example, when using a primer concentration of 19.5 nmoles, a concentration of 100 µM (100 µmoles/ L) requires dissolving the 19.5 nmoles (or 0.0195 µmoles) in 195 µL RT-PCR Grade Water using the following equations: $\frac{100\ \mu M}{0.0195\ \mu moles}$= 5128.21 and $\frac{1\ l}{5128.21}$= 0.0001949998 L = 195 µL.

**Table 8: Reagents and materials used for PCR.**

| Reagents | Vendor | Lot./Primer Nr. | Exp. |
|---|---|---|---|
| Bioneer Accupower TaqPCR PreMix | Bioneer | 141320111J | 10/2016 |
| RT-PCR Grade Water | Ambion | 1610071 | - |
| cDNA (see 4.3.2) | - | - | - |
| CD14 forward | Invitrogen | 335853A10 | - |
| CD14 reverse | Invitrogen | 335853A11 | - |
| CD31 forward | Invitrogen | 335853A08 | - |
| CD31 reverse | Invitrogen | 335853A09 | - |
| CD44 forward | Invitrogen | 335853A04 | - |
| CD44 reverse | Invitrogen | 335853A05 | - |
| CD45 forward | Invitrogen | 335853A06 | - |
| CD45 reverse | Invitrogen | 335853A07 | - |
| CD73 forward | Invitrogen | 337295B09 | - |
| CD73 reverse | Invitrogen | 337295B10 | - |
| CD90 forward | Invitrogen | 337295B11 | - |
| CD90 reverse | Invitrogen | 337295B12 | - |
| CD105 forward | Invitrogen | 337295C01 | - |
| CD105 reverse | Invitrogen | 337295C02 | - |

For the preparation of the primer mix, 10 µL forward primer and 10 µL reverse primer were added to 80 µL RT-PCR grade water, which resulted in a total amount of 100 µL primermix. The primers used for the characterization of MMC 29 (4/5/2017), (4/20/2017), MMC 96A1 and MMC 96B1 (4/20/2017) are listed in table 9. Each primer pair produces PCR products which can be separated by their base pair size.

**Table 9: Sheep MSC characterization RT-PCR primer.**

| gene | forward | reverse | size |
|---|---|---|---|
| **CD14** | TGACACAATCAAGGCTCTGC | CGACACGTTACGGAGACTGA | 211 bp |
| **CD29** | TTGCAAGTGTCGAGTGTGTG | TATTGAAGGCTCGGCACTGA | 242 bp |
| **CD31** | CTGGAGTCTTCAGCCACACA | TCCCACTCTGCCACTCTCTT | 152 bp |
| **CD44** | CGTACTGCTTCAATGCCTCA | GTAGCCTCCTGACGTGCTTC | 245 bp |
| **CD45** | CCACGGGTATTCAGCAAGTT | CCCAGATCATCCTCCAGAAA | 244 bp |
| **CD73** | TGTCGTGTGCCCAGTTATG | CTTTCCACGCACCAAAGTTATG | 704 bp |
| **CD90** | GGTCCTCTACCTGTCCAATTTC | CTCACTCTCCATCAGGTCTCTA | 550 bp |
| **CD105** | TCTGCTCTGCACCATCATAAG | CCTGGATGAGTTCCACGATTT | 389 bp |

The PCR was performed by adding 18.5 µL RT-PCR grade water to 20 µL Bioneer Accupower TaqPCR PreMix. Thereafter, 0.5 µL cDNA and 1 µL of 10 µM primer mix were added and mixed by gently pipetting up and down. The thermal cycler was set up with the following

program: 94 °C for 2 minutes, 94 °C for 10 seconds, 60 °C for 20 seconds, 72 °C for 30 seconds. This scheme, excluding the initial 2 minutes at 94 °C, was repeated 34 times. Then, a final step at 72 °C for 7 minutes followed before the cycler cooled down to 4 °C until the PCR products were taken out and stored at -20 °C.

For imaging of the samples, an agarose gel was prepared (table 10). The PCR products were separated by electrophoresis and a SYBR Safe DNA gel stain was used to make the products visible.

**Table 10: Reagents and materials used for preparing the agarose gel.**

| Reagents | Vendor | Lot. | Exp. |
|---|---|---|---|
| SYBR Safe DNA gel stain | Invitrogen | 1771519 | - |
| Agarose | SaeKem | AG6086 | 05/2010 |
| 50x TAE Buffer Tris/ acetic acid/ EDTA | Biorad | Cat. P1610743 | - |

To prepare a 2% agarose gel (2%), 0.7 g agarose was added to 35 mL TAE Buffer (50 fold diluted in aqua dest) The liquid was heated in a microwave for 35 seconds. Afterwards, 3 µL dye was added. When the heated glass flask with the gel could be touched for 5 seconds, the suspension was ready to be poured into the frame. Two gels were loaded with the samples (5 µL each) of all three cell lines.

### 4.4 ELISA testing

In order to test the BDNF-secretion the BioSource BDNF ELISA Kit (Lot: 20160718, Exp. 01/2018, all reagents have the same Lot. and Exp.) was used. The standards were ready to use and the protocol was performed as described in the manual of the kit.

Medium from the cell lines MMC 29A, MMC 96A1 and MMC96 B1 was tested undiluted and at two different dilutions (1:2) and (1:10), in triplicate for each dilution. Former experiments showed a high interference of the cell culture medium and FBS for this assay. Therefore, the higher dilutions might be more accurate as long as they are in range. The ELISA test was performed in the provided 96-well plate according to the template depicted in table 11. A minimal volume of 540 µL medium from each cell line was needed to run the ELISA in triplicates with different dilutions. For one well, 100 µL sample was used. For 1:2 dilutions 50 µL sample + 50 µL diluent was used in the analysis. For 1:10 dilutions 10 µL sample + 90 µL diluent was used.

**Table 11: Sheep BDNF ELISA template**

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | Standard: 1000 pg/ml | Standard: 1000 pg/ml | MMC 29A (undiluted) | MMC 29A (undiluted) | MMC 29A (undiluted) | MMC 96A1 (1:10) |
| B | Standard: 500 pg/ml | Standard: 500 pg/ml | MMC 29A (1:2) | MMC 29A (1:2) | MMC 29A (1:2) | MMC 96A1 (1:10) |
| C | Standard: 250 pg/ml | Standard: 250 pg/ml | MMC 29A (1:10) | MMC 29A (1:10) | MMC 29A (1:10) | MMC 96A1 (1:10) |
| D | Standard: 100 pg/ml | Standard: 100 pg/ml | MMC 96A1 (undiluted) | MMC 96A1 (undiluted) | MMC 96A1 (undiluted) | D5+GF Medium |
| E | Standard: 50 pg/ml | Standard: 50 pg/ml | MMC 96A1 (1:2) | MMC 96A1 (1:2) | MMC 96A1 (1:2) | D5+GF Medium |
| F | Standard: 0 pg/ml | Standard: 0 pg/m | MMC 96A1 (1:10) | MMC 96A1 (1:10) | MMC 96A1 (1:10) | |
| G | PBS | PBS | MMC 96B1 (undiluted) | MMC 96B1 (undiluted) | MMC 96B1 (undiluted) | |
| H | | | MMC 96B1 (1:2) | MMC 96B1 (1:2) | MMC 96B1 (1:2) | |

The ELISA was analyzed using a Spectra Max i3 plate reader (Molecular Devices) and the results were calculated by Soft Max Pro 6.3.1. The BioSource HGF ELISA Kit Lot: 07/2016 Exp. 02/2017 (all reagents have the same Lot. and Exp.) was used. The standards were ready to use and the protocol was performed as described in the kit manual. The 96-well plate layout was designed as shown in table 12.

**Table 12: Sheep HGF ELISA template** The samples written in a black font were collected after 24 h of plating 3/29/2017. The samples written in a blue font were collected after 24 h of plating 3/16/2017.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| A | Standard: 20 ng/ml | Standard: 20 ng/ml | MMC 29A (undiluted) | MMC 29A (undiluted) | MMC 29A (undiluted) | MMC 96A1 (1:10) | MMC 96A1 (1:10) | MMC 96A1 (1:10) | MMC 96B1 (1:2) |
| B | Standard: 10 ng/ml | Standard: 10 ng/ml | MMC 29A (1:2) | MMC 29A (1:2) | MMC 29A (1:2) | MMC 29A (undiluted) | MMC 29A (undiluted) | MMC 29A (undiluted) | MMC 96B1 (1:2) |
| C | Standard: 5 ng/ml | Standard: 5 ng/ml | MMC 29A (1:10) | MMC 29A (1:10) | MMC 29A (1:10) | MMC 29A (1:2) | MMC 29A (1:2) | MMC 29A (1:2) | MMC 96B1 (1:2) |
| D | Standard: 2.5 ng/ml | Standard: 2.5 ng/ml | MMC 96A1 (undiluted) | MMC 96A1 (undiluted) | MMC 96A1 (undiluted) | MMC 29A (1:10) | MMC 29A (1:10) | MMC 29A (1:10) | MMC 96B1 (1:10) |
| E | Standard: 1.25 ng/ml | Standard: 1.25 ng/ml | MMC 96A1 (1:2) | MMC 96A1 (1:2) | MMC 96A1 (1:2) | MMC 96A1 (undiluted) | MMC 96A1 (undiluted) | MMC 96A1 (undiluted) | MMC 96B1 (1:10) |
| F | Standard: 0.625 ng/ml | Standard: 0.625 ng/ml | MMC 96A1 (1:10) | MMC 96A1 (1:10) | MMC 96A1 (1:10) | MMC 96A1 (1:2) | MMC 96A1 (1:2) | MMC 96A1 (1:2) | MMC 96B1 (1:10) |
| G | Standard: 0 Sample Diluent | Standard: 0 Sample Diluent | MMC 96B1 (undiluted) | MMC 96B1 (undiluted) | MMC 96B1 (undiluted) | MMC 96A1 (1:10) | MMC 96A1 (1:10) | MMC 96A1 (1:10) | D5+GF Medium |
| H | | | MMC 96B1 (1:2) | MMC 96B1 (1:2) | MMC 96B1 (1:2) | MMC 96B1 (undiluted) | MMC 96B1 (undiluted) | MMC 96B1 (undiluted) | D5+GF Medium |

The O.Ds were read and a standard curve was plotted as recommended in the manual.

### 4.5 Cytokine array

The Ray Bio Ovine Cytokine Array Q1(1) Kit (Lot: 71816 26001, Exp. 01/2017) was performed on 4/13/2017. The included cytokines for this kit were CXCL9 (MIG), Decorin, IFN-γ, lL-8, IL-17A, IL-21, IP-10, sFRP-3, TNF-α and VEGF-A. The array is a multiplexed sandwich ELISA-based quantitative array which enables the simultaneous detection of the above mentioned cytokines. The glass slide is divided into 16 wells and every well provides quadruplicates of identical cytokine antibody arrays. The main focus for data regarding this array was placed on VEGF (Vascular Endothelial Growth Factor) because it is known for effects in wound healing (Ziegler et al.,1997). All other cytokines were not tested with a specific purpose. The protocol was performed as described in the kit manual. Manuals were provided by Ray Bio and are available online (Ray Bio, 2017). For the materials, no exp. date was provided. Materials included in the kit are listed in table 13.

**Table 13: Materials for Ovine Cytokine Array**

| Materials | Lot |
|---|---|
| Glass Slide | Q0382615 |
| Quantibody Sample Diluent | Q0662116 |
| 20X Wash Buffer l | 0621616 |
| 20X Wash Buffer ll | 0612016 |
| Standard Mix | Q034031 |
| Biotynilated Antibody Cocktail | Q035415 |
| Cy3 equivalent dye-conjugated Streptavidin | Q0632216 |
| Slide Washer/ Dryer | - |
| Adhesive Film | - |

The cytokine array was set up at 4/12/17 according to the scheme provided in table 14 and 15. Per well a volume of 100 µL sample was used. The data was obtained using a GenePix Molecular Device equipped with a Cy3 wavelength for fluorescence detection.

**Table 14 and 15**: The template for the cytokine array is on the left (14). Each well is built up in spotted quadruplicates of antibodies for cytokine detection as depicted in the Table 15 on the right.

| | 1 | 2 |
|---|---|---|
| A | CNTRL | Standard 1 |
| B | Standard 2 | Standard 3 |
| C | Standard 4 | Standard 5 |
| D | Standard 6 | Standard 7 |
| E | D5 | MMC 29A undiluted |
| F | MMC 29A 1:2 | MMC 29A 1:10 |
| G | MMC 96A1 undiluted | MMC 96A1 1:10 |
| H | MMC 96B1 undiluted | MMC 96B1 1:10 |

| 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|
| POS 1 | | | | POS 2 | | | |
| CXCL9 | | | | Decorin | | | |
| IFN-γ | | | | IL-8 | | | |
| IL-17A | | | | IL-21 | | | |
| IP-10 | | | | sFRP-3 | | | |
| TNF-α | | | | VEGF-A | | | |

### 4.6 Cell differentiation

The cells were fixed and stained on 3/21/2017, 24 days after inducing cell differentiation. The dyes used to stain the cells to determine the degree of differentiation were already prepared and stored at RT, Lot numbers and expiry dates were not available for these solutions. For the adipocytes the Oil Red staining protocol was used. For the osteocytes the Alizarin Red staining protocol was used. All samples were washed 2 times in PBS and fixed in formalin for 30 minutes. After aspirating the formalin the cells were washed 3 times in PBS for 5 minutes.

For the Oil Red staining the cells were rinsed with aqua dest. twice. The whole amount of aqua dest. was aspirated. To cover all cells, 500 µL Oil Red staining solution was added to the wells of the 24-well plate. After 50 minutes incubation at room temperature, the solution was aspirated and the cells were washed with aqua dest. 3 times. The nuclei were stained with hematoxylin solution (500 µL) for 10 minutes. Then, the cells were rinsed twice using aqua dest. and left in PBS until imaging took place. Prior experiments showed that the staining resulted in blue nucleolus (hematoxylin) and red lipid droplets when the differentiation into adipocytes was successful.

For the Alizarin Red staining, PBS was aspirated and the Alizarin staining solution (500 µL) was added to the wells of the 24-well plate and incubated at room temperature for 2 minutes. Then, the cells were washed with aqua dest. once and with PBS twice. Until imaging took place, the cells were kept in PBS. A successful differentiation into osteocytes was shown by calcium deposits stained orange red.

## 5. Results

The cells for this study were cultured using the already existing protocols available in the laboratory. For this study, cells were kept in culture for a maximum of 24 days. During cell culture, the cell viability stayed constant for the complete duration of the study. Optical characteristics of the cells were judged by bright field microscopy. The conditions were continuously good and a contamination never occurred. Therefore, all cells cultured for this project could be used to establish and test the methods described above. MMC 29A grew faster at same passage number when compared to MMC 96B1. This is the reason why MMC 96B1 was excluded from the cell differentiation experiments. The successfully performed culturing of the cells provided the following results.

The results of the flow cytometry analysis from 3/9/2017 are summarized in figure 3. These antibodies were previously not tested in the laboratory. The protocol for performing flow cytometry has been used for human PMSC and showed positive results for CD90, CD73 and CD105.

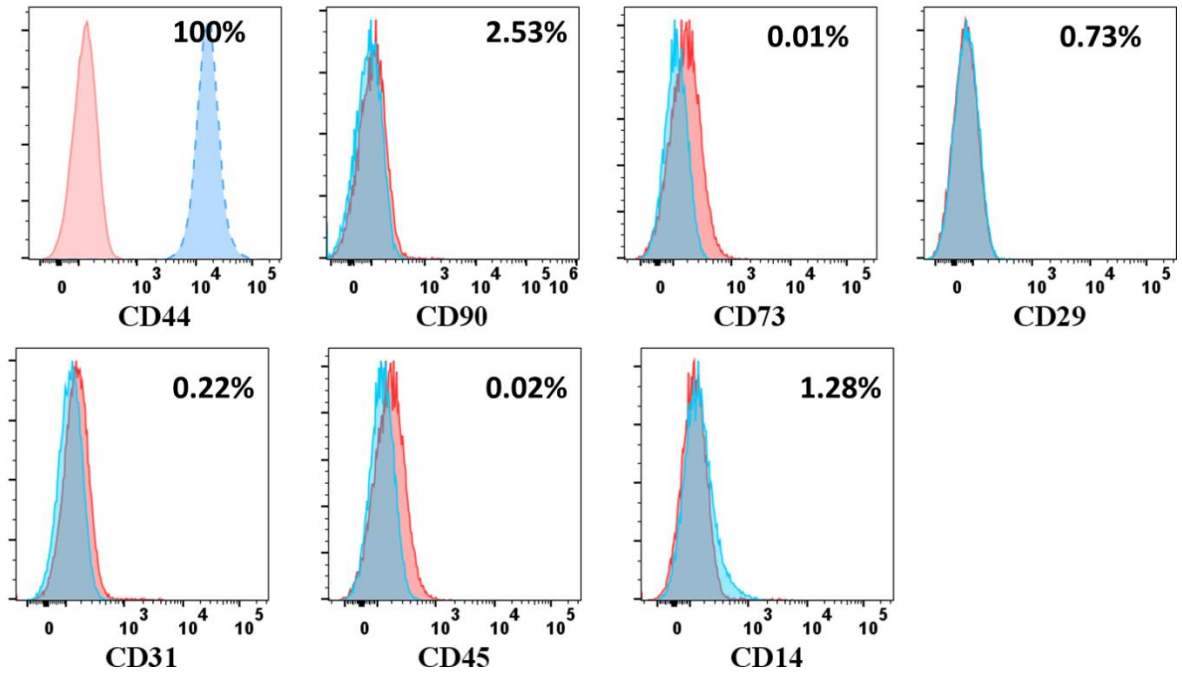



**Figure 3: Flow Cytometry results for MMC 29A.** The results showed the staining for the different cell surface markers as depicted underneath the x-axis. The histograms show the counted cells on the y-axis (vertical) and the detected amount of light gathered by the photomultiplier on the x-axis (horizontal). The red graphs depict the results of the unstained cells, the blue ones of the stained cells.

The results showed that the cells were 100% positive for CD44. In case of CD90 and CD73, which are markers that were expected to be positive, the stained and unstained samples cannot be clearly separated and the fluorescent detected light was only measured at a very

low and probably unspecific manner. The analysis was repeated for CD90 using different secondary antibodies (see table 5). The results of this flow cytometric analysis performed on 3/15/2017 again resulted in negative results for CD90 (data not shown). The other cell surface markers were negative, which correlated to the expected result (figure 3 and data not shown). Therefore, the flow cytometric analysis showed, that only CD44 could be used as a positive marker for these cells in this experiment. To prove, that the cells express genes for CD-markers, in order to meet the minimal criteria for MSCs, a novel PCR was set up on 4/5/2017. The samples were loaded onto the gel after amplification using the schedule depicted in table 16. The order of the cell lines used was **MMC 29A, 96A1 and 96B1**. The results of the PCR analysis are shown in figure 4.

**Table 16: Template used for the agarose gel electrophoresis.** MMC 29A samples were loaded in the first 16 lanes, MMC 96A1 samples in the second 16 lanes and MMC 96B1 in the last 16 lanes.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **MMC** | CD 90 | CD 90 | CD 73 | CD 73 | CD 105 | CD 105 | CD 44 | CD 44 | CD 45 | CD 45 | CD 29 | CD 29 | CD 31 | CD 31 | CD 14 | CD 14 |
| **29 A** | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- |
| **96A1** | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- |
| **96B1** | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- | RT+ | RT- |

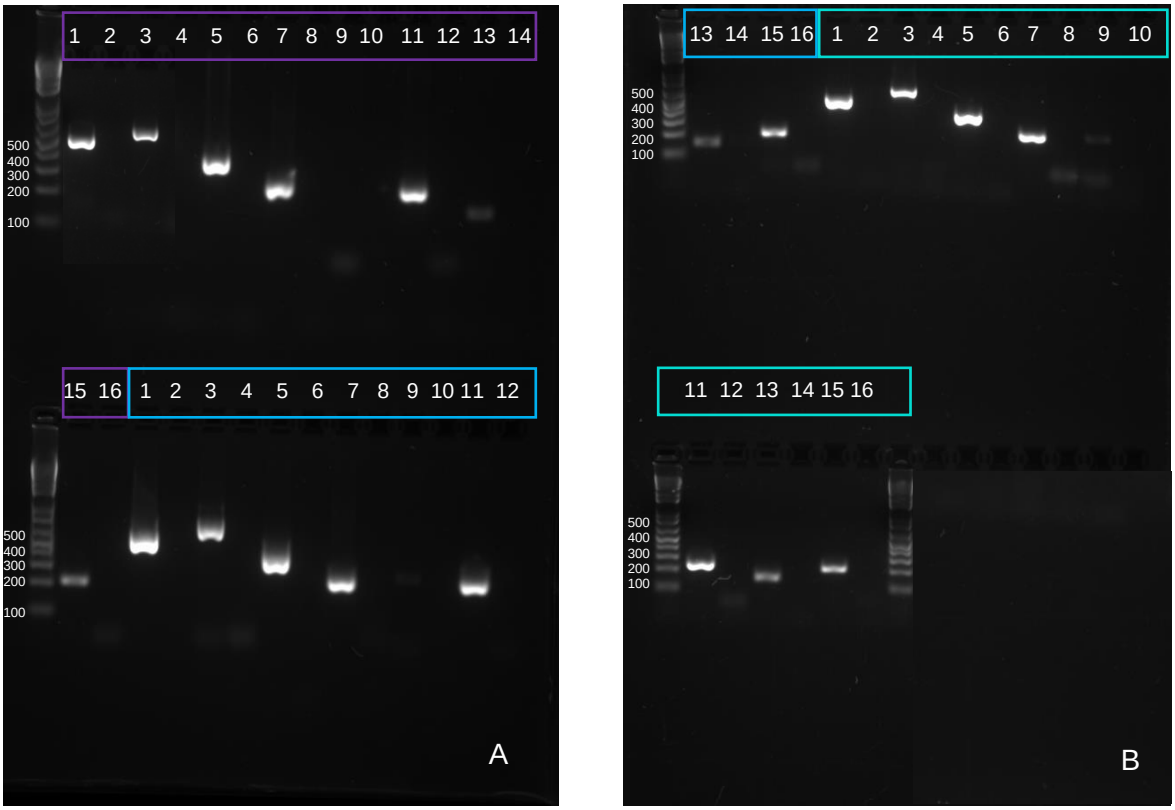



**Figure 4: PCR products on a stained 2% agarose gel.** A shows MMC 29A sample 1-16 and MMC 96A1 sample 1-12, B shows MMC 96A1 sample 13-16 and MMC 96B1 sample 1-16. The left lanes show the size in bp of amplified PCR products starting with 100 at the bottom.

All 3 cell lines showed amplified PCR products for CD90, CD73, CD105, CD44 and CD29 (MSC markers). Some bands in the lanes 9 for CD45, which is a leucocyte marker were weakly positive. All three cell lines showed a very faint band for CD31 (endothelial marker, lane 13) and CD14 (blood cell marker, lane 15). These data show that the cell lines express the expected MSC markers on the RNA level and in addition, some very low expression rates of other, non-MSC markers could be observed. Thereafter, ELISAs were performed to determine whether the cultured cells show a specific MSC secretory pattern. ELISA testing is a common method in the laboratory, but it was the first time testing this sheep reactive ELISA kit. The BDNF ELISA results are depicted in table 17-19. The standard curve was constructed by plotting the average O.D. for all standards on the vertical y-axis against the known concentrations on the x-axis. The recommended blank control for this test is PBS. As second control, the medium D5+GF was used to determine the interference of the medium on test results. A four-parameter logistic curve by Soft Max Pro 6.3.1. was created as recommended in the BDNF manual (figure 5). The concentrations were calculated corresponding to the mean absorbance from the standard curve by the use of a mathematical equation. The results are listed in table 18.

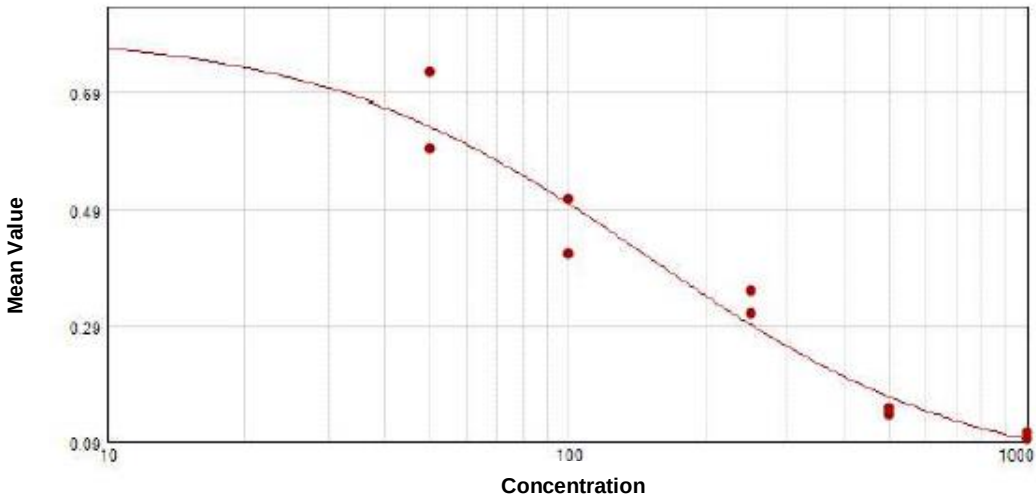



**Figure 5: A 4 parameter logistic curve was created.** By measuring multiple samples with known concentrations, the unknown samples were determined.

The 4 parameter logistic equation: $y = \mathrm{D} + \frac{\mathrm{A}-\mathrm{D}}{1+(\frac{x}{C})^{B}}$. The estimated value for the parameter A was 0.794, for parameter B 1.225, for parameter C 149.5 and for parameter D 0.025. In this equation Y is the response variable, X is the quantity of substance, A is the response at zero dose and D is the response at the highest dose. The midpoint dose where 50% of antibody-antigen binding happens stands for C and B is the slope factor. (Christopoulos and Diamandis,

1996) $R^2$ was calculated to be 0.966. In table 17, the known concentrations from the standards are compared with the O.D. values. From the duplicates, the mean values were calculated.

**Table 17: Known standard concentrations and O.D. values.**

| Standard | Conc. | Value | Mean Value |
|---|---|---|---|
| 1 | 1000 | 0.094<br>0.105 | 0.099 |
| 2 | 500 | 0.147<br>0.135 | 0.142 |
| 3 | 250 | 0.350<br>0.312 | 0.331 |
| 4 | 100 | 0.507<br>0.413 | 0.460 |
| 5 | 50 | 0.725<br>0.597 | 0.561 |
| 6 | 0 | 0.833<br>0.745 | 0.789 |

**Table 18: Results of the BDNF ELISA** (calculated by Soft Max Pro 6.3.1)

| Sample | Value | Result | Mean Result | SD | Adjusted Results |
|---|---|---|---|---|---|
| MMC 29A (undiluted) | 0.180<br>0.177 | 458.30<br>466.88 | 452.59 | 6.07 | |
| MMC 96A1 (undiluted) | 0.186<br>0.197<br>0.187 | 442.79<br>413.31<br>493.40 | 431.84 | 16.13 | |
| MMC 96B1 (undiluted) | 0.228<br>0.241<br>0.224 | 345.62<br>322.02<br>352.72 | 340.12 | 16.07 | 0.422 |
| MMC 29A (1:2) | 0.245<br>0.310<br>0.296 | 318.83<br>229.84<br>245.11 | 264.59 | 47.59 | 529.18 |
| MMC 96A1 (1:2) | 0.285<br>0.315<br>0.294 | 257.96<br>224.59<br>247.98 | 243.54 | 17.08 | 487.09 |
| MMC 96B1 (1:2) | 0.296<br>0.292<br>0.294 | 245.22<br>249.72<br>247.17 | 247.37 | 2.26 | 494.74 |
| MMC 29A (1:10) | 0.395<br>0.397<br>0.422 | 158.95<br>157.34<br>141.67 | 152.66 | 9.55 | 1526.55 |
| MMC 96A1 (1:10) | 0.449<br>0.470<br>0.492 | 126.37<br>115.01<br>104.67 | 115.35 | 10.85 | 1153.49 |
| MMC 96B1 (1:10) | 0.317<br>0.440<br>0.328 | 222.97<br>131.22<br>212.27 | 188.82 | 50.17 | 1888.20 |
| D5+GF | 0.226<br>0.177 | | | | |
| PBS | 0.739<br>0.573 | | | | |

The results of the 1:10 dilutions contained the lowest amount of medium, which showed interference in the test (table 18). These dilutions were all in range and could therefore be used for the calculations. The D5 interference was not considered and the averaged PBS blank control values were calculated as samples (43.23) and subtracted. Values were calculated using the following mathematical equation:

$$y = \mathrm{d} + \frac{\mathrm{a} - \mathrm{d}}{1 + (\frac{\mathrm{x}}{\mathrm{c}})^2}$$

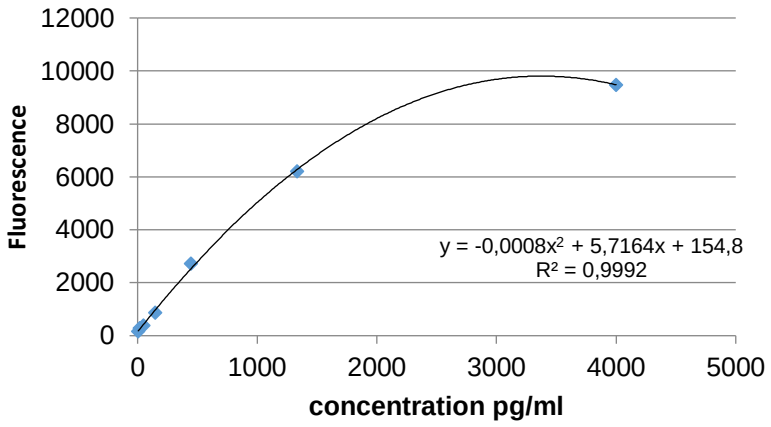
The levels of BDNF were calculated in pg/mL, these values are shown in table 19.

**Table 19: Calculated BDNF levels in pg/mL**.

| BDNF | pg/mL |
|---|---|
| MMC 29A (1:10) | 1482.77 |
| MMC 96A1 (1:10) | 1110.26 |
| MMC 96B1 (1:10) | 1844.97 |

The results of the HGF ELISA showed that almost all values were below the lowest standard and therefore out of range. Medium only (D5+GF) showed high interference in the analysis (data not shown). This was the first time the sheep reactive HGF ELISA was performed.

To obtain a more complementary picture of the secreted cytokines by these cell lines, a cytokine array was performed. In the scope of this Bachelor thesis, the focus is placed on Decorin, VEGF-A, and sFRP-3, which was secreted in detectable amounts by the cells in culture. Standard curves and values were gained for Decorin, sFRP-3 and VEGF-A. A calculation is shown for Decorin as an example (figure 6 and table 20). The results for all parameters analyzed are shown in table 21.




| Standards pg/ml | RFU |
|---|---|
| 4000 | 9480.333 |
| 1333 | 6198.000 |
| 444 | 2730.333 |
| 148 | 878.6667 |
| 49 | 394.3333 |
| 16 | 276.6667 |
| 5 | 157.3333 |
| 0 | 130.6667 |

**Figure 6: Standard curve for Decorin and table depicting the analyzed RLUs for the standards.**

**Table 20: Calculation for all final values at the example of Decorin.** The final values were calculated by multiplying with the dilution factors.

| | RFU | conc. | dilution | final value pg/mL |
|---|---|---|---|---|
| D5+GF | 2798.333 | (497) | | |
| MMC 29A undiluted | 5140.667 | 1016.93 | 1 | 1016.93 |
| MMC 29A 1:2 | 10668.67 | 1482.36 | 2 | 2964.72 |
| MMC 29A 1:10 | 6266 | 1308.75 | 10 | 13087.5 |
| MMC 96A1 undiluted | 11822 | - | | |
| MMC 96A1 1:10 | 6870.667 | 1482.36 | 10 | 14823.6 |
| MMC 96B1 undiluted | 10699.67 | - | | |
| MMC 96B1 1:10 | 12448.67 | - | | |

As this was the first time the ovine cytokine array was tested, the D5+GF medium only was analyzed to detect fundamental interference of the medium on the test, but this value was not subtracted from the final values because of the different dilutions.

**Table 21: Calculated results from the cytokine array.** Dilution factors are already included in these calculations.

| Cells | Decorin (pg/mL) | VEGF-A (pg/mL) | sFRP-3 (pg/mL) |
|---|---|---|---|
| MMC 29A undiluted | 1016.93 | 2.47 | 491.78 |
| MMC 29A (1:2) | 2964.72 | 20.65 | 850.82 |
| MMC 29A (1:10) | 13087.5 | 34.86 | 2827.73 |
| MMC 96A1 undiluted | above standard curve | 10.52 | 527.81 |
| MMC 96A1 (1:10) | 14823.6 | 41.10 | 2875.69 |
| MMC 96B1 undiluted | above standard curve | 40.28 | 1879.9 |
| MMC 96B1 (1:10) | above standard curve | 66.47 | 3399.75 |

Thereafter, the differentiation of the cell lines was determined using microscopic imaging. By using provided protocols from human PMSC cell differentiation, the process was performed for ovine cells the second time in the lab (prior testing was not successful, data not shown). The results showed for the linages MMC 29A and MMC 96A1 positive results for the osteogenic differentiation. In contrast, both controls were negative. The induction of the adipogenic differentiation was negative for both cell lines. Only the nuclei were stained by hematoxylin, but there are no red stained lipids in the preparations (figure 7).

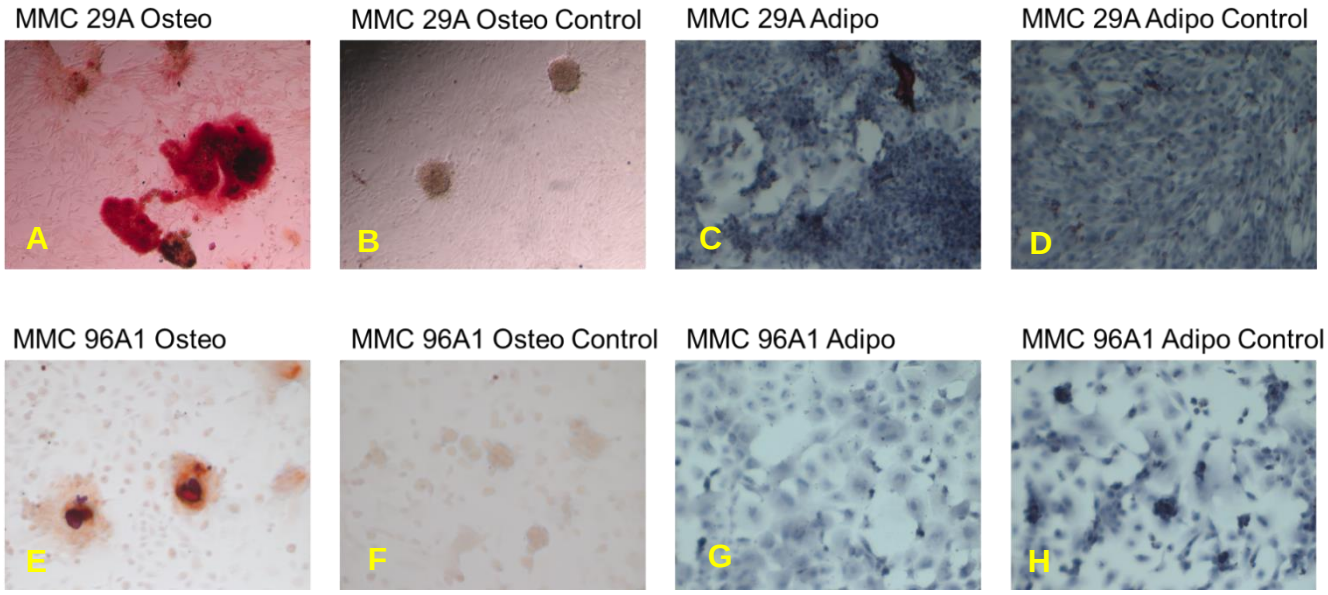



**Figure 7: Differentiation of MMC 29A (A, B, C, D) and MMC 96A1 (E, F, G, H).** A/E: Successful induced osteogenic differentiation. B/F: Negative control for osteogenic differentiation. C/G: Adipogenic differentiation was not induced. D/H: Adipogenic control

These results showed that the osteogenic differentiation could be induced successfully, whereas this was not possible for the adipogenic differentiation.

In summary, the results obtained with the complete array of different methods showed that accurate performed cell culturing provided sufficient amounts of cells for the different applications. CD44 was confirmed to be present on the cell surface of the tested cells (MMC 29A, MMC 96A1, MMC 96B1) by flow cytometry. By PCR, it was shown, that these cells produced the RNA and therefore also expressed the genes for the CD-markers to meet the minimal criteria for PMSCs (CD90, CD73, CD105, CD44 and CD29). A certain amount of BDNF was detected by ELISA testing and higher dilutions showed increasing amounts of BDNF. The secretion of HGF could not be determined, because most values were below the lowest standard and medium showed a high interference. By cytokine array, Decorin, sFRP-3 and VEGF-A was detected positive in all three samples. The results from differentiation showed that osteogenic differentiation was induced successfully, whereas the adipogenic differentiation could not be induced.

## 6. Discussion

In the scope of this Bachelor thesis, the aim was to develop several different novel laboratory-based techniques for the generation and analysis of PMSCs. The results showed that only a combination of several different methods and techniques provides a comprehensive picture on the quality, potential and status of the generated PMCS. For the described study, ovine cells were used, since the pre-clinical experiments to determine the proof-of-principle of using stem cells for the treatment of spina bifida are currently performed on sheep. The ovine model is not a very commonly used animal model. Therefore, the sheep reactive reagents needed for the different analysis techniques described in this study are not widely commercially available. This resulted in difficulties obtaining the full panels needed for a thorough analysis and of course also increases the costs of these experiments.

The flow cytometry results showed that CD44 was presented on the cell surface of ovine PMSCs. The other surface antigens that were analyzed (CD45, CD31, CD14, CD90, CD73 and CD29) were all negative. McCarty et al. published 2009 data of flow cytometry characterization of sheep PMSCs, their data showed that CD29 was tested positive in their ovine MSC characterization. Unfortunately, there are no details regarding the reagents and methods available in the published manuscript. Since there was a discrepancy between the results found in the scope of this thesis and those from McCarty et al., experiments were performed to increase the specific binding to by increasing the CD90-specific antibody by a factor 10. Moreover, also the amounts of the secondary antibodies were 10 times increased. These alterations did not affect the results (data not shown). Therefore, the species specific surface antigens could not be detected by the antibodies used in the present study. The staining procedure was performed as for human PMSCs, which confirmed the presence of CD90, CD73 and CD 105 prior. The confirmation of CD44 verified, that the staining procedure for flow cytometry worked, since all samples were treated equally. A new batch of antibodies should be tried in further flow cytometry experiments to check if the used antibodies are defective. The antibody used, was described as sheep reactive, but this could not be confirmed with the results presented above. These data also imply that the negative staining obtained for all other CD markers should be taken cautiously, since false negative results might have occurred. Next steps should include control experiments using sheep blood cells for the respectively negative markers to confirm the species-specific binding of the selected antibodies.

PCR analysis was performed to prove that the cells produce the RNA and therefore also express the genes for the CD-markers which are known to be the minimal criteria for PMSCs. All cell lines showed positive results for CD90, CD73, CD105, CD44 and CD29 RNA

expression, which are markers for MSCs. The presence of RNA is not to be equated with the presence of CD-markers. By PCR, the sequences which code for the requested CD-markers were found but this is not a proof that the cell surface markers are present on the outside of the cell. It is a common practice to compare the amount of mRNA to proxies to the amount of the corresponding proteins, but several different regulatory processes occur after mRNA is made. The series of action to produce cellular protein includes a lot of linked processes like synthesis and degradation of mRNAs, translation, localization, modification and the programmed destruction of proteins itself. Investigations on mRNA and protein levels in single cells showed that mRNA concentrations just explain ~54-77% of the variance in average protein levels. The difference in protein production and turnover depends on cellular conditions and principles governed by their regulation. (Vogel and Marcotte, 2012) That is why PCR cannot just replace flow cytometry and future trials should not neglect flow cytometry data. Some faint bands that were observed most probably stem from the primers. All three cell lines showed moderate positive results for CD31 (endothelial marker) and CD14 (blood cell marker). The population of cells can have up to 2% of CD45, CD31 and CD14 according to the minimal criteria of MSCs (Dominici et al., 2006). Unfortunately, a quantification of the cell populations was not possible using normal PCR. For this purpose, real time PCR should be performed. This quantification should be performed in a next step to assure that the amount of non-MSCs is within the boundaries to reach the minimal criteria.

The secretion of BDNF analyzed by ELISA showed that this protein was present at detectable amounts, which confirms the usual characteristics of PMSCs. The results of the 1:10 dilution were the most reliable ones, because the interference of the medium (D5) can be neglected at this dilution. Former trials showed a high interference in the ELISA depending on the concentration of FBS in the medium (D5+GF). For this reason, calculated results from undiluted samples and 1:2 dilutions are not considered to be accurate. Due to the competitive design of this ELISA, it could be observed that the signal increased with an increasing dilution factor. When aiming for a higher sensitivity, the dilution factors can be increased even further. Other ELISA methods were also tested for the sheep cells, but did not provide reliable results (data not shown). For further analysis, it is recommended to try other competitive ELISA systems if available. In addition, the development of a new ELISA could be taken into consideration to allow for a tailor made optimization process. In addition, the correlation coefficient of the standard should increase in further testing to at least 0.966 in order to obtain reliable data. The HGF ELISA (data not shown) showed most values below the lowest standard and the concentrations are therefore out of range. D5+GF medium only showed values slightly over the lowest standard. Concentrations and backgrounds could therefore not be separated. Relating to the gained data it could not be determined whether secretion took place or not. At

the time of use, the ELISA kit was expired. Therefore, it is recommended to try another HGF ELISA to obtain more reliable data.

The use of the cytokine array resulted in the detection of certain amounts of cytokines, but at varying concentrations. The dilutions gave significantly higher values than the undiluted samples. This was observed before when testing the BDNF ELISA. The cytokine array manufacturer recommends the use of serum-free conditioned media. The use of serum free media for culturing ovine MSCs was tried prior but with very limiting success. For future applications, a serum free cell culture medium should be considered, since a lot of antibody-antigen based testing applications recommend serum free culture conditions. Therefore, the medium was supposed to interfere in the analysis. To provide more consistent results in future experiments, the severity of rocking during the incubation steps should be decreased. To reduce the background signals for the negative controls, the manufacturer recommends that the wash buffer should be removed completely in each step. In the presented study, all suspensions were removed using a vacuum line. The glass slide should not be touched by the tip, but it should be completely dried which is in combination nearly impossible to perform.

There were several limiting factors according to the cytokine array. The glass slide provided just 8 wells for samples, whereas one well was used for D5+GF medium. The remaining 7 wells left no space for testing in duplicates or triplicates and different dilution series. Additionally, the high cost of this array was another limiting factor, furthermore it should be mentioned that the cytokine array was expired at the time of use. Therefore, another array should be used to gain more accurate data. However, the costs of this array are high and it is therefore difficult to optimize the system for the experimental set-up described in this thesis. The array contained cytokines known to play a role in inflammation, such as IL-21, IL-17, TNF-α and IL-8 (Watts et al, 2013). The absence of these cytokines in the cell culture supernatants was therefore expected. VEGF has been described to be secreted by PMSCs (Bhattacharya and Stubblefield, 2015), which could be confirmed with our data. Furthermore, the cytokine array was the only commercially available multiplex array suitable for ovine application at the time of the trial. The experiment showed the secretion of VEGF, sFRP-3 and Decorin by all cell lines tested. The secretion of sFRP-3 and Decorin has not been described before and their presence and mechanisms of action should be further explored.

Finally, the cell differentiation experiments worked for the osteogenic but not for adipogenic differentiation. Both samples were treated equal and the reasons for the different results are currently unknown. The ingredients of the differentiation medium are not provided by the manufacturer, further insight in these might improve our knowledge on the observed

discrepancies. Therefore, the cell differentiation experiments need to be repeated and further research has to be performed. This was not possible in the scope of the presented study.

## 7. Conclusion

The aim of this bachelor was, to develop different laboratory-based techniques to investigate PMSCs and their mechanism of action. The results presented in this study show that flow cytometry is a powerful method, but strongly depends on the availability of the right species-specific reagents. In addition, ELISA testing and the cytokine array were shown to be effective, but these methods also need the appropriate species-specific reagents. Nevertheless, the fetal ovine model is a valuable and proven animal model for spina bifida and the limiting factor regarding the rare availability of sheep reactive reagents should therefore be circumvented. Compared to ELISA and flow cytometry, PCR is species independent and an excellent method for investigating ovine PMSCs. The combination of all analytical methods used would be ideal in order to provide comprehensive data for the characterization and investigation of these cells. The experiments performed in this thesis were part of the preparatory work for autologous stem cell transplantation. The hypothesis that the use of novel lab-based methods will aid the further analysis of PMSCs used to improve paralysis in children affected by MMC was therefore confirmed.

## 8. References

Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A, 2016. Flow cytometry: basic principles and applications. Critical Reviews in Biotechnology 2017 37: 163-176

Adzick NS, Thom EA, Spong CY, Brock JW, Burrows PK, Johnson MP, Lori JH, et al., 2012. A randomized trial of prenatal versus postnatal repair of myelomeningocele. The New England Journal of Medicine 364: 993-1004

Bhattacharya N, Stubblefield PG, 2015. Regenerative medicine. Using Non-Fetal Source of Stem Cells. London, Springer: 10

Barlow S, Brooke G chatterjee K, Price G, Pelekanos R, Rossetti T, Doody M, Venter D, Pain S, Gilshenan K, Atkinson K, 2008. Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. Stem Cells Development 17: 1095-107

Christopoulos TK, Diamandis EP, 1996. Immunoassay. An Imprint of Elsevier. San Diego: Academic Press: 70

Dominici M, Le Blanc K, Mueller I, Slaper-Cortnbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM, 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy Position Statement. Cytotherapy 4: 315-317

Fung ET, 2004. Protein arrays. Methods and protocols. New Jersey: Humana Press Inc: 229

Gressner AM, Arndt T, 2013. Lexikon der Medizinischen Laboratoriumsdiagnostik. 2nd Edition. Berlin Heidelberg: Springer: 734

Hallbach J, 2011. Klinische Chemie und Hämatologie. Biomedizinische Analytik für MTLA und Studium. 3. Auflage. Stuttgart New York: Thieme: 69-71

Harding J, Roberts RM, Mirochnitchenko O, 2013. Large animal models for stem cell therapy. Stem Cell Research & Therapy 4: 23

Harrison MA, Rae F, 1997. General techniques of cell culture. Cambridge: Cambridge University Press: 2-11

Kizaki M, 2016. Molecular pathogenesis and treatment of chronic myelogenous leukemia. Japan: Springer: 117

Kück U, 2005. Praktikum der Molekulargenetik. Berlin Heidelberg: Springer: 226

Lankford L, Selby T, Becker J, Volodymyr R, Ling C, Farmer D, Wang A, 2015. Early gestation chorionic villi-derived stromal cells for fetal tissue engineering. World Journal of Stem Cells 7: 195-207

Mather JP, Roberts PE, 1998. Introduction to cell and tissue culture. Theory and technique. New York: Plenum Press New York: 1-7

McCarty RC, Gronthos S, Zannettino AC, Foster BK, Xian CJ, 2009. Characterisation and developmental potential of ovine bone marrow derived mesenchymal stem cells. Journal of Cellular Physiology 219: 324-333

McCarthy DA, Macey MG, 2001. Cytometric analysis of cell phenotype and function. Cambridge: Cambridge University Press: 38

Meierhenry JA, Ryzuk V, Miguelino MG, Lankford L, Powell JS, Farmer D, Wang A, 2015. Placenta as a source of stem cells for regenerative medicine. Current Pathobiology Reports 3: 9-16

Miki T, Lehmann T, Cai H, Stolz DB, Strom SC, 2005. Stem cell characteristics of amniotic epithelial cells. Stem Cells 23: 1549-59

Nanadakumar K, Robin A, Oluwatobi SO, Joshy KS, Sabu T, 2016. Nanomedicine and tissue engineering. State of the art and recent trends. Oakville: Apple Academic Press: 406-408

Nordheim A, Knippers R, Dröge P, Meister G, Schiebel E, Vingron M, Walter J, 2015. Molekulare Genetik. 10th ed. Stuttgart: Thieme: 536-540

Palmer MS, Roberts GW, Polak JM, 1995. Molecular neuropathology. Postgraduate Medical Science. Cambridge: Cambridge University Press: 22-24

Pamies D, Bal-Price A, Simeonov A, Tagle D, Allen D, Gerhold D, Yin D, et al., 2016. Good cell culture practice for stem cells and stem-cell-derived models. Altex 34: 95-132

Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, Hennerbichler S, et al., 2008. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international workshop of placenta derived stem cells. Stem Cells 26: 300-311

Pritchard TC, Alloway KD, 1999. Medical neuroscience. Integrated medical science. Madison Connecticut: Fence Creek Publishing: 64

Ray Bio, 2017. Ray Bio Ovine Cytokine Array Q1(1) kit manual. <http://www.raybiotech.com/files/manual/Antibody-Array/QAO-CYT-1.pdf> last check: 5/24/2017

Sneath RJ, Mangham DC, 1998. The normal structure and function of CD44 and its role in neoplasia. Molecular Pathology 51: 191-200

Stergiotou I, Borobio V, Bennasar M, Goncé A, Mula R, Nuruddin M, Soler A, Borrell A, 2016. Transcervical chorionic villus sampling: a practical guide. The Journal of Maternal Fetal Neonatal Medicine 29: 1244-51

Storhas W, 2013. Bioverfahrensentwicklung. 2nd Edition. Weinheim: WILEY-VCH:106

Sutton LN, Adzick S, Bilanjuk LT, Johnson MP, Crombelholme TM, Flake WA, 1999. Improvement in hindbrain herniation demonstrated by serial fetal magnet resonance imaging following fetal surgery for myelomeningocele. JAMA 282: 1826-1831

Thomas L, 1998. Labor und Diagnose. Indikation und Bewertung von Laborbefunden für die medizinische Diagnostik. Frankfurt: Dade Behring: 1139-1140.

Vogel C, Marcotte EM, 2012. Insights into the regulation of protein abundance from proteomic and trancriptomic analyses. Nature Reviews Genetics 13: 227-292

Wang A, Brown EG, Lankford L, Keller BJ, Pivetti CD, Sitkin NA, Beattie MS, Bresnahan JC, Farmer DL, 2015. Placental mesenchymal stromal cells rescue ambulation in ovine myelomeningocele. Stem cells translational medicine 4: 1-11

Watts RA, Conaghan PG, Denton C, Foster H, Isaacs J, Müller-Ladner U, 2013. Oxford Textbook of Rheumatology. 4nd Edition. Oxford: Oxford University Press: 367, 844

Wilkerson MJ, 2012. Principles and applications of flow cytometry and cell sorting in companion animal medicine. Veterinary Clinics of North America: Small Animal Practice 42: 53-71

Witwer KW, Buzas EI, Bemis LT, Bora A, Lässer C, Lötvall J, Nolte-t' Hoen EN, Piper MG, et al., 2013 Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. Journal of Extracellular Vesicles 2: 10.3402/jev.v2i0.20360.

Ziegler TR, Pierce GF, Herndon DN, 1997. Growth factors and wound healing. Basic Science and Potential Clinical Applications. Boston Massachusetts: Springer: 16

Özek MM, Cinalli G, Maixner WJ, 2008. The spina bifida management and outcome. Milan Italy: Springer: 84

## 9. Acknowledgements

At this point, I would like to thank all people who have accompanied me on my way. First and foremost, I have to thank Ms Oostingh, for actively supporting my international internship at the UC Davis medical center. Furthermore, for all the assistance in writing and processing data, I am very grateful. Without her involvement in every step, this bachelor thesis would have never been finished.

I would also like to show gratitude to Mr Wang, my Supervisor in Sacramento. He supplied a lot of precious information. My time at the research laboratory at the UC Davis medical center was highly productive and working with Lee Lankford was an extraordinary experience. His enthusiasm for the topic made a strong impression on me.

Special thanks are directed to the Marshall Plan Foundation. Without the scholarship, I would never have had the chance to perform stem cell research in California.

Most importantly, none of this could have happened without my family. I want to thank my son and my partner for having gone to Sacramento for a three-month internship with me, and my parents for all their personal and financial support. This was awesome.