Their potential is my passion!

Cover design:

Sarah Broeckx, Brenda Gorremans en Antoine Bouchier

About the cover:

The cover background represents $CD29^{+}$ adult multipotent stem cells from horses. The cells were stained using anti-CD29 FITC antibodies, which provided the green emission light.

During the final battle of the last King of the House of York (England, $15^{th}$ century), the horse of King Richard III was killed. Unable to flee and unable to gather more troops, he falls to the Earl Of Richmond who assassinates him, becoming the new King of England. This act, often quoted from Shakespeare, is from actual facts and King Richard III's last words were: a horse, a horse, my kingdom for a horse. Indeed, a kingdom is of little value if you are about to be defeated or killed just because you don't have a horse to flee. The horse in the back is Takashi van Berkenbroeck. With this horse I lived many adventures, but our paths separated because competitions and University studies were unfortunately not the best match. Because this horse is still my kingdom, I decided to dedicate the cover of my thesis to him!


Department of Comparative Physiology & Biometrics

# Equine peripheral blood- and mammary gland-derived multipotent stem cells: An update on their characterization and potential

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# LIST OF ABBREVIATIONS

**7-AAD:** 7-amino-actinomycin D

**AAEP:** American association of equine practitioners

**Ab:** antibody

**AD:** asymmetric cell division

**AF:** amnion fluid

**AP:** alkaline phosphatase

**AT:** adipose tissue

**b-FGF:** basic-fibroblast growth factor

**BM:** bone marrow

**BrdU:** bromodeoxyuridine

**BSA:** bovine serum albumin

**CDT:** cell doubling time

**CFU:** colony forming unit

**CK:** cytokeratin

**COMP:** cartilage oligomeric matrix protein

**DAB:** diaminobenzidine

**DJD:** degenerative joint disease

**DMEM:** Dulbecco's modified Eagle's medium

**EGF:** epidermal growth factor

**EMT:** epithelial-mesenchymal transition

**ESCs:** embryonic stem cells

**ER:** estrogen receptor

**FCS:** fetal calf serum

**FBS:** fetal bovine serum

**FDA:** food and drug administration

**G-CSF:** granulocyte-colony stimulating factor

**GF:** growth factor

**GFP:** green fluorescent protein

**HA:** hyaluronic acid

**HIF:** hypoxia inducible factor

**HS:** heparan sulphate

**HSCs:** hematopoietic stem cells

**IARC:** international agency for research on cancer

**ICM:** inner cell mass

**IHC:** immunohistochemistry

**IL:** interleukin

**iPS cells:** induced pluripotent stem cells

**ISCT:** International Society for Cellular Therapy

**LR:** load rate

**LREC:** label-retaining epithelial cells

**MaSC:** mammary stem/progenitor cell

**M-CSF:** monocyte-colony stimulating factor

**MFE:** mammosphere forming efficiency

**MHC:** major histocompatibility complex

**MiRNA:** microRNA

**MSC:** mesenchymal stem cell

**NO:** nitrogen oxide

**P:** passage

**PB:** peripheral blood

**PBMC:** peripheral blood mononuclear cell

**PBS:** phosphate buffered saline

**PDT:** population doubling time

**PF:** paraformaldehyde

**PO:** peroxidase

**PRP:** platelet-rich plasma

**PSGAG:** polysulphated glycosaminoglycans

**P/S/A:** penicillin/streptomycin/amphotericin B

**PVF:** peak vertical force

**rhTGF-β3:** recombinant human transforming growth factor-beta3

**RT:** room temperature

**SC:** stem cell

**SCID:** severe combined immunodeficiency

**SD:** symmetric cell division

**SDFT:** superficial digital flexor tendon

**SL:** suspensory ligament

**SLC:** small light staining cells

**SMA:** smooth muscle actin

**SP:** side population

**TEB:** terminal end bud

**TF:** transcription factors

**TGF-β3:** transforming growth factor-beta3

**TNF-α:** tumor necrosis factor-alfa

**UCB:** umbilical cord blood

**UCM:** umbilical cord matrix

**VEGF:** vascular endothelial growth factor

**VI:** vertical impulse

# CHAPTER 1

## General Introduction

Part of this introduction has been published:

Spaas JH, Guest DJ, Van de Walle GR, 2012. Tendon regeneration in human and equine athletes: ubi sumus-quo vadimus? Sports Medicine 42(10), 871-890.

## 1.1. Definition of stem cells

In a scientific context, the term "stem cell" (SC) was reported for one of the first times in the late 19th century by the German hematologist, Artur Pappenheim (Pappenheim, 1896) and a few years later by the Russian histologist, Alexander Maximov (Maximov, 1909; Ramalho-Santos and Willenbring, 2007). Maximov used this term to suggest the existence of hematopoietic SCs with the morphological appearance of a lymphocyte and capable of migrating throughout the blood to micro-ecological niches that would allow them to proliferate and differentiate. Nowadays, SCs are defined as cells that show self-renewal either with or without differentiation, depending on the symmetry of the division (Horvitz and Herskowitz, 1992). This means that SCs are able to form identical daughter cells (symmetric cell division, SD) or a SC and a more differentiated daughter cell (asymmetric cell division, AD) (Blanpain and Fuchs, 2009). Thanks to their proliferative nature, SCs are able to repopulate an entire organ. Since SCs resemble in this respect to an embryo and in order to better understand their developmental process, a short revision on embryology is advisable. After fertilization of the oocyte, SD forms the first SCs, which are called totipotent, because of their ability to form an embryo along with the extra-embryonic tissues such as the fetal membranes and the placenta. Subsequently, SD and AD form a blastocyst which consists of an inner cell mass (ICM) with pluripotent embryonic SCs (ESCs), which are able to differentiate into all cell types of the three germ layers of the embryo (Figure 1). At approximately day 16 after fertilization, the equine epiblast (now called primitive ectoderm) will give rise to all three germ layers of the embryo: ectoderm, mesoderm, and endoderm (Walter et al., 2010). The hypoblast (primitive endoderm) on the other hand, gives rise to extra-embryonic structures, such as the primary (primitive) yolk sac (providing early nourishment to the embryo). After the gastrulation process, the pluripotent SCs give rise to multipotent SCs which continue the cellular development with more specific tasks and

reduced plasticity (Figure 1). Interestingly, this process of further differentiation can be switched backwards, since Nobel Prize winners Yamanaka (2012) and Gurdon (2006) reported that differentiated adult non-pluripotent somatic cells (mainly fibroblasts) can be forced to express specific pluripotency genes and become so-called induced pluripotent stem (iPS) cells which is a physiological homeorhetic event (Figure 1). In order to obtain this return to pluripotency in a somatic cell, different pluripotency genes, such as Sox2, Oct4, Klf4, and c-Myc have to be stably integrated in the host's genome by means of viral vectors (especially retro or lenti viruses that randomly incorporate in the DNA) and this results in the reprogramming of somatic cells to a pluripotent state which can, before transplantation, be differentiated into the desired cell type (Hackett and Fortier, 2011; Takahashi et al., 2007).

The term "adult" SCs is used as a synonym for somatic SCs and refers to a population of undifferentiated cells that are residing all over the body after or during development of the tissues in juvenile or adult animals. In the following subchapters, two types of adult multipotent SCs will be discussed in more detail, i.e. the mesenchymal SCs (MSCs), derived from the mesoderm, and the mammary gland stem/progenitor cells (MaSCs), derived from the ectoderm. The term stem/progenitor cells is commonly used in the case of MaSCs (Choudhary and Capuco, 2012), because researchers are still doubting whether the isolated mammary cells are a pure SC population or a mixture with progenitor cells. In this regard, Dontu described the cultivation of human mammary gland-derived spheroid cell clusters, the so-called "mammospheres" which contained a mixture of MaSCs and their progeny (Dontu et al., 2003a).

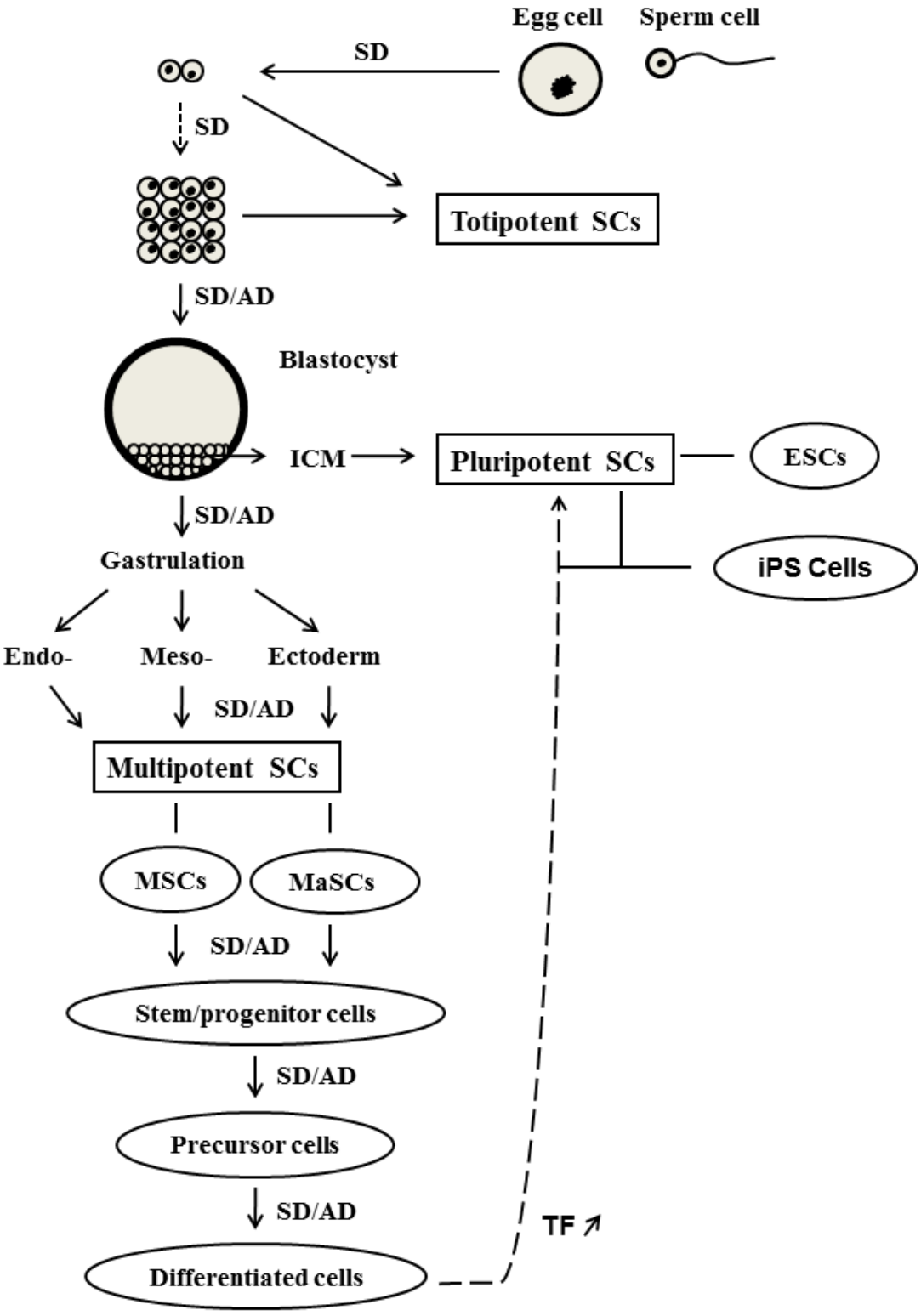


**Figure 1.** Schematic overview of the different types of stem cells (SCs) in vertebrates *in vivo* (Adapted from Spaas et al., 2012). After fertilization of the oocyte, symmetric cell division (SD) results in totipotent SCs, and subsequent SD and asymmetric cell division (AD) will lead to a blastocyst. The inner cell mass (ICM) of the blastocyst contains pluripotent embryonic SCs (ESCs). After gastrulation into three germ layers, multipotent SCs continue the development process. Mesenchymal SCs (MSCs) are mesoderm-derived SCs, whereas mammary SCs (MaSCs) are ectoderm-derived. Both SC types develop towards precursor and progenitor cells through SD or AD before they differentiate into organ-specialized cells. *In vitro*, differentiated cells can become induced pluripotent stem (iPS) cells through increased expression of specific transcription factors (TF).

Both MSCs and MaSCs have a common ancestor, and therefore, may share some properties such as cell morphology, receptor expression, colony forming unit capacity and possibly even differentiation capacities to similar cell types. Importantly, they both can be isolated from a highly accessible source, namely peripheral blood (PB) for MSCs and slaughterhouse material for MaSCs. The term "peripheral blood" covers the pool of circulating blood in veins, arteries and capillaries that is not sequestered within the lymphatic system, spleen, liver or bone marrow (BM). In practice, it is in most cases a synonym for venous blood, drawn from an easily accessible vein such as the *vena jugularis* in horses. Peripheral blood must be distinguished from blood from locations other than the "periphery", such as cardiac, coronary, splenic, umbilical cord and placental blood.

The present thesis describes the optimization of the isolation and characterization protocols of multipotent SCs in the horse, which can be derived from readily available sources. In addition, the fundamental research of this thesis provided a basis for the treatment of degenerative joint disease (DJD) in a horse. In the next paragraphs, the current state of the art is given concerning the isolation and characterization of equine MSCs and MaSCs, and their potential for either regenerative therapy (MSCs) or as a model for mammary gland physiology and tumorigenesis (MaSCs).

## 1.2. Mesenchymal stem cells (MSCs)

### *1.2.1. General characteristics*

In 1970, Alexander Friedenstein was one of the first to evidence the presence of non-hematopoietic cells in the bone marrow (BM) that were capable of self-renewal and bone differentiation (Friedenstein et al., 1970). These cells were typed as mesenchymal stem cells (MSCs) since they can differentiate into cells of the mesodermal germ layer (Friedenstein, 1970). Since the discovery of MSCs, their potential use in regenerative medicine has been

studied with increasing interest. The major differences of using adult multipotent MSCs instead of pluripotent embryonic stem cells (ESCs) for therapeutic purposes is the fact that MSCs, in contrast to ESCs, do not have the ability to form a teratoma *in vivo* (Fong et al., 2010; Lensch et al., 2007) and can be used autologous (in the same individual). In theory, therapeutic cloning could provide "autologous" ESCs for clinical applications (French et al., 2006; Vassiliev et al., 2011) but in practice ESCs are mainly being used in an allogeneic set-up (i.e. recovered from a different individual than the receptor), which raises the concern of immune rejection (Menendez et al., 2005). Nevertheless, it has been shown that human (allogeneic) ESCs do not express major histocompatibility complex (MHC) II, express only low levels of MHC I antigens and costimulatory molecules, are not recognized by natural killer cells and inhibit T-cell induced-stimulation (Menendez et al., 2005), which might indicate that ESCs are not very immunogenic. Interestingly, it becomes increasingly clear that also MSCs are not really immunogenic and could even have an immunosuppressive function, which creates new opportunities to use these cells in an allogeneic set-up as well. Indeed, the use of allogeneic multipotent MSCs without any adverse reactions has been described in both humans and horses (Carrade et al., 2011a; Carrade et al., 2011b; Fang et al., 2007; Ringden et al., 2006; Riordan et al., 2009). Moreover, MSCs can inhibit the innate immune activation by blocking dendritic cell maturation and suppressing macrophages and T-cell activity (Di Nicola et al., 2002; Djouad et al., 2007; English et al., 2008; Nasef et al., 2007; Ortiz et al., 2007). Furthermore, MSCs inhibit both B-cell proliferation and production of IgM, IgA and IgG, (Corcione et al., 2006).

In 2006, the International Society for Cellular Therapy (ISCT) has carefully determined the qualities human cells must possess in order to be defined as MSCs (Dominici et al., 2006). Combining this information with other reports (Majumdar et al., 2003; Pittenger et al., 1999), human MSCs have to fulfill the following requirements. First of all, they have to be plastic-

adherent, meaning that they should attach to a coated surface. Secondly, MSCs should be positive for typical stem cell markers (CD29, CD44, CD73, CD90 and CD105) and negative for adult blood cell markers (CD14 or CD11b, CD34, CD45, CD79a or CD19 and MHC II). Finally, MSCs have to be able to differentiate into different cell types of the mesodermal germ layer such as osteoblasts, chondroblasts and adipocytes. Indeed, a cell can only be named "stem cell" when it is able to perform not only symmetrical cell divisions, but also asymmetrical cell divisions with the formation of more differentiated and/or specialized daughter cells. For equine MSCs, no such guidelines have been described to date, although this would greatly benefit researchers working in this field (De Schauwer et al., 2011b).

Different sources of MSCs have been described in humans, equines and other mammal species. However, there is no clarity about which source is the most suitable for the treatment of which pathology or in which animal species. In horses, several sources of MSCs have been described, with BM, adipose tissue (AT), umbilical cord matrix (UCM) and umbilical cord blood (UCB) as being the best studied to date (Table I) (De Schauwer et al., 2011a; Guest et al., 2008a; Hoynowski et al., 2007; Koch et al., 2009b; Radcliffe et al., 2010). Remarkably, not every source delivers the same amount of MSCs and differences in marker expression and differentiation capacities have been noticed. Indeed, Kern reported that in comparison with 64.2% of human BM-derived colony forming units (CFUs), only 7.1% of the human AT-derived CFUs were able to differentiate into chondrocytes and adipocytes (Kern et al., 2006). This implicates that either the SCs' potential (functionality) differs between different sources, or that the purity of the cells is compromised in AT. On the other hand, it has been reported that BM- and AT-derived equine MSCs have chondrogenic, osteogenic and adipogenic capacities (Table I) (Ahern et al., 2011; Koerner et al., 2006). This confirms that both sources contain functional MSCs, so the reported discrepancy in the study of Kern was probably due to a lower recovery rate of functional SCs. Both BM- and AT-derived MSCs express CD13,

CD29, CD44, CD49d, CD73, CD90, CD105, CD106, CD146 and CD166 transcripts (Table II) (Ranera et al., 2011). However, significant differences in gene expression levels between BM and AT MSCs were observed for CD44, CD90, CD29 and CD34 (Ranera et al., 2011). In addition, both cell types were negative for CD45 and CD31 (Ranera et al., 2011). When comparing the chondrogenic potential between BM- and AT-derived MSCs, a superior differentiation potential of BM MSCs has been described based on an increased proteoglycan and collagen type II deposition in their extracellular matrix (Ahern et al., 2011; Kisiday et al., 2008). Recently, the amnion has been reported as a source of equine MSCs also (Table II). These amnion-derived MSCs expressed CD105 and were able to perform a trilineage differentiation towards cartilage, bone and fat (Table I) (Violini et al., 2012). Moreover, also the fluid present in the amnion has been reported to contain MSCs, which were able to perform a trilineage differentiation, expressed CD44, CD90 and CD105 and are negative for CD14, CD34 and CD45 (Table II) (Iacono et al., 2012).

In addition, UCM-derived MSCs were shown to be positive for CD54, CD90, CD105 and CD146 and negative for MHC II (Table II) (Hoynowski et al., 2007). These UCM-derived MSCs were able to differentiate towards chondrocytes, osteocytes, adipocytes and neuronal-like cells (Table I). For the immunophenotypic characterization of equine UCB-derived MSCs different markers, such as CD29, CD44 and CD90 (positive) and CD73 and CD105 (variable positive) and CD45, CD79α, Macrophage/Monocyte and MHC II (negative) have been described (Table II) (De Schauwer et al., 2011a; De Schauwer et al., 2012). Also these MSCs were able to perform a trilineage differentiation (Table I) (De Schauwer et al., 2011a; Koch et al., 2007).

**Table I.** Overview of the different sources of equine mesenchymal stem cells (MSCs) with the described markers and differentiation potential.

| Source | Cell marker | Differentiation | Reference |
|---|---|---|---|
| **BM** | ND | C | Hegewald et al., 2004 |
| | ND | O, C, A | Koerner et al., 2006 |
| | ND | O, A | Vidal et al., 2006 |
| | CD90 | O, C, A | Arnhold et al., 2007 |
| | ND | C | Vidal et al., 2008 |
| | CD14, CD29, CD44, CD79α, CD90, MHC I&II | O, C, A | Guest et al., 2008a |
| | ND | O, C | Colleoni et al., 2009 |
| | CD34 | O, T | Violini et al., 2009 |
| | CD11a, CD29, CD44, CD45, CD90 | O, C, A | Radcliffe et al., 2010 |
| | CD13, CD29, CD34, CD44, CD49d, CD73, CD90, CD105, CD106, CD146, CD166 | O, C, A | Ranera et al., 2011 |
| | ND | O, C, A | Ranera et al., 2013 |
| | CD44, CD49d | ND | Ranera et al., 2012 |
| **AT** | ND | C | Vidal et al., 2008 |
| | ND | O, C | Colleoni et al., 2009 |
| | CD13, CD44, CD90 | ND | de Mattos Carvalho et al., 2009 |
| | CD13, CD29, CD34, CD44, CD49d, CD73, CD90, CD105, CD106, CD146, CD166 | O, C, A | Ranera et al., 2011 |
| | CD44, CD49d | ND | Ranera et al., 2012 |
| **UC** | CD34, CD45, CD54, CD73, CD90, CD105, CD133, CD146, MHC I | O, C, A | Hoynowski et al., 2007 |
| | CD34 | ND | Cremonesi et al., 2008 |
| | ND | ND | Passeri et al., 2009 |
| | CD14, CD34, CD44, CD45, CD90, CD105 | O, C, A | Iacono et al., 2012 |
| **UCB** | ND | O, C, A | Koch and Betts, 2007 |
| | ND | O, C, A, M | Reed and Johnson, 2008 |
| | CD18 | O, C, A | Schuh et al., 2009 |
| | CD29, CD44, CD79α, CD90, MHC II | O, C, A | De Schauwer et al., 2011a |
| | CD14, CD34, CD44, CD45, CD90, CD105 | O, C, A | Iacono et al., 2012 |
| | CD29, CD44, CD45, CD73, CD79α, CD90, CD105, MHC II | O, C, A | De Schauwer et al., 2012 |
| **PB** | ND | O, C, A | Koerner et al., 2006 |
| | ND | O, C, A | Giovannini et al., 2008 |
| | CD13, CD34, CD44, CD45, CD90, CD117 | O, A, M | Martinello et al., 2010 |
| | CD51, CD90, CD105 | O, C, A | Dhar et al., 2012 |
| **Amnion** | CD105 | O, C, A | Violini et al., 2012 |
| **AF** | CD14, CD34, CD44, CD45, CD90, CD105 | O, C, A | Iacono et al., 2012 |

BM = Bone Marrow; AT = Adipose Tissue; UC = Umbilical Cord; UCB = Umbilical Cord Blood; PB = Peripheral Blood; AF = Amnion Fluid; ND = Not Described; O = Osteogenic differentiation; C = Chondrogenic differentiation; A = Adipogenic differentiation; M = Myogenic differentiation; T = Tenogenic differentiation

**Table II.** Overview of frequently described markers of equine MSCs derived from different sources.

| Marker | Source | Presence | Reference |
|---|---|---|---|
| **CD11** | BM | - | Iacono et al., 2012; Radcliffe et al., 2010 |
| **CD13** | BM | + | Ranera et al., 2011 |
| | AT | ± | de Mattos Carvalho et al., 2009; Ranera et al., 2011 |
| | PB | + | Martinello et al., 2010 |
| **CD14** | BM | - | Guest et al., 2008a |
| **CD29** | BM | + | Guest et al., 2008a; Radcliffe et al., 2010; Ranera et al., 2011 |
| | UCB | + | De Schauwer et al., 2011a; De Schauwer et al., 2012 |
| **CD31** | BM | - | Ranera et al., 2011 |
| | AT | - | Ranera et al., 2011 |
| **CD34** | BM | - | Violini et al., 2009 |
| | UC | - | Cremonesi et al., 2008; Hoynowski et al., 2007; Iacono et al., 2012 |
| | UCB | - | Iacono et al., 2012 |
| | PB | - | Martinello et al., 2010 |
| | AF | - | Iacono et al., 2012 |
| **CD44** | BM | + | Guest et al., 2008a; Radcliffe et al., 2010; Ranera et al., 2012 |
| | AT | + | de Mattos Carvalho et al., 2009; Ranera et al., 2012 |
| | UC | + | Iacono et al., 2012 |
| | UCB | + | De Schauwer et al., 2011a; De Schauwer et al., 2012; Iacono et al., 2012 |
| | PB | + | Martinello et al., 2010 |
| | AF | + | Iacono et al., 2012 |
| **CD45** | BM | ± | Radcliffe et al., 2010; Ranera et al., 2011 |
| | AT | - | Ranera et al., 2011 |
| | UC | - | Hoynowski et al., 2007; Iacono et al., 2012 |
| | UCB | - | De Schauwer et al., 2012; Iacono et al., 2012 |
| | PB | - | Martinello et al., 2010 |
| | AF | - | Iacono et al., 2012 |
| **CD49d** | BM | + | Ranera et al., 2012 |
| | AT | + | Ranera et al., 2012 |
| **CD51** | PB | + | Dhar et al., 2012 |
| **CD73** | BM | + | Ranera et al., 2011 |
| | AT | + | Ranera et al., 2011 |
| | UC | + | Hoynowski et al., 2007 |
| | UCB | ± | De Schauwer et al., 2012 |
| **CD79α** | BM | - | Guest et al., 2008a |
| | UCB | - | De Schauwer et al., 2011a; De Schauwer et al., 2012 |
| **CD90** | BM | + | Arnhold et al., 2007; Guest et al., 2008a; Radcliffe et al., 2010; Ranera et al., 2011 |
| | AT | + | de Mattos Carvalho et al., 2009; Ranera et al., 2011 |
| | UC | + | Hoynowski et al., 2007; Iacono et al., 2012 |
| | UCB | + | De Schauwer et al., 2011a; De Schauwer et al., 2012; Iacono et al., 2012 |
| | PB | + | Dhar et al., 2012; Martinello et al., 2010 |
| | AF | + | Iacono et al., 2012 |
| **CD105** | BM | + | Ranera et al., 2011 |
| | AT | + | Ranera et al., 2011 |
| | UC | + | Hoynowski et al., 2007; Iacono et al., 2012 |
| | UCB | ± | De Schauwer et al., 2012; Iacono et al., 2012 |
| | PB | + | Dhar et al., 2012 |
| | A(F) | + | Iacono et al., 2012; Violini et al., 2012 |
| **CD106** | BM | + | Ranera et al., 2011 |
| | AT | + | Ranera et al., 2011 |
| **CD117** | PB | + | Martinello et al., 2010 |
| **MHC I** | BM | + | Guest et al., 2008a |
| | UC | + | Hoynowski et al., 2007 |
| **MHC II** | BM | - | Guest et al., 2008a |
| | UCB | - | De Schauwer et al., 2011a; De Schauwer et al., 2012 |

+ = present; - = absent; ± = moderate expression; BM = Bone Marrow; AT = Adipose Tissue; UC = Umbilical Cord; UCB = Umbilical Cord Blood; PB = Peripheral Blood; AF = Amnion Fluid

Taken together, there have been reports of differentiation of equine MSCs from several sources into cartilage (Hegewald et al., 2004), bone (Vidal et al., 2006), fat (Koch et al., 2007), muscle (Martinello et al., 2010), tendon (Smith, 2008) and even neuronal-like cells (Hoynowski et al., 2007). Since obtaining BM and AT samples is a rather invasive procedure, the search for alternative sources has received more attention lately. As an alternative, UCB has been suggested, which in humans can be easily collected at birth at the hospital. However, in horses, autologous UCB is not always available and a sterile collection is only possible under highly hygienic circumstances, which is difficult to achieve under field circumstances. Moreover, the cost of storage of UCB- and UCM-derived MSCs for future therapeutic use over the lifetime of the animal/patient must also be considered.

The disadvantages of UCB can be avoided by using PB as a source for MSCs. Since collection of a sterile blood sample can easily be performed by any equine practitioner, PB is a readily accessible source of autologous MSCs when injuries occur. The isolation of equine PB-derived MSCs was described for the first time in 2006 and was based on morphology combined with a differentiation towards osteocytes and adipocytes (Koerner et al., 2006). Two years later, another research group managed to produce chondroblasts from equine PB-derived MSCs, although this was only achieved after 9 weeks and also here, no immunophenotypic characterization of the cells was performed (Giovannini et al., 2008). More recently, CD44 and CD90 have been used as positive immunophenotypic markers for equine PB-derived MSCs, but these cells were also positive for the hematopoietic SC marker CD117 and no differentiation towards chondroblasts was reported (Martinello et al., 2010). Also other researchers identified a MSC population in the PB using CD90 and CD105 as positive markers, but no further characterization was performed (Marfe et al., 2011). All this indicates that there is a need for more research to further characterize equine MSCs obtained from this readily available alternative source.

### *1.2.2. The MSC niche*

The stem cell (SC) niche is defined as the micro-environment where SCs reside and which contains important factors for SC regulation. The specific interactions between the residing mesenchymal SCs (MSCs) and their micro-environment determines whether or not a SC remains quiescent, starts to proliferate or differentiates into an adult cell (Becerra et al., 2011; Kuhn and Tuan, 2010; Scadden, 2006). The niche contains two main divisions: a biological and a physical (temperature, oxygen level, relative humidity,…) one. The biological niche can be divided into four subdivisions: surrounding cells, extracellular matrix components, growth factors and cytokines (Figure 2). Since MSCs mainly reside perivascular (as pericytes) in most tissues, they live in close contact with endothelial cells of the blood vessels and migrated macrophages (Figure 2) (Crisan et al., 2008; da Silva Meirelles et al., 2006; Kode et al., 2009; Zhou et al., 2010).

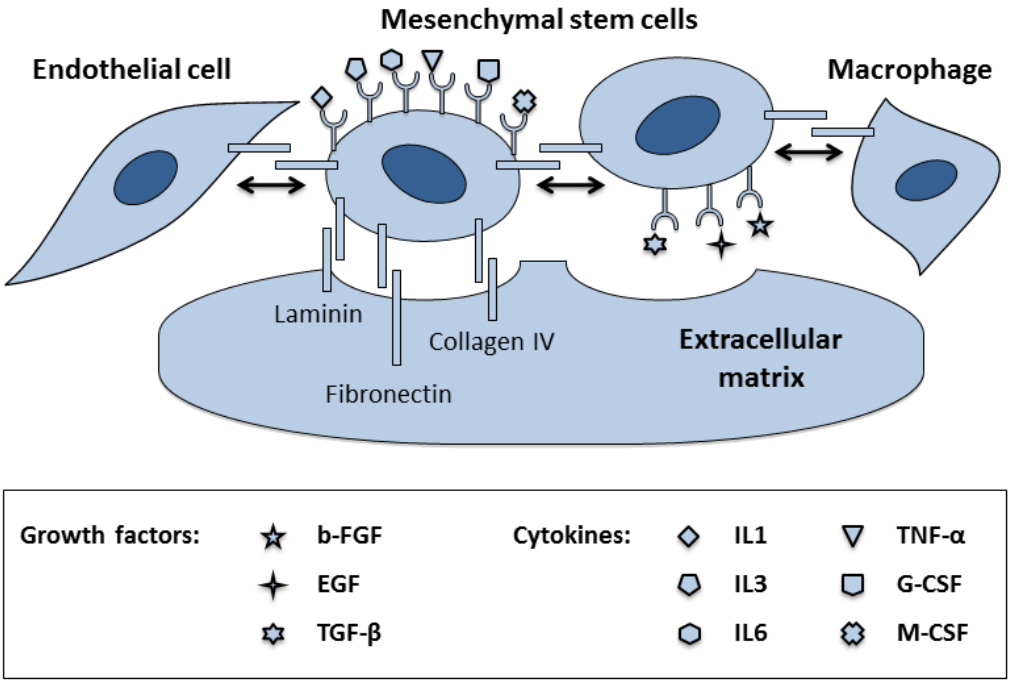


**Figure 2.** Different niche factors which are of importance in the regulation of MSCs.

Manipulation of the niche can as such represent an important tool to guide the further direction of development of the MSCs. In this regard, an influence of human umbilical vein endothelial cells on the osteogenic potential of human BM MSCs has been previously reported (Xue et al., 2009). This study describes that 5 days after the addition of endothelial cells to MSCs, the cell proliferation significantly increased and cellular bridges between the two cell types were present, as well as increased mRNA expression of alkaline phosphatase (AP). Moreover, they reported a greater effect on differentiation after the addition of the endothelial cells than when adding osteogenic factors such as dexamethasone, ascorbic acid and beta-glycerophosphate to the culture medium. Furthermore, AP activity and mRNA expression of various osteogenic markers significantly increased when human endothelial cells and BM MSCs were cocultured on materials with calcium phosphate scaffolds compared to tissue culture polystyrene or to MSCs alone (Bulnheim et al., 2012). In that study it was also observed that the expression of osteopontin and osteocalcin was highly sensitive to the used adhesion substrate, indicating the importance of cell surfaces as a regulating structure in the micro-environment. In addition, coculturing human BM MSCs with endothelial cells increased formation of microvessel-like structures *in vitro*, as confirmed by CD31 and CD146 expression (Kolbe et al., 2011) and vascular structures were formed as early as 48 hours after subcutaneous implantation within a starch-poly(caprolactone) biomaterial *in vivo* (Ghanaati et al., 2011). Also in humans, it has been described that co-cultivating adipose tissue (AT)-derived MSCs with skeletal myoblasts changes their protein expression (Mizuno, 2010). These studies implicate that the products MSCs secrete depend on the surrounding cells.

Furthermore, different growth factors have been described with proliferation or differentiation stimulating effects on human MSCs (Fong et al., 2011; Kuhn and Tuan, 2010). Hereby, the addition of basic-fibroblast growth factor (b-FGF) increased the mitosis capacity and maintained the growth and multilineage differentiation potential of human MSCs *in vitro*

(Kuhn and Tuan, 2010; Sotiropoulou et al., 2006; Tsutsumi et al., 2001). Also epidermal growth factor (EGF) and transforming growth factor-β3 (TGB-β3) would have an influence on human MSC growth and are considered valuable niche factors (Chieregato et al., 2011; Fong et al., 2011). In addition, after migration to the injury site, MSCs release all the aforementioned growth factors in order to stimulate local cells (and MSCs) to heal the damaged tissue (Shi et al., 2010). Several independent studies have suggested that pro-inflammatory cytokines, which are present at the injury site, would have an influence on the MSC behavior, mainly by maintaining their undifferentiated state (Kode et al., 2009; Pricola et al., 2009). Indeed, MSCs contain receptors for the most common pro-inflammatory cytokines, such as interleukin (IL)1, IL3, IL6 & Tumor Necrosis Factor-α (TNF-α), (Kode *et al.*, 2009; Kuhn and Tuan, 2010; Pricola *et al.*, 2009). Moreover, adding IL6 to MSC cultures enhanced their proliferation, but inhibited adipogenic and chondrogenic differentiation (Pricola et al., 2009). Furthermore, it has been reported that granulocyte- and macrophage-colony stimulating factor (G-CSF & M-CSF) may also affect MSCs, since these factors stimulate the BM MSC release (Dygai et al., 2009).

From the aforementioned it became clear that the homeostasis within different tissues, and maybe even within the entire body is sustained by a complex network of growth factors and transcription factors that orchestrate the proliferation and differentiation of MSCs. Increasing evidence recently indicated that microRNAs (miRNAs), small non-coding RNAs with a post-transcriptional regulating function, are one of the key players of this concert. Indeed, it has been reported that upregulation of miRNA-16 contributed to the differentiation of human bone marrow (BM) MSCs towards myogenic phenotypes in a cardiac niche (Liu et al., 2012). Moreover, miRNA-124 would play an important role in maintaining subventricular zone (brain compartment) homeostasis, by regulating the number of progenitors and the timing of neuronal differentiation (Cheng et al., 2009).

Regarding equine MSCs and their niche, many details remain unknown to date and most of the molecular characterizations of equine MSCs have been made at 20% $O_2$, a higher oxygen level than that surrounding the cells inside the organism. In this regard, it has been reported that hypoxia maintains the undifferentiated state and potency of different SC types, such as ESCs, hematopoietic SCs (HSCs), neural SCs and MSCs (Basciano et al., 2011; Mohyeldin et al., 2010). Concerning the influence of hypoxia on the differentiation capacities of equine MSCs, several studies have been performed. In a study of Ranera et al. (2012), no significant differences were found in long-term cultures for osteogenesis and adipogenesis between normoxic (20% $O_2$) and hypoxic (5% $O_2$) expanded equine BM-derived MSCs. However, chondrogenesis-related genes (COL2A1, ACAN, LUM, BGL, and COMP) were upregulated and the extracellular sulphated glycosaminoglycan content was increased under hypoxemic conditions. These results suggested that chondrogenesis was enhanced at a low oxygen level (Ranera et al., 2013). The same group made a comparison between the influences of low oxygen surroundings on BM-derived MSCs and AT-derived MSCs (Ranera et al., 2012). Hereby, it was reported that fewer BM MSCs were obtained in hypoxia (5% $O_2$) than in normoxia (20% $O_2$), as a result of significantly reduced cell divisions. Hypoxic AT MSCs proliferated less than normoxic AT MSCs. In contrast, another study reported that the isolation rate of equine umbilical cord blood (UCB)-derived MSCs was improved by reducing the oxygen tension from 20% to 5% (Schuh et al., 2009). Although flow cytometry revealed no considerable changes in protein expression of both MSC sources under both oxygen conditions, PCR showed that statistically significant higher levels of gene expression of CD49d in BM MSCs and CD44 in AT MSCs were found under normoxic circumstances (Ranera et al., 2012). Furthermore, hypoxic cells tended to display a higher marker expression, which confirms the aforementioned statement that hypoxia retains MSCs in an undifferentiated state. Furthermore, hypoxic preconditioning of MSCs significantly increased

the expression of hypoxia inducible factor-1 (HIF-1) alpha and chemokine receptor CXCR4 in MSCs and enhanced their migration *in vitro* (Yu et al., 2013). Additionally, hyperbaric oxygen treatment has been reported to enhance MSC recovery from peripheral blood (PB) in horses and to significantly increase CD90-positive cells (Dhar et al., 2012). Indeed, hyperbaric oxygen treatment has been reported before to stimulate BM release of HSCs (Thom et al., 2006). According to the authors, the increased SC release would be due to an increase in nitrogen oxide (NO) synthase. This would be a plausible explanation, since NO plays a key role in triggering progenitor cell mobilization from the BM through releasing a SC active cytokine (Kit ligand) (Aicher et al., 2003; Heissig et al., 2002).

From the above it is clear that a specific interaction exists between residing MSCs and their micro-environment. Although there has been significant progress in the field of MSC biology, there is still ongoing controversy and debate concerning the influence of niche factors on the *in vivo* capacities of MSCs. In fact, the exact *modus operandi* of SCs itself remains a question. Although various clinical applications using MSCs have been described, the mechanisms underlying their therapeutic effects are not well known and characterized. For this reason, MSC treatments sometimes give unsatisfactory results in clinical trials (Frisbie et al., 2009; Wilke et al., 2007). Nowadays, it is generally accepted that the positive effects of SC therapy are due to the products they secrete, and not necessarily to the cellular reconstitution of the injured tissue. In this regard, it has been reported that MSCs enhance the metabolic function of other cells (Yang et al., 2013) and that the regulation of biologically active peptide secretion by MSCs would determine the efficacy of tissue regeneration (Cabrera et al., 2012). On the other hand, it has to be mentioned that several studies report a stable and uniform integration of MSCs in the repair tissue, indicating some sort of structural support (Guest et al., 2008b, Martinello et al., 2013). Nevertheless, there are no reports on the effects of injecting SC antigens or their secreted factors alone. For all the aforementioned

reasons, more efficacious therapies may result from the possibility to exogenously regulate the protein secretion *in vitro*, and hence, discriminate between the different therapeutic effects of MSCs or the products they secrete *in vivo*. To reach this goal, however, the micro-environment of these cells should be tightly regulated. More specifically, identifying and using important niche factors which alter the protein secretion of patient MSCs *in vitro*, might prove invaluable to increase the efficacy of *in vivo* tissue regeneration and even standardize regenerative medicine, and also the clinical outcome of the injured patients.

### *1.2.3. MSCs in physiology and pathology*

Symptomatic therapies for some musculoskeletal injuries (depending on the location and severity of the damage) may last for long periods of time and in several cases no significant improvements in functionality of the tissues have been reported. Therefore, the use of causal treatments may be of substantial importance. Causal treatments, such as stem cell (SC) therapy aim to reestablish the tissue or metabolic pathway to a status before disease. With causal treatments, researchers aim to repair, replace, restore or regenerate damaged or diseased cells, tissues and organs. In this regard, the aforementioned SC niche plays an important role and might influence the *in vivo* outcome. From a physiological point of view, a difference has to be made between homeostatic and homeorhetic processes (Bauman and Currie, 1980). The first phenomenon indicates maintenance of physiological equilibrium or constancy of environmental conditions within the animal (Bauman and Currie, 1980). After a homeostatic regulation, the original status and tissue integrity should be reconstituted (*restitutio ad integrum*) and the damage was in fact reversible, which remains questionable in the case of naturally occurring injuries. Homeorhesis on the other hand, aims to orchestrate the metabolism of body tissues in order to support a physiological state, which is a more realistic description of what may be achieved after SC treatment (Bauman and Currie, 1980).

The broad field of causal treatments covers a variety of research areas that includes growth factors (GFs) and cell therapy. One of the most explored GF-based therapies in equine research is the use of platelet-rich plasma (PRP), whereas the cell-based therapies are largely focused on the use of SCs. These therapies will be further discussed in this subchapter, using the treatment of equine tendinopathies as an example.

*1.2.3.1. Equine tendon (patho)physiology*

Tendons are dense bands of fibrous connective tissue that primarily serve as force transmitters between muscle and bone. Tendons are highly compliant structures under moderate tension. Physiological loads usually cause less than 4% increase in the length of the tendon (Jozsa and Kannus, 1997) and under those circumstances, the tendon will be in the elastic phase or "toe" region. This represents the loss of the waveform on the tendon surface but is fully recoverable (Figure 3). Strains above 4% result in tendon damage with changes in the mechanical characteristics of the tendon and irreversible changes in the ground substance (Stromberg B., 1969). This is called the visco-elastic phase (Figure 3). The point of complete tendon rupture, in humans as well as horses, occurs at strains of around 8-12% (Crevier et al., 1996; Kader et al., 2002; Riemersma and Schamhardt, 1985; Riley, 2004) (Figure 3). Given that strains of up to 12-16% have been recorded *in vivo* at the gallop (Stephens et al., 1989), the equine superficial digital flexor tendon (SDFT) is therefore functioning close to its limits.

After acute damage to the tendon, the first reaction consists of an inflammatory phase that lasts several days to two weeks. The extent of the inflammatory response may determine the level of pain experienced, but it is not always directly related to the extent of tendon damage (Ketchum, 1979). Directly after the acute reaction, the repair phase starts and continues for several months. This phase is characterized by fibroplasia and angiogenesis (Kajikawa et al., 2007). Thirdly, there is the collagen phase which is of great importance for tendon healing,

since the type of collagen influences the tendon's mechanical properties and the re-occurrence rate of tendinopathies is mostly dependent on this phase (Birch et al., 1998; Manske et al., 1984; Richardson et al., 2007).

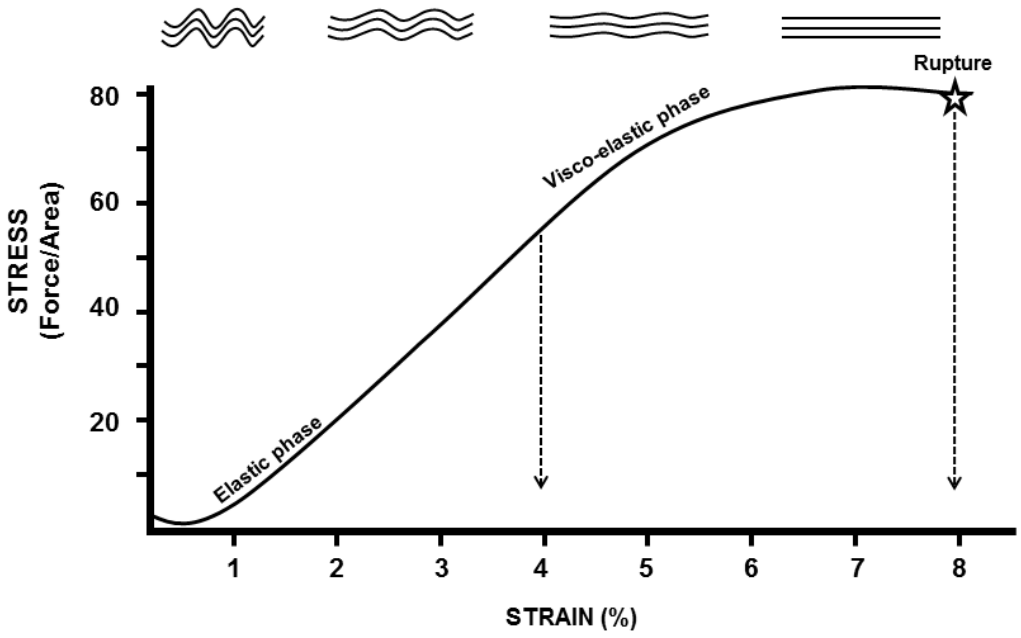


**Figure 3.** The physiological stress-strain curve of a tendon (adapted from Riley, 2004). This curve shows the relationship between stress (force per area) and strain (deformation due to stress) of the tendon. The star represents the moment when tendon rupture occurs.

Healthy tendon tissue is primarily composed of collagen type I (approximately 95% of the total collagen), which provides strength and elasticity. During tendon healing, random collagen type III deposition occurs (McCullagh et al., 1979), resulting in a much higher percentage (20-30%) of collagen type III compared to normal, functional tendon (1-3%) in horses and humans (Obaid and Connell, 2010; Williams et al., 1980). As collagen type III tends to produce smaller, less organized fibrils, it will provide a structural, but not a functional (homeorhesis) recovery of the tendon. Due to the loss of the original strength and elasticity, the risk of reinjury is increased (Birch et al., 1998; Manske et al., 1984). Finally, there is the remodeling or maturation phase of the repaired tissue. As the directional strain placed upon the tendon re-aligns the tendon fibers, collagen type III is partially replaced by

collagen type I. Limited exercise is therefore important to improve longitudinal orientation of tendon fibers (McCullagh et al., 1979), however, excessive exercise during this time can disrupt the early repair process (Ketchum, 1979; Rudolph et al., 1980).

Ligament and tendon pathologies are a common cause of injuries and have a high re-occurrence rate in sport horses. The group of Ely described that every year 22.8% of the horses were inflicted with these kind of injuries (Ely et al., 2009), and therefore, different regenerative therapies have been studied in this animal species. Chronic tendon injuries are caused by repetitive over-loading of the tendon. Their pathology differs to that of an acute injury in that there is no inflammatory stage. Instead there is an initial reactive stage where tendon cells proliferate and increase their production of proteoglycans. The tendon will increase in thickness but if the load is reduced it can, at this stage, revert to normal (Ohberg et al 2004). If the tendon continues to be overloaded, tendon dysrepair will occur. Tendon cells continue to proliferate and produce increased amounts of proteoglycans resulting in matrix disorganization. This can progress to the degenerative stage where the matrix changes are more pronounced with areas of acellularity, increased vascularity and reduced collagen (Cook & Purdam 2009).

*1.2.3.2. Equine clinical studies using growth factors (GFs)*

Based on positive laboratory results and promising *in vivo* results, the use of autologous GFs is gaining enormous popularity. These GFs have the *in vitro* potential to change collagen production and degradation by influencing matrix-regulating enzymes (Creaney and Hamilton, 2008; Mishra et al., 2009; Sampson et al., 2008). One of the most explored GF-based therapies in equine research is the use of PRP. This is an autologous concentrate of platelets in a small volume of blood plasma and contains various endogenous GFs, such as platelet-derived GF (PDGF), transforming GF-beta (TGF-β), insulin-like GF (IGF) and

vascular endothelial GF (VEGF) (Marx, 2001). These GFs play a central role in the healing process of tendon injuries, because of their capacity to stimulate cell proliferation and the synthesis of extracellular matrix (ECM) components (Molloy et al., 2003).

In horses, several studies on the use of PRP have been performed. *In vitro* studies with SDFT explant cultures have shown that PRP increases the expression of tendon matrix genes in these explants (Schnabel et al., 2007). The group of Bosch et al., have studied the *in vivo* effects, using several different parameters, of PRP injection one week after the creation of surgically-induced SDFT defects. In those placebo-controlled experimental studies, they found an increased neovascularization, as well as improved histological, biochemical and biomechanical properties of the tendons, suggesting that PRP treatment might be beneficial, at least for acute tendon lesions (Bosch et al., 2011; Bosch et al., 2010). The successful use of autologous PRP to treat chronic desmitis of the suspensory ligament (n=3) has also been reported, but due to the very limited number of patients in this study, no real conclusions on the benefits of PRP treatment for chronic tendon lesion can be made at present (Arguelles et al., 2008).

According to another group, using PRP in combination with BM-derived mononuclear cells for the treatment of chronic equine tendinosis may enhance equine tendon regeneration in clinical cases (Torricelli et al., 2011). Although this study was not placebo-controlled, the authors did demonstrate a statistically significant reduction in recovery time associated with increased platelet concentration. This latter study implicates the potential of a combined use of GFs together with cell-based therapies (as described below) for the treatment of chronic tendinopathies.

*1.2.3.3. Equine clinical studies using cell-based therapies*

Cell-based therapies are defined as the process of introducing new cells into a tissue in order to treat a disease or regenerate damaged tissue. Such therapies not only include the use of pluri- (1) or multipotent (2) SCs, but also include the transplantation of mature, functional cells (3). In general, it is accepted that these treatments have the highest success rate when administered immediately after the inflammatory phase of the initial tendon injury (before infiltration with fibroblasts and scar tissue formation), because of the ideal environment for cell growth at that point (Richardson et al., 2007). However, in clinical trials as well as under experimental conditions, acute as well as chronic tendon lesions have been studied to date.

*(1) Pluripotent stem cells*

Pluripotent SCs can differentiate into every cell type of the three germ layers (endoderm, mesoderm, and ectoderm). To date, there are two major groups of pluripotent SCs: embryonic SCs (ESCs), which are obtained from the inner cell mass (ICM) of a blastocyst, and induced pluripotent stem (iPS) cells, which are obtained from adult somatic cells (Chan et al., 2009; Paris and Stout, 2010). Over the last decade, the public opinion, especially in human medicine, has been ranging from increasing enthusiasm for SC therapy towards pronounced ethical concerns about using pluripotent ESCs, since this implies the destruction of embryos. In veterinary medicine, the objections against the use of SC therapy are not very much focused against the use of animal embryos, but are mainly based on the fact that SC therapies are being used in practice without being backed up by proper fundamental research. Since it is known that acute fetal tendon injuries can fully regenerate (Favata et al., 2006), the interest in ESCs has dramatically increased. These ESCs can be recovered from the inner cell mass of day 7-8 equine blastocysts (Li et al., 2006), but have not been proven yet to be real ESCs.

In contrast to horses, human ESCs have been fully characterized by the expression of pluripotency markers and different functional characteristics including telomerase activity,

embryoid bodies and teratoma formation (Kempf et al., 2011; Lensch et al., 2007; Marion and Blasco, 2010). The SCs derived from equine embryos have undergone a much more limited characterization (Guest and Allen, 2007) and interestingly, teratoma formation *in vivo* of these cells has been unsuccessful to date (Li et al., 2006). Still, equine embryonic-like SCs have already been used in an *in vivo* experimental study for the treatment of surgically created equine SDFT lesions, 1 week after inducing the lesion. The cells did not induce a cell-mediated immune response, nor did they form tumors in the 90 day time period studied (Guest et al., 2010). The ES-like cells survived in high, constant numbers for the duration of the study period and were capable of migrating to other areas of damage within the same tendon. In contrast, mesenchymal SCs (MSCs) derived from the bone marrow (BM) of these horses demonstrated very low cell survival within the damaged tendon. However, the effect of either cell type on tendon regeneration was not investigated in this study.

Fetal-derived SCs have also been investigated for acute (after 1 week) collagenase-induced SDFT injury treatment in the horse (Watts et al., 2011). This experimental *in vivo* study showed that tissue architecture, tendon size, lesion size and linear fiber pattern improved significantly in the treated tendons compared to controls and no tumor formation was reported. However, no differences in tendon matrix specific gene expression or total DNA, proteoglycan and collagen production were seen between the control group and the cell-treated group.

As aforementioned, the iPS cells are derived from somatic cells by the forced expression of a combination of specific transcription factors. This results in the reprogramming of these cells to a pluripotent state which allows them to differentiate into any desired cell type for autologous transplantation. This should decrease the risk of immune rejection, although a recent study in mice suggests that abnormal gene expression as a result of reprogramming may produce an immune response following autologous transplantation (Zhao et al., 2011).

On the other hand, Araki et al. (2013) reported a limited to no immune response after transplantation of terminally differentiated cells derived from mouse iPS cells. Nonetheless, iPS cells do raise fewer ethical concerns compared to ESCs and they have been successfully produced from both human and equine fibroblasts (Breton et al., 2013; Hackett et al., 2012; Hussein et al., 2011; Nagy et al., 2011). However, equine iPS cells have been shown to induce teratoma formation in mice models (Nagy et al., 2011), which most likely explains why no clinical applications with iPS cells have been reported in horses to date.

*(2) Multipotent stem cells*

Adult multipotent SCs can only differentiate into a limited number of tissues of the adult individual (De Schauwer et al., 2011b). MSCs from different sources are the most commonly used multipotent SCs for the treatment of orthopedic injuries, mainly because of their regenerative, anti-inflammatory and homing capacities (Fong et al., 2011; Iyer and Rojas, 2008; Shi et al., 2010).

In horses, the first report on the use of cellular BM to aid tendon repair was in 2001 (Herthel, 2001). In this study 84% (n=100) of horses with a naturally-occurring suspensory ligament desmitis returned to full work after BM treatment, in contrast to the control group, where only 15.2% (n=66) of the horses reached the same performance level as before. It must be noted, however, that in this study no information was given on the frequency of forelimb and hindlimb problems, nor the region of the injury, which are all of importance since they have different prognoses (Dyson, 2000; Dyson et al., 1995). In another study, the beneficial effects of BM-derived MSCs were evaluated in horses suffering from SDFT tendinosis and they found that 82% (n=168) of the horses treated with MSCs performed at their original level without reinjury in the next year (Smith, 2008), whereas 42-44% of the horses with SDFT tendinosis treated with conservative and reparative therapy with hyaluronic acid (HA) and polysulfated glycosaminoglycans (PSGAG) re-injured (Dyson, 2004). Here, it should be

mentioned that the documentation from the control group was from another study and spanned a longer time frame post-treatment (2 years). However, a recent 2 year follow-up study of 141 horses with SDFT tendinosis confirmed a significantly lower reinjury rate of 27% after BM-derived MSC treatment (Godwin et al., 2011).

The use of adipose tissue (AT)-derived MSCs has been described for the treatment of clinical cases of tendinopathy (Del Bue et al., 2008; Richardson et al., 2007). To date, there is only one study where they have evaluated the effects on tendon repair in an experimental equine model where therapy was initiated 1 week after inducing the lesion (Nixon et al., 2008). Unfortunately, the authors used an AT-derived cell mixture instead of a pure AT-derived MSC population in this study and although biomechanical properties of the repaired tissue were not measured, similar significant histological improvements, as described for BM MSCs (Schnabel et al., 2009), were noted along with a significant increase in COMP gene expression. The gene expression of collagen type I and III, on the other hand, were similar in the treated and control tendons (Nixon et al., 2008). Although equine AT-derived MSCs have been demonstrated to be inferior to BM MSCs in cartilage differentiation *in vitro* (Kisiday et al., 2008; Vidal et al., 2008) and the treatment of experimentally-induced osteoarthritis in horses (Frisbie et al., 2006), there has been - to our knowledge - no direct comparison reported to date on the ability of AT-derived versus BM-derived MSCs to generate tendon tissues.

Although there are no reports on the use of synovium-derived MSCs in horses, the use of human synovium-derived MSCs has been reported as a promising treatment for musculoskeletal disorders due to the high proliferation and differentiation capacities of these cells *in vitro* and *in vivo* (De Bari et al., 2003; Ju et al., 2008).

Recently, amniotic fluid (AF) has also been described as a useful source of MSCs in horses and in humans (Manuelpillai et al., 2011; Park et al., 2011a). In contrast to humans, where it

has been described that these cells may have clinical significance in prenatal and postnatal medicine (Shaw et al., 2011), no data on the clinical application of AF-derived MSCs has been reported in horses to date.

The isolation of MSCs from umbilical cord matrix (UCM) as well as umbilical cord blood (UCB) has been described in equines (De Schauwer et al., 2011a; Hoynowski et al., 2007; Koch et al., 2007; Zeddou et al., 2010). A recent *in vitro* study in horses proposed the use of UCB-derived MSCs for musculoskeletal regeneration based on the differentiation potential of these cells. Unfortunately, no differentiation of equine UCB-derived MSCs towards tenocyte-like cells was performed (Reed and Johnson, 2008). Moreover, the only *in vivo* experiments using equine UCM-derived MSCs to date report the injection of autologous and allogeneic UCM-derived MSCs into the radiocarpal joints and into the skin of healthy horses in order to evaluate any potential adverse effects (Carrade et al., 2011a; Carrade et al., 2011b). In both equine studies, no clinical signs of an immune response were detected after injection with autologous or allogeneic UCM-derived MSCs.

As described above, regenerative medicine has been intensively used for the treatment of tendinopathies (Table III).

**Table III.** Overview of reported multipotent mesenchymal stem cell (MSC) therapies to treat tendon injuries.

| Species origin | Tissue origin | In vivo model | Tendon studied | Reference |
|---|---|---|---|---|
| Horse | BM | Horse | SDFT | Schnabel et al., 2009 |
| Horse | BM | Horse | SDFT | Lacitignola et al., 2008 |
| Horse | BM | Horse | SL | Herthel, 2001 |
| Horse | BM | Horse | SDFT | Smith, 2008 |
| Horse | BM | Horse | SDFT | Godwin et al., 2011 |
| Horse | AT | Horse | SDFT | Nixon et al., 2008 |
| Horse | AT | Horse | SDFT | Del Bue et al., 2008 |

SDFT: Superficial digital flexor tendon; SL: Suspensory ligament

On the other hand, the administration of MSCs has also been proposed as a promising treatment for other diseases such as arthrosis (Wilke et al., 2007), bone fractures (Vidal et al., 2006), hepatic disorders (Petersen et al., 1999), pancreatic dysfunction (Santana et al., 2006), myocardial pathologies (Chen et al., 2006) and even skin wounds (Chen et al., 2012). Nevertheless, only limited clinical information is available or only a small number of animals have been included in these case reports.

*(3) Differentiated cells*

The use of autologous tenocytes has been reported for differentiated cell therapy and tenocytes have been isolated from tendon tissue of humans, rabbits, chickens and horses (Cao et al., 2002; Casey, 2011; Chen et al., 2007; Yao et al., 2006).

To date, there is only one report of equine autologous tenocytes used for the treatment of naturally occurred tendon lesions (Casey, 2011). In this study, 45 horses with discrete core tendon lesions were treated. The tenocytes showed a strong linear cellular alignment *in vitro* and showed no *in vivo* overgrowth of the cells/tendon tissue. Furthermore, no negative effects, such as hypersensitivity or hyperproliferation after sampling or re-injection were observed. In this study, 60% (27 out of the 45 treated horses) reached the same competition level as before. Unfortunately, no control group of horses with identical discrete core tendon lesions was included in this study. Moreover, the functionality of equine tenocytes after *in vitro* culturing as well as after *in vivo* application remains to be demonstrated. In addition, recently the use of ovine amnion epithelial cells has been described to produce tenocytes *in vitro* and to treat 15 horses with acute tendon lesions (Muttini et al., 2013). Here, they found that 80% (12 out of 15) of the patients were able to return to their previous performance level. However, the lack of control groups in this study does not allow to draw any definite conclusions concerning this xenogeneic treatment.

*1.2.3.4. Complications of MSC therapy*

Despite the positive effects of MSCs, an issue of debate is the risk of spontaneous transformation of these cells. For human AT-derived MSCs, this phenomenon has been reported after long-term *in vitro* passaging (Rubio et al., 2005). On the other hand, human BM-derived MSCs did not undergo transformation nor exhibited telomere maintenance mechanisms when culturing for a normal duration and less than 25 population doublings (Bernardo et al., 2007). Recently, clinical trials with 227 and 339 human patients treated for various orthopedic conditions with BM-derived MSCs failed to demonstrate any tumor formation on MRI at the re-implanted sites at 2 and 3 years after injection, respectively (Centeno et al., 2011; Centeno et al., 2010). Although long-term follow-up studies are lacking, these preliminary data implicate that MSCs are probably safe to use in humans. In 141 horses with overstrain injury of the SDFT treated with BM-derived MSCs and followed up for a 2 year period, there was also no evidence of any complications, differentiation to undesirable cell types or neoplastic transformation at any time point post treatment with these cells (Godwin et al., 2011). Therefore, all current evidence suggests that the use of MSCs is safe in both humans and horses. Nevertheless, future research will provide new insights into the immunogenicity and complications after clinical application of SCs.

Another drawback of SC therapy is the risk of possible disease transmission from animal-based serum (fetal calf serum), which is commonly used for the cultivation of SCs. Therefore, several researches have explored alternatives, and the use of autologous platelet lysate appeared to be a worthy replacement for fetal calf serum (Centeno et al., 2010; Rauch et al., 2011; Schallmoser et al., 2007). Defined cell culture media lacking any animal products has also been reported for the culture of human pluripotent SCs (Chen et al., 2011).

Finally, and more specifically related to the use of SCs for the treatment of tendinopathies, there is the issue of ectopic bone formation. To date, different human and equine studies

report no ectopic bone formation after the clinical use of MSCs (Centeno et al., 2011; Centeno et al., 2010; Crovace et al., 2007; Godwin et al., 2011; Richardson et al., 2007; Riordan et al., 2009; Smith, 2008). In rabbits, however, ectopic bone formation was reported in 28% of the animals (n=34) following injection of rabbit BM-derived MSCs into acute tendon lesions (Harris et al., 2004). Culturing these MSCs in a 3D collagen scaffold resulted in an even greater amount of ectopic bone formation (Harris et al., 2004). In this regard, other studies also implicate that the right scaffold or delivery vehicle is of great importance for each SC application (Bosch and Krettek, 2002; Koch et al., 2009a; Longo et al., 2011; Nixon et al., 2012).

*1.2.3.5. The horse as a model for human regenerative medicine*

The use of MSCs in regenerative therapy has been studied extensively in mice, to gain valuable information concerning the *in vivo* efficiency and safety of regenerative medicine in humans. In this regard, nude mice and murine severe combined immunodeficiency (SCID) mutation are being reported as the ideal animal models to assess the *in vivo* functionality of heterologous cells, because the immune system of these animals accepts foreign cells much more easily compared to any other animal (Gerling et al., 1994; Mahasiripanth et al., 2012). Thus, the mouse can be considered the best model as a highly efficient recipient of human or equine cells to engraft, proliferate and differentiate SCs. This unique feature offers a great opportunity for enhancing therapy researches of cancer, leukemia, visceral diseases, AIDS, and other diseases. It also provides applications for cancer, infection, regeneration, and hematology studies. Not only the short generation turnover and fast reproduction are great advantages for using small laboratory animals in clinical trials, but also their size, low cost and ease of handling benefits this animal species (Rosenthal and Brown, 2007).

However, for the evaluation of a new medicine against certain musculoskeletal diseases, such as osteoporosis, the food and drug administration (FDA) recommends the *in vivo* evaluation of the same product in two different animal species, namely a small laboratory animal and a second non-rodent large animal that has a similar tissue structure and remodeling pattern to that of humans (Food and Drug Administration, 1994). Indeed, the metabolism of horses, as well as the musculoskeletal physiology and pathologies resemble humans more closely than those of small laboratory animals. Moreover, there are many similarities between the weight bearing tendons of the horse and the human athlete (Smith and Webbon, 2005), with the tendons of both species showing a strong resemblance in function, matrix composition and nature of the injuries sustained (Smith and Webbon, 2005). Also the non-calcified cartilage thickness of the stifle joint in horses provides the closest approximation to humans, and this is considered to be relevant in pre-clinical studies of cartilage healing (Frisbie et al., 2006). Furthermore, in 2005, the FDA selected the horse as the most reliable animal model to evaluate new therapies on cartilage defects in human medicine. Additionally, the thickness, volume, composition, manipulation and exploration of equine cartilage is very similar to human cartilage (Frisbie et al., 2006). For all the aforementioned reasons, the evaluation of new treatments for musculoskeletal injuries in horses will be of advantage to human medicine. Of course, the equine animal model also directly benefits veterinary patients.

## 1.3. Mammary stem/progenitor cells (MaSCs)

### *1.3.1. General characteristics*

In 1959, the transplantation of mammary gland tissue into a cleared fat pad (mammary adipose tissue without mammary epithelium) of a genetically identical acceptor mouse gave rise to the development of an entirely functional mammary gland with the formation of ductal

and alveolar epithelial cells (Deome et al., 1959). With this finding, the hypothesis of the existence of a mammary stem/progenitor cell (MaSC) population was born. In 1983, the so-called cap cells were identified as possible MaSCs (Williams and Daniel, 1983). These cap cells are epithelial cells and are located at the tip of an elongating mammary duct in mice, which is also called terminal end bud (TEB) (Figure 4) (Hinck and Silberstein, 2005).

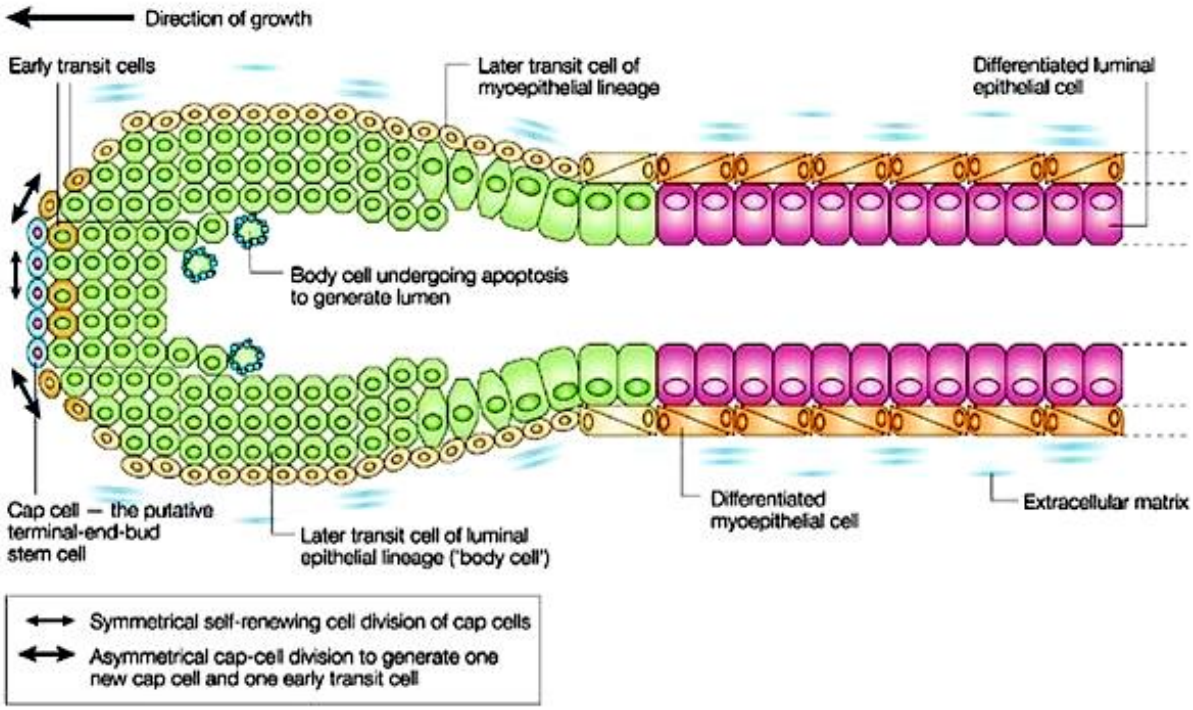


**Figure 4.** During rodent mammary gland development cap cells in the terminal end buds (TEBs) move through the fat pad and establish the different epithelial cell layers (Smalley and Ashworth, 2003).

This developmental unit in the mammary tissue is called "terminal ductal lobular unit" (TDLU) in other species, such as ruminants and humans, and is characteristic for postpubertal mammary development (Capuco et al., 2002; Telang et al., 1990). The ductal lumen is formed when central body cells undergo apoptosis and outer cells differentiate into luminal epithelial cells, and extracellular-matrix enzymes degrade the stroma in front of the TEB to enable it to move through the fat pad (Smalley and Ashworth, 2003). Fifteen years later it was reported that MaSCs and their progeny are located in the entire mammary gland (Kordon and Smith,

1998). Even though the existence of undifferentiated human mammary cells, which could survive in suspension as so-called mammospheres, was reported for the first time in 1986 (Soule and McGrath, 1986), it took some years before these cells were designated as MaSCs. These cells showed properties of bipotent cells based on their capacity to produce adult luminal epithelial on the one hand and myoepithelial cells on the other hand (Figure 5) (Petersen et al., 1992; Stingl et al., 2001). In line with what has been described for MSCs, MaSCs also need to express stem cell (SC)-specific markers and be negative for certain lineage-specific markers. Most information available comes from mice, and to a lesser extent humans, whereas not much is known on the immunophenotypic profiles in other mammalian species with the exception of bovine MaSCs. An overview of the markers most commonly used to immunophenotype murine, human and bovine MaSCs can be found in Table IV.

The differentiation of murine and human MaSCs into myo-epithelial cells and ducto-alveolar structures has been reported using a 3D culture model (Bai and Rohrschneider, 2010; Bandyopadhyay et al., 2012; Dontu et al., 2003a; Weaver and Bissell, 1999; Welm et al., 2003). Also in cattle, the differentiation of MaSCs towards alveolar epithelial cells (on collagen coated culture plates) and towards myo-epithelial cells (on normal culture dishes) has been reported (Li et al., 2009; Motyl et al., 2011), however, less information concerning their cell surface markers is available in this animal species.

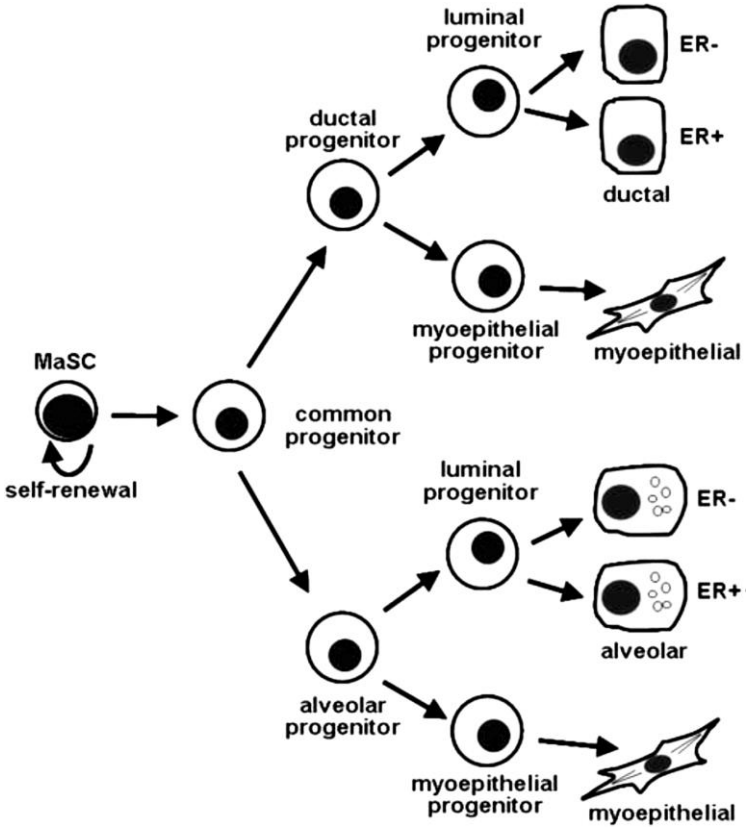


**Figure 5.** Schematic overview of the different cell types in the bovine mammary gland (Capuco et al., 2012). The mammary stem/progenitor cell (MaSC) can multiply through symmetric division with the formation of identical daughter cells. After asymmetrical cell division, a more differentiated progenitor cell rises from the MaSC. From a common progenitor cell, a ductal and alveolar progenitor cell can be produced. These can both give rise to myo-epithelial cells or ductal and alveolar epithelial cells with (+) or without (-) an estrogen receptor (ER), respectively.

To date, several *in vitro* assays have been described to identify, isolate and/or characterize MaSCs. In order to morphologically identify this hierarchy, transmission electron microscopy can be performed with an osmium tetroxide staining or immunohistochemistry can be done on tissue sections after a repeated intravenous Bromodeoxyuridine (BrdU) injection in cows or intraperitoneal thymidine injection in mice. In different studies with different animal species, such as mice, rats and cows, the results were similar: small light staining cells (SLCs) or BrdU or thymidine-label-retaining epithelial cells (LRECs) were identified and this population most likely encompassed the MaSCs (Capuco, 2007; Chepko and Smith, 1997; Smith, 2005; Smith and Chepko, 2001).

**Table IV.** Overview of most commonly used mammary stem/progenitor cell (MaSC) markers, their function and their presence in bovine, murine and human MaSCs.

| Marker | Function | Expression | Animal | Reference |
|---|---|---|---|---|
| **CD10** | Peptide | + | Mice | Han et al., 2006 |
| | degradation | + | Human | Bachelard-Cascales et al., 2010 |
| | | ND | Bovine | |
| **CD24** | Cell adhesion | ± | Mice | Bai and Rohrschneider, 2010 |
| | | ± | Human | Dey et al., 2009 |
| | | ± | Bovine | Rauner and Barash, 2012 |
| **CD29** | Cell adhesion | + | Mice | Wang et al., 2008 |
| | | + | Human | Jo et al., 2010 |
| | | + | Bovine | Li et al., 2009 |
| **CD31** | Cell adhesion | - | Mice | Booth et al., 2008 |
| | Cell migration | - | Human | Stingl, 2009 |
| | | - | Bovine | Rauner and Barash, 2012 |
| **CD34** | Cell adhesion | ± | Mice | Han et al., 2006 |
| | | - | Human | Dey et al., 2009 |
| | | ND | Bovine | |
| **CD44** | Cell adhesion | + | Mice | Dontu et al., 2005 |
| | Migration | + | Human | Dey et al., 2009 |
| | | ND | Bovine | |
| **CD45** | Signaling | - | Mice | Shackleton et al., 2006 |
| | molecule | - | Human | Hardt et al., 2012 |
| | | - | Bovine | Rauner and Barash, 2012 |
| **CD49f** | Cell adhesion | + | Mice | Bai and Rohrschneider, 2010 |
| | | + | Human | Stingl, 2009 |
| | | + | Bovine | Li et al., 2009 |
| **CD61** | Cell adhesion | + | Mice | Asselin-Labat et al., 2007 |
| | | + | Human | Leccia et al., 2012 |
| | | ND | Bovine | |
| **CD133** | Unknown | - | Mice | Sleeman et al., 2007 |
| | | - | Human | Lehmann et al., 2012 |
| | | ND | Bovine | |
| **ESA** | Epithelial | + | Mice | Amann et al., 2008 |
| | Cell adhesion | + | Human | Dey et al., 2009 |
| | | ND | Bovine | |
| **Sca1** | Mediates | + | Mice | Welm et al., 2003 |
| | adhesion | - | Mice | Stingl et al., 2006 |
| | & signaling | ND | Human | |
| | | ± | Bovine | Motyl et al., 2011 |
| **ALDH1** | Detoxification | + | Mice | Park et al., 2011b |
| | enzyme | + | Human | Ginestier et al., 2007 |
| | | + | Bovine | Rauner and Barash, 2012 |
| **CK14** | Cytoskeletal | ± | Mice | Wang et al., 2008 |
| | (Cell shape and size) | ± | Human | Dey et al., 2009 |
| | | ± | Bovine | Martignani et al., 2009 |
| **CK18** | Cytoskeletal | ± | Mice | Stingl et al., 2006 |
| | (Cell shape and size) | ± | Human | Dey et al., 2009 |
| | | ± | Bovine | Martignani et al., 2009 |

+ = present; - = absent; ± = moderate expression; ALDH = Aldehyde dehydrogenase; ND = Not Determined

Originally it was accepted that these SLCs and LRECs were lost lymphocytes, however, the presence of desmosomes and hemidesmosomes confirmed their epithelial nature (Ellis and Capuco, 2002). Moreover, these cells were undifferentiated because of the presence of only a few cytoplasmic organelles, small amounts of cell fibrils and a limited chromatin condensation in the nucleus. In addition, they were located in the basal layers of the mammary gland epithelium, a localization that corresponds with the expected MaSC niche (Verstappen et al., 2009). Based on the staining retention, also the progeny of the MaSCs has been identified: undifferentiated large light cells (progenitor cells), differentiated large light cells and large dark cells (Chepko and Smith, 1997).

Even though the staining and label-retaining techniques were of great value for the identification and localization of MaSCs, no viable MaSCs can be isolated with these techniques. Therefore, the use of the lipophilic, membrane permeable Hoechst (nuclear staining) has been described in order to isolate MaSCs. In contrast to differentiated cells, different types of SCs possess ABC membrane transporter proteins on their cell surface which are able to exclude different lipophilic drugs. Because MaSCs are capable of excluding Hoechst 33342, they can be identified as a negative side population (SP) (Figure 6) which can subsequently be separated by means of fluorescence activated cell sorting (Alvi et al., 2003; Dontu et al., 2003a; Woodward et al., 2005). In order to verify if the cells in the SP can truly exclude Hoechst, and therefore, contain the MaSCs, different membrane transporter inhibitors, such as Cyclosporin A or Verapamil can be added to the cell suspension and this would result in the disappearance of the SP (Figure 6) (Dey et al., 2009). A similar molecule, Rhodamine 123 has been described to generate a negative SP as well (Dey et al., 2009).

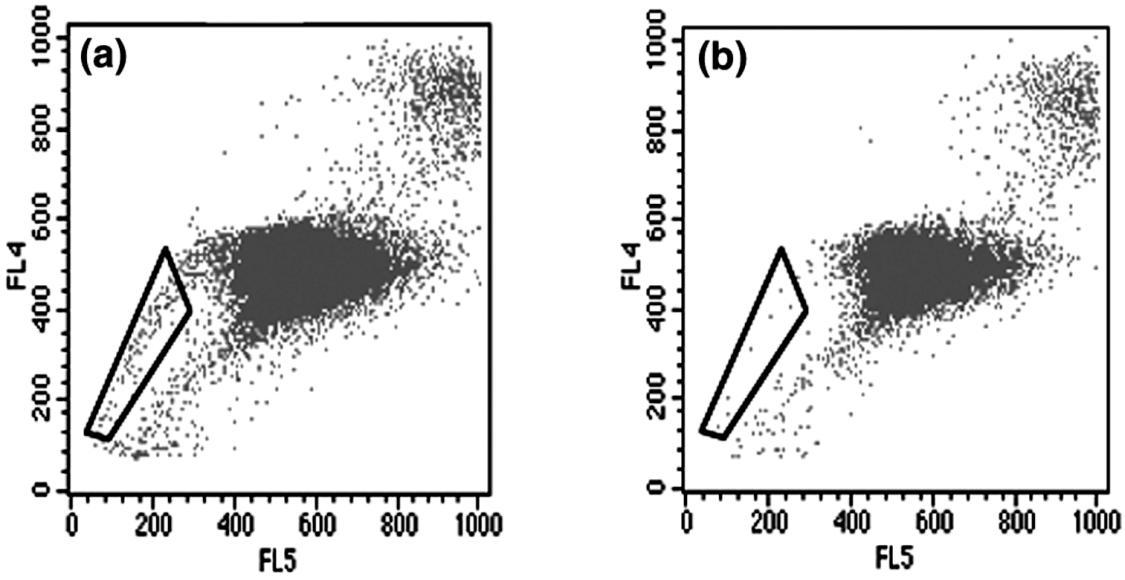


**Figure 6.** Flow cytometric analysis of human breast epithelial cells with Hoechst (Alvi et al., 2003). The cells in the black box represent the Hoechst negative side population (SP) before (**a**) and after (**b**) addition of Verapamil. This resulted in a 12-fold reduction of the SP.

Besides cell sorting, another technique for isolating MaSCs is the use of suspension cultures. In 1994, the first report on the influence of surface properties of the culture recipient on protein production (decreased in suspension) of murine mammary epithelial cells was published (Hurley et al., 1994). In this regard, a couple of years later Dontu described the culture of human mammary gland-derived spheroid cell clusters, the so-called "mammospheres" (Figure 7) which contained progenitor cells with MaSC properties (Dontu et al., 2003a). Unquestionably, there are many ways to identify, isolate and characterize MaSCs.

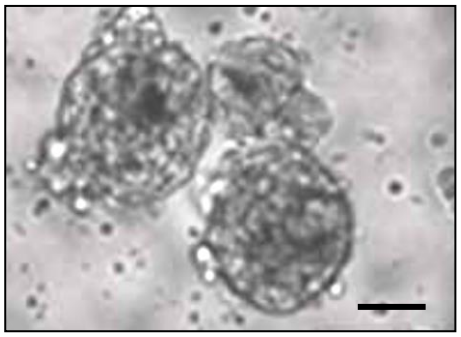

**Figure 7.** Human mammospheres. Bar represents 50µm (Bachelard-Cascales et al., 2010).

However, there is a need for uniformity, and guidelines for MaSC characterization should be constituted. Indeed, there are only a few reports on the functional characterization of MaSCs, although a more thorough study on the physiological (and pathological) behavior of these cells could provide valuable information regarding mammary gland physiology (and pathology).

### *1.3.2. The MaSC niche*

Mammary stem/progenitor cells (MaSCs) are recruited in response to specific physiological demands to regenerate, repair or maintain essential cellular components of tissues through activating signals which are part of a larger dynamic framework, the MaSC 'niche' (Joshi et al., 2012). This micro-environment responds to different stimuli, generating signals that activate MaSCs in order to expand and/or differentiate. Mammary epithelial fragments taken from any area of the gland can give rise to new ductal trees indicating that MaSCs and their niches are distributed throughout the ductal network (Daniel et al., 1971; Deome et al., 1959), or in other words, that the entire fat pad is in fact their original niche. This would explain why transplantation of a single MaSC into the cleared fat pad results in an elaborate epithelial outgrowth (Shackleton et al., 2006; Stingl et al., 2006). At the same time it implicates the necessity of stroma-derived signals in order to support the MaSCs. The exact nature of these stroma-derived signals is, however, still not elucidated. It is clear though that these signals must direct both symmetrical and asymmetrical division of the MaSCs in order to develop a functional ductal tree and that hormones play a key role in directing the fate of the MaSCs. Although mouse MaSCs themselves do not express steroid receptors (Asselin-Labat et al., 2006; Sleeman et al., 2007), ductal and alveolar growth is severely inhibited in ER knockout mice (Mallepell et al., 2006). Indeed, it has been reported that adding different hormones to MaSC cultures alter their destiny and determine the differentiation pathway they

will follow (Martignani et al., 2009; Visvader and Lindeman, 2006; Visvader and Smith, 2011). In this regard, it was found that culturing bovine and canine MaSCs with insulin and hydrocortisone induced ductal differentiation, and that alveolar differentiation with the production of milk proteins (casein) could be induced after supplementing prolactin (Cocola et al., 2009; Li et al., 2009).

Besides hormones, the mammary gland niche contains different cell types, such as adult epithelial cells, macrophages, eosinophils and stromal cells, such as fibroblasts and adipocytes (Gouon-Evans et al., 2002). It has been reported that the repopulating ability of murine MaSCs is compromised in the absence of macrophages (Gyorki et al., 2009). Indeed, macrophage-colony stimulating factor (M-CSF) deficiency in mice, caused defects in branching morphogenesis during pregnancy (Dai et al., 2002). Moreover, during postnatal mammary gland development, eosinophils are positioned around the top of TEBs and inhibiting the eosinophil infiltration, significantly reduced the total ductal branch numbers in murine mammary glands (Gouon-Evans et al., 2000). In addition, MSCs that are present in mammary adipose tissue (Zhao et al., 2012) produce IL6 and vascular endothelial growth factor (VEGF) which promote breast cancer cell migration through a more significant and more persistent activation of intracellular signaling pathways in these mammary cells (MAPK, AKT, and p38MAPK) (De Luca et al., 2012).

In conclusion, although morphological mammary gland development and interactions with different hormones and cell types is well described in several animal species, much remains to be understood about the interaction of MaSCs with other cells, growth factors, cytokines and extracellular matrix components. Moreover, to our knowledge, in horses there are no reports on MaSCs or their interaction with the micro-environment.

### *1.3.3. MaSCs in physiology and pathology*

In the following paragraphs, the role of mammary stem/progenitor cells (MaSCs) in mammary gland physiology is described mainly based on data gathered in rodents and ruminants, since studies on mammary gland development have primarily been conducted in these species.

The development of the mammary gland is an unusual phenomenon, since from birth till after puberty, in several animal species the mammary gland remains rudimentary with a relatively quiescent growth mainly consisting of ductal elongation (Tiede and Kang, 2011). Already *in utero*, the mammary gland develops from a relatively small number of stem cells (SCs) which multiply and form clonal regions in the mammary epithelium (Tsai et al., 1996). During mammary gland development, terminal end buds (TEBs) in rodents or terminal ductal lobular units (TDLUs) in other species ramify through the fat pad and establish inner luminal epithelial cell layers and outer myoepithelial cell layers (Brisken and Duss, 2007; Neville et al., 1998; Smalley and Ashworth, 2003). A unique and fascinating aspect of mammary gland biology is the requirement for MaSCs to grow and function in a stromal matrix referred to as the mammary fat pad (Neville et al., 1998). Crucial to this environment are the resident adipocytes (Hovey et al., 1999). Whereas this adipose depot was once accepted as a relatively inert tissue, it is now well-recognized that this is not the case (Neville et al., 1998). Besides the growth and function modulating properties of the adipocytes, this environment ultimately dictates to which extent the glandular epithelium of an individual can develop into a functional mammary gland (Hoshino, 1978).

Prepubertal mammary development mainly involves penetration of the fat pad by progressive increase in ductal structures (Meyer et al., 2006; Sinha and Tucker, 1969). This invasion is mainly attributable to growth factors and steroidal hormones. Insulin-like growth factor-type I (IGF-I) and estrogen cause cell multiplication at the tip of the ducts (lengthening

and branching) (Figure 8), while progesterone causes ductile cells to multiply, leading to ductile development (Figure 8) (Atwood et al., 2000; Russo and Russo, 1996). This elongating and branching process is by no means a random process and is controlled by "zones of inhibition" around each duct into which other ducts are not able to grow (Faulkin and DeOme, 1960). The regulatory factor in this process has been identified in mice as transforming growth factor-β (TGF-β) (Daniel et al., 1996).

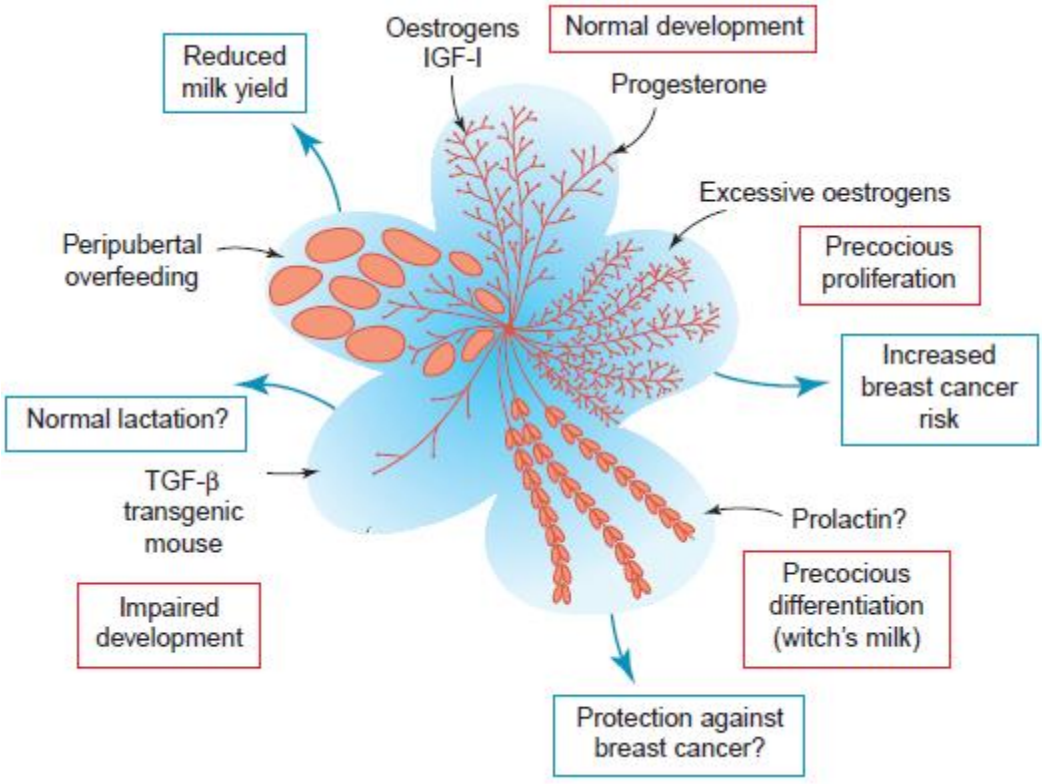


**Figure 8.** Schematic representation of the effect of growth factors and hormones on neonatal bovine mammary gland development (Adapted from Knight and Sorensen, 2001).

During puberty, the increasing ovarian activity stimulates ductal elongation, probably because of progenitor cell activation through their occupied estrogen receptors (Brisken and Duss, 2007; Capuco, 2007). During pregnancy TDLUs develop and form a cluster of alveoli, which is histologically recognizable as a lobuloalveolar unit. In this physiological phase the mammary gland undergoes intensive remodeling with alveolar growth and secretory

differentiation to initiate milk secretion, followed by involution with apoptosis and regression when lactation is terminated (Figure 9) (Lewis, 2000; Motyl et al., 2011; Tiede and Kang, 2011). Different researchers have suggested that these changes are driven by the coordinated division and differentiation of MaSCs in response to several growth factors and hormones (Kass et al., 2007; Shackleton et al., 2006; Tiede and Kang, 2011).

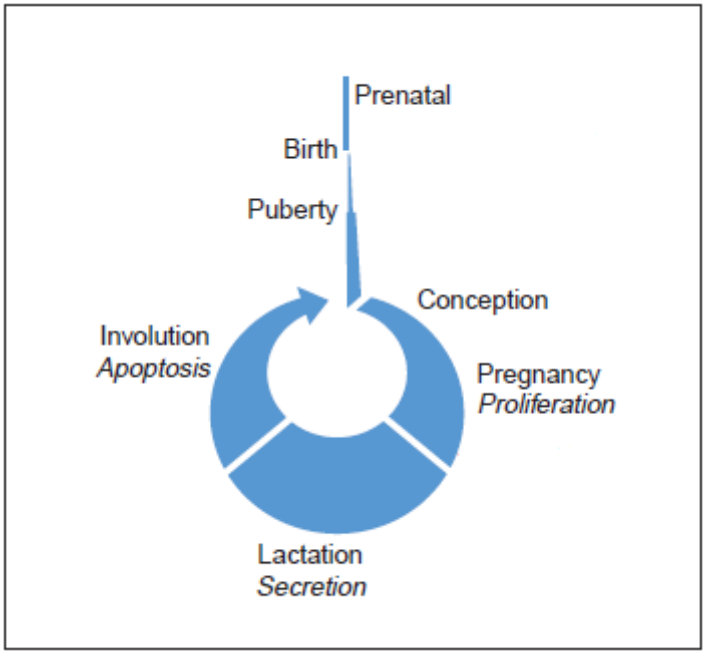


**Figure 9.** Schematic representation of mammary gland development (Knight and Sorensen, 2001). The width of the arrow represents the size of the gland.

As for many other organs, the mammary gland encloses parenchym and stroma. The parenchym contains alveoli and ductuli, whereas the stroma consists mainly of connective tissue, adipose tissue, blood vessels and lymph vessels. Based on the mammary gland morphology, it would be logic to anticipate that MaSCs populate the parenchym, whereas mesenchymal SCs (MSCs) and hematopoietic SCs (HSCs) would populate the stroma. In order to construct functional alveoli and ductuli, different epithelial cell types have to be generated from MaSCs, i.e. ductal epithelial cells (ductuli), alveolar epithelial cells (alveoli) and myo-epithelial cells (contraction for milk ejection).

Moreover, it has been reported that MaSCs are not inexhaustible, since they lose their proliferation and differentiation capacities after repeated cell divisions. This was originally noted after a series of transplantations of murine mammary epithelium in cleared fat pads (Chepko and Smith, 1997; Clarke and Smith, 2005; Smith, 2006). After an undefined number of transplantations, the epithelium can only form alveolar structures or ductuli or even lose their capacity to produce anything at all. Apparently, senescence appears first in actively proliferated epithelial cells and later on in resting epithelial cells (Chepko and Smith, 1997). This implicates that the previous number of mitoses determines the senescence rate of the cells. However, more research is needed to fully unravel the self-renewal rate and proliferation capacities of MaSCs.

The mammary gland is not only an organ which is important for lactation, it is also an important subject for research in tumorigenesis, especially in humans. Indeed, breast cancer is a devastating disease with in Belgium alone 5000 women who are being diagnosed and 2500 women that pass away each year (Beyens et al., 2002). According to the International Agency for Research on Cancer (IARC), Belgium has the highest prevalence of women that are being diagnosed with breast cancer compared to other countries (probably because of the high screening frequency as reported by the IARC). Therefore, researchers are looking for improved treatments and ultimately, a potential cure. Already since the beginning of time it is known that all diseases should be treated based on the cause, instead of treating the symptoms. In this regard, it has been hypothesized that MaSCs could be at the origin of the development of mammary tumors, leading to the so-called cancer SC hypothesis (Figure 10) (Sagar et al., 2007). Moreover, it has been shown that deregulation of normal self-renewal pathways in undifferentiated breast SCs or progenitor cells could alter their destiny, resulting in abnormally differentiated cells in human and rodent breast cancer cell lines (Dontu et al., 2003b; Reya et al., 2001). Indeed, because SCs live much longer than adult cells they have an

increased risk of genetic alterations or mutations (Marion and Blasco, 2010). In addition, SCs and tumor cells share many characteristics such as their: self-renewal rate, migration capacities, undifferentiated status and activated cytoprotective mechanisms (increased telomerase activity, increased transmembrane efflux capacities, etc.) (Ponti et al., 2005). For all these reasons, it is hypothesized that MaSCs could be at the basis of tumor development (Figure 10).

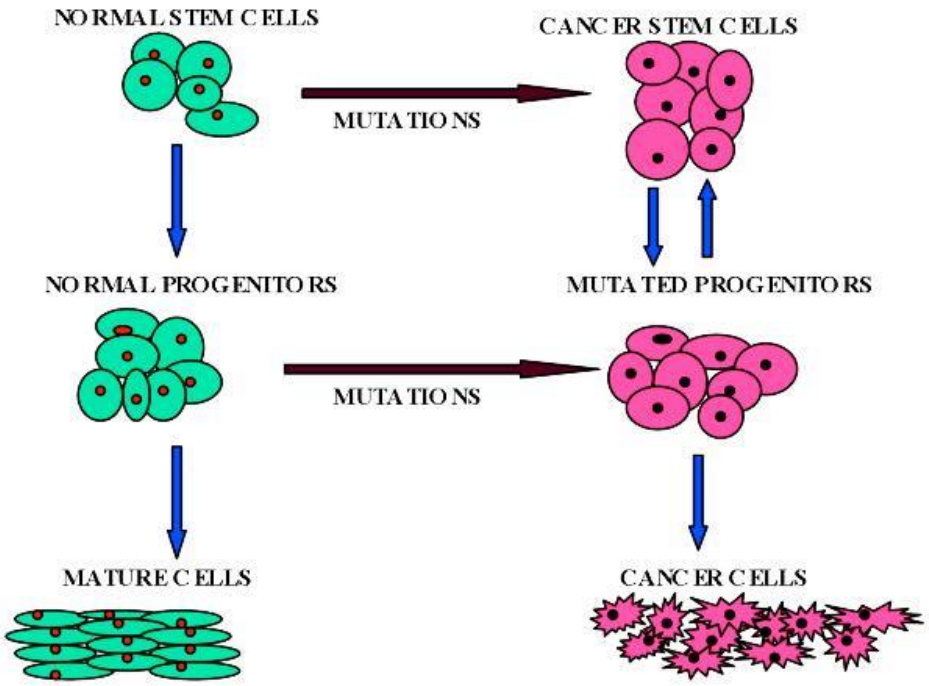


**Figure 10.** Schematic overview of the cancer stem cell (SC) hypothesis (Sagar et al., 2007). The cancer SCs may develop when normal mammary SCs or progenitor cells acquire mutations and are transformed by altering only proliferative pathways or by multiple oncogenic mutations. The changed morphology in cancer cells indicates uncontrolled proliferation.

Interestingly, mammary cancer is common in humans and carnivores (Munson and Moresco, 2007), whereas cows, sheep, pigs and horses only very rarely develop mammary tumors (Knight and Sorensen, 2001). Using this variation amongst different animal species, new insights could be gathered on the mechanisms underlying the functional behavior and regulation of MaSCs, and their role in tumorigenesis. Therefore, it is of importance to study MaSCs in as many species as possible. But in order to do that, proper isolation and

characterization procedures are needed to obtain MaSCs derived from animals with a low and high susceptibility to mammary cancer. In dogs, an animal species with high mammary tumor susceptibility, there are some reports on cancer MaSCs (Cocola et al., 2009; Hellmen et al., 2000). In cattle, an animal species with low mammary tumor susceptibility, MaSCs are mainly studied for their capacity to improve milk production (Capuco, 2007; Li et al., 2009; Martignani et al., 2009). In this regard, it should be taken into account that low milk production might be caused by inflammatory processes. In several mammals, such as horses, mastitis is usually caused by bacterial infection (McCue and Wilson, 1989). These infections may occur as a result of inadequate mammary wound healing. One of the determining factors whether or not a trauma heals properly might be the accurate proliferation and differentiation of MaSCs. In this regard, MaSCs could also have a protective effect on the occurrence of mastitis and more knowledge on the MaSC biology might provide us with a tool for the prevention or even treatment of mastitis. Unfortunately, there are no reports on the presence and function of MaSCs in horses. This is probably due to the low economic impact of milk production in this animal species and because equine mammary tumors and mastitis are rare (Hirayama et al., 2003; Jackson, 1986; McCue and Wilson, 1989; Seahorn et al., 1992). Moreover, a thorough immunophenotypic characterization of equine cells in general, and putative equine MaSCs in specific, is hampered in this species, since only about 4% of human antibodies react with the equivalent equine proteins (Ibrahim et al., 2007). Still, a thorough study of equine MaSCs could potentially extend the current knowledge of the mammary gland biology in this animal species and form a negative model for a comparative physiological approach of MaSC tumorigenesis.

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# CHAPTER 2

## Aims of the study

## Aims

When a fertilized egg cell develops into a multicellular organism, only highly proliferative pluripotent stem cells (SCs) are capable of driving this process. During further organ and tissue development, SCs become more specialized and less potent. However, multipotent SCs are still present in differentiated tissues of adult animals where they function to replace aged cells throughout an individual's life. So if SCs can be successfully isolated from these tissues and identified as such, they can form the basis for clinical applications in regenerative medicine and for more fundamental research on organ/tissue maintenance.

Mesenchymal SCs (MSCs) and mammary stem/progenitor cells (MaSCs) represent two entirely different populations of SCs and require different isolation techniques. In the horse, MSCs have an important potential for clinical applications in the repair of bone, cartilage and tendon, tissues which are frequently damaged in sport horses. Besides that, MaSCs are invaluable tools for research on mammary gland development and disease. The horse is an interesting species in this respect, since it has a very low incidence of mammary carcinoma and as such, could represent a non-rodent cancer-resistant animal species.

For the aforementioned reasons, the current thesis aimed:

1. To optimize the isolation and characterization of equine MSCs from peripheral blood (PB), since PB is a highly accessible source of MSCs, which is easy to collect under field circumstances (**Chapter 3**).
2. To optimize the isolation and characterization of equine MaSCs, since equine mammary gland tissue is highly accessible through slaughterhouse collection and MaSCs play a pivotal role in mammary gland development and (mal)functioning, (**Chapter 4**).

3. To demonstrate in a supplementary part of this thesis, how equine PB-derived MSCs can be applied clinically by injection into the pastern joint of a horse suffering from degenerative joint disease **(Chapter 5)**.

# CHAPTER 3

## Equine peripheral blood-derived mesenchymal stem cells

Adapted from:

Spaas JH, De Schauwer C, Cornillie P, Meyer E, Van Soom A, Van de Walle GR, 2013. Culture and characterization of equine peripheral blood mesenchymal stromal cells. The Veterinary Journal 195(1), 107-113.

## 3.1. Abstract

The use of mesenchymal stromal cells (MSCs) for the treatment of orthopedic injuries in horses has been described in several studies. In contrast to human MSCs, no official guidelines have been proposed to classify a particular cell as an equine MSCs. For peripheral blood (PB)-derived MSCs in specific, only a limited characterization has been reported to date. In the present study, we have increased the currently available data on PB-derived MSCs. To this end, MSCs were isolated from equine PB samples and colony forming unit (CFU) assays as well as population doubling time (PDT) calculations from $P_0$ to $P_{10}$ were performed. Hereby, two types of colonies, fingerprinted and dispersed, could be observed based on macroscopic as well as microscopic features. Moreover, after an initial lag phase as indicated by a negative PDT at $P_0$ to $P_1$, the MSCs divided rapidly as shown by a positive PDT at all further passages. Furthermore, an immunophenotypic characterization was performed with trypsin- as well as accutase-detached MSCs, to evaluate a potential trypsin-sensitive destruction of epitopes of certain antigens. Hereby, it was found that the isolated MSCs were positive for CD29, CD44, CD90 and CD105, and negative for CD45, CD79α, MHC II and a monocyte/macrophage marker, irrespective of the cell detaching agent used. Finally, a trilineage differentiation towards osteoblasts, chondroblasts and adipocytes was confirmed using different histological staining methods.

**Keywords:** Peripheral blood; Horse; Mesenchymal stromal cells

## 3.2. Introduction

Stem cells (SCs) are defined as cells displaying a self-renewal capacity either with or without differentiation, depending on the symmetry of the division (Horvitz and Herskowitz, 1992). More specifically, mesenchymal stromal cells (MSCs) are adult SCs derived from the mesodermal germ layer. In 2006, the International Society for Cellular Therapy (ISCT) has carefully determined the qualities human cells must possess in order to be defined as MSCs (Dominici et al., 2006). Hereby, human MSCs have to be (i) plastic-adherent; (ii) positive for the markers CD73, CD90 and CD105 and negative for the markers CD14 (or CD11b), CD34, CD45, CD79a (or CD19) and MHC II; and (iii) able to differentiate into different cell types of the mesodermal germ layer such as osteoblasts, chondroblasts and adipocytes. The use of other human MSC markers such as CD29 and CD44 was also reported (Majumdar et al., 2003; Pittenger et al., 1999).

For equine MSCs, no such guidelines have been described to date, although this would greatly benefit researchers working in this field (De Schauwer et al., 2011b). Sources of equine MSCs reported include bone marrow (BM), adipose tissue (AT), umbilical cord, amniotic fluid, umbilical cord blood (UCB), peripheral blood (PB), gingiva and periodontal ligament (Ahern et al., 2011; Carrade et al., 2011; Koch et al., 2007; Koerner et al., 2006; Mensing et al., 2011; Park et al., 2011). For MSCs isolated from equine BM, AT and UCB, the use of several MSCs markers and a successful trilineage differentiation have been described (Guest et al., 2008; Hoynowski et al., 2007; Koch et al., 2009; Radcliffe et al., 2010). In contrast, only a limited characterization of equine PB-derived MSCs has been reported to date, despite the existence of several papers describing their isolation. Hereby, only one group has described a more extended immunophenotypic characterization of PB-derived MSCs using two of the proposed positive markers, namely CD44 and CD90, and two of the proposed negative markers, CD34 and CD45 (Martinello et al., 2010). Nevertheless, for

the negative markers used in this study, no information was provided on the positive controls used to confirm cross-reactivity with equine cells and a potential influence of the detachment product on epitope expression was not evaluated. The latter might be of importance, since a recent paper by Hackett *et al.* describes a destructive effect of trypsin on the CD14 epitope of equine BM-derived cells, indicating that attentiveness is needed when evaluating negative SC markers on trypsin-detached cells (Hackett et al., 2011). Moreover, and aside from the immunophenotypic characterization, the results of different studies on the differentiation of equine PB MSCs into cartilage are contradictory (Giovannini et al., 2008; Koerner et al., 2006). All this indicates the need for more characterization of PB-derived equine MSCs.

Current clinical regenerative therapies with MSCs in horses mainly use BM-derived MSCs for the treatment of tendinopathies (Crovace et al., 2007; Smith, 2008; Smith et al., 2003) and BM-or AT- derived MSCs for the treatment of osteoarthritis (Frisbie et al., 2009). The most obvious disadvantages of BM and AT are the difficulty and invasiveness of the harvesting procedure. An excellent alternative would be blood, such as UCB collected at birth or PB from an adult horse. Despite the safety and high success rate of collecting UCB for use as a source for MSCs (Bartholomew et al., 2009), a potential disadvantage is the fact that autologous UCB is not always available at the moment of injury. In this case, the use of PB as a source for MSCs might prove a valuable alternative. Indeed, PB can be easily taken from the patient in a sterile manner, making this a readily accessible source of autologous MSCs when injuries occur and hence, indicates the potential of PB as a source of equine MSCs for regenerative therapies. Moreover, the first clinical applications of an heterogenous population of PB-derived SCs have been recently described for the treatment of ophthalmologic pathologies in horses (Marfe et al., 2011; Spaas et al., 2011). In order to standardize the promising results of this regenerative therapy, it is indispensable to use a well-characterized and homogenous SC population.

Therefore, the goal of the current study was to broaden the knowledge on the characterization of equine PB-derived MSCs by (i) determining the growth efficiency and proliferation rate of the cells, (ii) using a more wide-ranging set of complementary markers for their immunophenotyping and (iii) performing trilineage differentiation experiments.

## 3.3. Materials and Methods

### *3.3.1. Isolation of putative peripheral blood (PB)-derived mesenchymal stromal cells (MSCs)*

Ten mL of peripheral blood (PB) from the *vena jugularis externa* of four adult Warmblood horses was collected between 9 and 10 am in the spring season into EDTA tubes and transported at 4°C to the laboratory within 4 hours after sampling. The donor horses consisted of 1 mare (15 years-old), 2 stallions (4 years-old) and 1 gelding (5 years-old). The PB was centrifuged at 1000xg for 20 minutes at room temperature (RT). The buffy coat fraction was collected and diluted 1:1 with phosphate buffered saline (PBS). Subsequently, the cell suspension was gently layered on a Percoll gradient (density 1.080 g/mL; GE Healthcare) and centrifuged at 600xg for 15 minutes at RT, as previously described (De Schauwer et al., 2011a). The interphase was collected, washed three times with PBS by centrifuging at 200xg for 10 minutes, and cells were planted at $16x10^4$ cells/$cm^2$ in a $T_{75}$ flask in culture medium consisting of low glucose (LG) Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 30% fetal calf serum (FCS) (GIBCO), $10^{-11}$ M low dexamethasone, 50 µg/mL gentamicin, 10 µl/mL antibiotic-antimycotic solution, 250 ng/mL fungizone (all from Sigma) and 2 mM ultraglutamine (Invitrogen). The medium was refreshed twice a week and the putative MSCs were maintained at 37°C and 5% $CO_2$. At 70% confluency, cells were trypsinized with 0.25% trypsin-EDTA ($P_0$) and were further cultured for 10 subsequent passages ($P_1$ to $P_{10}$) in expansion medium, with the latter being identical to the culture medium but without dexamethasone.

*3.3.2. Colony forming unit (CFU) assay*

Ten, 50 and 100 MSCs were plated per 94 mm plate and fixed 8 days later at -20°C for 10 minutes using 90% ethanol. Crystal violet stainings were performed to visualize the colony forming units (CFUs) macroscopically and the total number of CFUs per plate were counted. These experiments were done in triplicate for all samples.

*3.3.3. Population Doubling Time (PDT) determination*

Cell doubling time (CDT) was calculated from $P_0$ to $P_{10}$ exactly as previously described (Hoynowski et al., 2007), using the following formula: $CDT= \ln(N_f/N_i)/\ln 2$ with $N_f$ as the final number of cells and $N_i$ the initial number of cells. For the population doubling time (PDT), the cell culture time (in days) was divided by the CDT (Hoynowski et al., 2007).

*3.3.4. Flow cytometric immunophenotyping*

To characterize the undifferentiated equine MSCs immunophenotypically, the expression of several MSC markers was evaluated simultaneously by flow cytometry. Cells were detached using either trypsin (Invitrogen) or accutase (Innovative Cell Technologies). Per series, $2x10^5$ cells were used and labeled with the following panel of primary antibodies: CD29-Alexa$^{488}$ (Biolegend, clone TS2/16), CD44-APC (BD, clone IM7), CD45-Alexa$^{488}$ (Serotec, clone F10-89-4), CD79α-Alexa$^{647}$ (Serotec, clone HM57), CD90 (VMRD, clone DH24A), CD105-PE (Abcam, clone SN6), MHC II (Serotec, clone CVS20) and a monocyte/macrophage marker-Alexa$^{488}$ (Serotec, clone MAC387). For the detection of the CD79α and monocyte/macrophage marker, fixation and permeabilization pretreatment was carried out with commercially available reagents (Invitrogen). In general, cells were incubated for 15 minutes on ice in the dark with the primary antibodies and washed twice in LG DMEM with 1% bovine serum albumin (BSA). Secondary Alexa$^{647}$-linked and PE-linked antibodies

(Invitrogen), again incubated for 15 minutes on ice in the dark, were used to label the CD90 and MHC II positive cells, respectively. In addition, viability assessment with the nucleic acid stain 7-amino-actinomycin D (7-AAD, Sigma) was performed on the non-fixed cells. At least 10,000 cells were acquired using a FACS Canto flow cytometer (Becton Dickinson Immunocytometry systems) equipped with a 488 nm solid state and a 633 nm HeNe laser, and these data were subsequently analysed with the FACS Diva software. To assess cross-reactivity of the differentiated blood cell markers, for which stem cells (SCs) should be negative, proper positive equine control cells consisting of equine peripheral blood mononuclear cells (PBMCs) and equine endothelial cells were used. In addition, cells were incubated with or without (autofluorescence) isotype-specific IgG's (mouse $IgG_1$, mouse IgM and rat $IgG_{2b}$) in parallel to establish the background signal.

*3.3.5. Trilineage cell differentiation*

For the osteogenic differentiation, $3x10^3$ cells/$cm^2$ were planted in a 4-well plate and incubated in expansion medium until cells were 70% confluent. At that point, osteogenic differentiation medium was added and refreshed twice a week. This medium consisted of LG DMEM (Invitrogen) supplemented with 10% FCS (GIBCO), 0.2 mM L-ascorbic acid-2-phosphate (Fluka), 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 µg/mL gentamycin and 10 µl/mL antibiotic-antimycotic solution (all from Sigma) (De Schauwer et al., 2011a; Koch et al., 2007). Three weeks later, differentiation was evaluated using Alkaline Phosphatase (Millipore detection kit) and Alizarin Red S staining in order to evaluate calcium phosphate deposition. For chondrogenic differentiation, $2.5x10^5$ cells/5mL were brought in a three-dimensional culture system, centrifuged at 150xg for 5 minutes at RT and resuspended in 0.5 mL chondrogenic inducing medium which was refreshed twice a week. This medium was based on the basal differentiation medium (Lonza), supplemented with 10 ng/mL

transforming growth factor-$\beta_3$ (Sigma). Differentiation was daily evaluated macroscopically and after 3 weeks of incubation, an Alcian Blue staining was performed on 8 µm histological sections after paraffin embedding of the chondrospheres. For adipogenic differentiation, $2.1x10^4$ cells/$cm^2$ were planted in a 4-well plate in expansion medium until the cells were 70% confluent and adipogenic inducing medium was added subsequently. After 3 days, this medium was replaced with adipogenic maintenance medium for 1 day. This cycle was repeated four more times after which the cells were refreshed twice with adipogenic maintenance medium. The adipogenic inducing medium consisted of LG DMEM (Invitrogen) supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 µg/mL recombinant human-insulin, 0.2 mM indomethacin, 15% rabbit serum, 50 µg/mL gentamycin and 10 µl/mL antibiotic-antimycotic solution (all from Sigma) (De Schauwer et al., 2011a; Koch et al., 2007). The adipogenic maintenance medium was identical but without dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine. Differentiation was evaluated after 3 weeks of cultivation using Oil Red O staining. As a control for the trilineage differentiation, MSCs were cultivated for 3 weeks in expansion medium at the same concentrations and in the same culture vessels, and all stainings were performed identically.

## 3.4. Results

### *3.4.1. Putative peripheral blood (PB)-derived equine MSCs are plastic adherent*

On average, the buffy coat of 10ml blood of the horses contained approximately $1x10^7$ peripheral blood mononuclear cells (PBMCs). The first plastic adherent colonies, approximately 8 for each horse, were noticed starting from 16-18 days after culturing these isolated fractions and around 21 days post seeding, cells became confluent with the formation of a monolayer (Figure 1A).

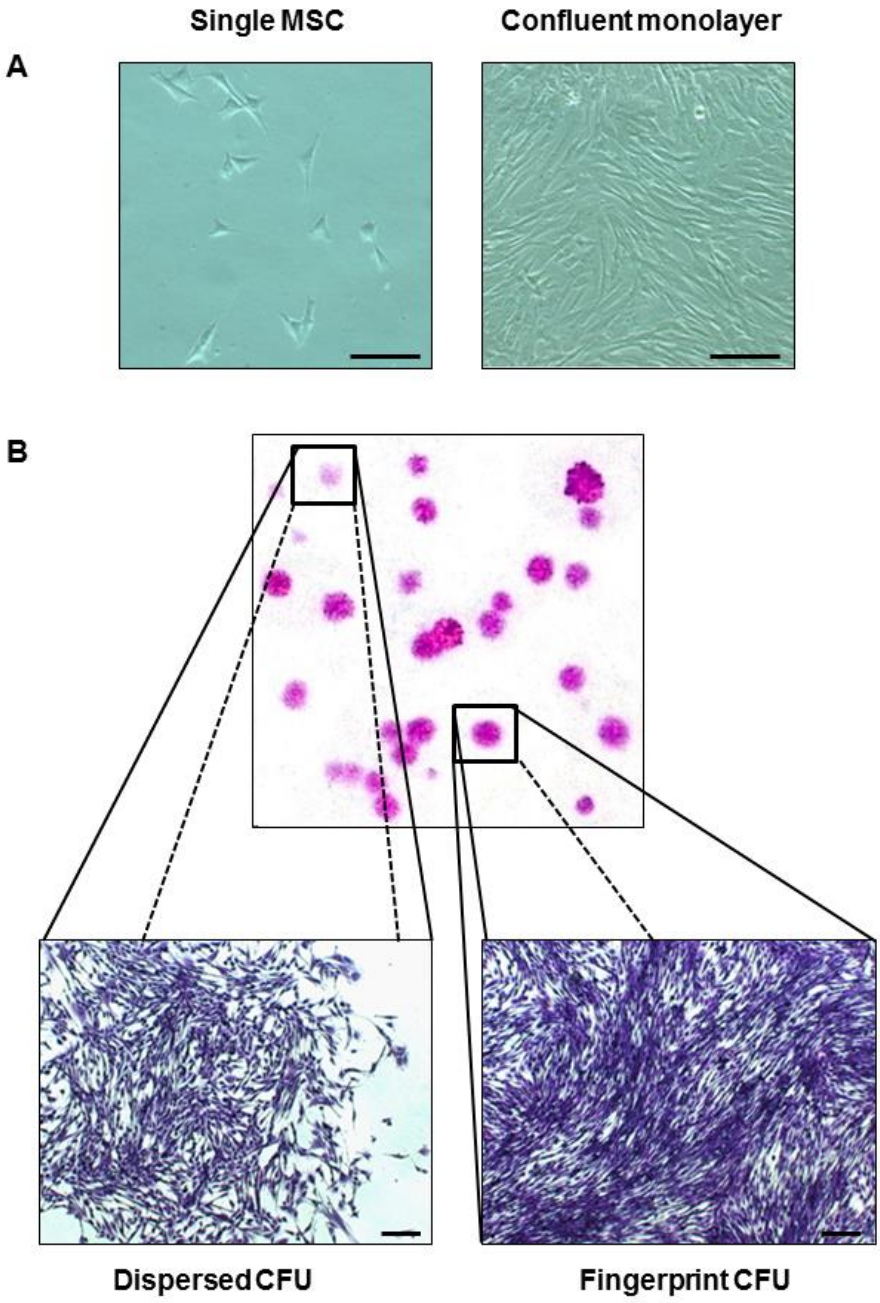


**Figure 1.** Adherent putative equine mesenchymal stromal cells (MSCs). (**A**) Representative pictures of putative single MSCs and a MSC monolayer. (**B**) Representative macroscopic and microscopic images of dispersed colony forming units (CFUs) and fingerprint CFUs after crystal violet staining. Scale bars represent 50µm.

### *3.4.2. Putative PB-derived equine MSCs have self-renewal growth properties*

Mesenchymal stromal cells (MSCs) can be evaluated using the well-characterized colony forming unit (CFU) assays. Hereby, a limited number of cells (10, 50 and 100) were seeded on a large surface and cultured for 8 days. At that time point, colonies in two different stages

could be observed macroscopically. First, there were dispersed CFUs identified by a spotted, vague macroscopic morphology and as rather scattered cells microscopically (Figure 1B). Second, darker and more packed CFUs were found with a microscopic fingerprint pattern (Figure 1B). In general, more dispersed compared to fingerprint CFUs were observed for all three seeding concentrations (Table I). To determine the growth efficiency and proliferation rate of the putative MSCs, population doubling times (PDT) in days were calculated from $P_0$ up to $P_{10}$. After an initial lag phase, indicated by a negative PDT at $P_{0\rightarrow1}$, the putative MSCs divided rapidly as shown by a positive PDT at all further passages tested (Table II).

**Table I.** Colony forming unit (CFU) assays of putative peripheral blood (PB)-derived equine mesenchymal stromal cells (MSCs) of four horses ($H_1$ to $H_4$). Data represent the means ± standard deviations.

| Number of seeded cells | Isolation | Fingerprint colonies | Dispersed colonies | Total colonies |
|---|---|---|---|---|
| **$CFU_{10}$** | **$H_1$** | 7±2 | 17±5 | 24±7 |
| | **$H_2$** | 6±1 | 11±4 | 17±5 |
| | **$H_3$** | 6±2 | 9±1 | 16±3 |
| | **$H_4$** | 9±5 | 10±4 | 19±9 |
| **$CFU_{50}$** | **$H_1$** | 20±4 | 47±5 | 68±4 |
| | **$H_2$** | 17±3 | 38±3 | 55±6 |
| | **$H_3$** | 17±3 | 38±6 | 55±5 |
| | **$H_4$** | 26±3 | 47±6 | 73±3 |
| **$CFU_{100}$** | **$H_1$** | 25±13 | 75±13 | 100±26 |
| | **$H_2$** | 32±2 | 82±6 | 114±6 |
| | **$H_3$** | 32±3 | 70±10 | 101±10 |
| | **$H_4$** | 32±9 | 65±11 | 99±12 |

**Table II.** Population doubling time (PDT) in days of the putative peripheral blood (PB)-derived equine mesenchymal stromal cells (MSCs) of four horses ($H_1$ to $H_4$).

| Passage (P) | PDT $H_1$ | PDT $H_2$ | PDT $H_3$ | PDT $H_4$ |
|---|---|---|---|---|
| **$P_{0\rightarrow1}$** | -5.46 | -6.25 | -3.29 | -3.72 |
| **$P_{1\rightarrow2}$** | 0.70 | 1.27 | 0.77 | 0.82 |
| **$P_{2\rightarrow3}$** | 1.21 | 0.98 | 0.75 | 1.17 |
| **$P_{3\rightarrow4}$** | 1.03 | 1.14 | 1.12 | 1.52 |
| **$P_{4\rightarrow5}$** | 1.49 | 1.02 | 0.98 | 0.92 |
| **$P_{5\rightarrow6}$** | 0.90 | 1.22 | 1.47 | 1.21 |
| **$P_{6\rightarrow7}$** | 1.35 | 1.00 | 1.23 | 1.41 |
| **$P_{7\rightarrow8}$** | 1.27 | 1.15 | 1.13 | 1.06 |
| **$P_{8\rightarrow9}$** | 0.74 | 1.02 | 1.01 | 1.92 |
| **$P_{9\rightarrow10}$** | 0.79 | 1.18 | 1.02 | 1.01 |

*3.4.3. Putative PB-derived equine MSCs are positive for MSC markers and negative for differentiated blood cell markers*

Flow cytometry, comprising a wide-ranging set of cellular markers, was used for the immunophenotypical characterization of the putative equine MSCs. Hereby, it was found that the cells were positive for the stem cell (SC) markers CD29, CD44, CD90 and CD105 (Figure 2).

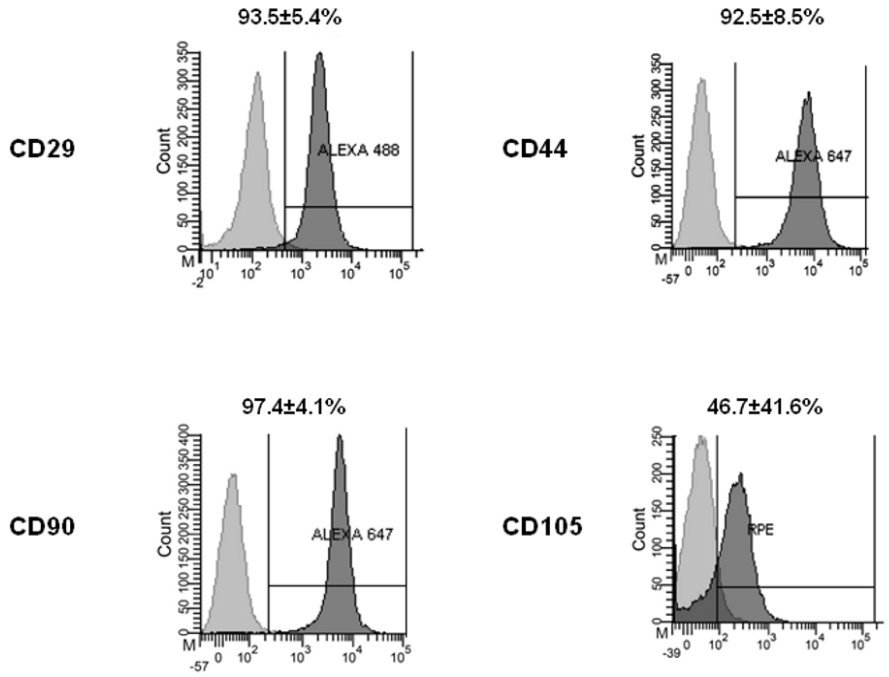


**Figure 2.** Immunophenotypic characterization with positive markers for equine mesenchymal stromal cells (MSCs). Two laser flow cytometry was performed with a set of 4 MSC positive markers: CD29, CD44, CD90 and CD105. Representative histograms show relative numbers of cells versus mean fluorescence intensity. The light and dark grey histograms represent the relevant isotype control staining and marker antibody staining, respectively with the corresponding mean percentage of positive cells ± standard deviation.

In addition, the putative MSCs were negative for the panleukocyte marker CD45, the B-lymphocyte marker CD79α, the monocyte/macrophage marker and a marker for MHC II, which is present on antigen presenting cells (Figure 3). Moreover, the negative results with the differentiated blood cell markers were due to the actual absence of these antigens on the

PB-derived equine MSCs since (i) these markers stained positive on the equine PBMC control cells, demonstrating cross-reactivity with the equine antigens (data not shown) and (ii) the results were virtually identical when using accutase-detached MSCs (Figure 3). In addition, no signal was detected with relevant isotype controls for all cell markers used (Figure 2 & 3).

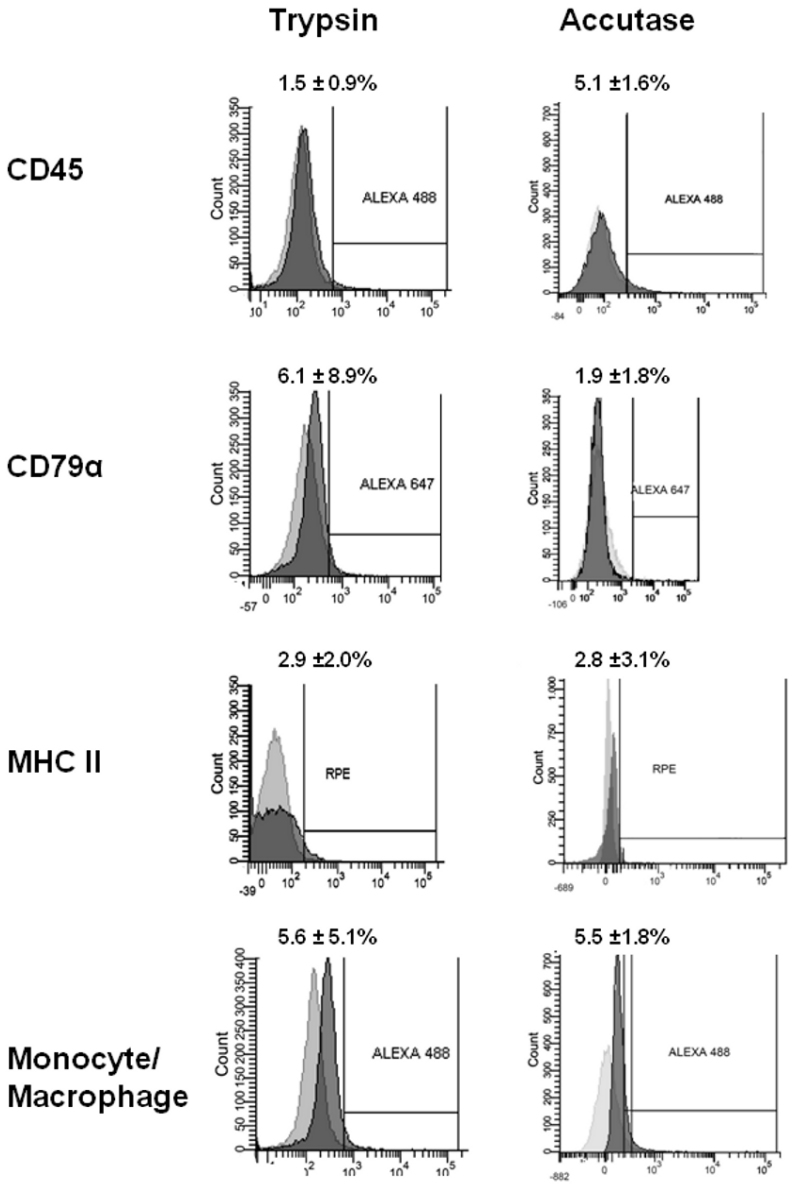


**Figure 3.** Expression of negative cell markers on trypsin- and accutase-detached putative equine mesenchymal stromal cells (MSCs). Two laser flow cytometry was performed with a set of 4 MSC negative markers: CD45, CD79α, MHC II and a monocyte/macrophage marker. Representative histograms show relative numbers of cells versus mean fluorescence intensity after trypsinization (histograms on the left) or detachment with accutase (histograms on the right). The light and dark grey histograms represent the relevant isotype control staining and marker antibody staining, respectively with the corresponding mean percentage of positive cells ± standard deviation.

*3.4.4. Putative PB-derived equine MSCs are capable of differentiating in vitro towards osteoblasts, chondroblasts and adipocytes*

The putative MSCs were further subjected to a functional characterization by differentiation experiments using selective media. After 3 weeks of culture in osteogenic medium, the morphology of almost all cultured cells changed from spindle-shaped to stellate and irregular (Figure 4B). Differentiated cells formed multiple individual clusters with a clear calcium deposition in the extracellular matrix and the presence of intracellular phosphatase as determined by Alizarin Red S (Figure 4A) and Alkaline Phosphatase (Figure 4B) stainings, respectively. The control MSCs on the other hand, maintained their spindle-shaped morphology with the formation of a monolayer and without any positivity for both staining methods (Figure 4A & B).

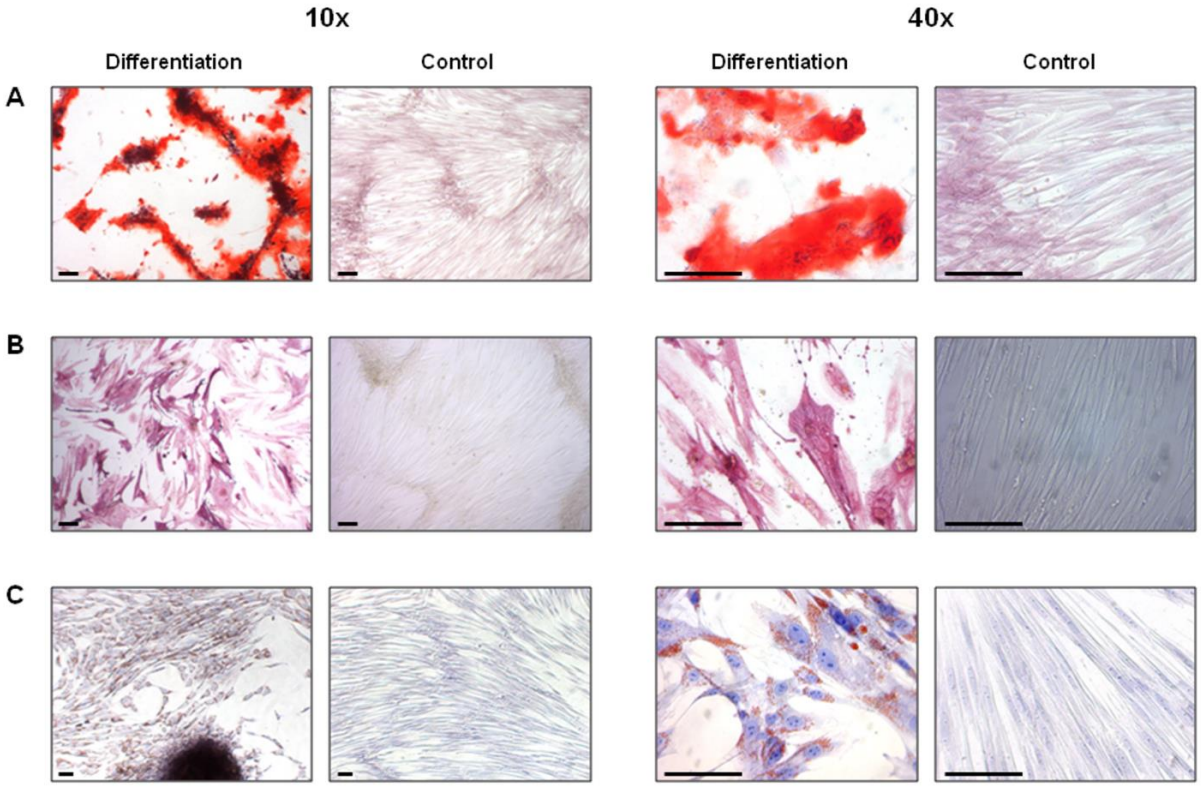


**Figure 4.** Osteogenic and adipogenic differentiation of putative equine mesenchymal stromal cells (MSCs). Representative microscopic images of the Alizarin Red S (**A**) and Alkaline Phosphatase staining (**B**) in order to confirm osteogenesis. The production of lipid droplets is illustrated using Oil Red O staining (**C**). Also the negative control cells are presented. Scale bars represent 50µm.

The differentiation towards adipocytes was performed using adipogenic inducing and maintenance media. The cell morphology changed from a spindle shaped towards a more round morphology during the differentiation process. Moreover, the production of lipid droplets was visualized using an Oil Red O staining (Figure 4C). For the chondrogenic differentiation, putative equine MSCs were grown in a three-dimensional culture system with chondrogenic medium and within 3 days of culture, spherical colonies, identified as chondrospheres, were already noted macroscopically (Figure 5A). The chondrospheres increased visually in size during the differentiation period and after staining with Alcian Blue, the presence of cartilage lacunes surrounded by sulphated acid mucopolysaccharides was clearly observed (Figure 5B). The size of the control pellet (Figure 5A) on the other hand, decreased gradually with packed cells without cartilage lacunes (Figure 5B). In addition, the control pellet stained negative for Alcian Blue which confirmed the absence of mucopolysaccharides (Figure 5B). Controls of non-differentiated MSCs subjected to the same detection methods maintained their spindle-shaped morphology with the formation of a monolayer (in adhesion) and stained negative (Figure 4 & 5).

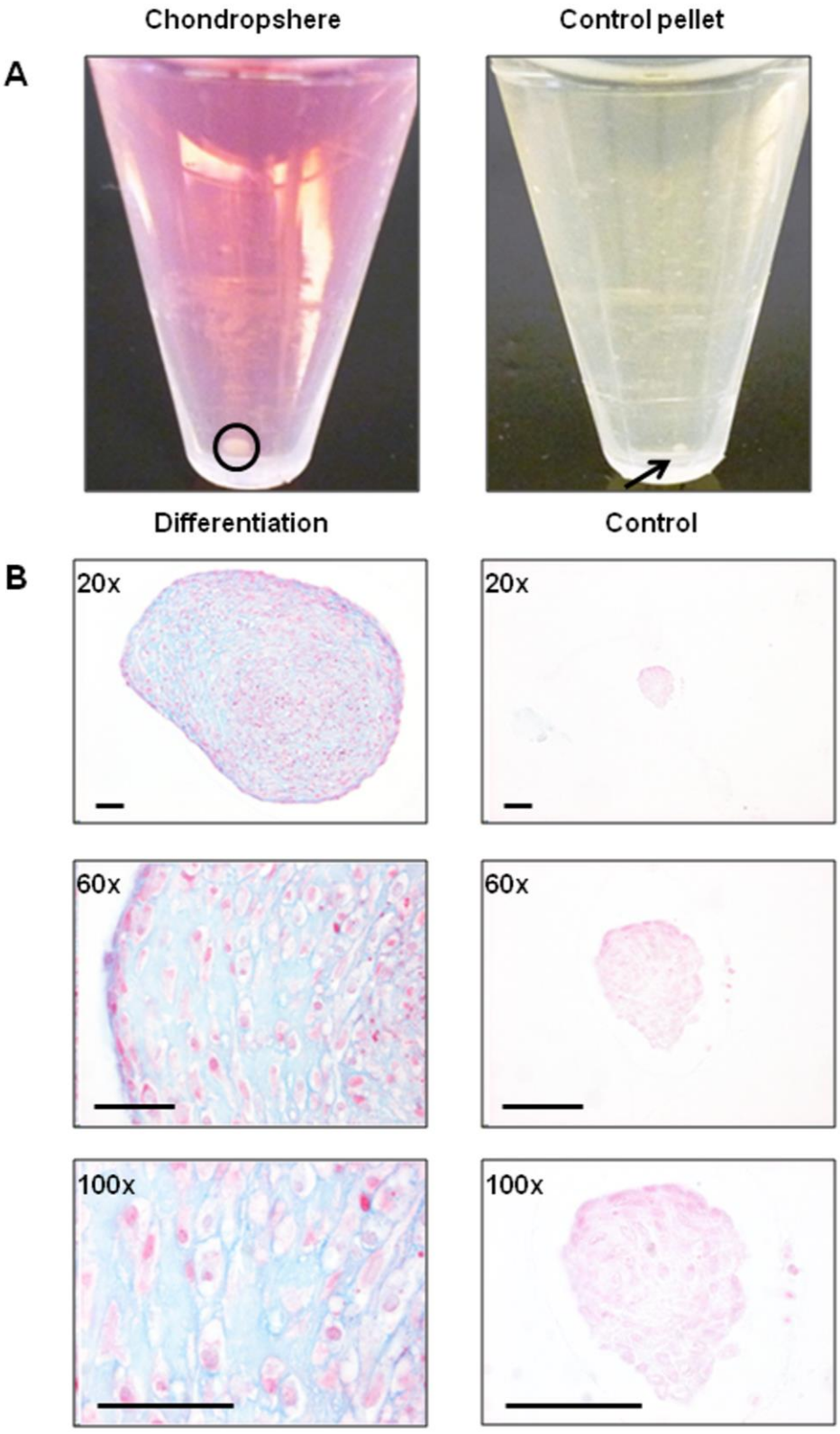


**Figure 5.** Chondrogenic differentiation of putative equine mesenchymal stromal cells (MSCs). A representative macroscopic image of an encircled chondrosphere and a control pellet, indicated with an arrow, at 2 weeks after cultivation (**A**). Alcian Blue staining indicated cartilage lacunes surrounded by sulphated acid muco-polysaccharides only in the differentiated chondrosphere pellets (**B**). Scale bars represent 50µm.

## 3.5. Discussion

Recently, the use of peripheral blood (PB) stem cells (SCs) has been reported as a valuable tool in equine regenerative medicine (Marfe et al., 2011; Spaas et al., 2011). However, in order to correctly evaluate their potential, an extensive characterization is warranted. Whereas in 1997 Lazarus et al. described that human mesenchymal SCs (MSCs) could not be recovered from PB, Zvaifler et al. reported in 2000 the first isolation of mesenchymal precursor cells from human blood based on morphological features, cell proliferation assays, positivity for the MSC marker CD105 and osteogenic differentiation (Lazarus et al., 1997; Zvaifler et al., 2000). For horses, the isolation of PB-derived MSCs was described for the first time in 2006 and was based on morphology combined with a differentiation towards osteocytes and adipocytes (Koerner et al., 2006). However, no immunophenotypic characterization of the cells was performed and the differentiation towards cartilage was unsuccessful. In 2008, another research group managed to produce chondroblasts from equine PB-derived MSCs, although this was only achieved after 9 weeks of differentiation and also in that study, no immunophenotypic characterization of the cells was carried out (Giovannini et al., 2008). More recently, CD44 and CD90 have been used as positive immunophenotypic markers for equine PB-derived MSCs, but these cells were also positive for the hematopoietic SC (HSC) marker CD117 and no differentiation towards chondroblasts was reported (Martinello et al., 2010). Also other researchers identified a MSC population in the PB using CD105 and CD90 as positive markers, but no further characterization was performed (Marfe et al., 2011). In the present study, we therefore aimed to perform a more extended characterization of PB-derived MSCs, both immunophenotypically as well as functionally.

The success rate of isolating equine MSCs from PB has been reported to range from 36.4 to 66.7% (Giovannini et al., 2008; Koerner et al., 2006; Martinello et al., 2010) and the underlying reasons for this remain elusive to date, although the age of the donor has been

proposed as a potential influencing factor. In the present study, we were successful in isolating PB-derived MSCs in all 4 horses, ranging in age from 4 to 15 years, and in addition, we were also successful in isolating PB-derived MSCs from a 4 month old foal (data not shown). This seems to indicate that donor age does not appear to influence the success rate of isolation, although it has to be mentioned that we did not statistically assess this hypothesis and only used a very limited number of horses, which makes it difficult to draw any true conclusions at present.

In order to produce valid data for an immunophenotypic characterization, it is necessary to use proper isotype controls to exclude aspecific antibody reaction and to use positive control cells for the confirmation of cross reactivity in equines, since only about 4% of human antibodies reacts with the equivalent equine proteins (Ibrahim et al., 2007). Another important feature to take into consideration when performing flow cytometric analyses is the fact that some epitopes can be destroyed by trypsin, resulting in a false negative result (Hackett et al., 2011). Since MSCs are not only phenotyped by the presence of SC markers but also by the absence of several differentiated cell markers, trypsin-sensitivity might be a concern. Indeed, recently it has been described that CD14, present on e.g. macrophages, neutrophils and dendritic cells and which is used as a negative marker for human MSCs, appears to be actually present on equine bone marrow (BM)-derived MSCs, but is absent when using trypsinized cells, indicating that this protein contains a tryspin-sensitive epitope (Hackett et al., 2011). So in order to evaluate whether the negative MSC markers which we used in the present study are truly absent and not just merely destroyed by trypsinization, MSC samples were detached using the cell detaching agent accutase and the expression of the negative cell markers were compared to trypsin-detached MSCs from the same horse. Since we did not observe any difference when using both cell-detaching agents, we conclude that the negative

cell markers we tested in our study recognize trypsin-insensitive epitopes and hence, the PB-derived equine cells fulfill all qualities to be immunophenotyped as MSCs.

The expression of different markers on equine BM-derived MSCs has been tested at different time points after harvesting and hereby, it was found that expression of MSC markers can vary during cultivation but stabilizes after 2 to 3 weeks post isolation (Radcliffe et al., 2010). Due to the late appearance of MSCs after seeding PB mononuclear cell (PBMC) fractions, we were unable to immunophenotype the PB-derived MSCs earlier than 3 weeks post isolation in order to evaluate if a similar variation in expression also occurs. However, the levels of expression of the cell markers of BM-derived MSCs at 3 weeks (positive as well as negative markers) were similar to the expression levels we found in the current study with equine PB-derived MSCs, indicating that the source of MSCs most likely does not influence the level of cell marker expression on these equine cells (Radcliffe et al., 2010).

In contrast to previous studies, where the differentiation towards chondroblasts was unsuccessful or only accomplished after 9 weeks of culturing in chondrogenic medium (Giovannini et al., 2008; Koerner et al., 2006), we could confirm in the present study the differentiation of PB-derived MSCs towards chondroblasts as early as 3 weeks post differentiation. First, we observed chondrospheres macroscopically in the 3-dimensional cultures as early as 3 days and secondly, a positive Alcian Blue staining at 3 weeks post culture confirmed the differentiation towards chondroblasts. A possible explanation for this discrepancy could be the use of commercially prepared chondrogenic differentiation medium, supplemented with transforming growth factor-$\beta_3$ in the present study, whereas previous studies used an in-house prepared culture medium. Furthermore, we supplemented our culture medium with dexamethasone already at passage 0 ($P_0$), which is in line with descriptions for culturing MSCs from equine umbilical cord blood (UCB) (De Schauwer et al., 2011a; Koch et al., 2007). Since dexamethasone is known to be essential for differentiation, adding this potent

synthetic glucocorticoid at the time of isolation might potentially have primed the equine MSCs for proper chondrogenic differentiation later on.

In the present study, population doubling time (PDT) experiments were performed, as this is a reliable method to determine the given cell proliferation rate under culture conditions and PDT is defined as the time by which a given cell population doubles in number (Eslaminejad et al., 2010). Our results are in agreement with previous reports where the PDT of equine MSCs over several passages were studied (Colleoni et al., 2009; Hoynowski et al., 2007). Hereby, it was found in the previous studies as well as in the present study, that the PDT at P0/P1 was negative, which can be explained by the fact that there are very few MSCs present in the original PBMC cultures at P0. Therefore, a negative value at P0/P1 points out towards the initial lag phase of the cells in culture and not necessarily a slow proliferation capacity. After this initial lag phase, all PDT values were positive (corresponding to the log phase) and remained approximately the same at later passages, indicating a stable proliferation capacity of the PB-derived MSCs in culture over time.

In conclusion, this study provides additional insights into the characterization of equine PB-derived MSCs which can prove to be valuable not only for future research on equine PB-derived MSCs in specific, but also on equine MSCs in general.

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# CHAPTER 4

## Equine mammary stem/progenitor cells

Adapted from:

Spaas JH, Chiers K, Bussche L, Burvenich C, Van de Walle GR, 2012. Stem/progenitor cells in non-lactating versus lactating equine mammary gland. Stem Cells and Development 21(16), 3055-3067.

## 4.1. Abstract

The mammary gland is a highly regenerative organ that can undergo multiple cycles of proliferation, lactation and involution. Based on the facts that (i) mammary stem/progenitor cells (MaSCs) are proposed to be the driving forces behind mammary growth and function and (ii) variation exists between mammalian species with regard to physiological as well as pathological functioning of this organ, we hypothesize that studying MaSCs from different mammals is of great comparative interest. Over the years, important data has been gathered on MaSCs of men and mice, although knowledge on MaSCs in other mammals remains limited. Therefore, the aim of this work was to isolate and characterize MaSCs from the mammary gland of horses. Hereby, our salient findings were that the isolated equine cells met the two *in vitro* hallmark properties of stem cells (SCs), namely the ability to self-renew and to differentiate into multiple cell lineages. Moreover, the cells were immunophenotyped using markers for CD29, CD44, CD49f and Ki67.

Finally, we propose the mammosphere assay as a valuable *in vitro* assay to study MaSCs during different physiological phases since it was observed that equine lactating mammary gland contains significantly more mammosphere-initiating cells than the inactive, non-lactating gland (a reflection of MaSC self-renewal) and moreover, that these spheres were significantly larger in size upon initial cultivation (a reflection of progenitor cell proliferation). Taken together, this study not only extends the current knowledge of mammary gland biology, but also benefits the comparative approach to study and compare MaSCs in different mammalian species.

**Keywords:** Mammary stem cells; Horse; Lactation

## 4.2. Introduction

The mammary gland is a unique organ because, although the stroma is established prior to birth, the mammary epithelium primarily develops postnatal and achieves full maturity in the adult pregnant individual (Motyl et al., 2011). In several animal species, from birth till after puberty, the mammary gland remains rudimentary with a relatively quiescent growth mainly consisting of ductal elongation (Tiede and Kang, 2011). Once pregnancy is initiated, this dynamic organ will undergo intensive remodeling with (i) alveolar growth and secretory differentiation, (ii) lactation, with milk secretion and (iii) involution, with apoptosis and regression (Lewis, 2000; Tiede and Kang, 2011). Growing evidence suggests that these changes are driven by the coordinated division and differentiation of mammary stem cell (SC) populations (Kass et al., 2007; Shackleton et al., 2006; Tiede and Kang, 2011). In 1986, Soule and McGrath reported for the first time the existence of undifferentiated human mammary cells which could survive in suspension (Soule and McGrath, 1986). A couple of years later, these cells were designated human mammary stem/progenitor cells (MaSCs) and showed properties of bipotent cells based on their capacity to produce adult luminal epithelial and myoepithelial cells (Petersen et al., 1992; Stingl et al., 2001). Now, if MaSCs are indeed the driving force behind the different remodeling phases, than it could be anticipated that the number or proliferation rate of MaSCs might vary depending on the physiological stage of this dynamic organ.

For understanding the functional role of MaSCs in normal mammary gland development, the cleared fat pad mouse model is frequently used (Bruno and Smith, 2011; Visvader and Smith, 2011). This *in vivo* model allows the transplantation and growth of mammary cells into their normal anatomical site and under the influence of a normal physiological environment (Visvader and Smith, 2011). Using this model, it has e.g. been demonstrated that MaSCs are a relative quiescent cell type, which only becomes activated under conditions of mammary

gland repopulation such as fetal growth or the pubertal growth phase (Molofsky et al., 2004; Reya et al., 2001; Visvader and Smith, 2011; Young et al., 1971). Also, this murine mammary gland-free fat pad transplantation system is the animal model of choice for human breast cancer research (Cardiff et al., 2002). Indeed, mice have played an indispensable and pivotal role in the study of breast cancer and this animal species will keep on being a major research resource in comprehending this devastating disease (Young, 2008). However, by studying (patho)physiological mechanisms in such model, some key insights might be lost due to the absence of variation. Intriguingly, whereas both in humans and other mammals, the mammary gland undergoes repeated cycles of development, function and dedifferentiation, changes proposed to be driven by MaSCs, the incidence of mammary cancer varies greatly amongst these species. Mammary cancer is common in humans and carnivores (Munson and Moresco, 2007), whereas cows, sheep, pigs and horses only very rarely develop mammary tumors (Knight and Sorensen, 2001). We now hypothesize that this variation amongst different animal species, namely the susceptibility for mammary gland cancer, might provide novel insights into the mechanisms underlying the functional behavior and regulation of MaSCs. Therefore, we believe it is of eminent importance to study MaSCs in as many species as possible.

In general, a thorough study on the isolation and characterization of MaSCs is a critical step towards elucidating MaSC functioning under different physiological as well as pathological circumstances in different species. Unfortunately, when looking at companion or production animals, not much information on MaSCs is available to date. This is primarily due to the lack of universal markers and *in vitro* systems to identify MaSCs in different mammals. Moreover, functional *in vivo* assays to detect MaSC activity are missing in these species, emphasizing the need for developing additional *in vitro* assays to better characterize these cells. Several reports describe the isolation and characterization of MaSCs from

mammary gland tissues of dogs, mostly in order to gain better insights into mammary tumorigenesis (Cocola et al., 2009; Hellmen et al., 2000; Michishita et al., 2011), a process that shares many features with human breast cancer (Kumaraguruparan et al., 2006a; Kumaraguruparan et al., 2006b; Uva et al., 2009). In cattle, on the other hand, MaSCs are being studied mainly to explore their potential to improve milk production efficiency (Capuco, 2007; Capuco et al., 2009; Li et al., 2009; Martignani et al., 2009; Motyl et al., 2011). In horses, however, the knowledge on mammary gland development is mainly limited to lactation physiology (Davies Morel, 2008; Leadon et al., 1984; Ousey et al., 1984) and no information on MaSCs in this species is available to date, although a thorough study of equine MaSCs could potentially extend the current knowledge of mammary gland biology and will aid in our comparative physiological approach to study and compare MaSCs in different mammalian species.

Therefore, the goal of the present study was to isolate and characterize MaSCs from equine mammary gland tissues and more specifically, MaSCs from non-lactating and lactating tissues in order to study potential differences between these two very important physiological mammary gland phases. To this end, previously described characterization experiments used in other species were optimized for identifying equine MaSCs. Hereby, equine MaSCs were enriched by culturing the cells under anchorage-independent condition, the so-called mammospheres. Next, equine MaSCs were immunophenotypically characterized using cross-reacting antibodies against SC markers and the cells were differentiated towards adult epithelial and myoepithelial cells using selective media. Moreover, colony forming unit (CFU) assays and mammosphere cycle assays were evaluated to see if these assays are valuable additional *in vitro* systems to better characterize MaSCs.

## 4.3. Materials and Methods

### *4.3.1. Sample collection*

Inactive, non-lactating mammary gland tissues (n=3) or active, lactating tissues (n=3) from adult Warmblood slaughterhouse mares (8-12 years old) were collected by excising two parts of 5cm$^2$ of tissue next to the median line of the two mammary gland compartments (Figure 1).

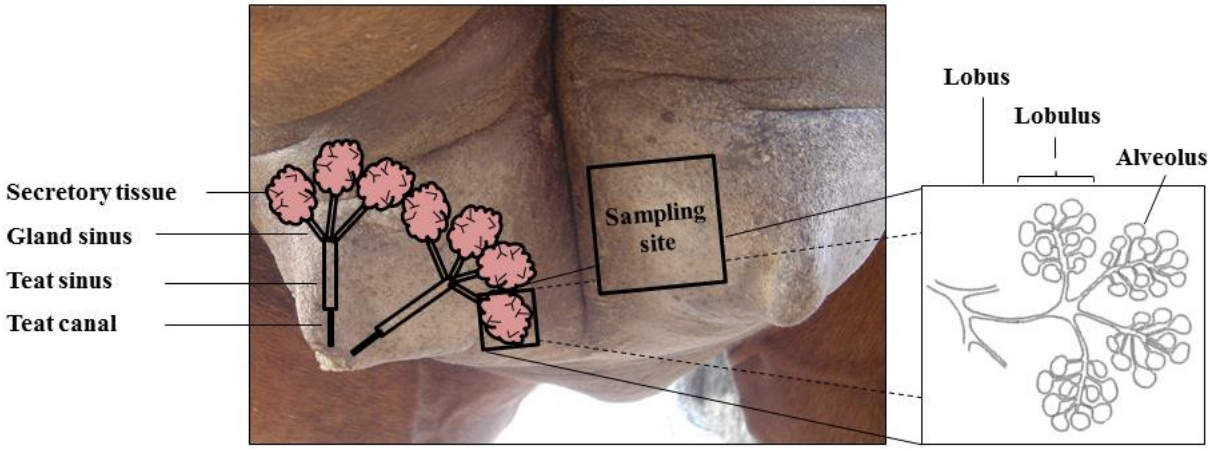



**Figure 1.** The mammary gland of a horse with tissue distribution and sampling site.

The samples did not show any macroscopic abnormalities and were transported to the laboratory on ice within four hours after sampling in phosphate buffered saline (PBS) 1x (without calcium and magnesium) with 1% of penicillin/streptomycin/amphotericin B (P/S/A, Sigma). A part of the sample was immediately fixed with 4% paraformaldehyde (PF) and hematoxylin-eosin stainings were performed on 8 µm histological sections after paraffin embedding for histological examination.

### *4.3.2. Collagenase digestion and mammosphere formation*

The isolation of equine mammosphere-initiating cells was based on the protocol of Dontu et al., with some modifications (Dontu et al., 2003). Upon arrival in the laboratory, mammary gland samples were dissociated mechanically with a sterile scalpel, followed by enzymatic

digestion with 0.1% collagenase III (Worthington Biochemical Corporation) at 37° C for 60 minutes. The cell suspension was subsequently filtered through a sterile 100µm and 40µm filter to obtain a single cell suspension, and centrifuged at 400xg for 10 minutes at room temperature (RT). Cells were resuspended in PBS with 1% P/S/A, centrifuged at 260xg for 10 minutes and resuspended in mammary stem/progenitor cell (MaSC) medium, consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 (50/50) supplemented with 10% of fetal bovine serum (FBS), 2% B27 (all from Invitrogen), 1% P/S/A (Sigma), 10 ng/ml basic-fibroblast growth factor (b-FGF) (BioVision) and 10 ng/ml epidermal growth factor (EGF) (Sigma). Approximately $5x10^5$ cells were seeded on a 6-well tissue culture dish for one hour to allow adherence of contaminating fibroblasts and this was repeated once more. The non-adherent cells were collected and seeded at approximately 20,000 cells/$cm^2$ on 6-well ultralow attachment plates (Corning, Elscolab). MaSC medium was refreshed twice a week by means of centrifugation of the mammospheres at 230xg for 6 minutes. For further experiments, mammospheres were seeded on adhesive tissue culture dishes in MaSC medium, unless where indicated otherwise.

*4.3.3. Flow cytometry*

To characterize mammosphere cells immunophenotypically, the expression of several stem cell (SC) markers, previously used for the phenotypic characterization of MaSCs in other species (Cocola et al., 2009; Dontu et al., 2003; Li et al., 2009; Michishita et al., 2011; Rauner and Barash, 2012; Smith, 2006; Stingl, 2009), was evaluated by flow cytometry. Per series, $2x10^5$ cells were used and labeled with following primary antibodies (Abs): mouse anti-human CD29-FITC $IgG_1$ (Southern Biotech, clone TDM29, 1:10), rat anti-mouse CD44-APC $IgG_{2b}$ (BD, clone IM7, 1:20), rat anti-mouse CD49f $IgG_{2a}$ (Novus Biologicals, clone GoH3, 1:10), and rabbit anti-human Ki67 IgG (Abcam, ab15580, 1:200). For the latter, cells were

fixed with 4% PF for 10 minutes and subsequently permeabilized with 0.1% Triton X for 2 minutes, both at RT. Cells were incubated with the primary Abs for 15 minutes on ice in the dark and washed twice in washing buffer, consisting of DMEM with 1% bovine serum albumin (BSA). For CD49f and Ki67, secondary goat anti-rat Alexa$^{488}$- and goat anti-rabbit Alexa$^{647}$-linked Abs (Invitrogen, 1:100), respectively, were used to identify positive cells after 15 minutes of incubation on ice in the dark. Finally, all cells were washed three times in washing buffer and in addition, viability assessment with 7-amino-actinomycin D (7-AAD, Sigma) was performed on the non-fixed cells. At least 10,000 cells were evaluated using a FACS Canto flow cytometer (Becton Dickinson Immunocytometry systems) equipped with a 488 nm solid state and a 633 nm HeNe laser, and these data were further analyzed with the FACS Diva software. All analyses were based on (i) autofluorescence and (ii) control cells incubated with isotype-specific IgG's, in order to establish the background signal. All isotypes were matched to the immunoglobulin subtype, conjugated to the same fluorochrome and used at the same protein concentration as the corresponding Abs.

*4.3.4. Differentiation experiments*

Differentiation of mammosphere cells towards the two major cell types present in the mammary gland, namely luminal epithelial and myoepithelial cells, was induced by culturing $2.5x10^3$ cells/cm² in a 24-well plate in differentiation medium, consisting of DMEM/F12 (50/50), 10% FBS, 2% B27, 1% P/S/A, 5µg/ml insulin, 1µg/ml hydrocortisone and 1µg/ml prolactin (Sigma), for 10 days. As a control, mammosphere cells were cultured in MaSC medium for 10 days. Media of the adherent cultures were refreshed every 3-4 days. Immunohistochemistry (IHC) was performed to evaluate the expression of markers present on differentiated cells (see below).

For evaluation of three-dimensional tubular structure formation, $5x10^3$ cells/$cm^2$ were seeded in a 6-well Synthemax plate (Corning, Elscolab) in differentiation medium supplemented with 50 pg/ml recombinant human transforming growth factor-beta3 (rhTGF-β3, Sigma) and cultured for 10 days. Control MaSCs were seeded on a normal tissue culture plate for 10 days as well. The formation of tubular/acinar structures was evaluated by light microscopic inspection of the wells.

For adipogenic differentiation, $2.1x10^4$ cells/$cm^2$ were seeded in 4-well plates in MaSC medium and cultured upon 70% confluency. At that moment, adipogenic inducing medium was added for 3 days, after which the medium was replaced with adipogenic maintenance medium for 1 more day. The adipogenic inducing medium consisted of DMEM (Invitrogen) supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 µg/mL recombinant human-insulin, 0.2 mM indomethacin, 15% rabbit serum and 10 µl/mL antibiotic-antimycotic solution (all from Sigma) (De Schauwer et al., 2011; Spaas et al., 2013). The adipogenic maintenance medium was identical but without dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine. As a control, cells were seeded at a concentration of $2.1x10^4$/$cm^2$ in 4-well plates in MaSC medium for 5 days. Adipogenesis was evaluated in all samples after 5 days of cultivation using Oil Red O stainings.

*4.3.5. Immunohistochemistry (IHC)*

Cells were fixed for 10 minutes with 4% PF and permeabilized for 2 minutes with 0.1% Triton X, both at RT. Cells were then incubated with 0.03% hydrogen peroxide for 5 minutes at RT and after washing with PBS, incubated for 2 hours at RT with the following primary mouse $IgG_1$ monoclonal Abs: anti-human cytokeratin (CK)18 (Abcam, clone C-04, 1:30), anti-human PanCK (Dako, clone, AE1/AE3, 1:50) and anti-porcine vimentin (Thermo Scientific, clone V9, 1:100); the mouse anti-human smooth muscle actin (SMA) IgG2a Ab

(Dako, clone 1A4, 1:200) and the rabbit anti-human IgG Ab casein kinase 2β (Abcam, clone EP1995Y, 1:50). All Abs were used at the concentrations as indicated by the manufacturer. After washing with PBS, secondary ready to use goat anti-mouse and anti-rabbit peroxidase (PO)-linked Abs (Dako) were added and incubated for 30 minutes at RT. Finally, 3,3'-diaminobenzidine (DAB) was added for 2-10 minutes and a counter staining with hematoxylin was performed to visualize the surrounding cells. As controls, identical stainings were performed on undifferentiated mammosphere cells and background staining was assessed by using the proper isotype-specific IgG's. All isotypes were matched to the immunoglobulin subtype and used at the same protein concentration as the corresponding Abs.

*4.3.6. Mammosphere cycle assay*

First cycle mammospheres, which developed from the inactive, non-lactating and lactating mammary gland tissue-derived cells on ultralow attachment plates (as described above), were counted at days 4, 7 and 11 post seeding. In addition, 10 mammospheres were photographed and average mammosphere areas were determined using *Image J* software (http://rsb.info.nih.gov/ij/). At day 11 post seeding, all mammospheres were collected and plated on adhesive tissue culture dishes in MaSC medium. Upon 80% confluency, the adherent cells were trypsinized with 0.25% trypsin-EDTA and seeded at a very low density of 4,000 cells/cm² in a 6-well ultralow attachment plate to initiate a second mammosphere cycle. These second cycle mammospheres were counted and evaluated exactly as described for the first cycle mammospheres. To calculate the mammosphere forming efficiency (MFE), the number of mammospheres was divided by the original number of single cells seeded and expressed as a percentage, exactly as previously described (Booth et al., 2010; Farnie et al., 2007).

*4.3.7. Colony forming unit (CFU) assay*

Approximately one mammosphere cell was seeded per cm² of a 6-well plate and fixed 7 days later with 90% ethanol for 10 minutes at -20°C. Crystal violet stainings were performed to visualize colony forming units (CFUs) macroscopically and the total number of CFUs per 6-well were counted. CFU assays were done in duplicate for three independent experiments.

*4.3.8. Statistical analysis*

Student's t-test for paired data was used to test for statistically significant differences in mammosphere numbers, areas and CFU assays between MaSCs from non-lactating and lactating mammary gland tissue. Data given are the means of three independent experiments and the bars show standard deviations.

## 4.4. Results

*4.4.1. Histological evaluation of the mammary gland tissue samples*

The physiological activity status of all mammary gland tissue samples used was confirmed by histology. Hereby, it was observed that the inactive, non-lactating mammary gland tissues consisted of small alveoli with a narrow lumen lined by small cuboidal cells (Figure 2A), whereas the lactating mammary gland tissues consisted of large and dilated alveoli containing milk secretion in their lumina (Figure 2B).

*4.4.2. Equine mammary gland tissue-derived cells are capable of mammosphere formation.*

Mammosphere formation is a frequently used *in vitro* cultivation system that allows for the propagation of mammary stem/progenitor cells (MaSCs) by culturing enzymatically digested mammary gland cells on ultralow attachment plates to prevent adhesion (Dontu et al., 2003).

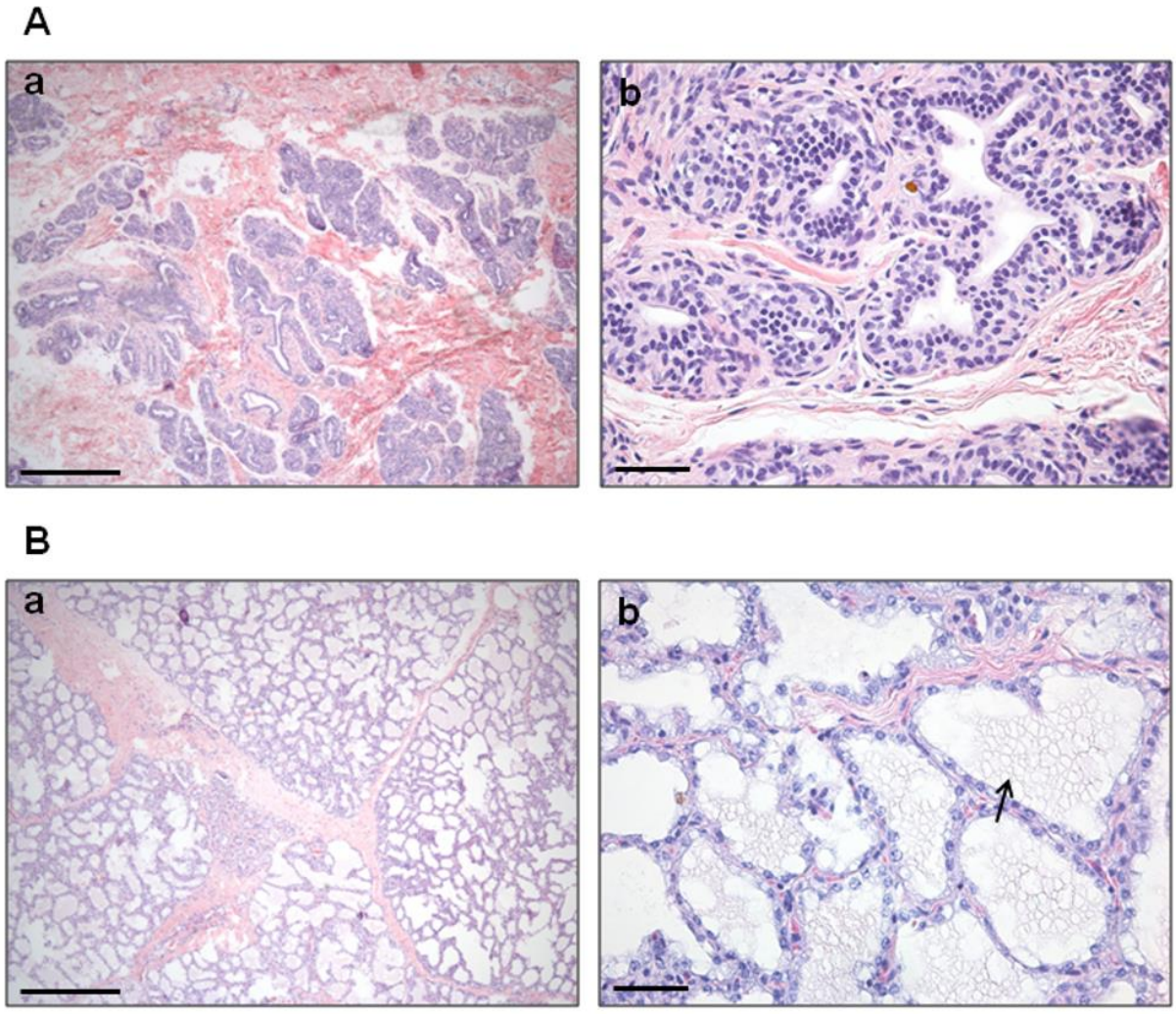



**Figure 2.** Hematoxylin-eosin staining of equine inactive, non-lactating (**A**) and lactating (**B**) mammary gland tissues. Small alveoli with a narrow lumen lined by small cuboidal cells were noticed in inactive mammary gland tissues, whereas large and dilated alveoli containing milk secretion (arrow) were visible in lactating mammary gland tissues. Bars represent 500µm (**a**) and 50µm (**b**).

The formation of mammospheres, typically consisting of non-adherent spherical clusters of cells, could be observed in all cultures as early as 4 days post seeding. Since mammospheres consist of a mixture of stem cells (SCs), their progeny and non-stem progenitor cells (Stingl, 2009), the observed equine mammospheres thus indicate that at least some mammary epithelial cells with SC characteristics, or MaSCs, could be obtained with this technique. In Figure 3 I & III, representative pictures of equine mammospheres are presented. These mammospheres could, upon trypsinization to obtain single cells again, be passaged on ultra-

low attachment plates up to passage (P) 6. From P6 onwards, the vitality of the cells rapidly declined and by P8, no more mammospheres could be observed.

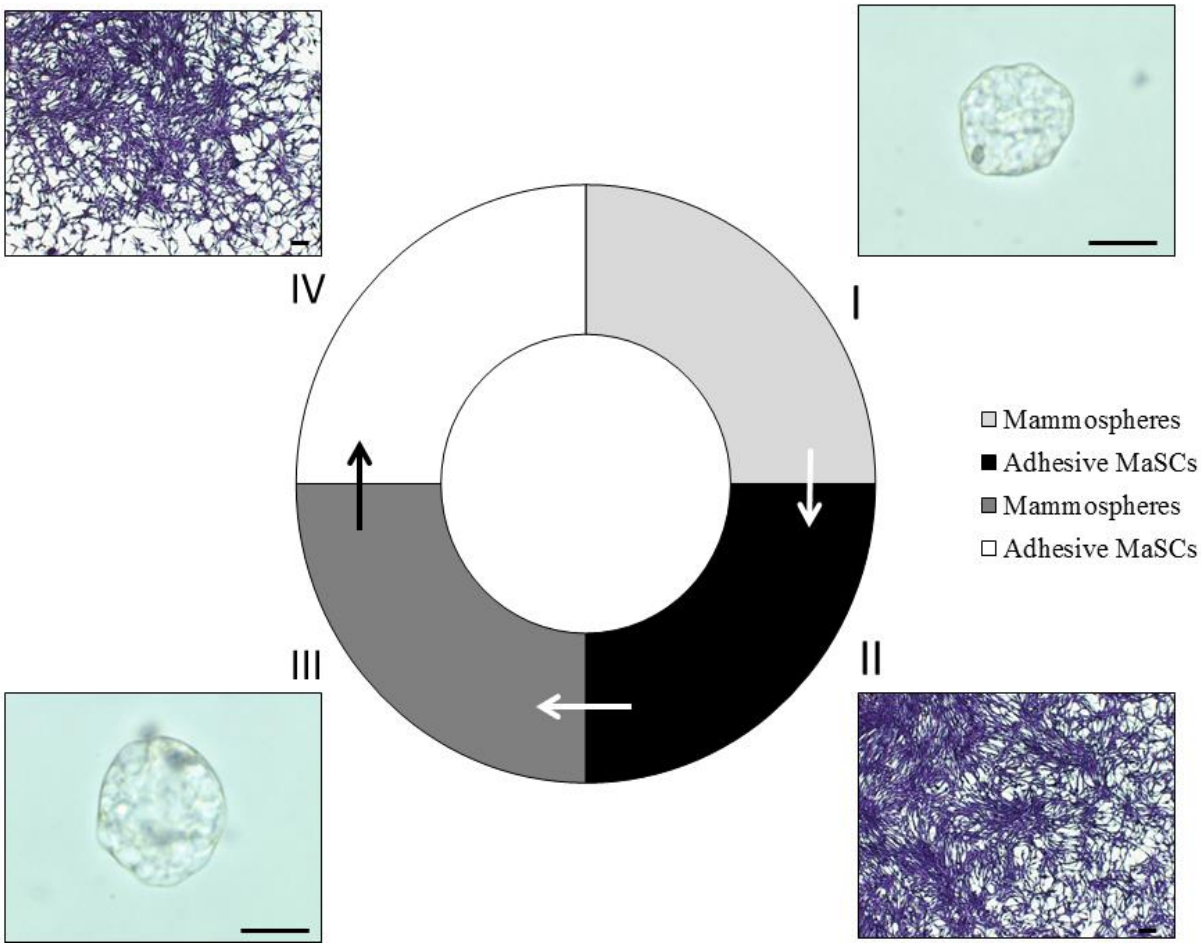



**Figure 3.** The mammosphere cycle assay. After mammosphere formation on ultralow attachment plates (first mammosphere cycle, **I**), mammosphere-derived cells are cultured on tissue culture dishes (**II**). Upon confluency, adherent cells are cultured on ultralow attachment plates (second mammosphere cycle, **III**), and subsequently cultured on tissue culture dishes (**IV**). Bars represent 50 µm.

*4.4.3. Equine mammosphere cells are positive for stem cell markers.*

Next, the mammosphere-derived cells were immunophenotypically characterized by flow cytometry and virtually all cells were positive for the SC surface markers CD29, CD44, CD49f and the proliferation marker Ki67, regardless of the activity status of the mammary gland (Figure 4). No fluorescence signal was obtained with the appropriate isotype controls (Figure 4).

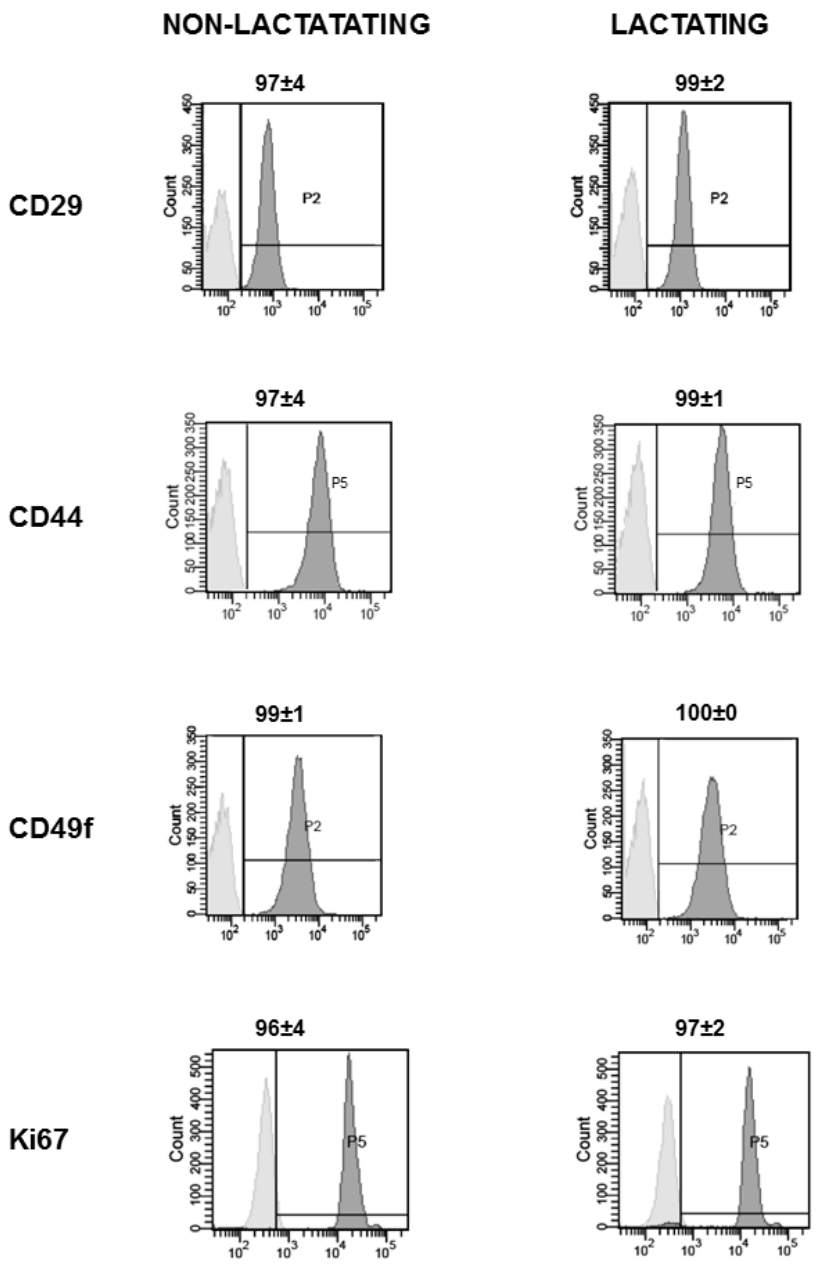



**Figure 4.** Immunophenotypic characterization of mammosphere cells from equine inactive, non-lactating and lactating mammary glands. Flow cytometry was performed with a set of four markers: CD29, CD44, CD49f and Ki67. Histograms show relative numbers of cells versus mean fluorescence intensity with the isotype control staining (light grey) and marker antibody staining (dark grey). Data represent the mean percentage of three experiments ± standard deviations.

### *4.4.4. Equine mammosphere cells can differentiate into luminal epithelial and myoepithelial cells and form 3-dimensional tubular structures.*

The mammosphere-derived cells were also subjected to a functional characterization by culturing the cells in selective media which was supplemented with insulin, hydrocortisone

and prolactin. After 10 days of culture, the structural organization of the cells changed from a monolayer (control cells) towards an acinar formation (differentiated cells) (Figure 5). The cells in these structures were immunohistochemically characterized as luminal epithelial and myoepithelial cells by using markers which were previously described for human and bovine MaSC differentiation studies (Cregan et al., 2007; Dontu et al., 2003; Hu et al., 2009; Li et al., 2009; Martignani et al., 2009; Zhao et al., 2010). Luminal epithelial cells showed a strong cytoplasmic staining for cytokeratin (CK)18 and a weak positive signal for the PanCK marker (Figure 5). For casein kinase 2β, both a nuclear and cytoplasmic immunolabeling was observed in these cells. A positive immunolabeling was not observed in mammosphere cells that had been cultured in normal MaSC medium (control cells), with the exception of casein kinase 2β, which showed a cytoplasmic staining (Figure 5). To characterize myoepithelial cells, an antibody against smooth muscle actin (SMA) was used and cells positive for this marker were found in the differentiated cell cultures, but not in the control cultures (Figure 5). Lastly, a staining for vimentin was also included and positive cells were found in the control cells as well as in the acinar differentiated cells (Figure 5). No signal was detected in the differentiated cell cultures when relevant isotypic controls were used for staining (Figure 5).

When culturing the mammosphere cells on Synthemax plates in the differentiation medium supplemented with recombinant human transforming growth factor (rhTGF)-β3, tubular, acinar and alveolar structures could be noticed in the cultures by light microscopy within 10 days of culture (Figure 6). Control cells were cultivated on normal tissue culture plates (Figure 6).

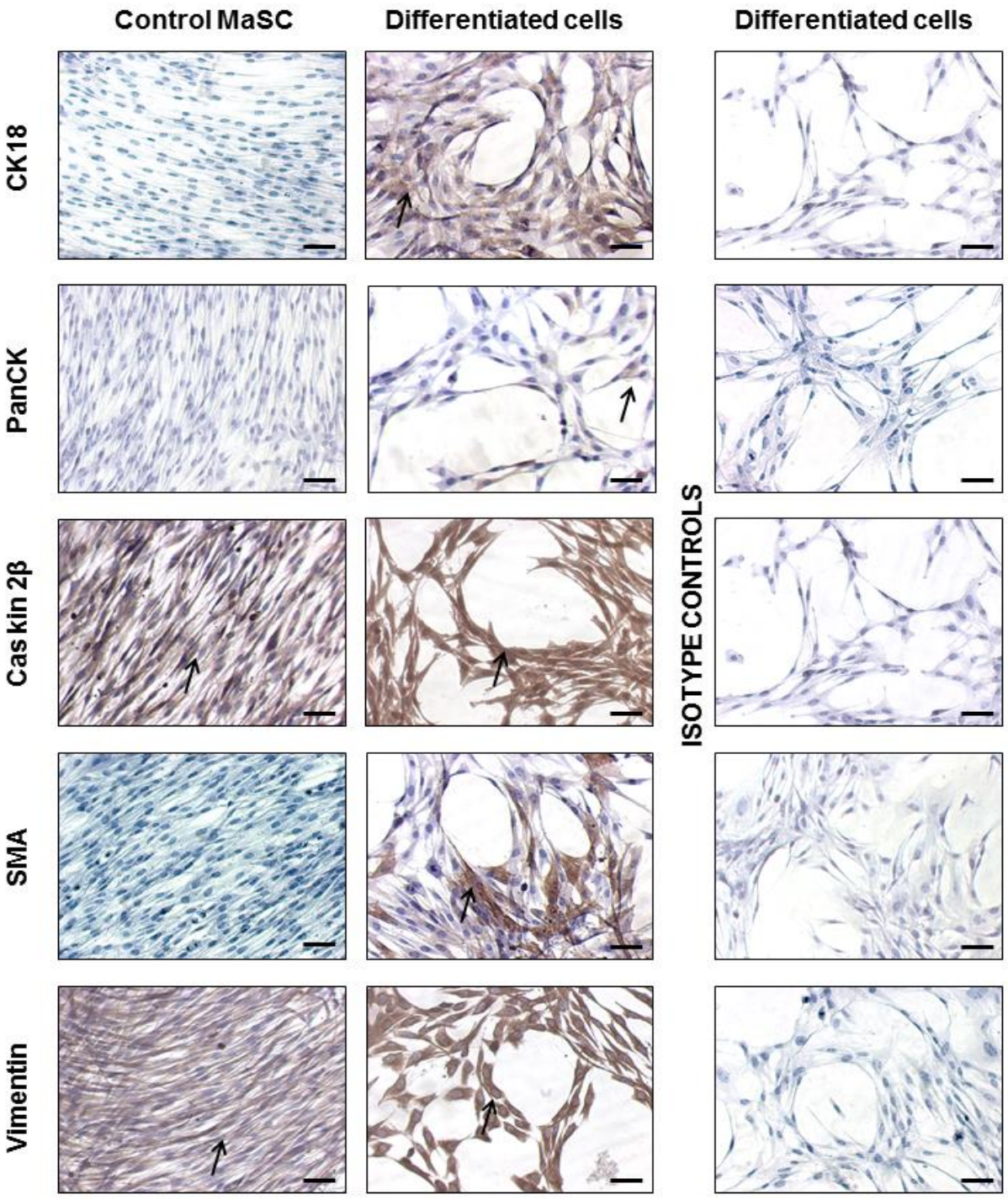



**Figure 5.** Differentiation of mammosphere cells into luminal epithelial and myoepithelial cells. Immunohistochemistry was performed on control and differentiated cells using cytokeratin (CK) 18, PanCK, casein kinase 2β, smooth muscle actin (SMA) and vimentin markers. Relevant isotype controls were also included. Arrows indicate positive stainings and bars represent 50μm.

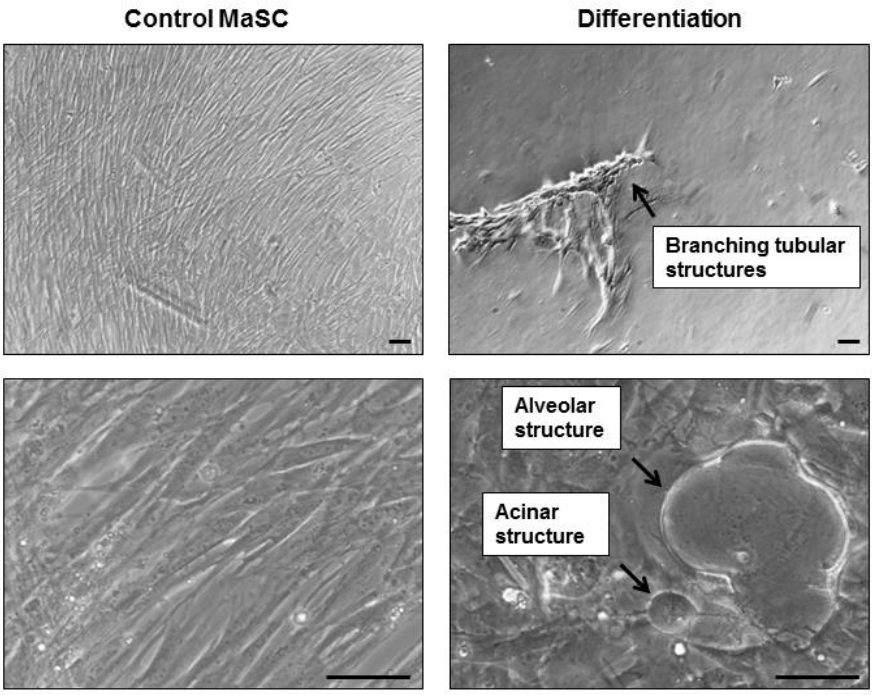



**Figure 6.** Three-dimensional tubular structure formation. Mammosphere-derived cells were cultured on Synthemax plates and acino-tubular structures were analyzed by light microscopy at different magnifications. Control cells were seeded on normal culture plates. Bars represent 50µm.

*4.4.5. Equine mammosphere cells can differentiate into adipocytes.*

In order to assess the SCs' potency, differentiation towards cell types of other germ layers can be performed (Nombela-Arrieta et al., 2011). Here, mammosphere-derived cells were cultured in a selective medium which is typically used to differentiate adult mesenchymal SCs (MSCs), originating from the mesodermal germ layer, into adipocytes (Spaas et al., 2013). Interestingly, within 5 days of culturing in such medium, the mammosphere cells changed morphologically from spindle-shaped to round cells and the production of small intracellular granules could be microscopically noticed in the cultures (Figure 7). Subsequent Oil Red O stainings confirmed the presence of lipids in these cells (Figure 7), indicating that mammosphere cells, which originate from the epidermal germ layer, are capable of differentiating into cell types of the mesodermal germ layer. Controls of non-differentiated cells subjected to the same detection method maintained their spindle-shaped morphology with the formation of a monolayer and stained negative (Figure 7).

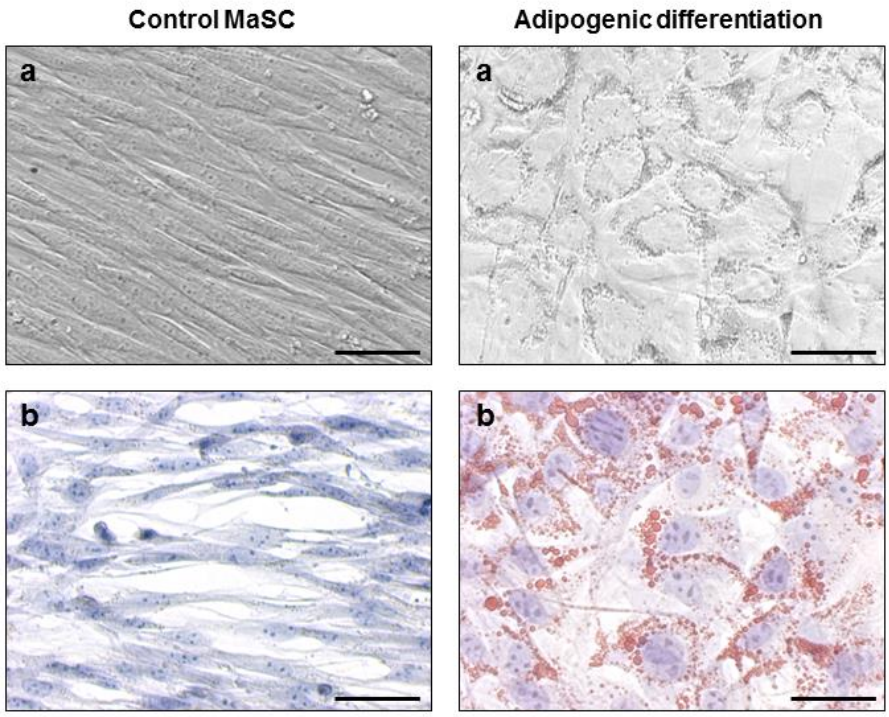
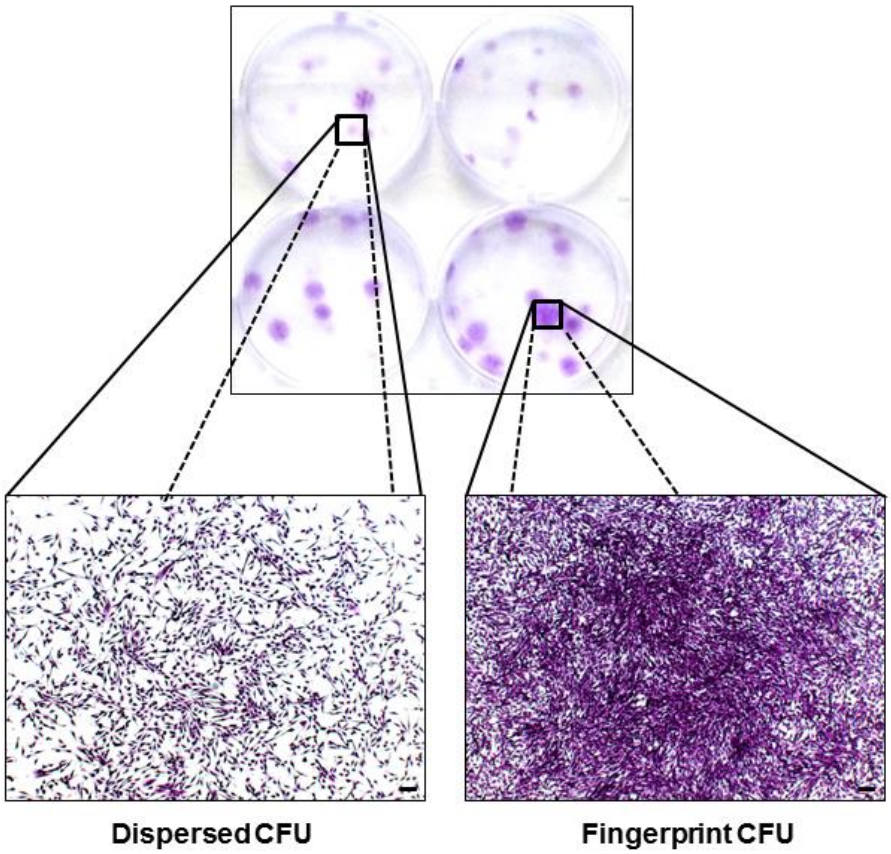



**Figure 7.** Adipogenic differentiation. Mammosphere-derived cells were cultured in adipogenic inducing and maintenance medium and analyzed by light microscopy (**a**) and Oil Red O stainings (**b**). Bars represent 50µm.

*4.4.6. Equine mammosphere cells from non-lactating and lactating mammary gland tissues exhibit similar clonogenic expansion capacities.*

To monitor and compare the clonogenic expansion of mammosphere cells from inactive, non-lactating versus lactating mammary gland tissues, colony forming unit (CFU) assays were performed. To this end, a limited number of cells was seeded at clonal density on a large surface (1 cell/cm$^2$ on a 6-well plate) and cultured for around a week. After cultivation, two different colony stages could be observed. One stage consisted of "dispersed" CFUs, identified by a spotted, vague macroscopic morphology and rather distant cells microscopically, whereas the other stage consisted of "fingerprint" CFUs, which were darker and more packed at macroscopic examination and with a microscopic fingerprint pattern. Overall, more dispersed colonies were observed compared to fingerprint CFUs (Figure 8). No differences between cells from inactive, non-lactating mammary gland tissues and cells from lactating mammary gland tissues were noted (Figure 8).

**A**




**B**

| CFUs | Dispersed | Fingerprint | Total |
|---|---|---|---|
| **Inactive mammary gland** | 9±3 | 3±1 | 12±3 |
| **Lactating mammary gland** | 10±2 | 4±0 | 14±2 |

**Figure 8.** Colony forming unit (CFU) assays of mammosphere cells derived from inactive, non-lactating and lactating mammary gland tissues. Macroscopic and microscopic images of dispersed and fingerprint CFUs after crystal violet staining (**A**). Bars represent 100µm. Numbers of CFUs were counted per 6-well and data represent the mean percentage of three experiments ± standard deviations (**B**).

*4.4.7. Cells from lactating mammary gland tissues generate more and larger mammospheres than cells from inactive, non-lactating tissues.*

Mammosphere cycle assays, as described in the Material & Methods section and Figure 3, were initiated to evaluate whether this assay could be a valuable additional *in vitro* system to better characterize MaSCs. Hereby, it was found that the number of mammospheres derived

from lactating mammary gland cells was higher compared to those from inactive, non-lactating tissues, a finding significant for all time points during the first cycle of the mammosphere assay (Figure 9A). In addition, the mammosphere forming efficiency (MFE) during the first mammosphere cycle varied between 0.8-1.5% for cells isolated from non-lactating versus 1.9-3.2% for cells isolated from lactating mammary glands (Figure 9A). During the first mammosphere cycle, a two-fold increase in number of mammospheres was noted between 4 and 7 days of culture, irrespective of the tissue type (Figure 9A). This finding is in good agreement with another study, where a two-fold increase was also noted in the number of mammospheres from freshly isolated Balb/c mammary epithelial cells cultured for 4 days and for 7 days (Booth et al., 2010). Moreover, when evaluating the size of the generated mammospheres, it was found that the first cycle mammospheres were significantly larger at day 4 for cells originating from lactating tissues ($P = 0.044$) (Figure 9B). At days 7 and 11, this difference was still noticeable, but no longer statistically significant (Figure 9B). During the second cycle mammosphere assay, significant differences in number, MFE or size of mammospheres from non-lactating and lactating mammary gland tissues were no longer apparent (Figure 9A & B).

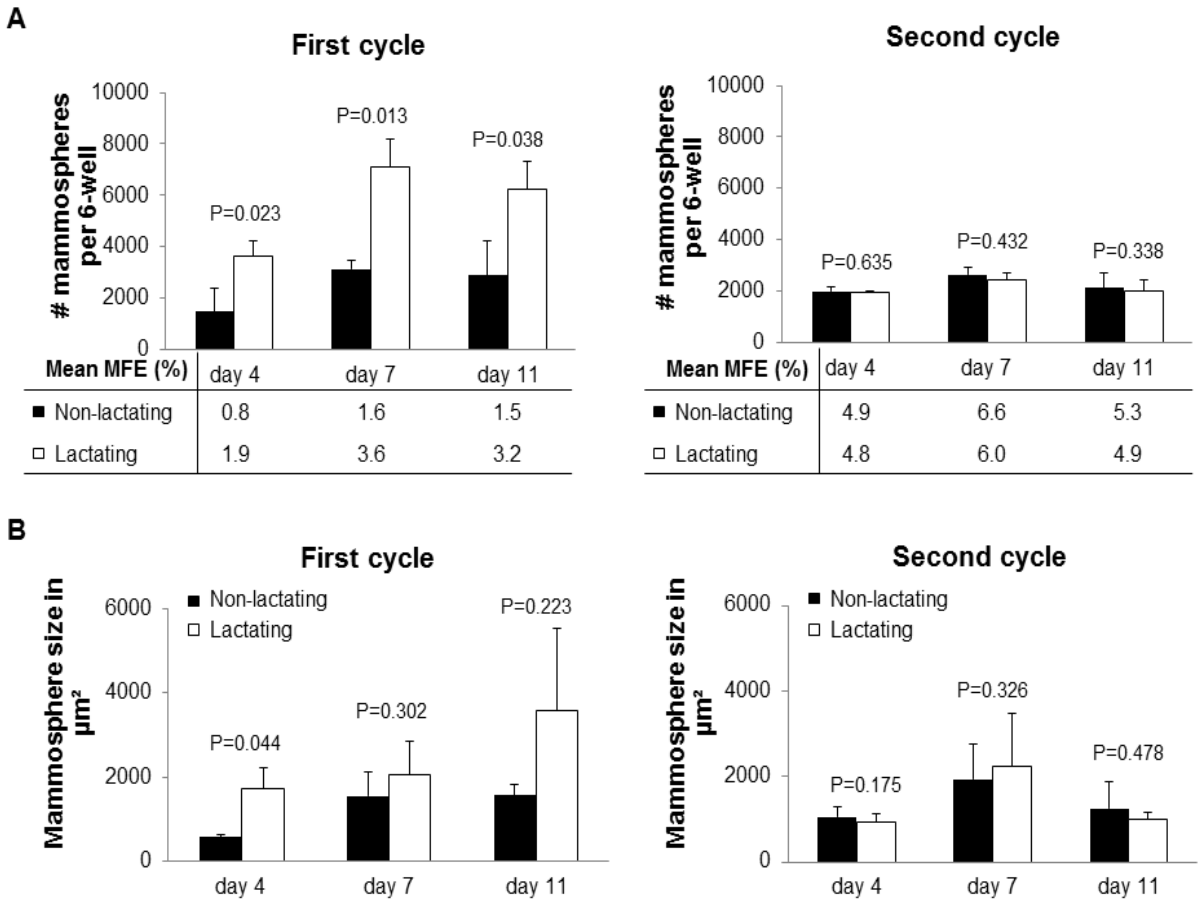



**Figure 9.** Mammosphere cycle assays of non-lactating and lactating equine mammary gland tissues. Numbers of mammospheres were counted per 6-well (**A**) and mammosphere sizes were calculated in µm² (**B**). To calculate the mammosphere forming efficiency (MFE), the number of mammospheres was divided by the original number of single cells seeded and expressed as a percentage. Calculations were made at three time points post seeding and data represent the mean of three experiments ± standard deviations.

## 4.5. Discussion

Much of the available data on mammary gland development comes from rodents (Sternlicht, 2006), although variation in mammary growth and function exist between mammals, especially with regard to the endocrine control of these processes (Forsyth, 1986; Lamote et al., 2004). Because (i) mammary stem/progenitor cells (MaSCs) are proposed to be the driving forces behind mammary growth and function and (ii) variation exists between mammalian species with regard to physiological as well as pathological functioning of this organ, we hypothesize that studying MaSCs from different species is of great comparative interest.

In the present study, we aimed to isolate and characterize MaSCs from the mammary gland of horses. Briefly, equine mammosphere-derived cells were immunophenotypically characterized by using stem cell (SC) markers CD29, CD44, CD49f and the proliferation marker Ki67. The latter was included to give an indication of the self-renewal rate of the cells, since this nuclear protein is only expressed during the late growth $(G)_1$-phase and the synthesis (S)-, mitosis (M)- and $G_2$-phase of the cell cycle (Coates et al., 1996). Moreover, a successful differentiation towards luminal (alveolar and ductal) epithelial and myoepithelial cells, the two major adult epithelial mammary gland cell types, was achieved. Luminal ductal epithelial cells, at least in mice, ramify through the fat pad before puberty and further proliferate under the influence of hormones in early pregnancy/gestation (Pitelka et al., 1973). During lactation, the myoepithelial cells will contract under the stimulation of oxytocin, thereby ejecting the milk from the luminal alveolar epithelial cells (Capuco et al., 2003; Dontu et al., 2003). For the latter, we found that after differentiating our equine mammosphere cells, which contained at least some mammary epithelial cells with SC characteristics, into adult luminal epithelial cells, these cells became positive for cytokeratin (CK) 18, whereas no expression was found on the undifferentiated control cells. This is in accordance with a human MaSC study (Dontu et al., 2003) and in contrast with a murine study, where it was found that undifferentiated MaSCs were CK18 positive (Stingl et al., 2006a). In several studies it was found that murine MaSCs were also positive for CK14 (Stingl et al., 2006a; Sun et al., 2010), however, we were unable to evaluate the expression of this cytokeratin on our equine mammosphere cells and their differentiated progeny, due to a lack of cross-reactivity of several CK14 antibodies tested (data not shown). In the present study, the differentiation of mammosphere cells towards both luminal and myoepithelial cells was achieved using one culture medium. This is in contrast to a study on bovine MaSCs where two different selective media were used (Li et al., 2009), but in line with what has been

previously reported for the differentiation of canine (Cocola et al., 2009) and human MaSCs (Stingl et al., 1998). Lastly, but interestingly, the equine mammosphere cells were capable of differentiating into adipocytes when cultured in adipocyte-inducing selective media (Spaas et al., 2013). Several independent studies report the differentiation of SCs into cell types of another germ layer (Ghaedi et al., 2012; Hermann et al., 2004). Here, we studied mammosphere cells, originating from the ectoderm, and showed that they were capable of differentiating into a cell type from the mesoderm, namely adipocytes. Since adipogenic transdifferentiation of human amnion and chicken oviduct adult epithelial cells has been reported (Khuong and Jeong, 2011; Murphy et al., 2010), the adipogenesis of epithelial mammosphere cells is not an unusual phenomenon.

It has to be noted, however, that some of the markers described above, including CD29, CD44 and CD49f, are not MaSC-specific but are also expressed on other SCs such as mesenchymal SCs (MSCs) (Semon et al., 2010; Smith and Chepko, 2001; Spaas et al., 2013). So, to ensure that the adipogenesis we observed was caused by epithelial cells with SC characteristics, or MaSCs, and not merely an effect of contaminating MSCs, we included the epithelial-specific marker casein kinase 2β (Deshiere et al., 2011). Flowcytometric analysis revealed that the equine mammosphere-derived cells were positive for this marker, in contrast to equine MSCs, which were casein kinase 2β-negative (data not shown).

The lack of *in vivo* functional assays has been pointed out as a vital drawback of current MaSC research (van Os et al., 2004). Indeed, because the use of a cleared fat pad model is not really feasible in horses, we aimed to evaluate additional *in vitro* assays to better characterize equine MaSCs. In our present study, we evaluated colony forming unit (CFU) assays and mammosphere cycle assays in equine cells from non-lactating versus lactating mammary glands. First, we assessed the clonogenic potential of mammosphere-derived cells derived from both physiological mammary gland stadia with the CFU assays and virtually identical

results were obtained, indicating that the *in vitro* clonogenic potential of MaSCs from lactating versus non-lactating mammary gland tissue is similar. Interestingly, two different colony morphologies were observed in the CFU assay at 7 days of culture (end of the experiments). The presence of different colony types has been described previously for human keratinocytes and other cell types: (i) holoclones: large colonies with lots of small cells which were shown to have the greatest reproductive capacity, (ii) paraclones: smaller colonies with large, flattened cells which have a short replicative lifespan and (iii) meroclones: a transitional stage between the holo- and paraclone which contains a mixture of cells with different growth potential (Barrandon and Green, 1987a). Based on these descriptions, we would like to hypothesize that the fingerprint colonies observed in the present study might represent holoclones, whereas the dispersed colonies resemble paraclones. Future experiments of subculturing the colony types we observed in the present study by transferring them to indicator dishes, as described in the paper of Barrandon and Green (Barrandon and Green, 1987b), will give more information on their growth potential and might prove an elegant method to determine the progenitor cell hierarchy in a given mammary gland sample under different (patho)physiological conditions. The existence of a progenitor cell hierarchy in the mammary gland has already been extensively studied in the human and murine mammary gland (Dontu et al., 2003; Stingl, 2009; Stingl et al., 2006b; Visvader and Lindeman, 2006), but information in other mammal species remains limited to date.

Second, the number as well as the size of the mammospheres was determined in both sample types using the *in vitro* mammosphere cycle assay. Hereby, the number of mammospheres formed upon serial passage reflects self-renewal of primitive MaSCs, whereas the mammospheres' size is a reflection of progenitor cell proliferation (Dontu et al., 2003). Interestingly, we found significantly more and larger mammospheres when cells were derived from lactating mammary glands compared to inactive, non-lactating mammary gland tissues,

at least in the first mammosphere cycle. Also, during the first cycle of the mammosphere cycle assay, a two-fold difference was found between the mammosphere forming efficiency (MFE) of the inactive, non-lactating (on average 1.3%) versus the lactating (on average 2.9%) equine mammary glands. A difference in MFE has previously been reported between epithelial cells from normal human breast tissue and breast tissue with ductal carcinoma in situ (DCIS), with the latter showing a significantly greater MFE (Farnie et al., 2007). The authors hereby suggested that a greater MFE, as seen in the DCIS samples, translates into the presence of a greater number of stem or progenitor cells (Farnie et al., 2007). So, based on the MFE data from our present study, the equine mammary gland appears to harbor a higher number of MaSCs during lactating compared to the inactive, resting state. Moreover, the significantly larger mammospheres indicate that MaSCs possess a higher progenitor cell proliferation rate during lactation. This is in line with what has previously been reported regarding lactation physiology in horses, where it was found that during lactation, cell divisions increase in line with milk production (Leadon et al., 1984; Ousey et al., 1984). This physiological process during lactation is most likely represented by MaSCs, since we demonstrated that MaSCs from lactating tissues are higher in number and show higher proliferation capacities. On the other hand, it has been proposed in rodents that murine MaSCs are relatively quiescent during lactation (Smith and Medina, 2008; Welm et al., 2002), which is in contrast to the increased proliferation rates, depicted by the larger mammosphere sizes, which were observed with the equine lactating mammary gland tissues. Hence, these results emphasize once more the importance of studying mammary gland physiology in general, and MaSC biology in specific, in different mammalian species.

For the second cycle of the mammosphere assay, spheres from the first cycle were collected, plated for one passage on an adherent plate and after trypsinization, replated at 4000 cells/cm$^2$. Hereby, it was observed that mammospheres derived from mammary gland

tissue had the capacity to form new generations of mammospheres in the second mammosphere cycle, with a similar regeneration capacity (MFE of 5,6% versus 5,2% on average) between the two tissue samples (non-lactating versus lactating, respectively). This is in contrast to the study with the normal human breast and DCIS tissues. There they found that DCIS mammospheres were capable of regenerating mammospheres at a significantly higher regeneration ratio compared to normal breast mammospheres (Farnie et al., 2007). A potential explanation for the lack of any difference in the second mammosphere cycle, observed in the present study, could be due to the fact that for this second cycle, mammosphere-derived cell populations were used after one passage on an adherent plate. It would be interesting to see if reseeding mammospheres as single cells directly under non-adherent conditions, would result in a difference between non-lactating and lactating tissues. Interestingly, the lack of any difference in the second mammosphere cycle reflects the lack of any difference observed in the CFU assays. For the latter, one mammosphere-derived cell obtained from an adherent culture was seeded per $cm^2$ of a 6-well plate. It would be interesting to see whether mammosphere populations where a difference in CFUs is observed, will also display the same difference in the second mammosphere cycle assay. Future work will therefore focus on further optimizing and validating these assays to determine the number and MFE of MaSCs present in the mammary gland at certain (patho)physiological stadia such as mastitis and mammary tumor formation not only in horses, but also in other mammalian species.

In conclusion, this study is the first to report on the isolation and characterization of equine MaSCs. Furthermore, we describe the valuable use of additional *in vitro* assays to compare mammospheres and mammosphere-derived cells in the mammary gland at different physiological stages.

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# CHAPTER 5

## Treatment of equine degenerative joint disease with autologous peripheral blood-derived mesenchymal stem cells: a case report

Adapted from:

Spaas JH, Oosterlinck M, Broeckx S, Dumoulin M, Saunders J, Van Soom A, Pille F, Van de Walle GR, 2012. Treatment of equine degenerative joint disease with autologous peripheral blood-derived mesenchymal stem cells: a case report. Vlaams Diergeneeskundig Tijdschrift 81, 11-15.

After extensively characterizing the isolated equine peripheral blood (PB)-derived mesenchymal stem cells (MSCs), as described in Chapter 3, these cells were injected into the pastern joint of a horse suffering from chronic degenerative joint disease. Since the injured horse was only clinically evaluated without the presence of a sham-treated control horse, repetition or any functional data, this report should be considered as purely descriptive.

## 5.1. Abstract

A 5-year-old German Warmblood stallion with chronic lameness, attributable to degenerative joint disease (DJD) of the pastern joint unresponsive to medical treatments, was treated with autologous mesenchymal stem cells (MSCs). These MSCs were isolated from the peripheral blood (PB) of the patient and injected into the pastern joint, at a concentration of $2.5x10^6$ cells, twice with an 8-week interval. The positive response to this MSC treatment was documented by visual gait evaluation as well as objective pressure plate analyses. This paper is the first to describe the use of autologous PB-derived MSCs to treat a horse suffering from chronic DJD. The favorable outcome of this single case may stimulate further research on the use of equine PB as a source of autologous MSCs in equine regenerative medicine.

**Keywords:** peripheral blood; mesenchymal stem cells; horse; degenerative joint disease

## 5.2. Introduction

Degenerative joint disease (DJD) is a major cause of diminished athletic function and wastage in performance horses (Frisbie 2005; Jeffcott et al. 1982; McIlwraith 1982). Ideally, any treatment should be based upon a good knowledge of the anatomy and physiology of normal joints and the processes occurring during degeneration and repair (Goodrich and Nixon 2006; McIlwraith and Vachon 1988; Nizolek and White 1981). Initially, such treatment should include some degree of rest or exercise restriction. Medical treatments for DJD may include anti-inflammatory and analgesic drugs to reduce inflammation and pain, and so-called disease modifying drugs such as glucosamine or chondroitin sulphate or hyaluronic acid (Goodrich and Nixon 2006; Malone 2002; Nizolek and White 1981). In the presence of severe cartilage and bone changes, the use of articular cartilage curettage, osteophyte removal and even arthrodesis could be suitable in some cases (Malone 2002; Zubrod and Schneider 2005). Nevertheless, the aforementioned therapies are merely palliative or may aim for an enhanced repair, albeit without actual regeneration of the affected joint. Over the last decades, the field of equine regenerative medicine is getting increased attention and the use of stem cells (SCs) to treat joint pathologies appears to be a promising strategy to regenerate injured tissues by differentiation towards cells with a hyaline-like cartilage morphology and which are able to produce cartilage-specific components, such as collagen type II and glycosaminoglycans (Berg et al. 2009; Koch and Betts 2007).

SCs are defined as cells displaying a self-renewal capacity either with or without differentiation, depending on the symmetry of the division (Donovan and Gearhart 2001). More specifically, mesenchymal SCs (MSCs) are adult SCs derived from the mesodermal germ layer. Current clinical regenerative therapies with MSCs in horses mainly use bone marrow (BM)-derived MSCs for the treatment of tendinopathies (Crovace et al. 2007; Smith 2006, 2008; Smith et al. 2003; Violini et al. 2009) and BM- or adipose tissue (AT)-derived

MSCs for the treatment of osteoarthritis (Frisbie et al. 2009). In general, obtaining BM and AT samples is invasive and quite difficult to harvest. As an alternative, scientists have suggested umbilical cord blood (UCB), which in humans can be easily collected at birth. However, in horses, autologous UCB is not always available and a sterile collection is only possible under highly hygienic circumstances, which is difficult to achieve under field circumstances. The disadvantages of UCB can be avoided using peripheral blood (PB) as a source for MSCs. Since collection of a sterile blood sample can easily be performed by any equine practitioner, PB is a readily accessible source of autologous MSCs when injuries occur.

This paper is the first to describe the use of autologous PB-derived MSCs to treat a horse suffering from chronic lameness attributable to DJD.

### 5.3. Case history

One year before injection of autologous peripheral blood (PB)-derived mesenchymal stem cells (MSCs), a 5-year-old German Warmblood stallion was presented with severe unilateral forelimb lameness (grade 4/5 according to the American Association of Equine Practitioners (AAEP) scale), attributable to DJD of the pastern joint. The diagnosis was made based on clinical and radiographic examination and a positive response to intra-articular anesthesia. Dorsopalmar and lateromedial radiographes revealed periarticular new bone formation (Figure 1). Initial medical treatment with oral non-steroidal anti-inflammatory drugs and box rest during 2 months, and subsequent intra-articular administration of steroidal anti-inflammatory medication and hyaluronic acid did not improve the impaired locomotion. Two months later, an additional period of 1 month of complete box rest and limited movement in a small paddock for the following 5 months did not give any improvement of the lameness. Given the lack of response to therapy after almost one year, the owners opted for treatment

with autologous PB-derived MSCs. The ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2010-147) approved the experimental design.

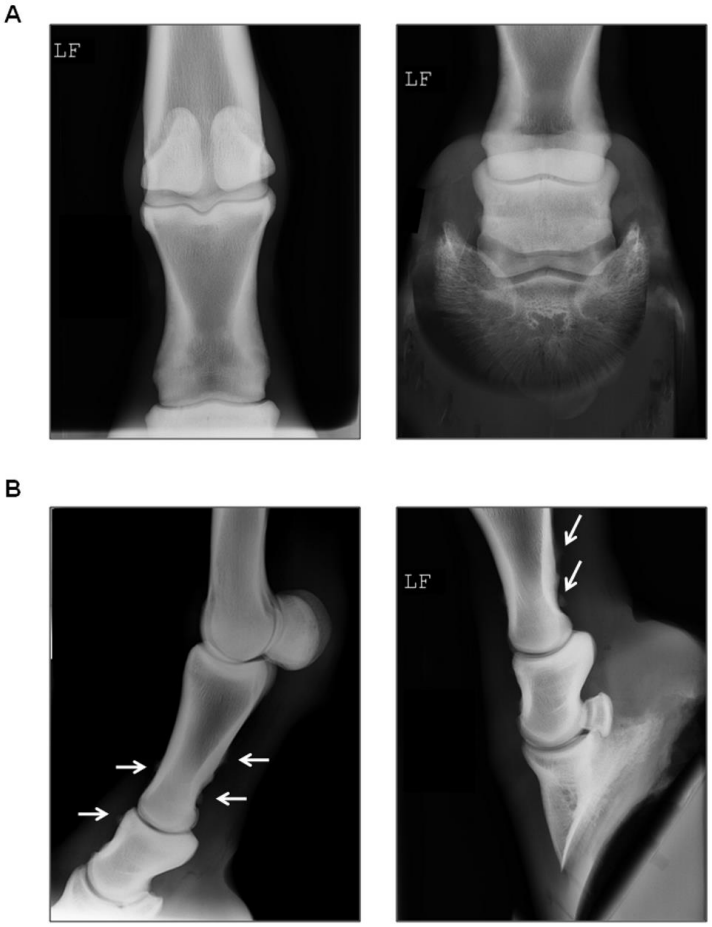



**Figure 1.** Dorsopalmar (**A**) and lateromedial (**B**) radiographic images of the pastern joint. The images on the left are weight-bearing and on the right using the podoblock. White arrows indicate the periarticular new bone formation.

## 5.4. Treatment

Ten mL of blood was taken from the *vena jugularis* of the patient and centrifuged at 1000xg for 20 minutes at room temperature (RT). The buffy coat fraction was collected and diluted 1:1 with phosphate buffered saline (PBS). Subsequently, the cell suspension was gently layered on a Percoll gradient (density 1.080 g/mL; GE Healthcare) and centrifuged at 600xg for 15 minutes at RT. The mesenchymal stem cells (MSCs) were maintained and characterized by the presence or absence of different immunophenotypic markers and by *in*

*vitro* differentiation towards osteoblasts, chondroblasts and adipocytes as previously described (De Schauwer et al. 2011).

For the first intra-articular injection, $2.5x10^6$ autologous peripheral blood (PB)-derived MSCs of passage 1 ($P_1$) were resuspended in 2.5 mL of sterile PBS with 50 µg/mL gentamicin (Sigma-Aldrich, Bornem, Antwerp, Belgium) and injected into the pastern joint of the patient. A similar injection was repeated 8 weeks later with cryopreserved MSCs of $P_1$ which were further cultured up to $P_3$. The intra-articular injections were performed after clipping and aseptic preparation of the skin, as is routinely done before any intra-articular treatment.

Figure 2 gives an overview of the timing of the injections with the autologous PB-derived MSCs and all evaluations of the patient, as described below.

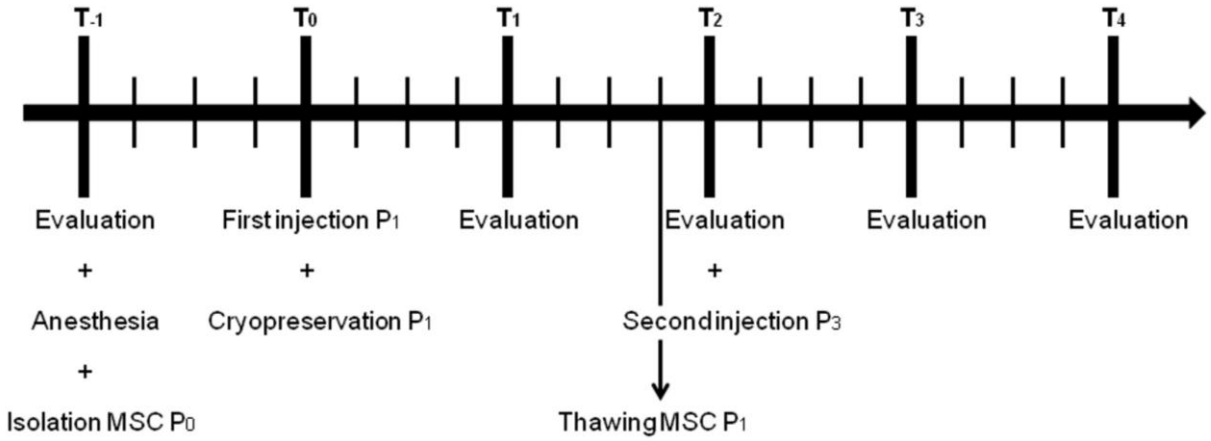



**Figure 2.** Timeline of equine mesenchymal stem cells (MSCs) injections and evaluation intervals. A 5-year-old stallion with chronic lameness attributable to degenerative joint disease (DJD) of the pastern joint was treated with autologous peripheral blood (PB)-derived MSCs. Clinical evaluations and pressure plate analyses were performed 4 weeks before ($T_{-1}$) and at 4-week-intervals ($T_1$= 4 weeks, $T_2$= 8 weeks, $T_3$= 12 weeks and $T_4$= 16 weeks) after the first injection. P = passage.

## 5.5. Outcome

### *5.5.1. Clinical assessment*

Clinical evaluation was performed 4 weeks before injection ($T_{-1}$), and at 4-week-intervals after the first injection ($T_1$= 4 weeks, $T_2$= 8 weeks, $T_3$= 12 weeks and $T_4$= 16 weeks),

according to the scoring system of the American Association of Equine Practitioners (AAEP) by the same team of veterinarians. Hereby, a grade 0 corresponds to soundness and a grade 5 to a non-weight-bearing lameness. At every time point, flexion tests of the affected lower limb were performed also and the results were graded from 0 (no response) to 3 (severe lameness).

Four weeks before the first injection ($T_{-1}$), the lameness was evaluated and localized to the pastern joint using intra-articular anesthesia. A score of 4/5 was given, which implied that the horse showed a severe lameness at the walk and the trot in a straight line, with a marked head nod and shortened stride. Moreover, the flexion test of the distal limb was strongly positive (3/3). Two days after the first injection with autologous mesenchymal stem cells (MSCs), a mild diffuse swelling was observed at the pastern. This swelling disappeared within a day. After 3 days of box rest, gradual hand-walking was initiated. Four weeks after the first injection ($T_1$), the lameness was obviously decreased although it could still be observed consistently at the trot under all circumstances tested, and therefore, a score of 3/5 was given. The flexion test at that time point was only slightly positive (1/3). After the second MSC injection, a mild, localized swelling together with a mild, transient lameness was observed at the walk. At all subsequent evaluation time points thereafter ($T_2$, $T_3$ & $T_4$), only a residual irregularity at the trot could be noticed when lunging the horse (grade 2/5) and the flexion tests were negative (0/3).

### *5.5.2. Pressure plate analysis*

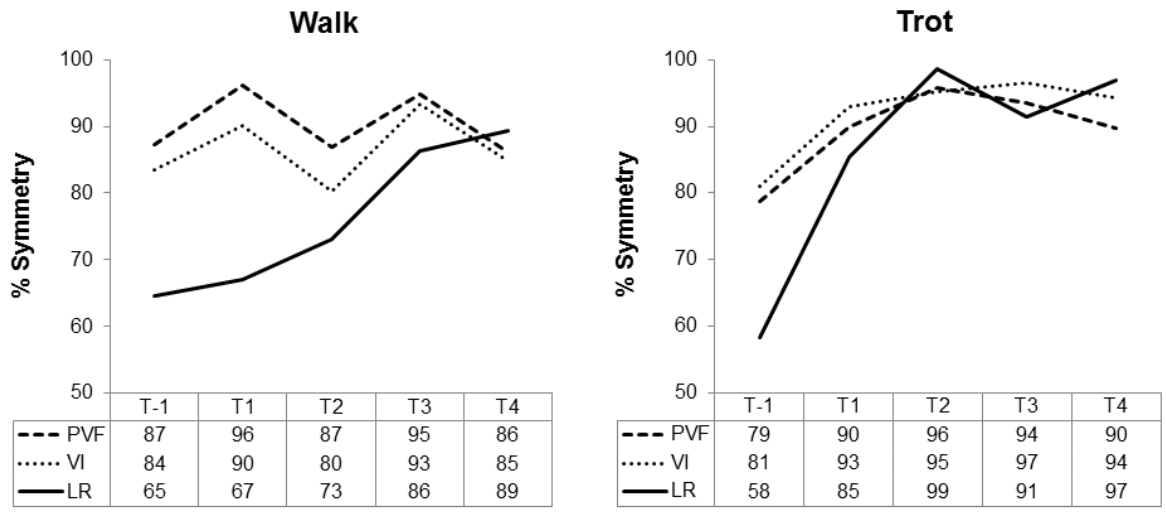
Recently, pressure plate analysis has been proposed as a useful tool to quantify equine locomotion and to evaluate the effects of a certain therapy in horses (Oosterlinck et al. 2010a; Oosterlinck et al. 2010b). Indeed, this technique allows for simultaneous analyses of different limbs and provides detailed information on the loading of the different portions of the foot

during a complete stance period. In this case, the horse was walked and trotted over a pressure plate (RSscan 3D 2m-system, RSscan International, Olen, Belgium) in a custom-made walkway, as described previously (Oosterlinck et al. 2010a; Oosterlinck et al. 2010b). A trial was considered valid only if a complete hoof print of at least one forelimb was recorded while velocity was within a preset range. A measuring session was limited to the number of trials necessary to obtain five valid measurements of both forelimbs. The following kinetic variables were calculated for both forelimbs at the walk and at the trot, and expressed as % symmetry (left/right x 100%): (1) peak vertical force (PVF), i.e. the maximal vertical force value throughout the stance phase; (2) vertical impulse (VI), calculated by time integration of the force-time curves and (3) load rate (LR) of the vertical force curve.

The PVF, VI and LR symmetry ratios at all time points are presented in Figure 3. Already after the first injection with the autologous PB-derived MSCs, an increase in the LR symmetry ratio was observed in both the walk and the trot, which even further increased considerably after the second injection (Figure 3). Clear improvements were also seen in the PVF and VI symmetry ratios, although these effects were more pronounced at the trot than at the walk (Figure 3).

### *5.5.3. Medical imaging*

Routine dorsopalmar and lateromedial radiographic evaluation of the pastern joint did not reveal considerable changes after the autologous MSC therapy compared to pre-treatment radiographs (data not shown). Four weeks before stem cell therapy, an ultrasonographic examination was performed and was normal. Therefore, no further ultrasonography was performed.




| Walk | T-1 | T1 | T2 | T3 | T4 |
|---|---|---|---|---|---|
| PVF | 87 | 96 | 87 | 95 | 86 |
| VI | 84 | 90 | 80 | 93 | 85 |
| LR | 65 | 67 | 73 | 86 | 89 |

| Trot | T-1 | T1 | T2 | T3 | T4 |
|---|---|---|---|---|---|
| PVF | 79 | 90 | 96 | 94 | 90 |
| VI | 81 | 93 | 95 | 97 | 94 |
| LR | 58 | 85 | 99 | 91 | 97 |

**Figure 3.** Pressure plate analyses at the walk and the trot. The patient was evaluated 4 weeks before ($T_{-1}$) and at 4-week-intervals ($T_1$= 4 weeks, $T_2$= 8 weeks, $T_3$= 12 weeks and $T_4$= 16 weeks) after the first injection with autologous peripheral blood (PB)-derived mesenchymal stem cells (MSCs). The following parameters were measured: peak vertical force (PVF), vertical impulse (VI) and load rate (LR).

## 5.6. Discussion

Mesenchymal stem cells (MSCs) represent a very promising therapeutic tool for certain types of degenerative or traumatic diseases in different animal species, because of their enormous plasticity and differentiation capacities. The *in vivo* use of MSCs in equine veterinary medicine has been studied intensively, with several independent studies reporting regenerative effects, mainly in tendon and ligament injuries (Crovace et al. 2007; Smith 2008). Equine degenerative joint disease (DJD) is one of the most common causes of early retirement and reformation of sport horses, and therefore, the use of MSCs is currently being explored for its regenerative potential in this musculoskeletal injury (Berg et al. 2009; Koch and Betts 2007). To our knowledge, there are only reports on the *in vivo* use of equine adipose tissue (AT)-derived and bone marrow (BM)-derived MSCs for the treatment of osteoarthritis (Frisbie et al. 2009; Wilke et al. 2007). Hereby, osteoarthritis was experimentally induced and the MSCs were injected in the acute phase of the lesion (within 14 days). In all these studies, a short-term clinical improvement was noticed after MSC therapy, although this was not the case for the long-term follow-up of the horses. In contrast, a more recent study of McIlwraith

showed no evidence of any clinically significant improvement in the joints injected with BM MSCs, but arthroscopic evaluation confirmed a significant increase in aggrecan production, repair tissue firmness and a trend for better overall repair tissue quality in BM MSC-treated joints (McIlwraith et al. 2011). This latter study emphasizes the importance of combining clinical evaluation with more scientifically-based parameters to evaluate the efficacy of MSC treatment in horses.

In the present case report, we describe for the first time the use of peripheral blood (PB)-derived MSCs to treat a horse suffering from chronic lameness attributable to DJD of the pastern joint. A positive response to the therapy could be demonstrated already at 4 weeks after the first injection, as assessed by clinical evaluations and pressure plate analyses. For the latter, it was proven that the load rate (LR) symmetry ratio increased considerably in both gaits, indicating an increased speed of loading at the walk as well as at the trot. Moreover, a clear improvement in peak vertical force (PVF) and vertical impulse (VI) symmetry ratios was evident at the trot, indicating increased symmetry of the weight-bearing function of the forelimbs. The lack of an overall increasing improvement in PVF and VI symmetry ratios at the walk is most likely associated with a lower reproducibility of pressure plate analysis at the walk than at the trot, as previously reported (Oosterlinck et al. 2010a). At one year after MSC treatment, the stallion was not showing any signs of recurrent lameness and was competing again (personal communication with the horse owner). Half a year later, joint stiffness increased (noticeable on the circle), leading to a third MSC injection. Within four weeks the horse was ready to compete again and is still clinically sound at two and a half years after the first injection. Still, in order to truly confirm the effectiveness of PB-derived MSCs, a double blinded standardized model should have been used with exactly the same induced lesions in a large number of horses, including control groups. Moreover, and in analogy to previously performed experiments in horses where the fate of injected BM MSCs was studied in an

equine tendon injury model (Guest et al. 2010), fluorescently-labelled PB MSCs could be used to prove that the *in vivo* cartilage regeneration is attributable to the MSC itself or rather to the products they secrete.

In conclusion, this case report is the first to describe the successful treatment of a patient suffering from chronic lameness attributable to DJD using autologous PB-derived MSCs. Clearly, this single case study does not allow us to draw definite conclusions on the clinical efficacy of the treatment protocol, nor does it allow for an extrapolation to other equine pathologies beyond DJD. However, the results of this case report are encouraging to further evaluate the efficacy of autologous PB-derived MSCs in equine regenerative medicine.

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# CHAPTER 6

## General Discussion

The present thesis describes the isolation and characterization of 2 different types of adult multipotent stem cells (SCs) in horses: the mesenchymal SCs (MSCs), derived from the mesodermal germ layer, and the mammary gland stem/progenitor cells (MaSCs), derived from the ectodermal germ layer. Since both SC types are able to constitute a tissue or an organ, they are at the origin of normal functioning of the organ, and defects may consequently result in pathologies (Kenyon and Gerson, 2007). The potential use of adult SCs in regenerative medicine has been the main reason for the increasing interest in MSCs (Kuo et al., 2009; Torricelli et al., 2011). On the other hand, MaSCs are mainly studied for their possible correlation with breast cancer (Ercan et al., 2011; Ponti et al., 2005; Tiede and Kang, 2011). In horses, both MSCs and MaSCs can be derived from highly accessible sources, namely the peripheral blood (PB) for MSCs and slaughterhouse material for MaSCs. Therefore, the focus of our research was to optimize the isolation and characterization techniques of PB-derived MSCs and mammary gland-derived MaSCs in the horse. In addition, we report the treatment of a horse suffering from chronic degenerative joint disease with autologous PB-derived MSCs. The following subchapters will discuss the different aspects described in this thesis.

## 6.1. Isolation of equine stem cells

*Mesenchymal stem cells*

In order to fulfill the first Aim of the present thesis, we performed the sampling of 10ml of venous blood of 4 horses resulting in a successful mesenchymal stem cell (MSC) isolation in all 4 cases (success rate of 100%). However, when we initiated the isolation of MSCs under identical culturing conditions in a larger number of horses (n = 10), this resulted in an isolation success rate of only 40% (unpublished data). Indeed, the greatest hurdle for using equine peripheral blood (PB) as a source of MSCs is the varying isolation success rate, as also

evidenced by other researchers. Koerner et al. (2006) described a successful isolation in 12 out of 33 attempts (36.4%), starting from 36ml of equine blood per isolation (Koerner et al., 2006). Two years later, Giovannini et al. reported a success rate of 66.7% (Giovannini et al., 2008). The aspirated blood volume did not seem to determine whether or not the isolation was successful, since Martinello et al. used 100ml of blood per isolation and MSCs were only recovered in 11 out of 25 horses (44%) (Martinello et al., 2010). In order to further optimize the isolation technique, we performed an experiment in 3 horses using different initial seeding concentrations of peripheral blood mononuclear cells (PBMCs) ($1.35x10^5/cm^2$ vs $2.7x10^5/cm^2$), however, without any significant differences in success rate (unpublished data). In all our studies a basic isolation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 30% of fetal calf serum (FCS), dexamethasone and a combination of amino acids, antibiotics and antimycotics was used and the first medium changes took place after overnight incubation.

In the study of Martinello et al. a similar medium was used, except for the lower concentration of FCS (20%) and the omission of dexamethasone (Martinello et al., 2010). Although a much higher seeding concentration of $4\text{-}6x10^6$ PBMCs/cm² was used in their study, no considerable difference in the MSC isolation percentage could be noticed in comparison to our preliminary experiment in 10 horses (44% vs 40%). Even though Giovannini reports an almost 2-fold increase in isolation success rate in comparison to Koerner, both studies used a similar set-up consisting of a similar isolation medium as Martinello with the addition of F12, an initial seeding concentration of $1.5x10^5$ PBMCs/cm² and first medium changes at 10-14 days later. Another option to optimize the isolation success could be the addition to the medium of different substances which have been shown to stimulate MSC proliferation. In this regard, it has been reported that adding fibroblast growth factor (FGF) to SC medium increases the mitose capacity with the conservation of

differentiation capacities (Kuhn and Tuan, 2010; Sotiropoulou et al., 2006; Tsutsumi et al., 2001) and that heparan sulphate (HS) can increase the initial MSC retrieval and stimulate SC proliferation (Dombrowski et al., 2009; Helledie et al., 2012). In our hands, medium supplementation with FGF and/or HS did not result in significant improvement of isolation success (data not shown).

A potential explanation for the variable success rate of MSC isolation from PB could be a difference in the physiological condition of the donor horse, which could cause a change in the number of MSCs in the PB at the time of sampling. In this regard, it could be postulated that the age, gender and general condition of the donor horse can influence the number of circulating MSCs. However, in all studies published so far, the evidence for this assumption is low to non-existing, mostly due to the lack of appropriate statistical analysis and/or low numbers of animals. In order to show a significant increase in isolation success rate of for example 20% starting from the reported 40% in horses with an age ranging from 5-15 years old versus horses younger than 5 yours old, 2 groups of 107 horses in each group would be necessary. If there would only be a difference of 10% in isolation success rate between both age groups, 2 groups with 408 horses per group would be needed.

Interestingly, we found that for 3 donor horses that were used in our study (Spaas et al., 2013) in which the isolation was successful, sampling of the same horses at a later time point (i.e. 4 months) was unsuccessful in isolating MSCs, with the exception of 1 horse, where the isolation was successful at both time points. This observation indicates that individual differences or the moment of blood sampling might be of vital importance. Hereby, it is known that the production in horses of different bone marrow (BM) activating substances depend on the seasonal and circadian rhythm (Cordero et al., 2012). Future research could focus on the success rate of MSC isolation at different time points in correlation with the physiological and hormonal status of the donor horses. In humans, it has been reported that

high corticosteroid levels induce BM activation (Dror et al., 2000) and also that the administration of granulocyte colony stimulating factor (G-CSF) and/or macrophage inflammatory protein-2 (MIP-2) before taking blood would enhance the BM release of SCs in humans (Kanold et al., 1998) and mice (Wang et al., 1997). Besides growth factors and cytokines, the use of hyperbaric oxygen treatments has been recently proposed to have a similar progenitor cell mobilizing effect, hereby increasing the amount of MSCs in PB and MSC recovery in mares. Indeed, Dhar et al. described an initial success rate of only 2 out of 6 mares without such treatment, whereas 3 hyperbaric oxygen treatments in the same mares resulted in a 100% success rate (Dhar et al., 2012). Still, larger studies should confirm this finding and a hyperbaric oxygen chamber is not always available in field circumstances.

*Mammary stem/progenitor cells*

In order to fulfill the second Aim of the present thesis, equine mammary biopsies were collected and an optimized isolation technique allowed us to isolate mammary epithelial cells (Figure 1, unpublished data) in 11 out of 11 tissue samples (Spaas et al., 2012; unpublished data). Mammosphere formation was evaluated in 8 out of the 11 samples and was successful in all trials.

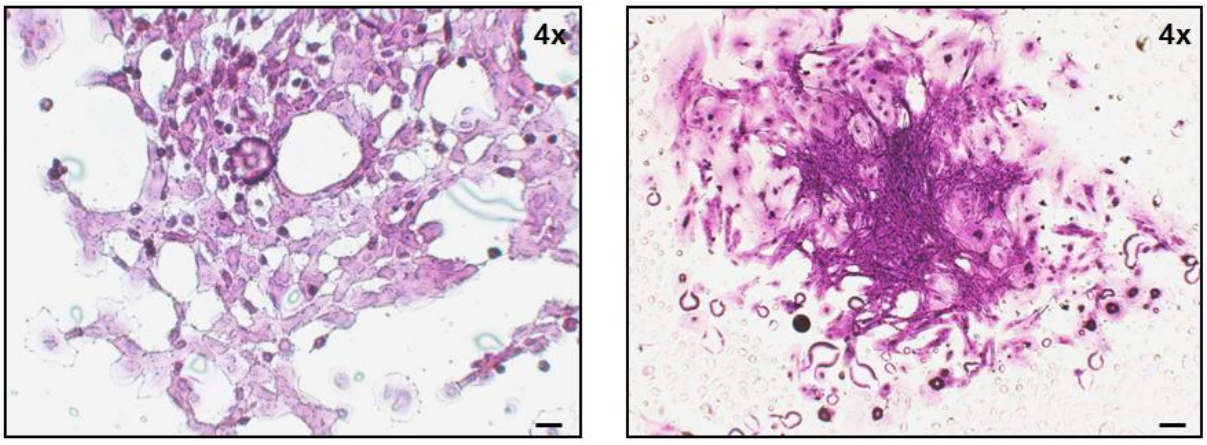


**Figure 1.** Mammary gland-derived adult cells at 2 weeks after isolation. Scale bars represent 100µm.

In the present thesis, epidermal growth factor (EGF) and FGF were added to the isolation medium for mammary stem/progenitor cells (MaSCs) and omission of these substances led to a substantial lower recovery rate of mammospheres after 7 days of cultivation (data not shown). Also in other animal species, the addition of FGF and EGF to the culture medium lead to a successful mammosphere formation after approximately 1 week (Dontu et al., 2003; Li et al., 2009). However, it is important to note that mammospheres do not solely consist of MaSCs, but also contain progeny cells and non-stem progenitor cells (Stingl, 2009). Nevertheless, the mammosphere formation described in this thesis indicates that at least some equine cells with MaSC characteristics could be successfully obtained with the present isolation technique. In this regard, the group of Dontu et al. described that seeding the isolated mammary cells at clonal density would increase the number of MaSCs per mammosphere, or in other words "the pureness" of the isolated cells (Dontu et al., 2003).

Still, after dissociating the mammospheres into single cells, only 4 out of 1000 cells were able to form new mammospheres in that study. In the present thesis, we report an average mammosphere forming efficiency (MFE) of 4.85% (at the second mammosphere cycle) after dissociating the adherent culture of MaSCs into single cells. This 10-fold increase in MFE in comparison to Dontu might be due to a different initial plating concentration, the use of another animal species (human vs horse) or the fact that the mammospheres in our study were first cultured on adherent plates before a second mammosphere cycle was initiated. This implicates that the number of mammosphere forming cells might fluctuate and depend on variable parameters. Nonetheless, the exact number of MaSCs per mammosphere remains a difficult parameter to determine. Therefore, future research may focus on defining the exact culture conditions that are necessary in order to obtain a pure MaSC population and whether or not MaSCs would need the surrounding cells in order to form mammospheres.

## 6.2. Characterization of equine stem cells

Although research in adult multipotent stem cells (SCs) is a hot topic to date, the exact functional as well as immunophenotypical blueprints of these cells are not completely unraveled. Indeed, if the isolates are not well characterized, there is the possibility of contamination with other cell types, which might give unsatisfactory results in different SC studies. For this reason, both Aim 1 and 2 of the present thesis do not only focus on the isolation, but also on the characterization of the obtained cells. Even with the generated data, it remains a major hurdle in equine SC research to find the appropriate tools (i.e. cross-reacting antibodies) to properly characterize these cells (Borena et al., 2013; De Schauwer et al., 2011).

*Mesenchymal stem cells*

In 2006, the International Society for Cellular Therapy (ISCT) has carefully determined the qualities human cells must possess in order to be defined as mesenchymal SCs (MSCs) (Dominici et al., 2006). No such guidelines have been established for equine MSCs, which makes it sometimes difficult to compare results on equine MSCs between different studies. In the present thesis, a thorough characterization of equine peripheral blood (PB)-derived MSCs was performed and several interesting observations were made. In contrast to previous studies, where only a limited number of SC markers were tested and chondrogenic differentiation was unsuccessful or only accomplished after 9 weeks (Giovannini et al., 2008; Koerner et al., 2006), we tested the expression of 8 different markers on PB-derived MSCs and the cells could be successfully differentiated into chondroblasts within 3 weeks. Nevertheless, it has been reported that the expression of MSC markers can vary during culture (Radcliffe et al., 2010). Therefore, it would be interesting to evaluate the evolution of marker expression at different stages of the culture process and after differentiation towards different

adult cell types. In addition, any possible influence of the used media (for culture) or physiological status of the donor horses on the marker expression and differentiation capacities could be an interesting addition to the presented data. In this regard, preliminary experiments revealed that the chondrogenic capacities of MSCs might vary from one donor to another (Figure 2, unpublished data). Indeed, MSCs of a healthy donor were capable of differentiating into chondroblasts in lacunes with the production of an extracellular matrix (blue glycosaminoglycan deposition), whereas the MSCs of an injured horse were not able to perform chondrogenesis under identical culturing circumstances (Figure 2, unpublished data). However, this was not the case for MSCs from other injured horses. Whether this was a coincidence, a genetic anomaly or determined by the physiological condition of the horse at that time, remains to be determined. Further research could provide valuable insights in the mechanisms affecting MSC chondrogenesis.

Also the influence of age, sex and hormonal status on the SC potential and differentiation capacities could provide us with valuable knowledge before therapeutic use. In this preliminary experiment both donors had the same age, indicating that also young horses might provide dysfunctional SCs. Whether aging increases the possibility of SC dysfunction should be tested in a large group of horses. In humans, it has been described that SCs of old donors have lower differentiation capacities (Zhang et al., 2012). Hereby, it has been reported recently that adipose tissue (AT)-derived MSCs of infants showed a higher expression of angiogenetic factors (VEGF and FGF-2) and responded more profound to osteogenic induction (alkaline phosphatase and alizarin red staining and bone-related gene expression, such as RUNX-2 and osteocalcin) than AT-derived MSCs of older donors (Wu et al., 2012). Moreover, it has been reported that the age-related disease, osteoporosis was correlated with enhanced human MSC mRNA expression of osteoporosis and osteoclastogenesis associated genes (Benisch et al., 2012). Whether the lower estrogen levels in elderly osteoporotic women

is the determining factor in changing the mRNA expression, however, needs to be determined.

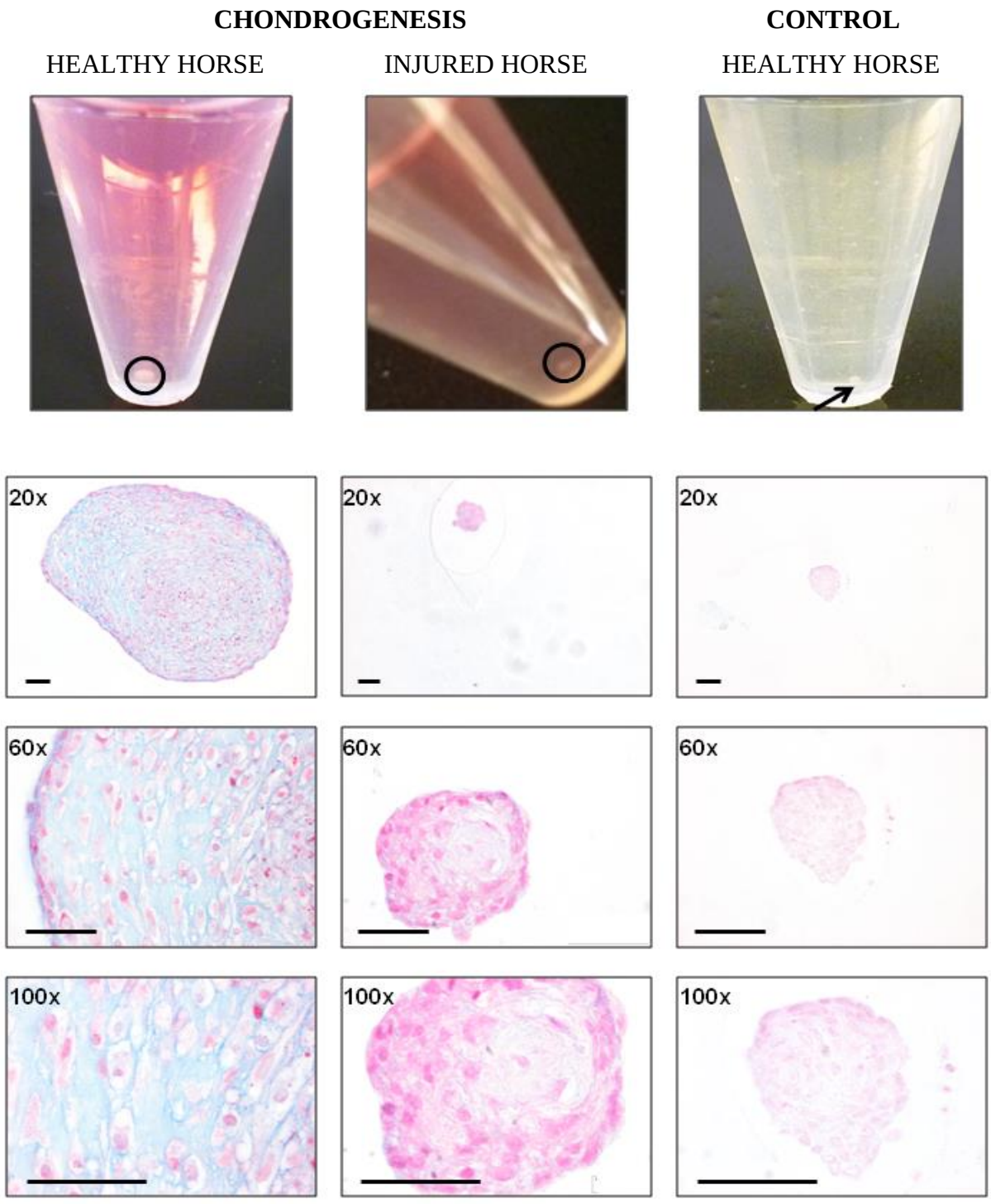


**Figure 2.** Macroscopic images of chondrogenic differentiation (left and middle panels) and undifferentiated mesenchymal stem cells (MSCs) as a control (right panels). Alcian blue stainings on all the samples confirmed chondrogenesis in a differentiated MSC sample of a healthy horse and absence of chondrogenesis in a differentiated MSC sample of an injured donor horse as well as in undifferentiated control MSCs. Scale bars represent 50µm.

Furthermore, other factors that are known to affect osteoblast activity, such as parathyroid hormone (PTH), have also been shown to affect SCs. Indeed, treatment of mice with parathyroid hormone has been described to have beneficial effects on the hematopoietic SC pool (Rashidi and Adams, 2009). In addition, it has been described that cell orientation has an important influence on MSC migration and behavior. For example, it has been shown that multilineage differentiation of immature human MSCs can be directed by changing tissue elasticity using a controlled elastic *in vitro* model (Engler et al., 2006). Indeed, soft matrices that mimic brain tissue induced neurogenesis, whereas stiffer matrices that mimic muscle tissue induced myogenesis and the most rigid matrices induced osteogenesis (Figure 3).

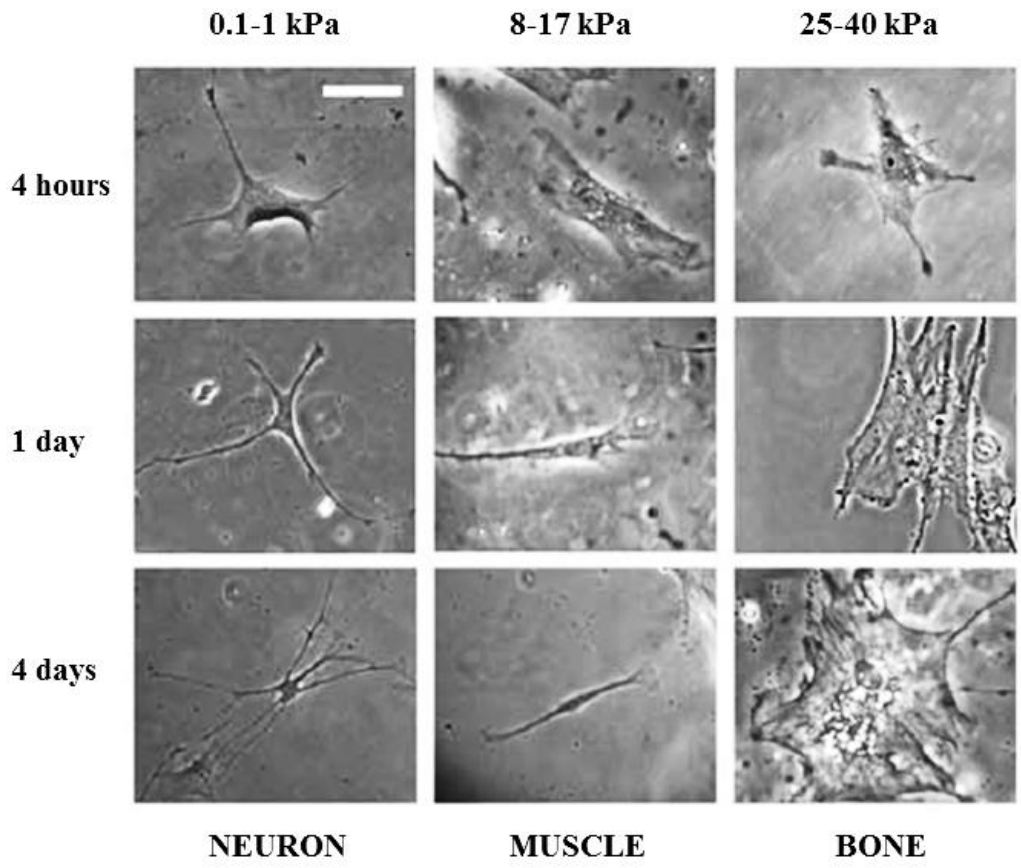


**Figure 3.** Naïve human mesenchymal stem cells (MSCs) become more branched, spindle or polygonal shaped when grown in matrices in the range of brain elasticity (0.1-1 kPa), muscle elasticity (8-17 kPa) or stiff cross-linked collagen matrices (25-40kPa), respectively (Engler et al., 2006). Scale bar represents 20µm.

In the study of Engler, responses were observed at all levels from RNA to protein production and from morphology to cell stiffness, indicating the overall impact of matrix elasticity on the MSC's biology. Unfortunately no data concerning the influence of matrix elasticity on equine MSCs are available to date. Besides the physical micro-environment, also biological factors, such as extracellular matrix components play a pivotal role on the SC's behavior. Indeed, ligation of the basement membrane component laminin to cell membrane anchored integrines would drive osteogenic differentiation (Klees et al., 2005; Klees et al. 2007). Moreover, as already mentioned in the General Introduction, several growth factors and cytokines that are present in the niche of the SC have a directing or preserving function on these cells. For all the aforementioned reasons, researchers should be encouraged to increase the knowledge of the niche of cells in general, and of MSCs in specific. Not only the fundamental science of MSC regulation would benefit from this knowledge, but also the efficacy of cell-based therapies might improve.

*Mammary stem/progenitor cells*

In contrast to MSCs, no uniform guidelines are available to properly define mammary stem/progenitor cells (MaSCs), not even in human MaSC research (Borena et al., 2013). Although a plethora of different isolation/characterization techniques are used for MaSCs across species, it is generally accepted that MaSCs have to be capable to form colony forming units (CFU) from single cells and to differentiate into different cell types of the epidermal germ layer, such as luminal epithelial cells (ductal and alveolar) and myo-epithelial cells. Furthermore, MaSCs should express SC-specific markers after multiplication in order to verify their stemness under *in vitro* cultivation (cfr. Table IV of the General Introduction). In the present thesis, we have evaluated cells isolated from equine mammary gland tissue for all

the requirements described above and our results strongly indicate that we have been successful in obtaining equine MaSCs.

In the present thesis, the functional characterization of equine MaSCs was performed by directing their differentiation *in vitro*. Still, the ultimate proof of the "stemness" of MaSCs is the *in vivo* outgrowth into a functional mammary gland. In this regard, MaSCs injected into the mammary gland could be marked with e.g. a fluorescent protein in order to be able to identify the implanted MaSCs and to discriminate them from the MaSCs which are already present in the mammary gland. Another option would be the use of a cleared fat pad model (cfr. General Introduction), where the MaSCs of an individual would be injected into the adipose tissue of a cleared mammary gland from another individual. By doing so, the repopulation activity, multilineage differentiation capacities and self-renewal potential of MaSCs can be assessed *in vivo*. The cleared fat pad is considered the "gold standard" assay of functional MaSCs, because the mammary fat pad is essential for the development of mammary epithelium, provides signals that mediate ductal morphogenesis, and stimulates alveolar differentiation (Neville et al., 1998). Although such an approach is difficult to achieve in horses, the transplantation of human normal and neoplastic mammary tissue (Outzen and Custer, 1975) and even human MaSCs (Vafaizadeh et al., 2012) into cleared fat pads of nude mice have been successfully reported. This should encourage scientists to adapt the cleared fat pad model for other mammals or to develop other assays to evaluate MaSC functioning.

In the course of mammary gland development and differentiation, it has been shown that MaSCs continue to proliferate by means of symmetric cell divisions in the mammary fat pad until the ducts reach the margins, at least in mice, indicating the transitive nature of this micro-environment (Woodward et al., 2005). For the bovine mammary gland, it is hypothesized (Capuco et al., 2012) that during mammary gland development asymmetric cell

division would occur in a plane that is perpendicular to the basal membrane and is located more or less vertically on the symmetrical cell division orientation, similar to what has been reported in skin studies (Blanpain and Fuchs, 2009; Jones and Simons, 2008) (Figure 4). Indeed, the differences in cell orientation and growth patterns affects the destination of MaSCs. Still, what the exact impact of the MaSC niche is and how it influences the behavior of MaSCs has yet to be evaluated.

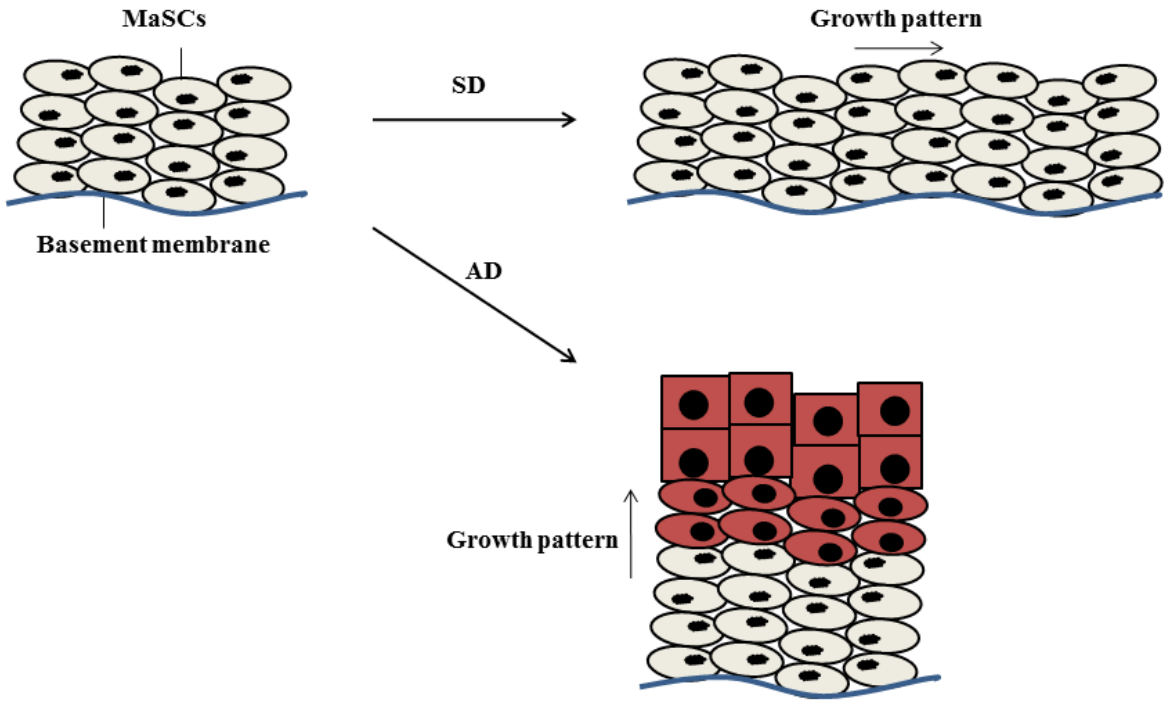


**Figure 4.** Hypothetical growth pattern of mammary stem/progenitor cells (MaSCs) after symmetric (SD) and asymmetric cell division (AD).

*MSCs and MaSCs: similarities and differences*

Interestingly, when comparing the results on MSCs and MaSCs obtained in the present thesis, it became clear that these two cell types shared many properties. Besides a similar CFU efficiency, both equine SC types expressed immunophenotypic markers such as CD29 and CD44 (Spaas et al., 2012; Spaas et al., 2013) and also the proliferation marker Ki67 was

present on MaSCs (Spaas et al., 2012) as well as MSCs (Figure 5, unpublished data). Moreover, we could demonstrate that both equine SC types share similar differentiation capacities, since both MaSCs and MSCs were capable to differentiate into adipocytes (Spaas et al., 2012; Spaas et al., 2013).

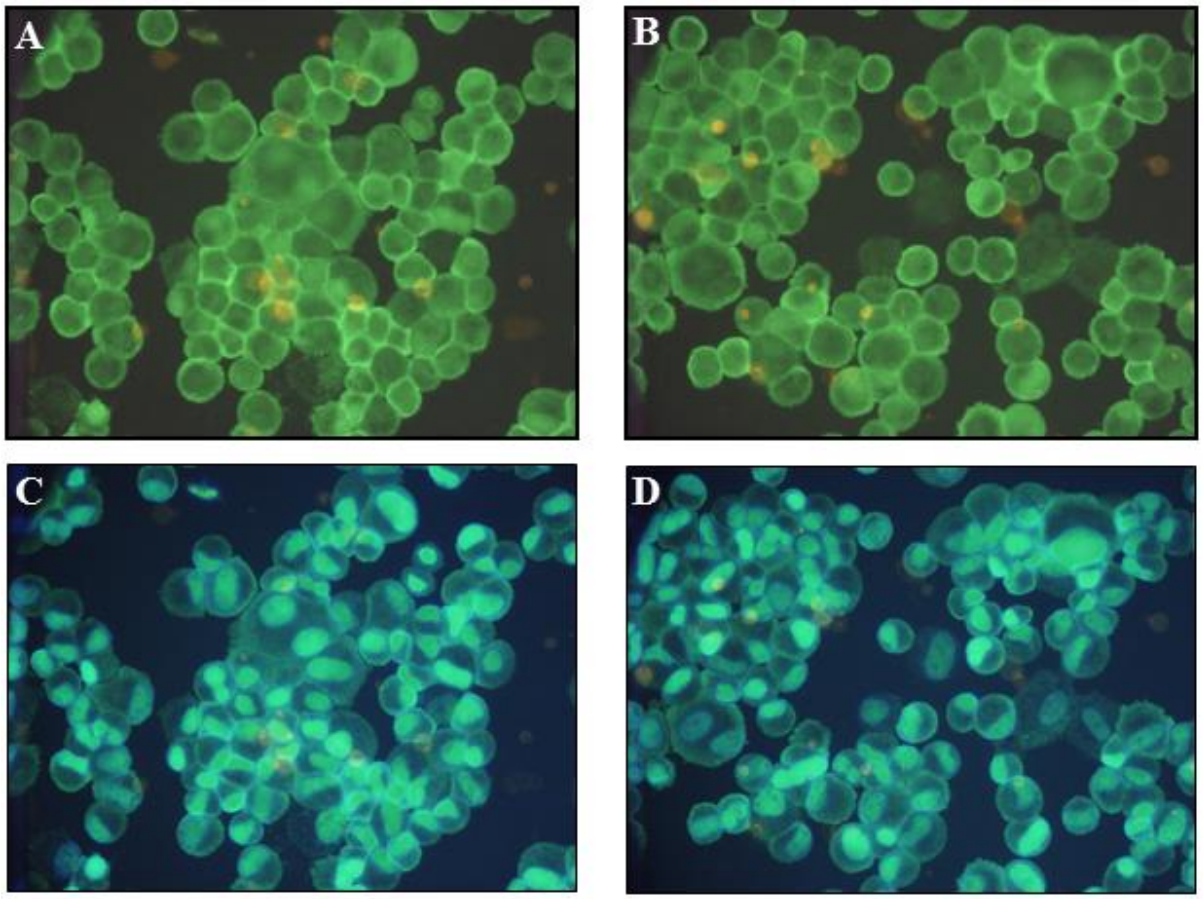


**Figure 5.** Immunofluorescence staining with the proliferation marker Ki67 (**A**-**D**) on cytospins of equine mammary stem/progenitor cells (MaSCs, **A** & **C**) and equine mesenchymal stem cells (MSCs, **B** & **D**). In panel **C** & **D** the nuclei were visualized after counterstaining with Hoechst.

On the other hand, differences in characteristics, i.e. marker expression, were also found. The equine MaSCs described in this thesis were positive for casein kinase 2β (Figure 6, unpublished data) and epithelial specific antigen (ESA) (Figure 7, unpublished data), which was to be expected, since they are two epithelial-specific markers, which is in contrast to equine PB-derived MSCs that need to be negative for these two markers (Figure 6 & 7). Not only do these SC types share several characteristics, it has been described in literature that

they can influence each other. In a study of Klopp et al., it was reported that human bone marrow (BM)-derived MSCs increased human mammosphere formation in a dose-dependent manner (Klopp et al., 2010). Moreover, these mammosphere-derived cells expressed less E-cadherin and more N-cadherin when compared to mammary cells cultivated without MSCs (Klopp et al., 2010). Based on this finding, the authors concluded that this was an indication that mammosphere-derived cells obtain a mesenchymal phenotype under the influence of MSCs.

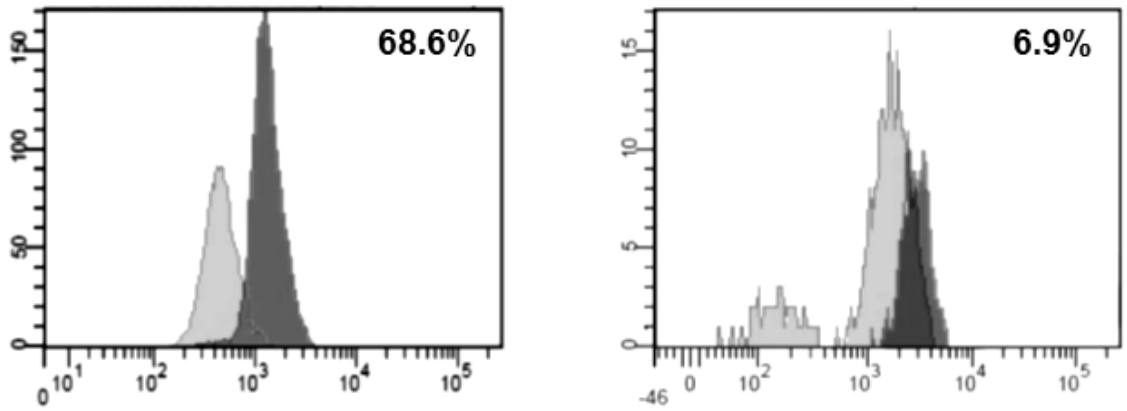


**Figure 6.** Flow cytometry of casein kinase 2 (dark grey histograms) demonstrated that 68.6% of the equine mammary stem/progenitor cells (MaSCs, left) were positive, whereas only 6.9% of the equine mesenchymal stem cells (MSCs, right) expressed this enzyme. Light grey histograms represent the isotype controls.

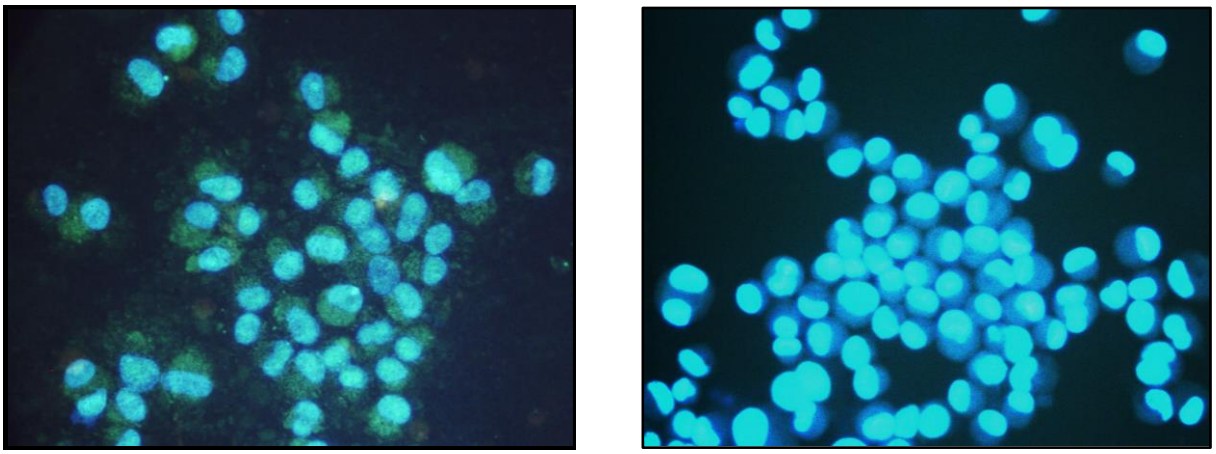

**Figure 7.** Immunofluorescence staining with the epithelial specific antigen marker (ESA) and nuclear counterstaining with Hoechst on cytospins of equine mammary stem/progenitor cells (MaSCs, left) and equine mesenchymal stem cells (MSCs, right).

A few years later, Xu et al. reported that this epithelial-mesenchymal transition (EMT), which is associated with cancer progression and metastases, was regulated by MSC-associated production of transforming growth factor-β1 (TGF-β1) (Xu et al., 2012). Since it has been reported that TGF-β1 reduces epithelial cell proliferation within the mammary gland (Lamote et al., 2004), one might expect an antagonistic effect on tumor growth. Nonetheless, inhibiting TGF-β1 effects of BM MSCs disrupted the cytokine network mediating the interaction between MSCs and breast cancer cells (Shangguan et al., 2013). Consequently, BM MSCs significantly inhibited growth and metastasis of co-inoculated cancer cells, whereas the opposite was the case before TGF-β1 inhibition. On the contrary, it has been reported that the conditioned media of human adipose-derived MSCs displayed an antiproliferative activity on human mammary tumor cells, where coculturing both cell types stimulated the proliferation of the tumor cells as reported for BM MSCs (Trivanović et al., 2013). As such, preactivation of human BM MSCs with tumor necrosis factor (TNF)-α enhanced their tumor-suppressive properties and may represent a useful strategy to develop MSC-based approaches for the treatment of cancer (Lee et al., 2012). By treating both normal and malignant breast epithelial tissue with human recombinant TNF-α alone, a specific cytotoxicity towards malignant cells could be noticed (Dollbaum et al., 1988). In addition, adding TNF-α to healthy rat mammary epithelial cells might even stimulate their proliferation (Ip et al., 1992). Therefore, one could postulate that TNF-α and undoubtedly also other (pro)-inflammatory cytokines play an important specific communicative and regulating role which determines the destination and even function of SCs and their progeny. For all the aforementioned reasons, studying the interaction between MSCs and MaSCs might not only lead to novel insights into tissue regeneration, but also in the mechanisms of tumor progression or regression.

Aside from the interaction between MSCs and MaSCs, there still are several questions that remain unanswered regarding how these interactions might influence the SC niche or vice versa, how the niche can influence these interactions. For example, it is well known that a correct spatial and temporal interaction between different cell types, such as SCs, and between cells and their environment is essential for proper functioning of cells and an efficient tissue regeneration, as has been shown in the case of MSCs (Borjesson and Peroni, 2011; Fortier and Smith, 2008). Taken together, more research is definitely needed to study (i) the common and specific characteristics of MSCs and MaSCs, (ii) mutual interactions and (iii) their interaction(s) with the micro-environment or niche.

### 6.3. The role of equine stem cells in regenerative therapy

The safety, efficacy and complications of stem cell (SC) therapy has divided public opinion. To date, clinical applications of embryonic SCs (ESCs) have been limited, mainly because of the risk of *in vivo* teratoma formation (Chou and Yabuuchi, 2011; Fong et al., 2010) and immune rejection (Menendez et al., 2005), at least in humans. However, an *in vivo* study using an equine model of acute surgically induced superficial digital flexor tendon (SDFT) injury revealed no teratoma formation or immune rejection following the injection of undifferentiated equine ESCs (Guest et al., 2010). As a result one could postulate that equine ESCs might not have the same properties as ESCs from other species that have the potential to form teratomas *in vivo*. Nonetheless, the authors used the term embryonic stem-“like” cells, indicating that further characterization of the isolated cells was warranted in order to confirm their potency and pureness. Still, the risk of immune rejection remains present and might be reduced by using induced pluripotent stem (iPS) cells, which can be derived from the patient itself, although the effect of inducing pluripotency genes on the host’s immune system has not

been studied yet in horses and *in vivo* teratoma formation was observed upon injection of equine iPS cells in mice (Nagy et al., 2011).

In order to avoid teratoma formation and potential immune rejections, adult SCs from the patient itself, also named autologous SCs, have been proposed. Several studies report the therapeutic use of autologous mesenchymal SCs (MSCs) in horses (Smith, 2008; Wilke et al., 2007). On the other hand, the safe use of MSCs from a donor which is different from the recipient, also named allogeneic MSCs, has been described in both humans and horses (Carrade et al., 2011a; Carrade et al., 2011b; Fang et al., 2007; Guest et al., 2008; Ringden et al., 2006; Riordan et al., 2009). The use of autologous as well as allogeneic MSCs has been shown to induce joint swelling and lameness for a short period of time in treated horses (Carrade et al., 2011b). Hereby, an inflammation within the synovial fluid which induced an increase in white blood cell count and protein concentration has also been reported (Carrade et al., 2011b). The third Aim of the present thesis was to clinically evaluate autologous MSCs in an equine patient with a non-induced orthopedic injury. After the second injection with autologous MSCs, a localized swelling of the treated joint was noticed. The exact reason for the observed swelling in the pastern joint remains unanswered. However, a potential explanation could be that the presence of MSC debris initiated a short period of swelling and pain at the injury site. On the other hand, one could postulate that cell attachment and defect filling may stimulate local nerves and create a short inflammatory reaction after transplantation. It also needs to be mentioned that the MSCs were expanded in a medium containing fetal calf serum (FCS), and residues of bovine proteins could cause an immune response after repeated injections in another animal species. Nevertheless, the exact reason for the noticed swelling after the second treatment in the present case report remains to be determined.

Although MSCs are able to differentiate into cells of the damaged tissue *in vitro*, the question remains if they can regenerate injured tissues *in vivo*. In this thesis, we report the first use of peripheral blood (PB)-derived MSCs to treat a horse suffering from chronic degenerative joint disease (DJD). It has to be noted that besides this single case report with a positive clinical outcome of DJD after MSC therapy, only a few placebo-controlled double blind studies have been performed in horses and those had a more reserved outcome (Frisbie et al., 2009; Mcllwraith et al., 2011; Wilke et al., 2007). The major advantage of our study was the application of MSCs in a patient with a chronic naturally occurred DJD of the pastern joint, rather than experimentally inducing cartilage lesions. The clinical improvement in this one case report might have been attributable to cartilage regeneration or to the inhibition of pro-inflammatory cytokines, as previously reported (Frisbie et al., 2009; Jing et al., 2003). Nevertheless, based on a single patient as reported in the present thesis, no definite conclusions can be made concerning the clinical effects of MSC treatment on DJD in horses.

In order to determine whether MSCs really regenerate the injury or stimulate the surrounding cells to do the work for them, MSCs should be labeled and histological sections could confirm which cells were at the origin of a possible regenerative response. In this regard, it has been recently described in donkeys that carpal joint arthrosis clinically improved at 2 months and 6 months after treatment with bone marrow (BM)-derived MSCs and that green fluorescent protein-labelled MSCs integrated in the cartilage, which indicated that the MSCs participated in the healing process of the damaged tissue (Mokbel et al., 2011). In the present case report no information was available on the amount of equine MSCs that remained in the injury site after intra-articular injection. In horses with naturally occurring tendon lesions, it has been reported recently that only 24% of the Technetium99m-labeled MSCs were still present at 24 hours after intralesional injection (Becerra et al., 2013). A similar setup has been previously used for the evaluation of MSCs transfected with a green

fluorescent protein (GFP)-expressing plasmid in tendon lesions in horses (Guest et al., 2008). In the latter study, the authors demonstrated that autologous as well as allogeneic MSCs integrated in the damaged tendons. However, after 34 days only $10^3$ of $10^6$ transplanted cells, i.e. 0.1% of the injected cells, were localized within the lesion whereas the majority of the cells had migrated into the surrounding tissue, indicating a strong need for optimizing current transplantation techniques.

Hereby, it has to be mentioned, that BM mononucleated cells and BM-derived MSCs had similar effects on tendon extracellular matrix (type I/III collagen ratio and COMP) (Crovace et al., 2010; Lacitignola et al., 2008). One could expect that any cell transplantation or maybe even a single epitope might be sufficient to induce the observed effects. Nonetheless, it has been reported that acellular BM also has the capacity to stimulate COMP synthesis in SL explants, even more than growth factor-based treatments, such as platelet-rich plasma (PRP) or platelet-poor plasma (PPP) (Schnabel et al., 2008). This confirms the earlier mentioned hypothesis (cfr. General Introduction) that the products MSCs secrete might be at the basis of the observed effects. For all these reasons, it is warranted to investigate the *modus operandi* of cell-based therapies and to identify healing enhancing cytokines, growth factors or metabolites. In conclusion, we can state that research to develop new and better regenerative therapies is a rapidly expanding field, making efficient treatments in the field of musculoskeletal injuries a reality for human as well as equine athletes in the nearby future.

To our knowledge, no clinical applications using mammary stem/progenitor cells (MaSCs) have been reported to date. Nevertheless, because MaSCs provide mammary cell renewal and turnover, several application possibilities have been pointed out in different studies. In this regard, it has been reported that MaSCs could benefit milk yield and persistency (Capuco et al., 2003; 2009; 2012), repair of damaged mammary tissue caused by mastitis or trauma

(Borena et al., 2013; Capuco et al., 2012), and maybe even provide a cure for breast cancer by studying tumoral transformation (Bierla et al., 2012; Borena et al., 2013; Sagar et al., 2007).

In chapter 4 of the present thesis, we described the isolation of MaSCs from slaughtered mares, however, in field circumstances taking tissue samples of 5cm² would not be ethical nor meaningful. For this reason, one might suggest to optimize the isolation technique for smaller biopsies or propose the use of allogeneic MaSCs, in line with what has been reported for MSCs. In the case of tissue damage or breast cancer studies this could be defendable after further investigation of the MaSCs for rejection proteins, possible side effects and carcinogenic properties. Furthermore, it has to be noted that mammary gland epithelial cells are likely to play an important role in the defense against intramammary infection by increasing pro-inflammatory substances, such as interleukin (IL)-8, tumor necrosis factor (TNF)-α, serum amyloid A and Lactoferrin (Wellnitz and Kerr, 2004). Increasing the inflammation is desirable in the case of pathogen exposure and in the light of the aforementioned tumor-suppressive properties, although leading to more tissue damage followed by a decrease in milk production (De Olives et al., 2013). In order to counteract an unnecessary decline in milk production due to an excessive inflammatory response, it would be interesting to illuminate the role of MaSCs or MSCs as an immunomodulating agent, as previously reported for the latter (Kode et al., 2009). It should be mentioned though, that such an approach is quite controversial in mammals with a high risk for breast cancer development (i.e. humans and carnivores) and should be interpreted with caution. Indeed, it has been recently reported by several groups that the immunosuppressive effects of human MSCs would possibly be correlated with tumor growth in breast cancer (Ljujic et al., 2013; Mandel et al., 2013). Therefore, future studies should focus on whether or not the immature MaSCs also possess these immunosuppressive properties and should be considered as a possible target for future cancer therapies (besides cancer SCs). Indeed, elucidating the *modus*

*operandi* of MaSCs might bring us one step closer to understanding their different roles in the (mal)functioning of this complex organ.

On the other hand, in order to increase milk production of healthy mammary glands in herbivores (especially cows and horses for economic purposes), the use of techniques which could stimulate or activate MaSCs *in situ*, could be a valuable option. As such, it has been found that an intramammary infusion of xanthosine, which suppresses asymmetric SC division by p53 inhibition, resulted in an increased number of MaSCs by promoting symmetric SC divisions (Capuco et al., 2009; Choudhary et al., 2012). In addition, a large number of treatments or agents have been shown to increase milk production and/or cell proliferation and it would be interesting to study whether this is caused by a direct or indirect effect on MaSCs. For example, increasing the milking frequency (Bar-Peled et al., 1995) and photoperiods (longer period of light per day) (Dahl et al., 1997) induces an increase in milk production. Daily injections with bovine somatotropin was shown to increase cell proliferation during late gestation in sheep (Stelwagen et al., 1993), as well as mammary growth during mid-lactation in goats (Knight et al., 1990). In both studies this phenomenon was followed by an enhanced milk yield. In addition, insulin-like growth factor-I (IGF-I) infusion into the local arterial mammary gland supply of goats increased milk synthesis as well (Prosser et al., 1990; Prosser and Davis, 1992). Since the ability of IGF-I to induce cell proliferation has been demonstrated in several studies, a link between cell proliferation and milk yield is easily made (Baumrucker and Erondu, 2000; Hadsell et al., 2002; Peaker and Linzell, 1974).

Based on all the cited studies, it could be hypothesized that cell proliferation instead of a higher secretory capacity or activity of the mammary cells lies at the basis of the noted enhanced milk yield, and therefore, that regulation of the MaSC pool might be sufficient to increase milk production. It has to be mentioned though, that estrogen and progesterone

induce proliferation of the mammary epithelium throughout gestation (Vangroenweghe et al., 2005), and MaSCs do not express receptors for these sex steroid hormones (Capuco et al., 2012). In this regard, one might postulate that instead of MaSCs, the progenitor cells are responsible for intramammary epithelial cell proliferation, because they do express estrogen and progesterone receptors (Capuco et al., 2012). However, it is not excluded that these steroid hormones are providing indirect signals to the MaSCs through an intercellular communication network that is still not elucidated.

Since it has been proven that cell proliferation within the mammary gland is at least partially driven by MaSCs (Capuco et al., 2009; Vaillant et al., 2011) and that the MaSC pool increased 14-fold due to maximal progesterone levels during the luteal phase in mice (Joshi et al., 2010), it might be postulated that estrogen and progesterone receptor positive cells release the major mitogen(s) for MaSC expansion in the mammary gland. In addition, the results of the present thesis confirm that a significant increase in mammosphere forming cells could be noticed after harvesting mammary glands from lactating mares (in comparison to non-lactating mares). This supports the theory that MaSCs are indeed the forces driving mammary gland development.

For all the aforementioned reasons, future studies should definitely focus on intercellular communication mechanisms between MaSCs and their surrounding counterparts. More research is not only warranted to provide more insights in the mammary gland physiology but also to the effects of these mediators on breast cancer (stem) cells.

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# SUMMARY

Stem cells (SCs) are undifferentiated cells which have the capacity to divide without differentiation or to form more specialized daughter cells. Traditionally, they are divided into 2 groups. The first group contains embryonic SCs (ESCs) that are able to differentiate into all the different cell types that are present in the body (pluripotency). The second group consists of adult or tissue specific SCs that have the capacity to mainly form cell types of their own germ layer (multipotency). The present thesis focuses on 2 different types of adult multipotent SCs in horses: (i) peripheral blood (PB)-derived mesenchymal SCs (MSCs), for their potential use in regenerative medicine and (ii) mammary gland stem/progenitor cells (MaSCs), which can represent an important tool for research on mammary gland development and disease, ie. breast cancer. More specifically, the present thesis focuses on the optimization of the isolation and characterization techniques of PB-derived MSCs and MaSCs in the horse, followed by a preliminary study describing a clinical application of characterized PB-derived MSCs.

Although equine MSCs can be easily isolated from bone marrow, adipose tissue and umbilical cord blood, there is the need for a less invasive and more practitioner-friendly source of MSCs. In this regard, PB represents an elegant and highly accessible source of MSCs. However, for therapeutic use, a major setback is that although several clinical trials with MSCs have been performed in horses, in most cases they are lacking a fundamental background. Indeed, this technology is still in an early stage, mainly because of the lack of horse specific markers for SC identification and because of the absence of a thorough fundamental characterization. Nevertheless, a fair knowledge of the SC biology is indispensable for the development of rationally sound SC therapies. For all the aforementioned reasons, an isolation and characterization of equine PB-derived MSCs was performed in chapter 3 which increased the currently available data on PB-derived MSCs. To this end, the properties of the isolated cells were assessed by means of different characterization techniques consisting of a functional and immunophenotypic component. The

functional characterization consisted of colony forming unit (CFU) assays as well as population doubling time (PDT) calculations in order to evaluate the self-renewal capacities of the cells on the one hand, and a trilineage differentiation towards osteoblasts, chondroblasts and adipocytes on the other hand. Secondly, an immunophenotypic characterization was performed in order to evaluate typical MSC markers (CD29, CD44, CD90 and CD105) and adult blood cell markers (CD45, CD79α, MHC II and Mφ marker). Because of this optimized characterization, the quality of PB-derived MSCs can now be thoroughly assessed before therapeutic application.

To date, there are no data available about the presence of MaSCs in the equine mammary gland. However, the mammary gland is a highly regenerative organ that can undergo multiple cycles of proliferation, lactation and involution, processes that are driven by MaSC proliferation and differentiation. Over the years, important data has been gathered on MaSCs of women and mice in the light of breast cancer research. Also canine and bovine MaSCs are receiving increased attention with regard to mammary tumors and increasing milk production, respectively. Studying the potential of equine MaSCs, however, could be of interest for understanding the SC biology and functioning in this dynamic organ at different physiological stages. Therefore, this thesis also covers the isolation and characterization of equine MaSCs in chapter 4. Similar to MSCs, this characterization consisted of a functional as well as immunophenotypic component in order to confirm the SC properties of the isolated cells. The isolated equine cells were able to self-renew and to differentiate into multiple cell lineages. Moreover, the cells were immunophenotyped using markers for CD29, CD44, CD49f and Ki67. Afterwards, MaSCs of non-lactating and lactating mares were compared. Here, we found that lactating mares contained significantly more mammosphere forming cells which were in a higher proliferative state. As a result, the mammosphere assay was proposed as a valuable assay to study MaSCs during different physiological stages. Taken together, chapter

4 is the first report of MaSC isolation in horses and extends the current knowledge of mammary gland biology.

In chapter 5 the clinical use of equine PB-derived MSCs was preliminary evaluated in a non-induced orthopedic injury. To this end, autologous PB-derived MSCs were injected locally in the pastern joint of a horse suffering from naturally occurred chronic degenerative joint disease (DJD). Since the injury was only clinically evaluated without the presence of sham-treated control horse or any functional data, this case report should be considered as purely descriptive, yet providing a basis for further research.

In conclusion, the present thesis describes the isolation and characterization of PB- and mammary gland-derived equine adult multipotent SCs which fulfilled all the requirements to be typed as MSCs and MaSCs, respectively. Nevertheless, controlled and scientifically sound studies concerning the biological properties and regenerative capacities of equine SCs are of vital importance in order to achieve the goals which were set for regenerative medicine and cancer research.

# SAMENVATTING

Stamcellen (SC'n) zijn ongedifferentieerde cellen die enerzijds onbeperkt kunnen delen zonder te differentiëren en anderzijds meer gespecialiseerde cellen kunnen aanmaken. Traditioneel worden ze ingedeeld in twee groepen: embryonale SC'n die in staat zijn om te differentiëren tot alle mogelijke celtypes van het lichaam (pluripotentie) en adulte of weefselspecifieke SC'n met een differentiatiecapaciteit naar voornamelijk celtypes van hun eigen kiemlaag (multipotent). Het huidige proefschrift beschrijft twee verschillende typen van adulte multipotente SC'n bij paarden: mesenchymale SC'n (MSC'n) die potentieel hebben voor gebruik in de regeneratieve geneeskunde, en melkklier stam/voorlopercellen (MaSC'n) die kunnen aangewend worden in studies gericht op de verhoging van de melkproductie of op de pathogenese van borstkanker. Aangezien beide SC typen een weefsel of orgaan kunnen genereren, liggen ze aan de basis van de normale werking van dit orgaan. Defecten in hun normale fysiologie kunnen daarom leiden tot afwijkingen zoals ongebreidelde vermenigvuldiging (kanker). Om voorgaande redenen is het huidige proefschrift gericht op het optimaliseren van de isolatie van MSC'n uit het perifeer bloed (PB) en MaSC'n uit de melkklier, en een typering van de karakteristieken van beide stamceltypen, gevolgd door een klinische toepassing van PB MSC'n.

Hoewel bij paarden MSC'n gemakkelijk geïsoleerd kunnen worden uit beenmerg, vetweefsel en navelstrengbloed, bestaat er een nood aan een minder invasieve en meer gebruikersvriendelijke bron van MSC'n. In dit opzicht is het PB een elegante en zeer toegankelijke bron van MSC'n. Ook al werden er reeds verschillende klinische studies met MSC'n beschreven, toch ontbreekt de fundamentele basiskennis in veel gevallen. Dit komt mede doordat deze technologie zich nog in een vroeg ontwikkelingsstadium bevindt, vooral door het gebrek aan paarden-specifieke merkers voor de identificatie van SC'n en door het ontbreken van een diepgaande fundamentele karakterisatie van deze cellen. Toch is een

kennis van de stamcelbiologie onontbeerlijk voor de ontwikkeling van rationeel-gefundeerde stamceltherapieën.

Om al deze redenen werd de isolatie en karakterisatie van paarden PB MSC'n uitgevoerd in hoofdstuk 3. De eigenschappen van de geïsoleerde cellen werden bepaald door een functionele en immunofenotypische karakterisatie. Functionele parameters waren de volgende: (i) kolonievormende eenheid assays, (ii) populatieverdubbelingstijd en (iii) differentiatie naar osteoblasten, chondroblasten en adipocyten. De immunofenotypische karakterisatie evalueerde typische MSC-merkers (CD29, CD44, CD90 en CD105) en volwassen bloedcelmerkers (CD45, CD79α, MHC II en Mφ merker). Dankzij deze optimalisatie kan de kwaliteit van MSC'n geïsoleerd uit het PB nu grondig geëvalueerd worden vooraleer ze therapeutisch toe te passen bij paarden.

Tot op heden zijn er nog geen gegevens bekend over de aanwezigheid van MaSC'n in de paardenmelkklier. De melkklier is een zeer regeneratief orgaan dat meerdere cycli van proliferatie, lactatie en involutie kan ondergaan, allemaal processen die worden aangedreven door MaSC proliferatie en differentiatie. In de loop der jaren zijn er belangrijke gegevens verzameld over MaSC'n van vrouwen en muizen in het kader van borstkankeronderzoek. Ook MaSC'n van hond en rund krijgen meer aandacht respectievelijk als model voor mammatumoren en als onderzoeksonderwerp voor het verhogen van de melkproductie. Het bestuderen van paarden MaSC'n zou van belang kunnen zijn voor het begrijpen van de stamcelbiologie en het functioneren van dit dynamische orgaan in de verschillende fysiologische stadia.

Om voorgaande redenen behandelt dit proefschrift ook de isolatie en karakterisatie van paarden MaSC'n in hoofdstuk 4. Zoals voor MSC'n, bestaat de karakterisatie van MaSC'n uit een functionele en immunofenotypische component om de stamceleigenschappen van de geïsoleerde cellen te bevestigen. Hierbij waren de geïsoleerde paardenmelkkliercellen in staat

om zichzelf te vermeerderen en te differentiëren naar meerdere cellijnen. Bovendien werden de cellen met verschillende merkers (CD29, CD44, CD49f en Ki67) immunofenotypisch gekarakteriseerd. Daarna werden de MaSC'n van niet-lacterende en lacterende merries met elkaar vergeleken. Hierbij werd vastgesteld dat bij lacterende merries significant meer mammosfeervormende cellen geïsoleerd werden die zich in een hogere proliferatieve toestand bevonden. Bijgevolg werd de mammosfeer assay voorgesteld als een waardevolle assay om MaSC'n te bestuderen tijdens verschillende fysiologische stadia. Deze studie is de eerste die rapporteert over de isolatie van paarden MaSC'n en vormt een belangrijke uitbreiding op de huidige kennis van de melkklierbiologie.

In hoofdstuk 5 wordt de klinische toepassing van equine PB MSC'n preliminair geëvalueerd in een niet-geïnduceerd orthopedisch letsel. In dit verband werden autologe PB MSC'n geïnjecteerd in het kroongewricht van een paard met een natuurlijk voorkomende chronische degeneratieve gewrichtsaandoening. Aangezien het letsel enkel klinisch geëvalueerd werd zonder controlegroepen of functionele data, moet deze case report enerzijds beschrijvend en anderzijds als basis voor verder onderzoek beschouwd worden.

In conclusie beschrijft dit proefschrift de isolatie en karakterisatie van volwassen multipotente SC'n, geïsoleerd uit het PB en de melkklier, die aan alle eisen voldoen om getypeerd te worden als MSC'n en MaSC'n. Toch zijn gecontroleerde en wetenschappelijk onderbouwde studies over de biologische eigenschappen en regeneratieve capaciteiten van paardenstamcellen van vitaal belang om dergelijke SC'n voor regeneratieve geneeskunde en kankeronderzoek te kunnen gebruiken.

# CURRICULUM VITAE

Jan Spaas werd op 6 januari 1986 geboren in Bilzen. Hij doorliep de eerste 2 jaar van zijn middelbare school in het Biotechnicum in Bocholt en vervolgde zijn middelbare schoolopleiding in de richting Latijn-Wiskunde op het Sint-Augustinusinstituut in Bree.

Vanaf 2004 volgde hij de opleiding diergeneeskunde aan de UGent, waar hij in 2007 het diploma van Bachelor in de Diergeneeskunde met onderscheiding wist te behalen. In 2010 werd hij Master in de Diergeneeskunde met onderscheiding. Zijn masterproef handelde over paardenstamcellen en hun eigenschappen en toepassingsmogelijkheden. Hiervoor heeft hij een periode doorgebracht in het laboratorium van Experimentele Geneeskunde aan de Faculteit Geneeskunde van de Universiteit van Rome (Italië). Deze samenwerking heeft geleid tot het winnen van de prijs voor jonge auteurs van het Vlaams Diergeneeskundig Tijdschrift.

Vervolgens behaalde hij een strategische onderzoeksbeurs van het Agentschap voor Innovatie door Wetenschap en Technologie (IWT) en trad hij in dienst aan de Vakgroep Vergelijkende Fysiologie en Biometrie van de Faculteit Diergeneeskunde (UGent). Zijn onderzoek spitste zich toe op stamcellen van perifeer bloed en de melkklier bij het paard. Daarnaast leverde hij ook een bijdrage aan het praktisch onderwijs en begeleidde verschillende masterstudenten. Op deze manier werd Jan Spaas auteur van meerdere publicaties in internationale tijdschriften en presenteerde hij zijn onderzoek op internationale congressen.

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