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Characterization of Stem Cell Phenotype and Adipogenic Potential of Human and Rat Primary Adipose-Derived Mesenchymal Stem Cells

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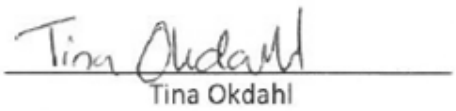

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ABSTRACT

Background: Cutaneous wound healing is a complex process, which can be disrupted by various pro-inflammatory factors within the area. Development of chronic wounds, which stalls in an inflammatory state, is a substantial health care problem with growing number of incidences. Mesenchymal stem cells (MSCs) are somatic stem cells found in various adult tissue. Research have shown that MSCs possess potent anti-inflammatory properties brought on mainly by paracrine mechanisms. Application of MSCs in a novel chronic wound treatment strategy aimed to force the inflammatory state of the injured tissue towards an anti-inflammatory microenvironment is an intriguing future perspective.

Aim: The aim of the present study was to characterize the stem cell phenotype of primary human and rat adipose-derived mesenchymal stem cells (hASCs and rASCs). Furthermore, the adipogenic potential of the cells was investigated.

Methods: Primary ASCs isolated from human and rat abdominal fat tissue were cultured in various induction media in the attempt to induce adipogenesis, osteogenesis, and chondrogenesis. Differentiation was subsequently investigated both histochemically and transcriptionally. Proliferation rate and proportion of colony-forming units in the cell populations were likewise investigated. Lastly, *in vitro* endothelial cell scratch assays were conducted in order to evaluate the angiogenic potential of the isolated cells.

Results: Adipogenic and chondrogenic differentiation were successful in hASC cultures, whereas only adipogenesis was achieved in rat culture. Both hASCs and rASCs proved to have high proliferative potential as well as being clonogenic in nature. Conditioned medium (CM) harvested from hASC cultures failed to accelerate healing of the endothelial scratch in the *in vitro* setup. In contrast, application of rASC-CM significantly reduced healing time of the rat endothelial cell monolayer.

Conclusion: In summary, the phenotypical analyses of isolated hASCs and rASCs indicate that the cells were in fact active and viable mesenchymal stem cells. Further attempts on tri-lineage differentiation are, however, necessary in order to validate the multipotency of the cells. The reduced healing time of rASC-CM-treated endothelial cells indicate that the rASCs secrete factors important for angiogenesis, which is relevant for future application of the cells in a diabetic rat chronic wound model.

ABBREVIATIONS

ASC	Adipose-derived mesenchymal stem cell
bFGF	Basic fibroblast growth factor
C-AIM	Continuous adipogenic induction medium
CFU	Colony-forming unit
CIM	Chondrogenic induction medium
CM	Conditioned medium
EGF	Endothelial growth factor
ESC	Embryonic stem cell
EVs	Extracellular vesicles
hASC	Human ASC
HDMEC	Human dermal microvascular endothelial cell
IFN-γ	Interferon-γ
M1	Classically activated pro-inflammatory macrophage
M2	Alternatively activated anti-inflammatory macrophage
MSC	Mesenchymal stem cell
OIM	Osteogenic induction medium
P-AIM	Phased adipogenic induction medium
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
rASC	Rat ASC
REC	Rat endothelial cell
Tregs	Regulatory T-cell
VEGF	Vascular endothelial growth factor

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1 - INTRODUCTION

1.1 - HISTOLOGY OF SKIN

Despite being often simply regarded as “the wrapping”, the skin plays an important role in the organism. Not only by being the largest organ of the body, but also for its many diverse functions, e.g. regulation of body fluids and temperature, activation of vitamin D, as well as being subject to tactile sensations. In addition, the skin plays a vital role as the initial barrier against the outside environment protecting the organism from physical and chemical harm as well as intrusion of pathogens. (1,2).

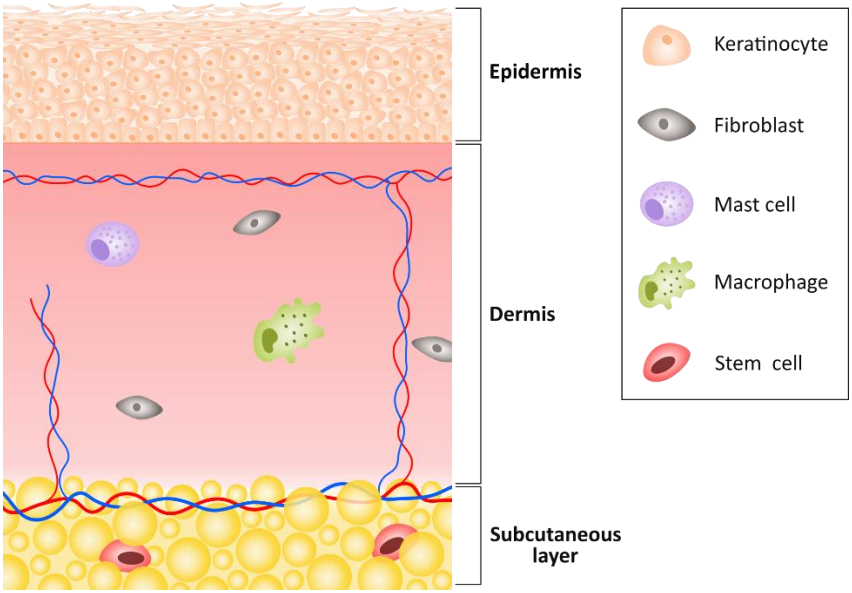

Figure 1.1 - Histological Layers of the Skin. The outer layer, the epidermis, is mainly made up of keratinocytes, which ascend gradually from the basal membrane towards the outer surface, where they are expelled. Connective tissue including collagen and elastin forms the inner layer known as the dermis. In addition to fibroblasts, macrophages and Mast cells, the dermis contains blood vessel, glands and nerves. The underlying subcutaneous layer is mainly composed of adipose tissue.

The skin can be divided into two histologically distinct layers; the dermis and the epidermis (fig. 1.1). The outermost layer, the epidermis, is constantly exposed to tear and wear from the outside environment, thus developing strategies towards enduring mechanical stress. The dominant cell type, the keratinocyte, maintains this task by two means. Firstly, by continuously renewing at the basal level followed by ascension towards the outer surface, whilst differentiating into more and more flattened and durable cell types. In this manner, the epidermis becomes a stratified self-renewing layer with an average turnover rate of about one month. Secondly, the keratinocytes produce the protein keratin, which accumulates in the ascending cells and provides the skin with its resistance towards mechanical stress. The inner layer, the dermis, is composed of

connective tissue primarily consisting of collagen and elastin, which provide the skin with tensile strength and elasticity, respectively. The connective tissue is produced by the fibroblasts, which reside in the dermis. In addition to fibroblasts, the dermis is also inhabited by macrophages and mast cells, which participate in the innate immune response of the skin. Furthermore, the dermis contains blood vessels, nerves, glands, and hair follicles. The dermis is interconnected with a deeper subcutaneous layer primarily consisting of adipose tissue in which somatic stem cells reside. (1,2).

1.2 - CUTANEOUS WOUND HEALING

Whenever the integrity of the skin is compromised by an injury, a highly complex process is initiated in order to restore the homeostasis of the skin. This wound repair process involves a high number of different cell types and bioactive substances, which together orchestrate the restoration of the crucial barrier against the outside environment. Factors such as size, depth, location, cause of the initial injury, and overall health status of the individual influence how long the wound healing process lasts. By convention, the healing of cutaneous wounds is divided into four overlapping phases; (1) Hemostasis, (2) Inflammation, (3) Proliferation and (4) Remodeling (fig. 1.2) (3,4).

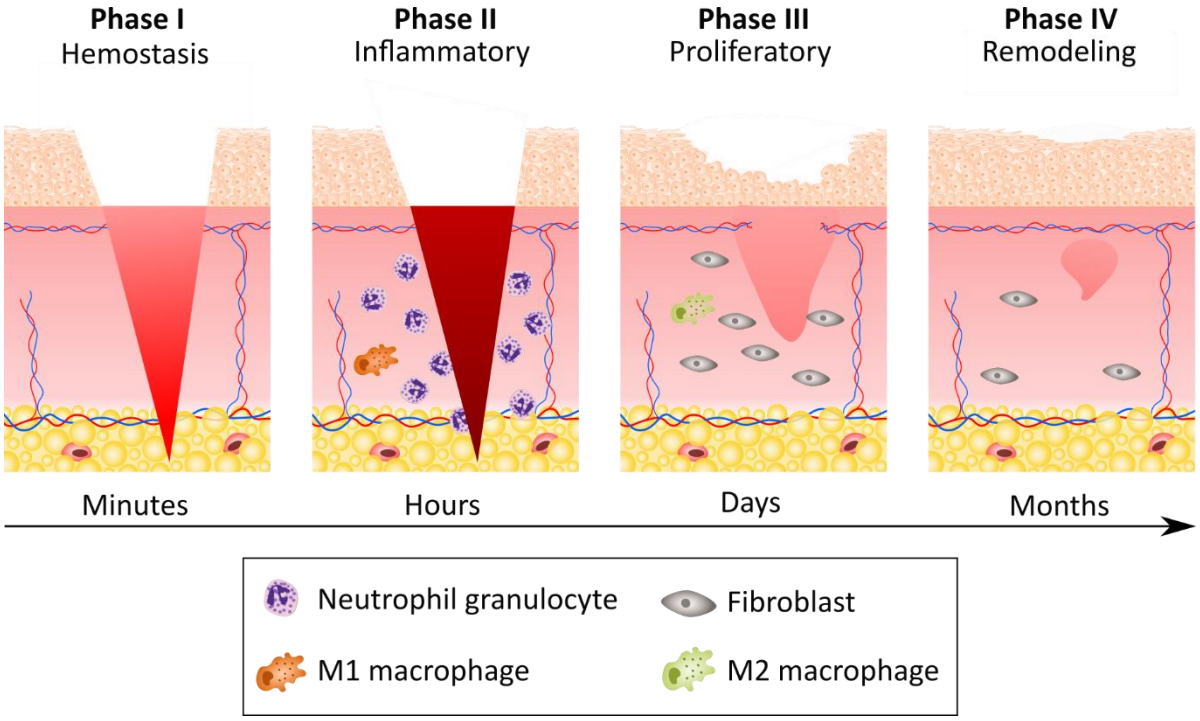

Figure 1.2 - Wound Healing Phases. The first phase of wound healing is initiated within few minutes thus ensuring limited blood loss though a process known as hemostasis. In the inflammatory phase, the wound area is invaded by large numbers of neutrophil granulocytes, which clear the wound of debris and pathogens. Pro-inflammatory M1 macrophages also participate in this process. Within days, the inflammatory phase transcends into a proliferatory phase in which the wound area is filled with immature granulation tissue. Fibroblasts, anti-inflammatory M2 macrophages, angiogenesis and keratinocyte reepithelization is involved in this process. In the remodeling phase, the granulation tissue is replaced with mature tissue and the original properties of the area are restored. This process may last for months or even years.

1.2.1 - HEMOSTASIS

The first event following an injury to the skin is the activation of the coagulation process in order to minimize blood loss from the wound (3). This process is known as hemostasis and is initiated only a few minutes after the injury. Hemostasis involves contraction of the vessel wall, production of fibrin fibers via the coagulation cascade and activation of platelets, all of which contribute to the formation of a blood clot within the injured vessels. In addition to contributing to hemostasis, the fibrin fibers form a network within the injury, which are crucial for the migration of cells in the subsequent phases of wound healing (5). Furthermore, the activated platelets produce proteins, which work as attractors of neutrophil granulocytes, resulting in the influx of a high amount of this cell type to the wound area, thus marking the transition to the next phase (3,4).

1.2.2 - INFLAMMATORY PHASE

Within the first 24 hours, the inflammation phase is initiated (6). The objective in this phase is to clear the wound of potential pathogens as well as cellular and extracellular debris. Initially, this task is carried out by the large numbers of neutrophils mentioned above. These are recruited from the circulation to the wound site by diapedeses brought on by expression of adhesion molecules on the endothelium (3,7). A few days later, monocytes from the circulation are recruited to the wound site and are activated into mature pro-inflammatory M1 macrophages, which participate in the cleaning of the wound site by phagocytosis. In addition to this, the macrophages are crucial for the regulation of the wound healing process and the progression to the next phase. This is achieved by their secretion of various growth factors and cytokines (8).

1.2.3 - PROLIFERATIVE PHASE

Once the injured site has been cleared of cellular and extracellular debris, the inflammation resolves and the proliferative phase begins. This happens approximately after 72 hours (4). This transition is influenced by the switch of the pro-inflammatory M1 macrophage towards the M2 macrophage phenotype with anti-inflammatory properties (3,4,9). The M2 macrophage has a different cytokine profile than the M1 macrophage, resulting in promotion of fibroblast migration and proliferation (3). The fibroblasts produce various components of the immature granulation tissue, which slowly fills the wound (4,5). Another key event in the proliferation phase is the re-epithelialization of the wound. This occurs when the keratinocytes in the epidermal edges of the wound start to proliferate and migrate across the newly formed granulation tissue, eventually leading to closure of the injury (3). The fibroblast and keratinocyte proliferation and migration is highly dependent on sufficient oxygen and nutrient delivery. Angiogenesis is therefore crucial for the proliferation phase to be successful. Factors within the wound area such as low oxygen tension and vascular endothelial cell growth factor (VEGF) participate in the promotion of a network of newly formed capillaries, which are able to accommodate the high oxygen demand from the rapidly diving cells in the wound (3,5). The proliferative phase lasts for approximately two weeks where it slowly transcends into the remodeling phase.

1.2.4 - REMODELING PHASE

In the last phase of wound healing, the purpose is to restore the original properties of the injured tissue. This is achieved by slowly replacing the newly formed granulation tissue with stronger and more mature tissue (5). This happens through remodeling of the granulation tissue. The keratinocytes, which covered the wound in the proliferation phase, begin to produce a new epidermis with the stratified characteristics of the original tissue (5). Collagen III is slowly degraded and replaced with cross-linked parallel-orientated collagen I fibers, which ensure an increased tensile strength of the tissue (3,5). The newly formed network of wound capillaries

disappear and the conventional blood flow to the area is restored (5). The remodeling phase may last for months or even years by the end of which reestablishment of the integrity of the skin barrier is achieved (3).

1.3 - CHRONIC WOUNDS

As the simplified summary above indicates, the process of restoration of homeostasis after cutaneous injury is highly complex and depending on numerous cell types and coordinated timing of key events in order to eliminate the potential life-threatening breach in the protective barrier against the outside environment.

Impaired healing potential of the skin is a substantial health care problem in the Western World (10). A chronic non-healing wound is defined as a wound, which persists for over three months and which do not follow the normal healing phases described above (11,12). Instead of proceeding towards the proliferative phase after a few days, the wound stalls in the inflammatory phase resulting in the microenvironment within the area to become incompatible with healing (13).

Chronic wounds affect a growing number of patients due to increased prevalence of risk factors such as Modern Lifestyle-associated pathologies including obesity, diabetes, and hypertension (12). In addition to decreased quality of life and social isolation, patients are at high risk of developing serious infections in the non-healing wound, potentially resulting in amputation of the affected limb or life-threatening sepsis (11). Furthermore, malignant transformation of the tissue due to the chronic inflammation in the area is a known complication to non-healing wounds (12).

A chronic wound is associated with specific local factors within the wound area, e.g. infection, hypoxia, necrosis, and elevated amounts of inflammatory cytokines (8,11). These local factors are often caused by underlying pathologies such as vasculitis, cutaneous malignancies, vascular insufficiency, or diabetes (8,12,14).

1.3.1 - DIABETIC FOOT ULCERS

Diabetes is a general term covering several metabolic pathologies with impaired insulin production and/or function, resulting in a hyperglycemic state of the blood (15).

Diabetes is associated with many long-term complications, one of which is the development of chronic wounds (3,15). Most commonly, a chronic wound of a diabetic patient develops in the lower extremities, more specifically on the foot. 25% of all diabetic patients will suffer from a chronic foot ulcer during their lifetime, and 15% of these will eventually require amputation of the affected limb. Diabetic foot ulcers are thus a substantial complication to diabetes in terms of both prevalence and severity (3).

Underlying causes for the development of diabetic foot ulcers include continued pressure or trauma to the foot, which is not perceived and eliminated due to peripheral sensory neuropathy (3). The acute wound is at risk of transcending into a chronic wound due to the diabetic state, which have several effects on the healing potential of the skin (16). Firstly, hyperglycemia causes accumulation of so-called advanced glycation end-products (AGEs) (3). AGEs are naturally occurring proteins or lipids with non-enzymatically bound sugar molecules (15). These modified molecules possess altered functional properties that interfere with normal physiological processes (16). Importantly, AGEs seem to play a substantial role in the increased systemic inflammatory level associated with diabetes (3). In regards to wound healing, AGEs have been reported to interfere with the polarization of M1 macrophages towards M2 macrophages, causing a prolonged inflammatory response (16). In addition, *in vitro* studies have shown that fibroblasts cultured with high levels

of AGEs exhibited decreased proliferation rate and collagen synthesis as well as increased apoptosis rate (16). Furthermore, diabetic wounds show decreased angiogenic potential, partly caused by deposition of AGEs in the vascular basement membrane (17). Moreover, the cellular composition in a diabetic wound is altered compared to a normal acute wound. One such difference is the increased neutrophil count (3). As mentioned earlier, infiltration of large numbers of neutrophils to the wound is essential in the inflammatory phase of wound healing. In diabetic mice, however, a prolonged and enhanced neutrophil response can be observed, contributing to the wound becoming chronic (18).

Macro- and microvascular dysfunction, another common complication of diabetes, further complicate the healing process in a diabetic wound (3). The diminished blood flow to the extremities causes not only impaired recruitment of circulatory immune cells to the wounded tissue, but also a hypoxic state with insufficient oxygen supply and increased oxidative stress (3,15).

1.3.2 - CHRONIC WOUND TREATMENT

Treatment of non-healing wounds is currently based on securing optimal conditions for healing in the affected area. This includes removal of necrotic tissue, a process known as debridement, and ensuring ideal moisture balance in the wound by applying various kinds of wound dressings (13,19). Furthermore, monitoring and early treatment of bacterial infections in the wound is crucial for a good prognosis and for preventing bacterial spreading to surrounding tissue or even systemic involvement (13,19). Despite these interventions, 50% of all chronic wounds fail to respond to the traditional treatment regime (8,14) and may remain non-healing for several years (Li).

Novel treatment strategies for non-healing wounds are currently under investigation. Common for many of these is the attempt to push the chronic inflammatory state in the wound area towards the proliferatory state of healing through various immunomodulatory strategies (11,19). In the following, two such promising novel approaches in chronic wound treatment will be introduced.

1.4 - MESENCHYMAL STEM CELLS

Regenerative medicine is an escalating field in medical research concerning restoration of damaged or diseased tissues and organs through methods such as tissue engineering and stem cell therapies (20). The pluripotent embryonic stem cell (ESC), which can be isolated from the inner cell mass of a blastocyst originally seemed like an obvious candidate for regenerative application, because of its ability to differentiate into all types of cells in the adult organism (21). However, limitations to the use of ESCs include ethical considerations and risk of malignant development as well as limitations in supply of human cells (20,22). Focus in regenerative medicine has therefore shifted towards the application of somatic stem cells. These cells represent a more differentiated stage than that of ESCs and are therefore denoted multipotent rather than pluripotent (10). These somatic stem cells are thought to be present in most, if not all, tissue found in the mature organism, where they are responsible for the continued renewal of specialized cells through asymmetric division (20).

Especially relevant to the field of regenerative medicine is the multipotent somatic mesenchymal stem cell (MSC) (10,20,22). As the name suggests MSCs, possess multi-lineage differentiation potential within the mesodermal lineage. Originally, MSCs were discovered in bone marrow, but have later been found in much diverse adult tissue including umbilical cord, dental pulp and adipose tissue (22). Especially relevant to regenerative medicine, is the fact that MSCs have been shown to be highly involved in the healing process

following tissue injury (23). Intense research on the mechanisms behind this ability have led to the conclusion that the regenerative capacities do not mainly rely on the ability to differentiate into various mature cell types. More crucial is the paracrine mechanisms by which the MSCs influence nearby cells. Studies have shown that MSCs effectively promote crucial events in tissue healing such as angiogenesis and proliferation (10). Furthermore, MSCs have proven to be efficient modulators of the immune system by conveying an anti-inflammatory microenvironment through secretion of important regulators of the immune response (20,22,23).

The secretome of the MSCs is highly diverse and capable of adapting to local needs. Firstly, MSCs secrete soluble factors, which include a vast amount of different growth factors, cytokines and chemokines, which are able to interact directly with receptors located on the cell membrane of nearby recipient cells (24,25). In addition to this, MSCs utilize shedding of extracellular vesicles (EVs) as a mean of paracrine communication (24). EV is a term covering different bilipid-membrane-surrounded structures e.g. exosomes (50-100 nm) and microvesicles (100-1000 nm) (26). The biogenesis of EVs enables molecules such as proteins, lipids and microRNA (miRNA) originating from the MSC cytoplasm to be transported though the extracellular matrix in a protected fashion followed by uptake through phagocytosis by recipient cells (24). EVs may even be directed at specific receptors on e.g. immune cells via surface-exposed ligands (27). The secretome of MSCs are thus a powerful tool by which the stem cells are able to regulate the behavior of other cell types important for homeostasis and tissue regeneration (24,28).

An international set of criteria (table 1.1) proposed by the International Society for Cellular Therapy in 2006 is commonly used to define MSCs in order to distinguish them from other cell types such as fibroblasts, with which they share a morphological resemblance. Firstly, the MSCs must show adherence to plastic surfaces under standard culturing conditions. Secondly, cells must express certain surface markers (CD73, CD90 and CD105) and lack the expression of others (e.g. CD45 and HLA-DR). Lastly, cells must be able to differentiate to adipocytes, osteoblasts and chondroblasts when cultured in certain differentiation induction media. (34).

TABLE 1.1 - CRITERIA FOR MSC IDENTIFICATION

1	Adherence to plastic surfaces under standard culture conditions	
2	**≥ 95% of cells positive for:** CD73 CD90 CD105	**≥ 2% of cells negative for:** CD34 CD45 CD14 or CD11b CD79α or CD19 HLA-DR
3	***In vitro* differentiation into:** Adipocytes Osteoblasts Chondroblasts	

Being the original location of MSC discovery, the bone marrow MSCs (BM-MSCs) were initially the focus of much regenerative medicine research (35). However, it has become evident that other subclasses of the MSCs may be more appropriate for therapeutic application. Especially the adipose-derived mesenchymal stem cells

(ASCs) are interesting candidates due to several important features (23,35). Firstly, adipose tissue are more easily accessible than bone marrow, and the extraction process is less invasive and painful for the donor (35). In addition, substantial amounts of adipose tissue are available as waste products from cosmetic liposuctions (23). Furthermore, adipose tissue is a more efficient MSC provider with 1 gram of lipoaspirate on average yielding 5 to 30 times more stem cells than 1 mL of bone marrow aspirate (35).

1.4.1 - ROLE OF MSCs IN WOUND HEALING

The regenerative nature of MSCs plays an important role in normal wound healing. In fact, research point towards the fact that MSCs regulate several key-events in all wound healing phases (4).

The immunomodulatory capacities are particularly important in the inflammation phase, where MSCs have been shown to induce a decrease in pro-inflammatory cytokines such as interferon-γ (IFN-γ) and IL-2 along with an increase in the anti-inflammatory IL-4 and IL-10 cytokines (4,10). Moreover, the MSC secretome actively induce a shift in macrophage phenotype towards the anti-inflammatory M2 version (4,36). This transition of macrophages is, as described earlier, crucial for the progression of a wound towards healing.

In the proliferation phase, the MSCs support the process of tissue repair by secretion of various important growth factors e.g. epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and VEGF. VEGF promotes the neovascularization process, which is vital for sufficient oxygen and nutrient flow to the area, and MSCs have been associated with increased angiogenic potential of surrounding endothelium (37). In addition to growth factors, MSCs secrete chemokines, which attract fibroblasts and keratinocytes to the wound, and mitogens resulting in increased proliferation of these cells (38–40). Furthermore, MSCs support the formation of granulation tissue by manipulating fibroblasts to upregulate the production of collagen I (39). In the remodeling phase, where the healing process is finished, the MSCs reduce the formation of scar tissue through paracrine mechanisms (4).

1.4.2 - MSCs AS CHRONIC WOUND TREATMENT

As described earlier, the development of chronic wounds is a substantial healthcare problem. Along with the discovery of MSCs being highly involved in normal wound healing, a research field regarding application of this cell type for chronic wound treatment has developed (10,35,41). The theory is that the anti-inflammatory capabilities of MSCs may be able to force a chronic wound, in which a pro-inflammatory microenvironment is dominating, towards the proliferative stage of healing.

It has become evident, however, that the therapeutic applicability of MSCs vary substantially between donors (29–32). Factors such as donor age, gender and Body Mass Index seem to influence the characteristics of the isolated cells (30,32,33). Furthermore, in regards ASCs the fat deposit site from which the cells are isolated largely affects the phenotype (29). The molecular basis behind these differences are yet to be fully elucidated, and much research are currently dedicated to expanding the knowledge on this issue. To this end, preclinical platforms on which the optimal MSCs donor for a specific therapeutic application can be identified are a crucial step towards developing novel and efficient treatment strategies.

The pathophysiology of chronic wounds are too complex to mimic in *in vitro* setups and animal models are therefore crucial for investigations on novel wound healing therapies such as stem cell application. In many such studies, small rodents including rats have been the animal of choice. Rats are popular in animal models, because of their low cost, easy handling and large availability (42). The induction of chronic wounds on these

animals are commonly achieved by injection of a substance toxic to the insulin-producing β-cells of the pancreas resulting in the animal developing diabetes after which a dermal wound is applied to the animal (43).

Several studies conducted on diabetic rat models have been able to show decreased healing time of a chronic wound when allogenic MSCs were injected into the edges of the wound (37,44–46). Furthermore, evidence of increased angiogenesis and proliferation of fibroblasts in experimental groups compared to controls were found (37,46). Other studies conducted on animal wound models have been able to show a wound healing promoting effect of MSC secretome application to the wound area in terms of decreased healing time (47,48) and induction of an anti-inflammatory microenvironment (36).

1.5 - PROBIOTICS

Another novel and interesting approach to chronic wound treatment involve manipulation of the gut microbiome. Though still experimental, studies suggests that ingestion of so-called probiotics may have beneficial effects on dermal wound healing (49,50). According to the World Health Organization, probiotics is defined as "live microorganisms, which when administered in adequate amount confer a health benefit to the host" (51). Recently, evidence of these health benefits to expand beyond the GI tract have been found (49,50,52,53). Systemic effects, which have been linked to gut homeostasis, include protection against hypertension, obesity, and cancer (52). The mechanisms behind the favorable properties is yet to be fully elucidated though it has become clear that probiotics possess potent immunomodulatory abilities (54–57). Animal models have shown that absence of microbial inhabitants in the GI tract results in an immature and compromised immune system (58,59). The induction of regulatory T-cells (Tregs) by beneficial intestinal bacteria have been proposed to be a crucial mechanism by which probiotics are able to convey their systemic health benefits (56,58,59). In a clinical study, oral consumption of probiotic yoghurt resulted in increased levels of circulating Tregs as well as an anti-inflammatory cytokine profile of the blood of the participants (60).

Topical administration of probiotics to dermal injuries has been the focus of several animal studies in recent years with overall positive findings such as reduced healing time and reduced inflammation in the area. (49,61–64). Conversely, reports of oral probiotics for wound healing applications have been sparse despite the systemic immunomodulatory effects of a healthy intestinal microbiome stated above. A meta-analysis from 2017 regarding efficacy of probiotics as treatment of cutaneous wounds identified only one study with oral administration of the bacteria (65). In this study, mice, which received purified lactobacilli in their water supply, showed significantly decreased healing time of a dorsal full thickness skin biopsy wound compared to the control group drinking regular water. Furthermore, the research group found an accelerated collagen deposition in the probiotic group as well as decreased levels of neutrophils and increased levels of Tregs in the wound area.

The systemic anti-inflammatory effects of a healthy intestinal microbiome as well as the notion that modern lifestyle and diet seem to have a negative influence on gut homeostasis (49) are making probiotics a highly interesting research field in modern medicine including chronic wound treatment.

1.6 - PRESENT STUDY

The present study is the initial step in a long-term plan at the Laboratory of Stem Cell Research at Aalborg University to establish a rat model in which the combined effects of stem cell injections and oral supplementation of probiotics in chronic wound treatment can be investigated (fig. 1.3).

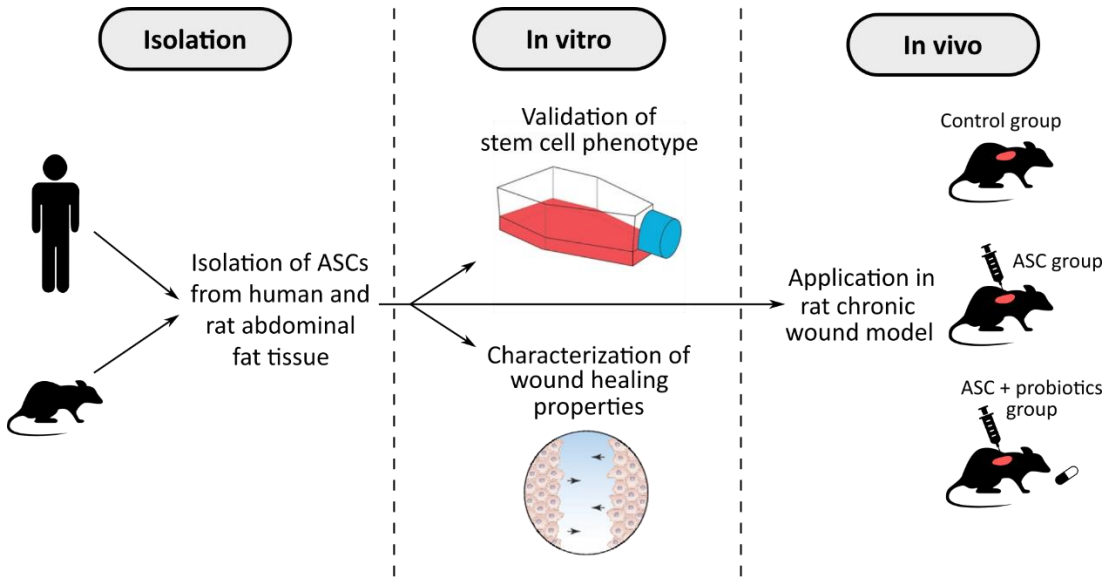

Figure 1.3 - Overview of the Long-Term Plan. In the initial step, ASCs from human and rat abdominal fat tissue are isolated. The isolated cells will be expanded and subjected to *in vitro* characterization assays. The final step is to apply the isolated cells in a chronic wound model in streptozotocin-induced diabetic rats. The *in vivo* study will also include application of oral supplementation of probiotics.

The first phase, which the present study is a part of, involves isolation of human and rat ASCs (hASCs and rASCs) followed by *in vitro* characterization of the stem cell phenotype including multipotency validation. Furthermore, wound healing promoting properties of the isolated cells are evaluated by *in vitro* scratch assays. These investigations will form the basis for proceeding onto animal trials. In the second phase, for which an application is currently under review by the Danish Committee on Animal Research (appendix 3), involves *in vivo* investigation of stem cell and probiotic treatment on chronic wound healing in streptozotocin-induced diabetic rats. Furthermore, the interspecies effect of human ASCs injection in a rat chronic wound model will be examined in order to establish a preclinical platform on which the inter-donor variability in regards to wound healing promoting properties of hASCs can be investigated.

2 - AIM

In the present study, characterization of human and rat ASCs in regards to stem cell characteristics and adipogenesis promoting abilities will be carried out.

For this purpose, rASCs will be isolated from seven adult Sprague-Dawley rats. These cells, along with previously isolated hASCs from a single healthy donor, will be subjected to investigations related to the conventional mesenchymal stem cell phenotype. These include adipogenic, osteogenic, and chondrogenic differentiation potential as well as proliferation rate and clonogenicity. Furthermore, the ability of the ASC secretome to induce proliferation of endothelial cells will be investigated in *in vitro* scratch assays.

3 - METHODS

3.1 - ISOLATION AND CULTURE OF ADIPOSE-DERIVED STEM CELLS

3.1.1 - HUMAN ADIPOSE-DERIVED STEM CELLS

Primary human ASCS were isolated from subcutaneous lipoaspirate from a single healthy donor. The isolation procedure was performed as described earlier (66) prior to the present study. After the isolation procedure, cells were expanded appropriately in expansion medium consisting of Minimum Essential Medium Alpha with GlutaMAX (α-MEM; Gibco), 5% Human Platelet Lysate (hPL; Cook Medical) and 1% Penicillin-Streptomycin (P/S; P: 10,000 U/mL, S: 10 mg/mL; Gibco) and subsequently cryopreserved in Human Albumin (HA; 200 g/L; CSL Behring) and dimethyl sulphoxide Hybri-Max (DMSO; Sigma) until further investigations. Once thawed, hASCs were cultured in growth medium consisting of α-MEM, 10% fetal calf serum (FCS; Gibco) and 1% P/S (unless otherwise stated) in a humidified atmosphere at 37°C, 20% O_2 and 5% CO_2. TrypLE (Gibco) was used to induce cell detachment for all ASC passage procedures.

3.1.2 - RAT ADIPOSE-DERIVED STEM CELLS

Adult male Sprague-Dawley rats (n=7) were used for the harvest of primary rat ASCs. The rats were terminated and the abdominal cavities were opened under sterile conditions. Visceral fat was carefully harvested, minced, and washed 3 times in phosphate buffered saline (PBS). The adipose tissue was stored in PBS + 1% P/S at room temperature overnight. The following day Collagenase NB 4 Standard grade (SERVA electrophoresis) in Hank's Balanced Salt Solution (HBSS; Invitrogen) was added to the samples, followed by a 45 minutes of incubation period at 37°C under rotation. The solution was filtered through a 100 µm Steriflip filter and centrifuged for 10 minutes at 400g. The cell pellet was resuspended in expansion medium (see above) and filtered through a 60 um Steriflip filter. Finally, the solution was centrifuged for 10 minutes at 400g and again the cell pellet was resuspended in the expansion medium and expanded appropriately. Subsequently, cells were cryopreserved in HA and DMSO until further investigations. Once thawed, rASCs were cultured in growth medium consisting of α-MEM, 10% FCS, and 1% P/S (unless otherwise stated) in a humidified atmosphere at 37°C, 20% O2 and 5% CO2. TrypLE was used to induce cell detachment for all ASC passage procedures.

3.2 - CHARACTERIZATION OF STEM CELL PHENOTYPE

Stem cell properties of both isolated hASCs and rASCs were investigated in regards to differentiation potential, proliferation rate and proportion of colony-forming units (CFUs) as explained below (fig. 3.1).

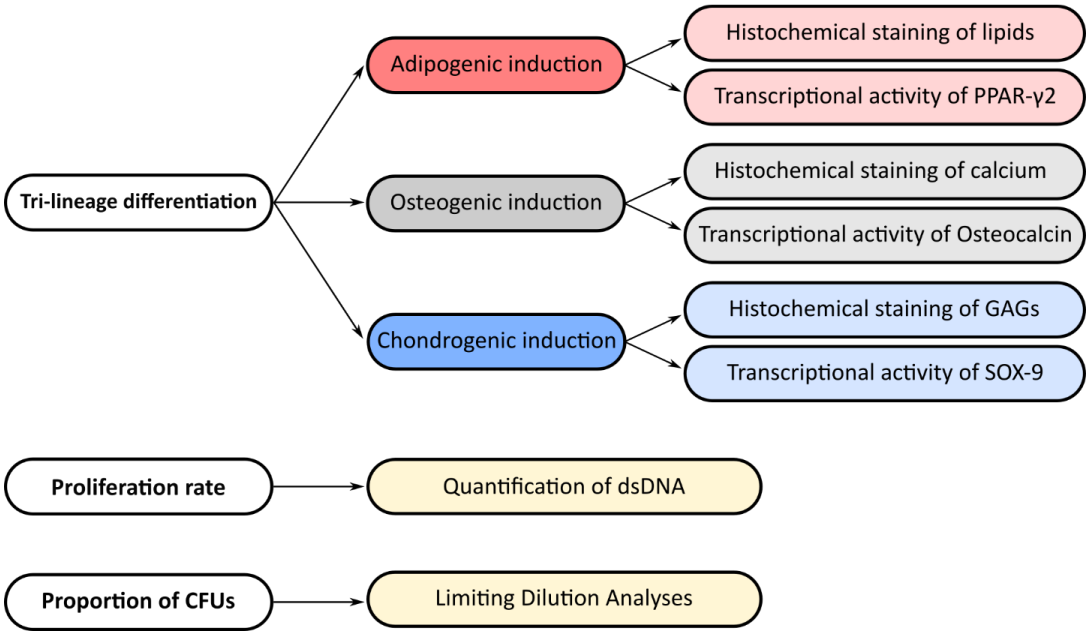

Figure 3.1 – Overview of the Characterization of Stem Cell Phenotype. Tri-lineage potential is investigated both histochemically and transcriptionally. Proliferation rate is calculated based on increase in dsDNA over a 12-day period. Lastly, proportion of CFUs is investigated through a limiting dilution setup.

3.2.1 - TRI-LINEAGE DIFFERENTIATION

The multi-lineage differentiation potential of the isolated ASCs were investigated by culturing cells in various induction media. Content of these media can be seen in table 3.1. Adipogenic, osteogenic, and chondrogenic differentiation were attempted as described below.

ADIPOGENESIS

In order to induce adipogenesis, ASCs (human: passage 3; rat: passage 1) were seeded at 18,750 cells/cm^2 in 6- and 48-well plates (Costar, Corning) and cultured in ASC growth medium until 100% confluent after which the differentiation process was initiated.

Two different adipogenic induction approaches were used in this project. Firstly, a continuous method in which cells received identical medium throughout the differentiation process, and secondly, a phased approach in which the applied medium switched between an induction and a maintenance composition in the following manner: Induction media (72h), maintenance media (24h), repetition of this cycle four times, and lastly maintenance media (1 week) for a total of 24 days of culture. Contents of the different media is shown in table 3.1. Negative controls received ASC growth media throughout the period. Media was changed every 3rd day unless otherwise stated.

Adipogenic differentiation was investigated by Oil Red O staining. Monolayers of the 48-well plates were fixed in 4% formaldehyde (Bie & Bernsen) at 4°C for 1 hour and stained with Oil Red O (Sigma-Aldrich) for 15 minutes at room temperature after which photos were taken using phased-contrast microscopy (Olympus CKX41 inverted microscope, Olympus; PL-A782 digital camera, PixeLINK; software PixeLINK Capture). Cells in the 6-well plates were lysed using a solution of 1% β-mecaptoethanol (Bio-Rad) in 70% ethanol. Lysates were stored at -80°C until RNA-isolation.

TABLE 3.1 - MEDIA USED FOR TRI-LINEAGE DIFFERENTIATION

Continuous Adipogenic Induction Medium (C-AIM)	Phased Adipogenic Induction Medium (P-AIM)		Osteogenic Induction Medium (OIM)	Chondrogenic Induction Medium (CIM)
	Induction	Maintenance		
α-MEM (Gibco)	DMEM (25 mM glucose) (Gibco)	DMEM (25 mM glucose) (Gibco)	α-MEM (Gibco)	DMEM (25 mM glucose) (Gibco)
10% FCS (Gibco)	10% FCS (Gibco)	10% FCS (Gibco)	10% FCS (Gibco)	1% P/S (Gibco)
1% P/S (Gibco)	1% P/S (Gibco)	1% P/S (Gibco)	1% P/S (Gibco)	1% ITS (Gibco)
0.1 µM dexamethasone (Sigma-Aldrich)	1 µM dexamethasone (Sigma-Aldrich)	1% ITS (Gibco)	0.1 µM dexamethasone (Sigma-Aldrich)	0.1 µM dexamethasone (Sigma-Aldrich)
0.45 mM IBMX (Sigma-Aldrich)	0.5 mM IBMX (Sigma-Aldrich)		50 µM L-ascorbic-acid (Sigma-Aldrich)	50 µg/mL L-ascorbic-acid-2-phosphate (Sigma-Aldrich)
0.17 µM insulin (Sigma-Aldrich)	1% ITS (Gibco)			40 µg/mL L-prolin (Sigma-Aldrich)
0.2 mM indomethacin (Sigma-Aldrich)	0.1 mM indomethacin (Sigma-Aldrich)			10 ng/mL TGF-β1* (Sigma-Aldrich)

Abbreviations: α-MEM, Minimum Essential Medium Alpha with GlutaMAX; FCS, Fetal calf serum; P/S, Penicillin-Streptomycin; IBMX, 3-Isobutyl-1-methylxanthine; ITS, Insulin-transferrin-selenium; DMEM, Dulbecco's Modified Eagle Medium; TGF-β1, Transforming growth factor beta 1.
* Due to instability in the media TGF-β1 was freshly added prior to each CIM change.

OSTEOGENESIS

For osteogenic induction, ASCs (human: passage 3; rat: passage 1) were seeded at 12,500 cells/cm^2 in 6- and 48-well plates and cultured in ASC growth media until 100% confluent. Hereafter, osteogenic induction

medium (OIM) (table 3.1) was added to the cells and they were cultured for 28 days with media-change every 3rd day. Negative controls received ASC growth media throughout the period.

Osteogenic differentiation was investigated by Alizarin Red staining. Cells in the 48-well plates were fix in ice-cold ethanol for 15 minutes and stained with Alizarin Red (Bie & Bernsen) for 5 minutes at room temperature. Photos were taken using phased-contrast microscopy. Cells in the 6-well plate were lysed using a solution of 1% β-mecaptoethanol (Bio-Rad) in 70% ethanol. Lysates were stored at -80°C until RNA-isolation.

CHONDROGENESIS

In order to induce chondrogenesis, ASCs (human: passage 5, rat: passage 1) were seeded in a 96-well plate with v-shaped bottom (Costar, Corning) with 225,000 cells/well in order to promote the formation of a cell pellet. Chondrogenic induction medium (CIM) (table 3.1) was added to the experimental wells and ASC growth medium to the negative controls. The plate was cultured until a cell pellet with an estimated size of 1-2 mm were visible (21 days).

For visualization of chondrogenesis, cell pellets were embedded and stained with Alcian Blue 8GX (Sigma-Aldrich). Briefly, pellets underwent a dehydration process by immersion in increasing concentrations of ethanol. Subsequently, pellets were submerged in Tissue-Tek Tissue-Clear (Sakura) and embedded in paraffin wax. 5 µm sections were cut on a microtome and mounted on glass slides. Sections were rehydrated in decreasing concentrations of ethanol before staining with Alcian Blue for 30 minutes and Mayer's Hematoxylin (Sigma-Aldrich) for 2 minutes. Lastly, sections were dehydrated and coverslips were mounted using Pertex Mounting Medium (HistoLab). Photos were taken using phased-contrast microscopy. Remaining cell pellets were lysed using a solution of 1% β-mecaptoethanol (Bio-Rad) in 70% ethanol. Lysates were stored at -80°C until RNA-isolation.

3.2.2 - TRANSCRIPTIONAL ACTIVITY OF LINEAGE-SPECIFIC GENES

In addition to staining, tri-lineage differentiation was furthermore investigated on the transcriptional level by detection of linage-specific genes by qRT-PCR. PPAR-γ2, Osteocalcin, and Sox9 expression were quantified for verification of adipogenic, osteogenic, and chondrogenic differentiation, respectively. Results were normalized against the YWHAZ housekeeping gene.

RNA from collected lysates was isolated using the Aurum Total RNA mini kit (Bio-Rad) according to the manufacturer's instructions. Concentration and purity of the isolates were determined by spectrophotometry (Nanodrop ND-1000) and RNA quantity were normalized among samples. cDNA synthesis was achieved using the iScript cDNA synthesis kit (Bio-Rad) followed by thermal cycle incubation (GeneAmp PCR system 2400; Perkin Elmer). Samples of cDNA were diluted 1:15 in Milli-Q water before mixing with iTag Universal SYBR Green Supermix (Bio-Rad) as well as forward and reverse primers for the specific genes. Samples were loaded in duplicates into a 96-well plate (Bio-Rad) sealed with optical plate cover (Bio-Rad) before centrifugation for 5 minutes at 1000 rpm. The CFX Connect Real-Time PCR Detection System (Bio-Rad) were used for the q-PCR process with a thermal cycle protocol set to: Initial denaturation for 3 min at 95°C followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing/extension for 30 seconds at primer-specific temperature (table 3.2). The CFX Manager 3.0 software (Bio-Rad) was used for data extraction. Gene expression ratios were calculated using the Pfaffl method. (qPCR Data Output in appendix 2).

TABLE 3.2 - PRIMERS FOR qRT-PCR

HUMAN PRIMERS			
GENE	**FORWARD**	**REVERSE**	**TEMP.**
PPAR-γ2	5'-CCC CTA TTC CAT-3'	5'-AAT GGC TTT CTG-3'	60°C
Osteocalcin	5'-TTC TAG CCC TGG TG-3'	5'-TGC CAC ATA CTC CG-3'	65°C
SOX-9	5'-TTC GGT TAT TTT TAG-3'	5'-CAC ACA GCT CAC TCG-3'	62°C
YWHAZ	5'-ACT TTT GGT ACA-3'	5'-CCG CCA GGA CAA-3'	60°C
RAT PRIMERS			
GENE	**FORWARD**	**REVERSE**	**TEMP.**
PPAR-γ2	5'-CCT TTA CCA CGG TTG ATT TCT C-3'	5'-GCA GGC TCT ACT TTG ATC GCA CT-3'	61°C
OSTEOCALCIN	5'-TCT CTG CTC ACT CTG CTG-3'	5'-GTC TAT TCA CCA CCT TAC TGC-3'	n/a
SOX-9	5'-CAA AGG CCG CCA AGA TAT AA-3'	5'-GAA ATT AGG CAG CAA CAT-3'	n/a
YWHAZ	5'-CTA CCG CTA CTT GGC TGA GG-3'	5'-TGT GAC TGG TCC ACA ATT CC-3'	60°C

3.2.3 - PROLIFERATION RATE

The proliferative potential of the isolated human and rat ASCs was investigated by quantitative measurement of DNA content during a 12-day period. Cells (human: passage 5, rat: passage 3) were seeded with a density of 300 cells/cm^2 in 48-well plates and cultured in growth medium with medium change every 3rd day. Every 24th hour four replicate wells were treated with 0.05% Sodium Dodecyl sulfate (SDS; Sigma-Aldrich) in order to lyse the cells present in the wells at the specific time point. Lysates were kept at -20°C until all wells were processed (12 days). DNA content of the lysates was quantified using Quant-iT PicoGreen dsDNA reagent (Invitrogen). Briefly, samples were diluted in 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and transferred to a black 96-well microtiter plate (Corning). PicoGreen reagent was added to each sample and the plate were allowed to incubate for 10 minutes before measurement of fluorescence intensity (excitation: 485 nm, emission: 535 nm) by a microplate reader (EnSpire). Standards consisted of six dilutions of λ-DNA (0.0-500 µg/mL; New England Biolabs) in 1X TE buffer.

Microsoft Excel (Office Package 2013) was used for the data analysis. Based on the DNA standards, the mean amount of dsDNA in the quadruplicates of each time point were calculated. DNA content was plotted as a function of time and the regression line of the exponential growth phase (expressed as $y = a * e^{kx}$) were used as basis for calculation of the doubling time (T_D) using the equation $T_D = \ln\left(\frac{2}{k}\right)$.

3.2.4 - COLONY-FORMING UNITS

The ability of the isolated ASCs to form colonies were investigated by limiting dilution. Cells (human: passage 5, rat: passage 3) were seeded in 96-well plates (Corning) with 30 to 1 cell per well and cultured in growth medium for 14 days with media change every 3-4 days. On the day of the analysis, cells were fixed in 4% formaldehyde for 5 minutes (AppliChem) and stained with 0.05% Crystal Violet (Sigma-Aldrich) for 30 minutes. Wells positive for colonies were counted and the proportion of colony-forming units were calculated using the L-Calc Software (Stem Cell Technologies).

3.3 - WOUND HEALING PROPERTY OF ASC SECRETOME

For investigations related to the wound healing potential of the ASC secretome, conditioned medium (CM) were collected. Cells (human: passage 3; rat: passage 1) were seeded in T25 culture flasks (CellStar, Greiner Bio-One) at a density of 8,000 cells/cm^2 and cultured in growth medium until 80% confluent. Cell conditioned media were harvested and stored at -20°C until further investigations.

3.3.1 - SCRATCH ASSAYS

Angiogenic potential of the ASCs were evaluated by *in vitro* scratch assays involving human and rat endothelial cells (fig. 3.2).

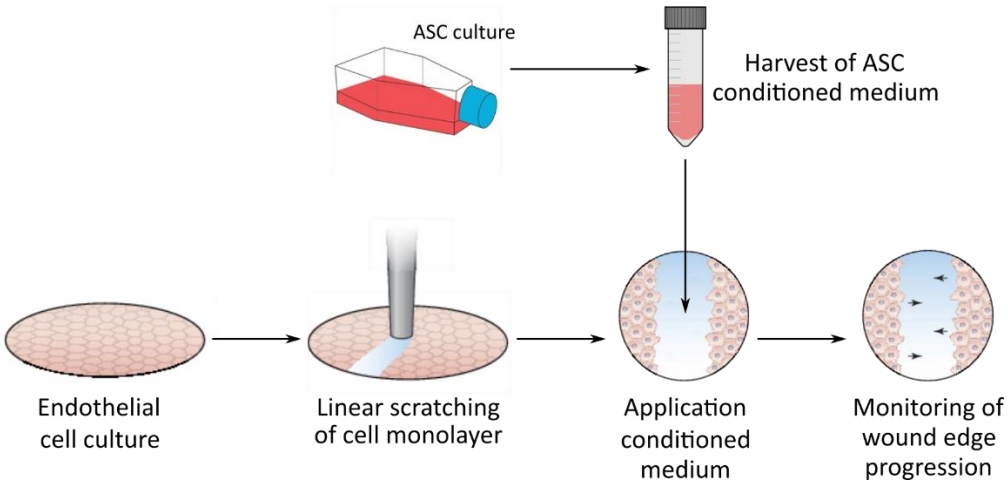

Figure 3.2 - Overview of *in vitro* Scratch Assay. Conditioned medium are harvested from sub-confluent cultures of ASCs. Endothelial cells are seeded in wells and grown until 100% confluent. A linear scratch is made with the Wounding Pin Tool and the ASC conditioned medium is applied to experimental wells. Progression of the wound edge is monitored for 30 hours with regular capturing of images.

HUMAN ENDOTHELIAL CELLS

Human Dermal Microvascular Endothelial Cells (HDMEC; PromoCell) (passage 8) were seeded in a 96-well plate at a density of 12,000 cells/cm^2 and cultured in Endothelial Cell Growth Media MV2 (HCMEC medium; PromoCell) until 100% confluent. A linear scratch was made in each well using a Wounding Pin Tool (V&P Scientific) before rinsing in PBS. CM from human ASCs (passage 3) were applied to the experimental wells and pictures were taken at regular time intervals for a total of 30 hours using phase-contrast microscopy (Olympus CKX41 inverted microscope). The plates were cultured in a humidified atmosphere at 37°C, 20% O_2 and 5%

CO_2 between capturing of images. ASC growth medium was applied as vehicle control and HDMEC medium as positive control. Images were uploaded to the ImageJ software (version 1.51j8) and the cell-free area were measured in pixels. Serial images were normalized to the image taken at 0 hours in order to eliminate differences between samples in initial scratch area.

RAT ENDOTHELIAL CELLS

Primary rat endothelial cells (RECs) (passage 2) isolated from the brains of 2-3 weeks old Sprague-Dawley rats (67) were seeded in a 96-well plate coated with a solution containing collagen type IV (Sigma-Aldrich) and fibronectin (Sigma-Aldrich) to enable cell attachment to the culture plate. Cells were seeded at a density of 32,000 cells/cm^2 and cultured in DMEM/F12 (Glutamax) with 10% plasma-derived bovine serum (PDS; First Link), ITS (Roche), bFGF (Roche) and gentamycin (Lonza Copenhagen) (PREC medium) until 100% confluent. The Wounding Pin Tool (V&P Scientific) were used to create a linear scratch in each well. CM from rat ASCs (passage 1) and human ASCs (passage 3) were applied to experimental wells. Positive controls consisted of REC medium and ASCs growth medium were applied as vehicle control. Images were captured and analyzed as described for the HDMEC scratch assay.

3.4 - STATISTICAL ANALYSES

All statistical analyses were performed using the GraphPad Prism software (version 6.01). Significance levels was set to 5% ($p<0.05$) for all analyses. In cases with two groups the *Independent samples t-test* was applied, whereas *One-way ANOVA* was applied in cases with multiple groups.

4 - RESULTS

4.1 - STEM CELL CHARACTERISTICS

Stem cell characteristics including morphology, tri-lineage differentiation potential, proliferation rate, and proportion of CFU were investigated in both human and rat ASCs.

4.1.1 - MORPHOLOGY

ASCs from both species showed a fibroblast-like spindle-shaped morphology typical for MSCs grown under standard culture conditions (fig. 4.1A). However, whereas hASCs demonstrated contact inhibition after reaching confluency, the rat cells continued to expand beyond monolayer structure, eventually resulting in the formation of macroscopically visible 3D structures (fig. 4.1B).

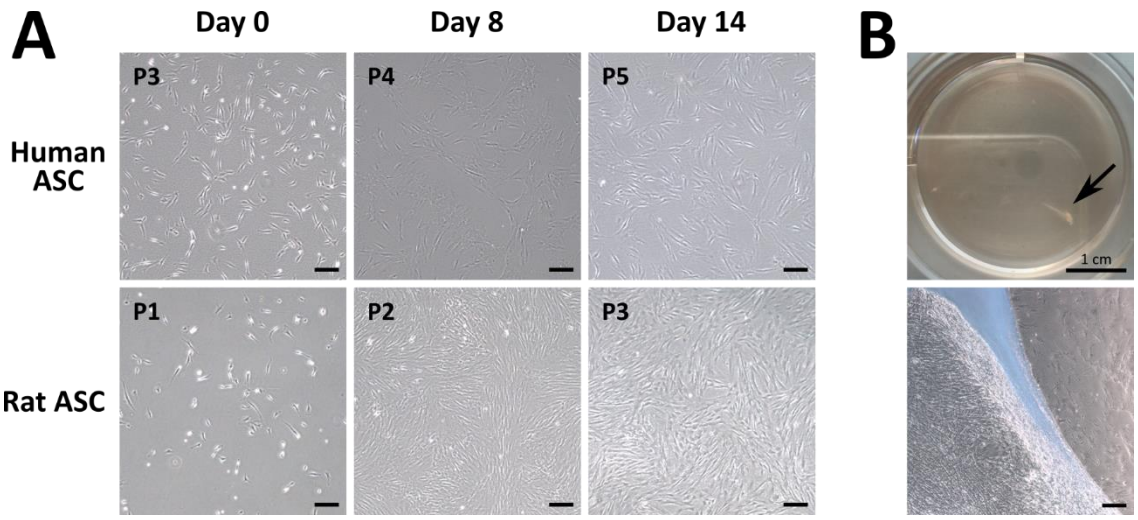

Figure 4.1 – Morphology of hASCs and rASCs *in vitro*. (A) Representative images of hASCs and rASCs at days 0, 8 and 14 after seeding. Passage number is indicated at each image. Scale bar = 200 µm. (B) rASCs culture at 16 days of growth after reaching 100% confluency. Bottom image scale bar = 200 µm.

4.1.2 - TRI-LINEAGE DIFFERENTIATION POTENTIAL

ADIPOGENIC DIFFERENTIATION

For adipogenic differentiation, two different induction media were applied in a continuous (C-AIM) and phased (P-AIM) approach, respectively. Both induction methods were able to induce formation of rounded lipid-containing adipocytes in hASC culture confirmed by Oil Red O (fig. 4.2A). Transcriptional activity of the adipogenic differentiation marker PPAR-γ2 was significantly higher in the C-AIM group compared to the control group ($P<0.01$). Despite the positive staining of lipids in the P-AIM group, no statistically significant difference was seen between this group and the control ($p=0.08$) (fig. 4.2B).

Rat cells grown under both continuous and phased induction conditions both stained positive for lipids, and some cells exhibited a more rounded morphology compared to the common elongated morphology of ASCs. The highest amount of positive-stained cells were seen in the wells subjected to C-AIM (fig. 4.2A). Both induction methods promoted a significantly higher gene expression of PPAR-γ2 compared to the control ($p<0.01$) (fig. 4.2B).

OSTEOGENIC DIFFERENTIATION

Calcium staining by Alizarin Red was unable to identify any osteogenic differentiation of neither hASCs nor rASCs (fig. 4.2A). Likewise, transcriptional activity of osteogenic differentiation marker Osteocalcin revealed no significant difference between human OIM-treated cells and the control. No detectable signal of Osteocalcin expression were obtained in the rat samples (fig. 4.2C).

CHONDROGENIC DIFFERENTIATION

For chondrogenic differentiation, a combination of CIM application and cell pellet promoting culture conditions was used. Microscopic sections of the cell pellets were stained with Alcian Blue for detection of glycosaminoglycans. The human cells formed spherical pellets with distinct boarders and dense inner masses, which clearly stained positive by Alcian Blue. In comparison, the human control pellet, grown in regular ASC media, was smaller, more irregular and lesser stained (fig. 4.2A). This observation of chondrogenic differentiation in human CIM-pellets were further confirmed by quantification of the transcriptional activity of chondrogenic differentiation marker SOX-9. The CIM-pellet contained significantly more SOX-9 mRNA compared to the control pellet ($p<0.01$) (fig. 4.2D).

Rat CIM-pellets were irregular, disorganized and fragmented (fig. 4.2A). Rat control cells were unable to form a sustainable pellet, and thus could not be subjected to microscopic investigations. Transcriptional activity of SOX-9 in rat samples did not yield any detectable signal (fig. 4.2D).

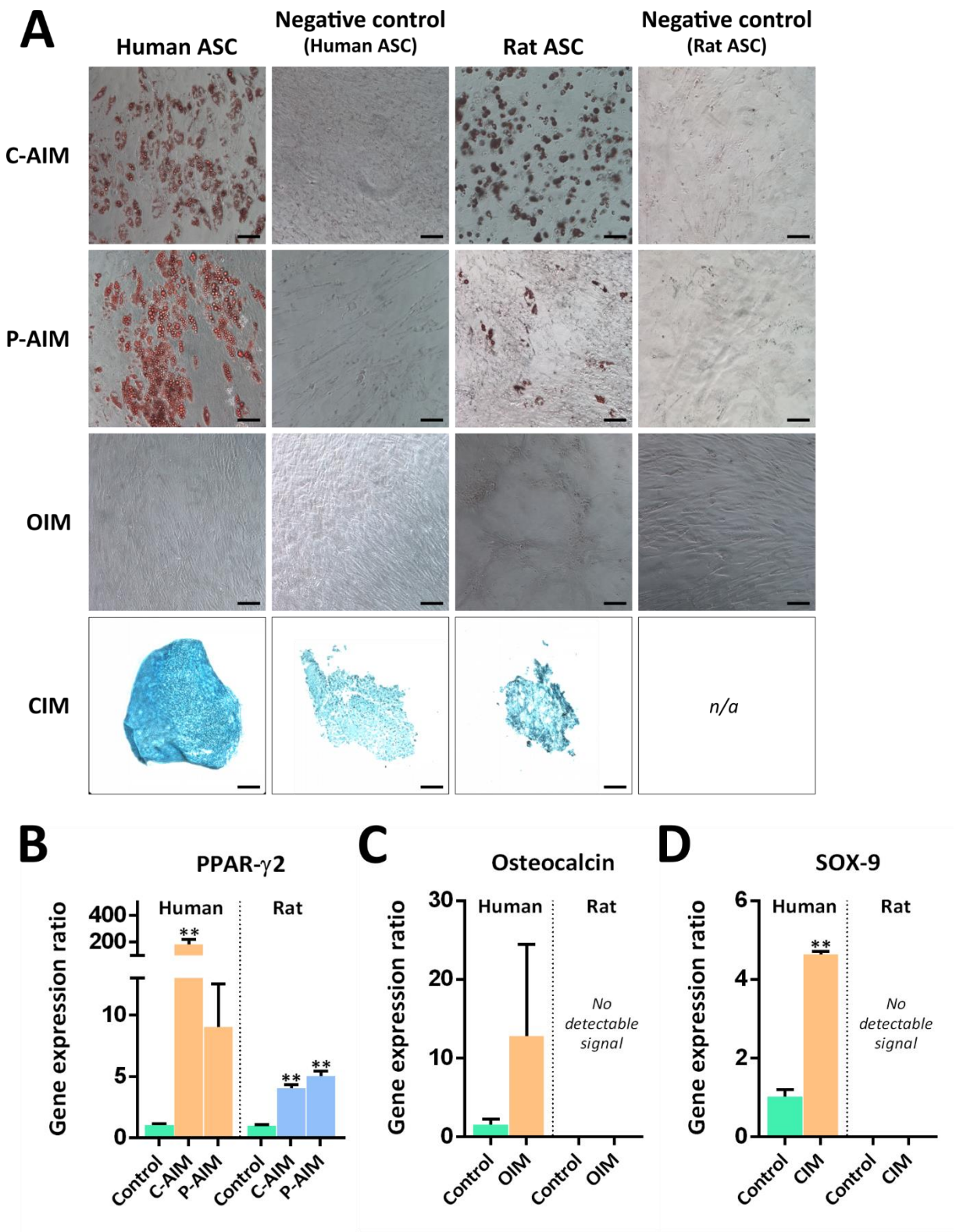

Figure 4.2 – Tri-Lineage Differentiation Potential of hASCs and rASCs. (A) Histochemical analysis of adipogenic, osteogenic and chondrogenic differentiation. Scale bar = 100 µm. (B), (C), (D) Transcriptional activity of differentiation markers PPAR-γ2, Osteocalcin and SOX-9. Data are based on triplicate samples for PPAR- γ2 and Osteocalcin and duplicate samples for SOX-9 and are presented as mean + SEM. ** Statistically different from control group ($p<0.01$).

4.1.3 - PROLIFERATION RATE AND CFU PROPORTION

Proliferation rate of hASCs and rASCs were investigated by quantification of dsDNA isolated from cells over a 12-day period. Throughout the period, the rat cultures had a significantly higher level of dsDNA ($p<0.01$). The exponential growth phase lasted for 5 days in the rat cultures and 6 days in the human cultures (fig. 4.3A). The doubling time of each cell type was calculated from the regression line based on the exponential growth phase to be 29 hours for human cells and 23 hours for rat cells (fig. 4.3B).

The proportion of CFUs in hASC and rASC cultures were examined through a limited dilution setup. rASCs had a slightly larger CFU proportion of 73% compared to 60% for human cells. The difference was, however, not significant (fig. 4.3C).

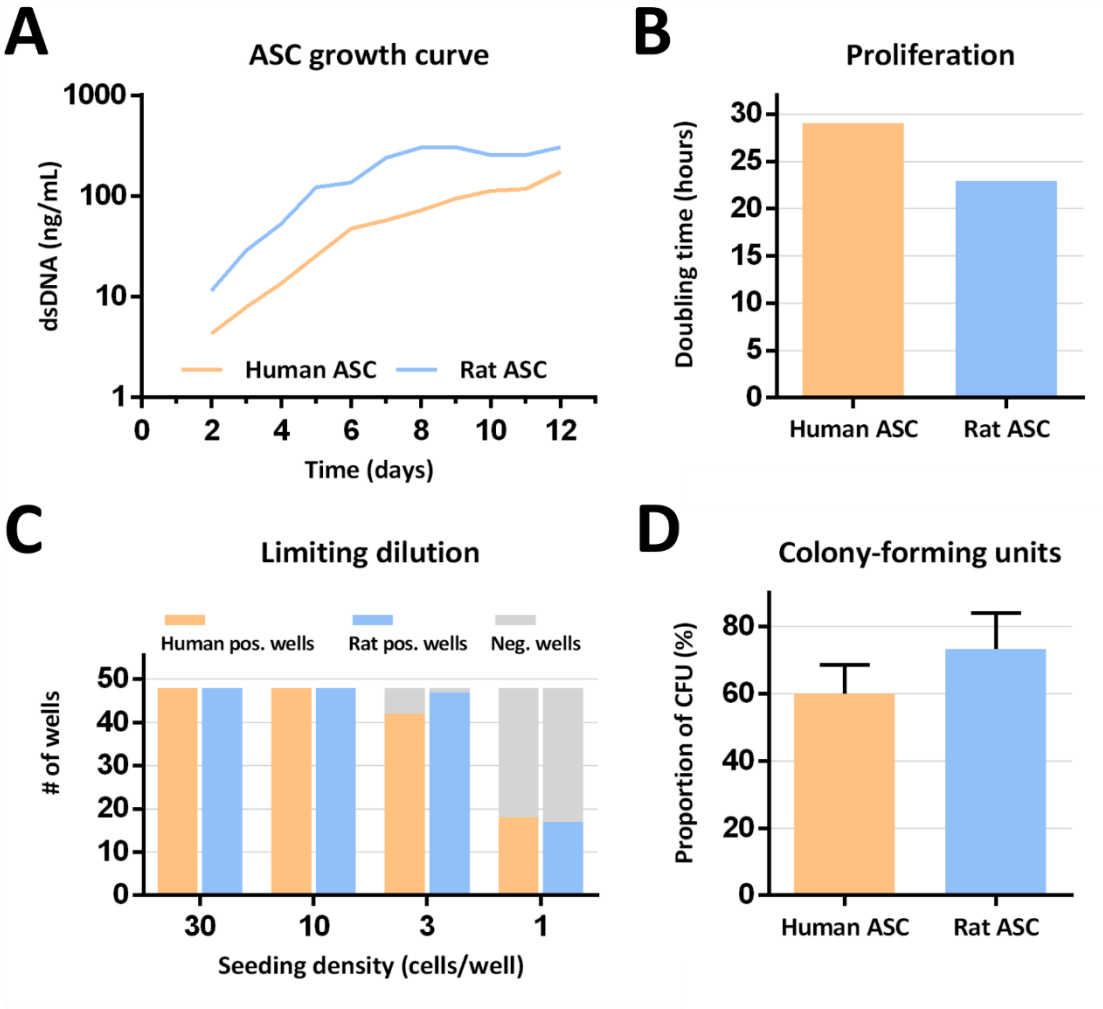

Figure 4.3 – Proliferation Rate and Proportion of CFUs of hASCs and rASCs. (A) ASC culture growth measured by content of dsDNA over a 12 days period. Graph is presented as mean of four replicate samples from each time point. (B) Doubling time calculated from the exponential growth phase seen in (A). (C) Distribution of wells positive or negative for ASC colonies after 14 days of culture at various seeding densities (D) Proportion of CFUs based on data from (C). Data presented as mean + SEM.

4.2 - PROANGIOGENIC POTENTIAL OF ASC SECRETOME

In vitro scratch assays conducted on endothelial cells were applied in order to investigate the proangiogenic potential of hASC-CM and rASC-CM.

Scratched monolayers of HDMEC subjected to hASC-CM showed no significant difference in healing time when compared to control samples subjected to pure ASC growth media (fig. 4.4A-B). hASC-CM was likewise unable to promote healing in a scratch assay conducted on REC. Contrary to this, rASC-CM induced significantly reduced healing time of the REC scratch with 75% healing after 30 hours compared to 23% healing in controls ($p<0.01$) (fig. 4.5A-B).

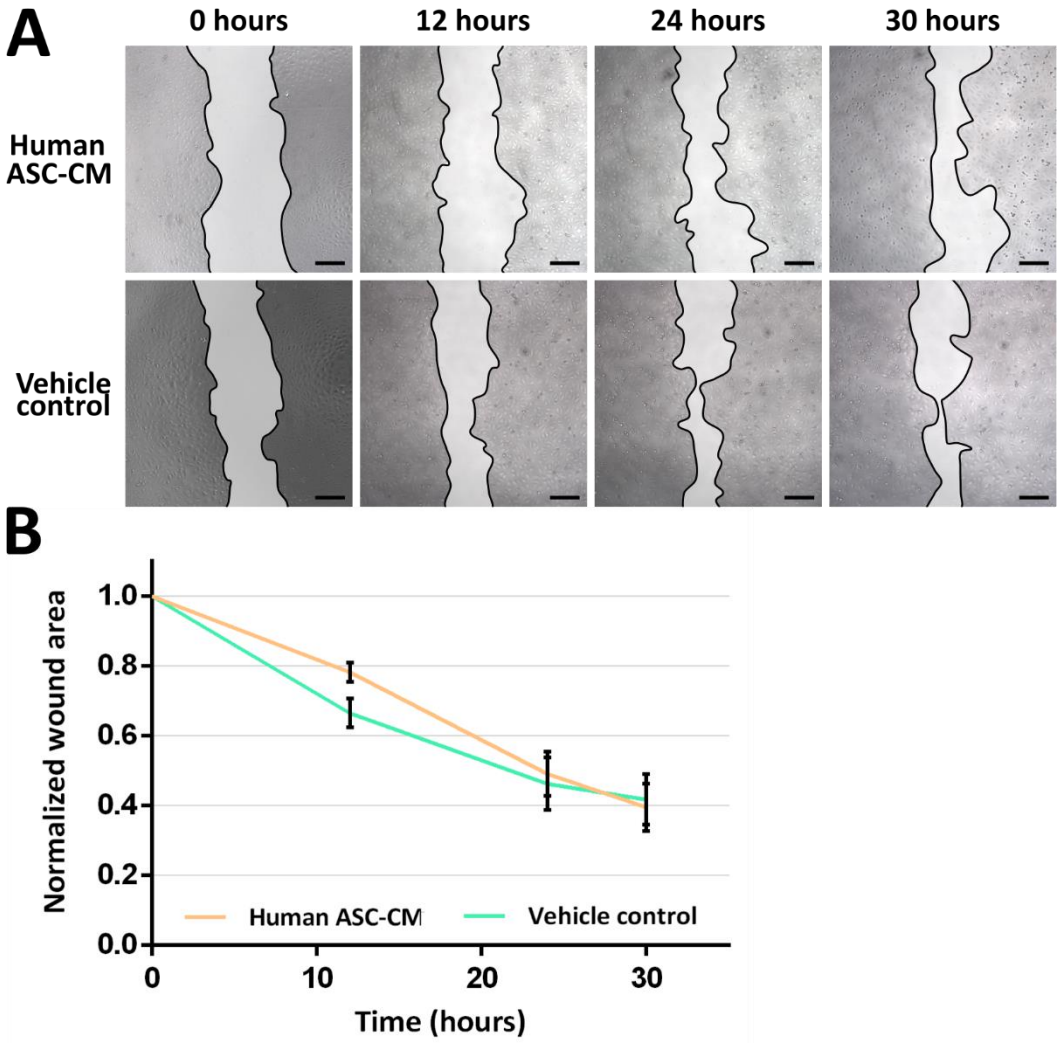

Figure 4.4 – Scratch Assay Conducted on HDMECs. (A) Representative images of scratch area over time. Cell-free zone is marked by black lines and grey area. Scale bar = 200 µm. (B) Normalized wound area over time. Human ASC-CM (n=8), Vehicle control (n=7). Data presented as mean ± SEM.

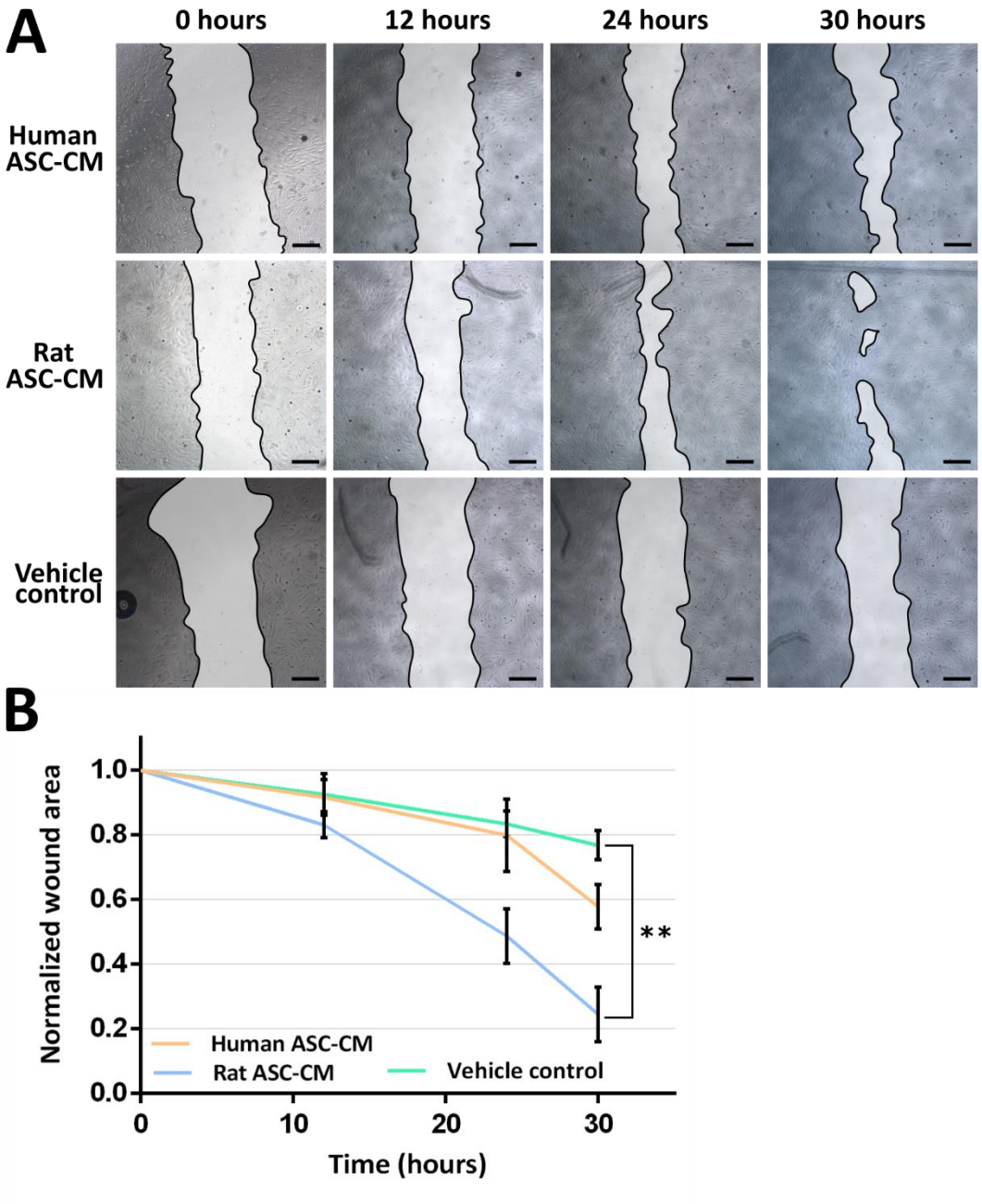

Figure 4.5 – Scratch Assay Conducted on RECs. (A) Representative images of scratch area over time. Cell-free zone is marked by black lines and grey area. Scale bar = 200 µm. (B) Normalized wound area over time. Human ASC-CM (n=3), Rat ASC-CM (n=5) Vehicle control (n=4). Data presented as mean ± SEM. ** Statistical difference ($p<0.01$).

5 - DISCUSSION

In the present study, hASCs and rASCs were put under investigations in regards to stem cell properties and angiogenesis promoting abilities. In order to validate the multipotency of the isolated cells, adipogenic, osteogenic, and chondrogenic differentiation were attempted *in vitro* by culturing the cells under the influence of different induction media. The differentiation was subsequently assessed by histochemical staining of cell-specific components along with quantitative RT-PCR for detection of the transcriptional activity of cell-marker genes. Two other stem cell characteristics, high proliferation rate and high proportion of CFUs, were likewise assessed. Additionally, for investigations on ASC angiogenic abilities, scratch assays, in which ASC-CM were applied to scratched monolayers of endothelial cells, were conducted.

Results of the performed investigations presented in the previous chapter are discussed below. Furthermore, aspects of MSC application in regenerative medicine are covered. Lastly, perspectives regarding the long-term plan, in which the current study has been the initial step, is further elaborated.

5.1 - PHENOTYPICAL CHARACTERISTICS OF hASCS AND rASCS

5.1.1 - TRI-LINEAGE DIFFERENTIATION

Adipogenic differentiation were successful for both hASCs and rASCs grown in C-AIM. In a prior attempt, rASCs were unable to undergo adipogenic transformation, when grown for 21 days in C-AIM (results not shown). A second attempt, in which the period of culture was increased to 28 days, effectively induced adipogenesis in both hASCs and rASCs confirmed both histochemically and transcriptionally. However, whereas the hASCs began morphological transformation after approximately 14 days of culture, the rASCs remained spindle-shaped for almost 21 days. As a result of this difference, only few areas within the rASCs culture were stained positive by Oil Red O, which corresponds well with the fact that the transcriptional activity of adipogenic differentiation marker PPAR-γ2 was many-fold higher in hASCs compared to rASCs. Other studies, which have achieved adipogenic differentiation of rASCs have kept cells in induction media between 14 and 26 days (68–70). The reason for the delayed differentiation process of rASCs in the present study is unclear.

In addition to an increased time of culture, the second attempt of adipogenic differentiation also included a phased induction approach, which was reported by Jin *et al*. to induce adipocyte transformation of rASCs (70). This approach was able to induce formation of lipid-containing cells in both human and rat cultures confirmed by Oil Red O staining. The transcriptional activity of PPAR-γ2 was, however, not significantly different from the negative control in human cultures. Inconsistency in PPAR-γ2 transcription rate between human samples, indicated by the large SEM (fig 4.2B), is likely the cause of this observation, rather than the inability of P-AIM to induce adipogenic differentiation of hASCs. An increased number of samples could possibly have ensured a detectable significant difference in transcriptional activity.

No osteogenic differentiation was achieved in the present study in either human or rat cultures in two separate attempts with 21 or 28 days of culture, respectively (results of the second attempt is presented in fig. 4.2). Previously, osteogenic induction of hASCs by the same procedure as attempted in the present study has been

successful in our laboratory (71). The reason for the failed differentiation is thus unclear, but inactivation of one or more OIM supplements might be suspected. Due to time limitations of the present study, a third attempt utilizing newly purchased osteogenic induction factors for OIM supplementation could not be carried out. Based on the fact that successful osteogenesis has been previously achieved in our lab, this would, however, be necessary before concluding that the hASCs lacked the ability to differentiate within the osteogenic lineage. No detectable signal of osteogenic marker transcriptional activity was seen in rASCs. Due to the lack of a positive control in the form of an actual rat osteoblast cell culture, it is unclear whether this observation is due to no transcriptional activity of Osteocalcin or to non-specific primer sequences. In a previous study, however, the Osteocalcin primer-pair had successfully identified osteogenic transcriptional activity in a rat model (72).

Chondrogenic differentiation was successful in hASC as seen by both Alcian Blue staining and transcriptional activity of chondrogenic marker SOX-9. For rASCs, however, the histochemical analysis revealed a, to some degree, positively stained, but disorganized and fragmented cell pellet. Comparison with a negative control was not possible, since no sustainable cell pellet formed in these cell cultures. This might be a sign of some form of chondrogenic induction in experimental rASCs cultures, but no definitive conclusion can be drawn from these observations. As it was the case in the osteogenesis setup, no detectable signal of transcriptional activity of the differentiation-specific marker was found in the rat samples. Based on the histochemical analysis, it can be suspected that the primers were unspecific for rat SOX-9, even though they have been reported to validate chondrogenic cell phenotype in a previous rat study (73). A chondrocyte cell culture would have been able to function as a positive control and thereby validating the specificity of the SOX-9 primer sequences.

5.1.2 - PROLIFERATION AND CFU

Apart from multipotency, MSCs are characterized by other distinct stem cell features, one of which being rapid proliferation (74,75). Quantitative measurements of increasing amounts of dsDNA in hASC and rASC cultures were conducted in the present study in order to investigate the doubling time of the cells. The results revealed a slightly faster doubling time of rASC (23 hours) compared to hASC (29 hours). Both results are consistent with those of other studies on human and rat stem cell properties (75,76). Another important feature of MSCs is their ability to establish colonies even when seeded at low cell densities. This ability is based on the high frequency of progenitor cells or CFUs within the MSC-population ensuring long-term self-renewal (74,75). In the present study, a limiting dilution setup was applied in order to evaluate the clonogenicity of hASCs and rASCs. The results showed a high proportion of CFUs in both hASC and rASC of 73% and 60%, respectively. Together the validation of a high proliferative potential and a potent self-renewal ability of both hASCs and rASCs support the notion of a MSC phenotype.

5.1.3 - SURFACE-MAKER EXPRESSION

Though originally applying to humane cells, the three criteria for identifying MSCs proposed by The International Society for Cellular Therapy have to some extend been transferred to other species as well. Apart from humane cells, the mostly studied MSCs are those originating from mice. Within this species it is widely accepted that two criteria, plastic adherence and tri-lineage differentiation, are also applicable (34). Several studies conducted on rats have likewise investigated the differentiation potential of rat MSCs in order to validate that experimental cells are in fact multipotent stem cells (46,68,70). The third criteria related to expression of different cell surface markers are possibly more difficult to transfer between species (34).

Despite this, presence of CD90 and absence of CD45 have been used as validation of the rat MSC phenotype in previous studies (37,44,45). Flow cytometric analysis of the isolated ASCs used in the present study could therefore be applied in order to identify the extent of specific surface marker expression and thereby further validating the stem cell phenotype of the cells.

5.2 - ANGIOGENIC POTENTIAL OF ASC SECRETOME

The angiogenic potential of the ASC secretome was investigated on both human and rat endothelial cells. hASC-CM failed to accelerate the healing process of HDMECs in an *in vitro* scratch assay. This result is in contrast with previous findings including one conducted in our lab (71,77). Here hASC-CM significantly reduced healing time of the cell-free area. hASC-CM was likewise unable to promote healing of REC. Although a tendency towards accelerated healing was seen after 30 hours, no significant difference from control samples could be detected. In contrast, rASC-CM did in fact facilitate healing in the *in vitro* REC scratch assay ($p<0.01$), with an average of 25% remaining wound area after 30 hours compared to 77% in controls. This finding is in agreement with previous research on hASCs (71,77) and indicates that the rASC-CM contained active angiogenic factors, which in turn bodes well for the applicability of the isolated rASCs in future rat chronic wound models.

There seems to be no obvious explanation for the lack of angiogenic potential of the hASC-CM. Even though transdifferentiation of MSCs into ECs have been reported, the main mechanism by which MSCs are believed to induce angiogenesis in surrounding endothelium is thought to be by paracrine secretion. Secretome analyses for identification of specific components might be able to shed some light as to why no accelerated healing time was observed. Especially investigations on concentration of VEGF in the CM would be of interest, as this the most potent promoter of endothelial growth (78).

When conducting experiments in which CM is collected for downstream analyses or assays, considerations regarding the most appropriate setup must be made. Some setups include a period of serum starvation of the cell culture just prior to harvest of the CM. This excludes contamination of the harvested sample by unknown substances in the supplementary serum, which might influence the result of downstream analyses (79). This approach, however, induces a stressful environment for the cells, due to deprival of essential nutrients and growth factors. The response of the cells to this starvation might include changes in gene activity resulting in a secretome, which do not correspond to that of baseline production (80). Thus, both serum supplementation and serum starvation might cause the harvested CM to contain an altered composition of substances. When choosing between serum supplementation and starvation in CM harvest setups, one has to consider, which strategy is more appropriate in the required investigations. In the present study, serum supplementation was chosen in order to ensure optimal support of endothelial growth during the scratch assay. Since all scratched monolayers, including negative controls, received media with equal amounts of FCS any changes between groups originate from ASC secreted substances and thus reflect the angiogenic potential of the secretome.

5.3 - MSC APPLICATION IN REGENERATIVE MEDICINE

Application of MSCs for various therapeutic purposes is a growing research field within regenerative medicine (10,20,22). Mainly the secretome of MSCs have been shown to possess potent paracrine abilities including immunomodulation as well as pro-angiogenic and anti-apoptotic factors (20,22,23). It has become evident, however, that there is a substantial amount of interdonor variation in regards to these MSC abilities.

Moreover, origin of the MSC, e.g. bone marrow and adipose tissue, as well as donor gender, age and overall health status also seem to influence the therapeutic potential of isolated cells (29–33). Interestingly, even MSCs isolated from the same donation such as a single lipoaspirate or a single bone marrow aspirate have been shown to contain a mixture of phenotypical distinct MSCs (71,81). Much research is currently dedicated to understanding the heterogeneity in a group of MSCs from the same donor by linking specific surface markers to distinct cell features. The ultimate goal is subdivision of MSCs by cell sorting in groups with specific therapeutic ability, e.g. angiogenic potential or immunomodulation etc. in order to choose appropriate MSC donors for a specific clinical purpose (71). As an example, a study conducted in our own lab have reported a connection between ASC expression of surface marker CD146 and paracrine support of angiogenesis (71).

Highly relevant to MSC therapeutic application is the ability of the cells to escape immune recognition when used for allogenic transplantation (82). The possibility of identifying donors from which MSCs optimal for specific therapeutic purposes can be harvested and subsequently used for application in patients with various pathologies is intriguing. In regards to chronic wound healing, allogenic transplantation of MSCs from healthy donors might be of certain interest since these patients are often elderly and suffering from other illnesses (12), which might have a negative influence on the therapeutic potential of their own MSCs (32). The lack of immune response activation furthermore allows for interspecies studies in which e.g. human MSCs are utilized in rat models. This allows for pre-clinical *in vivo* evaluation of the therapeutic potential of MSCs from different human donors. Relevant to the present study, wound healing promoting abilities of human MSCs have been reported in rat wound models (83,84).

Another aspect of optimizing MSC application for therapeutic purposes is through so-called preconditioning of the cells. Several studies have reported an enhanced immunomodulatory effect of MSCs, which have been exposed to various factors, with which an inflammatory environment is associated. These include hypoxia, IFN-γ, TNF-α and LPS. This exposure is thought to mimic *in vivo* inflammatory environment, which induces activation of an active immunomodulatory phenotype of the MSCs (8,85). Increases in secreted anti-inflammatory cytokines as well as angiogenic potential and induction of macrophage polarization towards M2 have all been reported as a result of preconditioning (36,86).

5.4 - FUTURE PERSPECTIVES

The anti-inflammatory effects of MSCs are well established and much research are currently dedicated to utilizing this effect in a novel chronic wound treatment regime (10,20,22). As described earlier probiotics have likewise been shown to be potent inducers of an anti-inflammatory immune response (54–57). Based on these observations, it can be proposed that allogenic MSCs application in combination with oral supplementation of beneficial bacterial strains have a role to play in the refinement of a safe and efficient treatment strategy for chronic wounds.

As mentioned in the introduction, the long-term plan for the present study is to utilize the isolated hASCs and rASCs in a diabetic rat model in which the combined effects of MSC injections and oral supplementation of probiotics on chronic would healing can be investigated.

Prior to moving onto the animal study platform, however, several aspects regarding wound healing abilities of the isolated ASCs could be further investigated. These include scratch assays conducted on keratinocytes and fibroblasts in order to evaluate the healing promoting abilities of key cell types in skin histology. Furthermore, secretome analyses would provide insight as to the composition of wound healing promoting factors.

6 - CONCLUSION

I the present study, characterization of stem cell phenotype of primary ASCs isolated from human and rat abdominal fat tissue was conducted. Adipogenic and chondrogenic differentiation were successfully induced in hASCs, whereas only adipogenic differentiation was achieved for rASCs. Osteogenic differentiation was unsuccessful for both human and rat cells. In addition to multipotency, the proliferative potential and proportion of CFUs were likewise investigated. Results from these assays revealed a low doubling time as well as potent clonogenicity of both hASCs and rASCs.

Taken together, the validation of two phenotypical characteristics of stem cells, high proliferation rate and high proportion of CFUs as well as the successful adipogenic differentiation indicate that the investigated ASCs were in fact active and viable mesenchymal stem cells. However, further attempts of osteogenic differentiation, and for rASCs also chondrogenic differentiation, are needed in order to verify the multipotency of the isolated cells.

Adipogenic potential of the isolated cells was investigated by *in vitro* scratch assays conducted on both human and rat endothelial cells. hASC-CM failed to facilitate accelerated healing of the scratch in both human and rat endothelial cell cultures. In contrast to this, addition of rASC-CM significantly reduced healing time in the rat endothelial cell scratch assay. This indicates that the rASCs actively secreted factors important for endothelial proliferation. Based on this result, it can be proposed that the isolated rASCs are appropriate for application in a future rat chronic wound model. Further *in vitro* studies are, however, essential prior to progressing onto animal studies.

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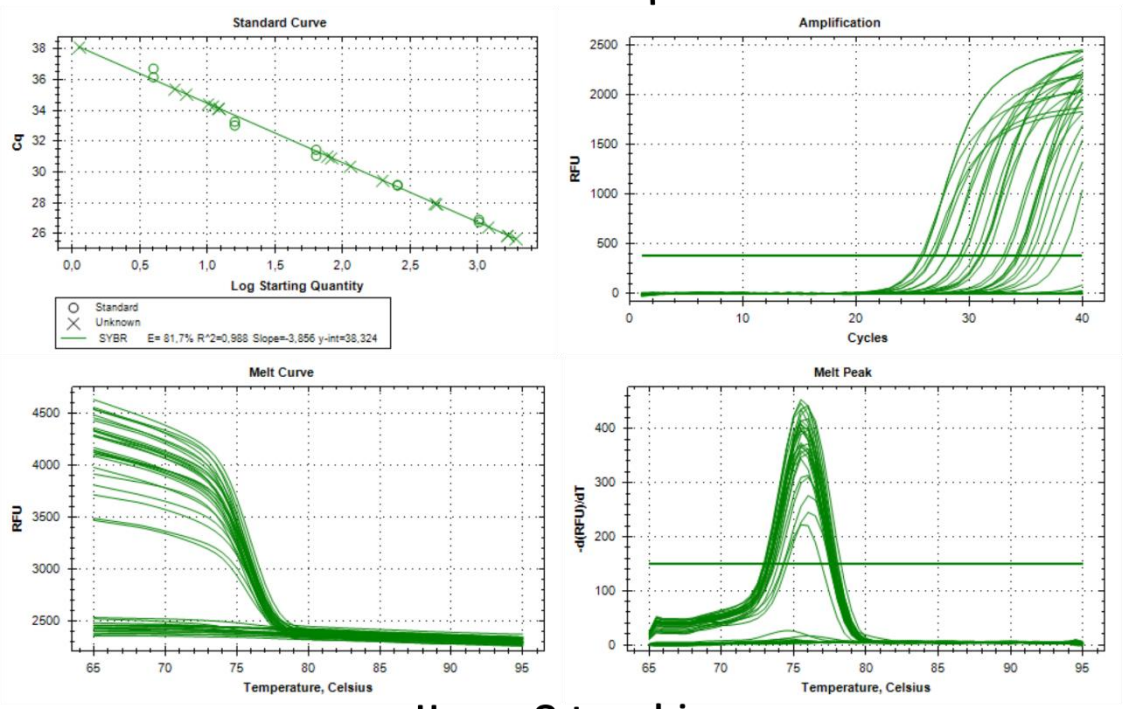
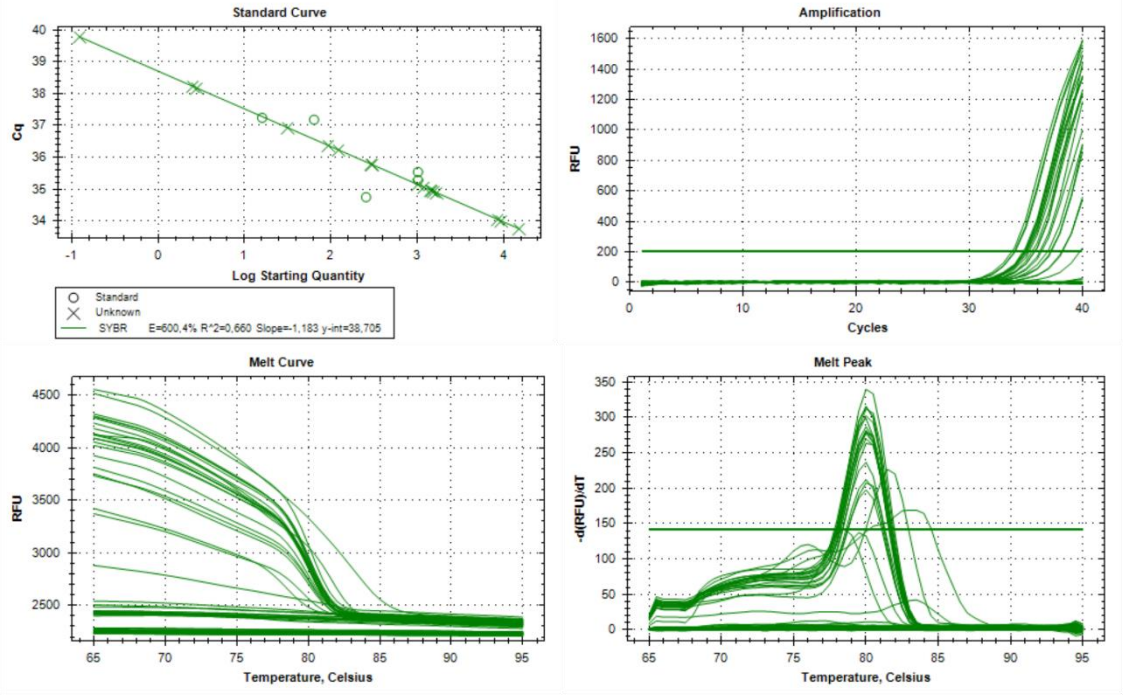
APPENDIX 1 - qRT-PCR

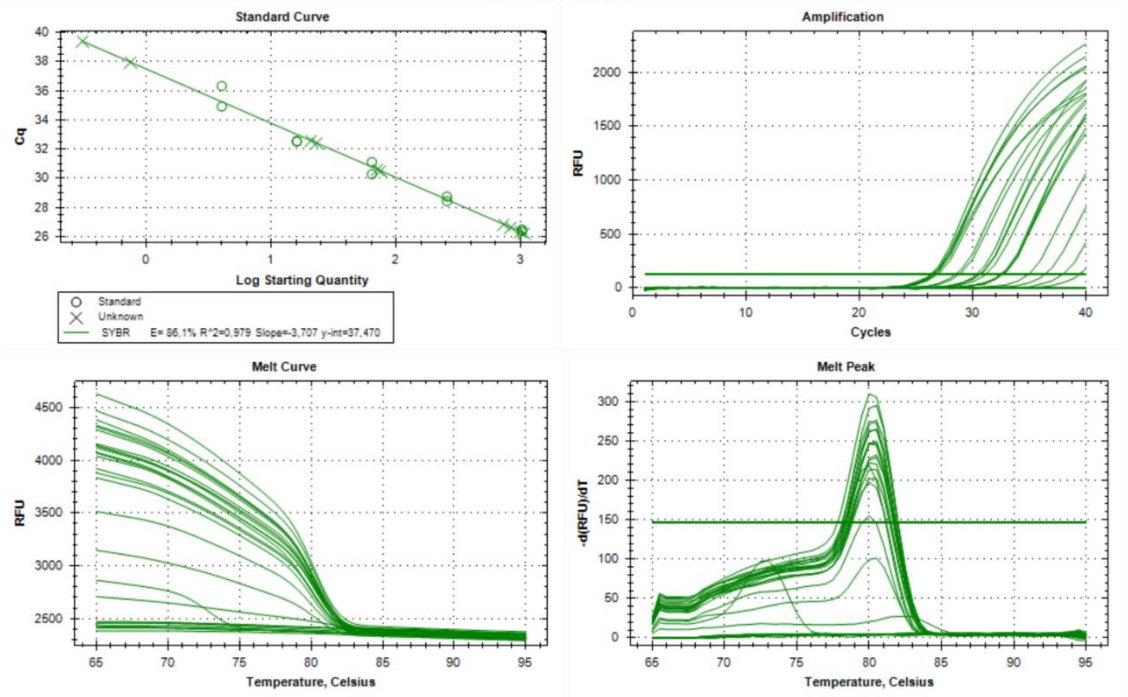
Human PPAR-γ2



Human Osteocalcin



Human SOX-9



Rat PPAR-γ2