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Assessment of Cell Based Therapies for Cutaneous Wound Healing in Rabbits and Horses

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Dissertation submitted in fulfillment of the requirements for the
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Cover design

The background picture of the cover pages of the thesis shows hematoxylin and eosin stained epithelial-like stem cells isolated from horse skin.

Dedication

To my husband Mr. Tesfaye Defar and My children: Sufeni Tesfaye and Firegnu Tesfaye

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Bizunesh

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List of Abbreviations

AdSC	Adult Adipose-derived Stem Cell
ASC	Adipose-derived Stem Cell
ATMP	Advanced Therapy Medical Product
bFGF	basic Fibroblast Growth Factor
BI	injection of bone marrow nucleated cells into wound margin
BM	Bone Marrow
BMDC	Bone Marrow-Derived Cell
BM-MNC	Bone Marrow Mononuclear Cell
BM-MSC	Bone Marrow Mesenchymal Stem Cell
BMNC	Bone Marrow Nuclear Cell
BMP	Bone Morphogenic proten
BSA	Bovine Serum Albumin
BT	administration of bone marrow nucleated cells topically
CD	Cluster of Differentiation
CFA	Colony forming Assay
CK	Ctyokeratine
CK	Cytokeratin
cm	centimeter
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGF	Epidermal Growth Factor
EMA	European Medicines Agency
EPC	Endothelial Precursor Cell
EpiASC	Epidermal Adult Stem Cell
EpSC	Epithelial (like) Stem Cell
ESC	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor

FITC	Fluorescein isothiocyanate
g	gram
G	Gravitational force
GCP	Good clinical Practice
GF	Growth Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GTP	Good Tissue Practice
HaCaT	immortalized human keratinocyte
hBM-MSC	human Bone Marrow Mesenchymal Stem Cell
HBSS	Hank's Balanced Salt Solution
HE	Hematoxylin and Eosin
hESC	human Embryonic Stem Cell
HGF	Hepatocyte Growth Factor
HLA-DR	Human Leukocyte Antigen D-related
hMSC	human Mesenchymal Stem Cell
Hr	Hour
HSC	Hematopoietic Stem Cell
hSTSC	human Scar Tissue Stem Cell
IGF	Insulin-Like Growth Factor
IHC	Immunohistochemistry
IL	Interleukin
ISCT	International Society for Cellular Therapy
kg	kilogram
KGF	Keratinocyte Growth Factor
mg	milligram
MHC	Major Histocompatibility complex
mL	milliliter
MMP-2	Matrix Metalloprotinase-2
MNC	Mononuclear Cell
MSC	Mesenchymal Stem Cell
NC	Nuclear Cell
NS	Normal saline
P	Passage

PB-MSC	Peripheral Blood-derived Mesenchymal Stem Cell
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PDT	Population Doubling Time
PI	Povidone Iodine
PRP	Platelet Rich Plasma
rbBM-MSC	rabbit Bone Marrow Mesencymal Stem Cell
SC	Stem Cell
SCBP	Stem Cell Based Product
SFE	Sphere Forming Efficiency
SKP	Skin-derived Precursor
SMA	Smooth Muscle Actin
SVF	Stromal Vascular Fraction
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
UCB-MSC	Umbilical Cord Blood Mesenchymal Stem Cell
UCM-MSC	Umblical Cord Matrix Mesenchymal Stem Cell
VEGF	Vascular Endothelial Growth Factor
VG	Van Gieson

CHAPTER 1

General Introduction

1.1 Regenerative medicine and cell therapy

Skin wounds remain a challenging clinical problem with early or late complications presenting a frequent cause of morbidity and mortality (Natarajan et al., 2000). Both humans and animals experience different types of wounds during the course of their lives. Particularly, veterinary patients are frequently affected by complex skin wounds (Theoret, 2009b). During the past few decades, various wound healing technologies for promoting cell activity or minimizing scar formation have been developed and some of them are being actively used at present (You and Han, 2014). Regenerative medicine is an emerging and rapidly evolving field of research developed with therapeutics aim to restore, maintain and improve body functions (Polak et al., 2008). Ultimately, this might provide a treatment for conditions where current therapies are failing or are inadequate (Ramakrishna et al., 2011). Cell therapy is a part of the broad field of regenerative medicine and can be defined as a set of strategies which use live cells with therapeutic purposes (Yolanda et al., 2014).

1.2 Definitions and types of stem cells

Cells used in cell therapy can be categorized as stem cells and adult/differentiated cells. In traditional textbooks, it was taught that stem cells were at the origin of all major tissue types and that once cells partly or totally differentiated into a cell type they became terminal cells and could not be dedifferentiated. However, now it is known that stem cells are 'plastic' and indeed dedifferentiate (You and Han, 2014). There are two major types of stem cells, the embryonic stem cells (ESC), which are derived from the inner cell mass of an early embryo, and the adult stem cells (ASC), which have been found in almost every tissue of every human and animal (Figure 1.1). ASC are the prime source for cell-based therapies as they are relatively easy to obtain through *in vitro* culturing and they escape the ethical issues associated with ESC (Volk and Theoret, 2013a). So far, fibroblasts, keratinocytes, adipose-derived stromal vascular fraction cells, bone marrow (BM) stem cells and platelets have been used to stimulate wound healing in clinical practice (You and Han, 2014). Details of cell based skin wound therapy can be found in Chapter 2 of this thesis.

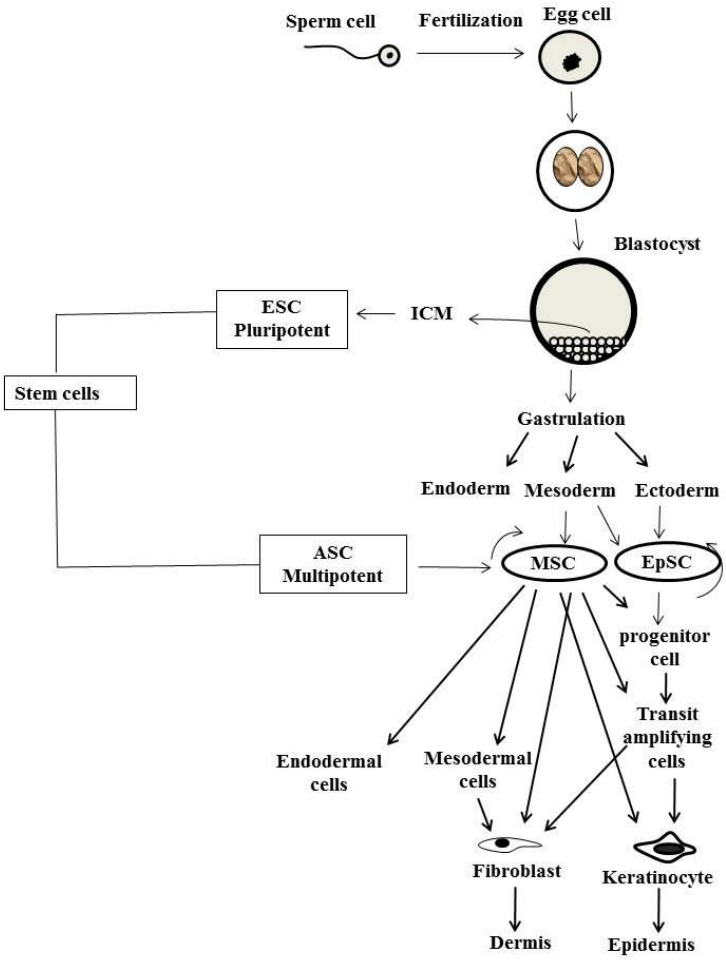

Figure 1.1 Classification of stem cells. Schematic overview of embryonic stem cells (ESC) and adult stem cells (ASC) with more emphasis on mesenchymal stem cells (MSC) and epithelial stem cells (EpSC) and their differentiation potential to most commonly cited skin cells. The zygote formed following fertilization undergoes serial cleavage to form the inner cell mass (ICM) of a blastocyst, which contains ESC. ASC can be isolated from various tissues of the three germ layers. MSC are derived from the mesoderm, whereas skin EpSC are isolated from dermis (mesodermal origin) and epidermis (ectodermal origin). Both stem cell types can be induced to differentiate into various adult cell types including keratinocytes and fibroblasts.

1.3 Animal models for stem cell based skin wound therapy

Different experimental animal models are currently being used to establish relevant preclinical data for developing human (regenerative) therapies (Campillo et al., 2014). Veterinary patients, including companion animals (dogs, cats and horses) and farm animals

(cattle, sheep, goats and pigs) are increasingly being used and recognized as translational models for the evaluation of novel agents for the treatment of human diseases. Compared to rodents and rabbits, all of the above mentioned animals are considered as "large" experimental animal models (Volk and Theoret, 2013a). "Small" animals, such as mice, rats and rabbits, have been extensively used as models for a number of preclinical and clinical studies evaluating cell-based therapies for cutaneous wounds. The advantages of these "small" species are the different available strains, low costs, short reproduction turnover and less ethical concerns in comparison to "large" animal models. The shorter life cycle and smaller size of rodents enhance the power of studies within a shorter timeframe. However, it is questionable whether results from "small" animal studies can be translated/extrapolated to humans (Volk and Theoret, 2013b). In comparison, "large" animal studies are assumed to generate results that are more alike results to be expected in humans. Indeed, the basic biochemical and physiological processes in large animal models resemble more closely those in humans compared with rodents (Parker et al., 2010). Moreover, studies show that some 292 canine, 163 feline, 142 bovine and 109 equine genetic diseases are homologous with human genetic defects (Lenffer et al., 2006). Nevertheless, in some cases the pathophysiology and resulting phenotype of such mutations are still undefined or may vary from that in humans

The advantages of "large" animal studies include the longer lifespan, availability of outbred offspring and exposure to external and environmental factors. Furthermore, long-term follow-up and repeated biologic sampling are more feasible in "large" animal models and enhance the ability to detect side effects of novel therapies which minimize both veterinary and human patient risks. In addition, the increase in demand for sophisticated, state-of-the-art care for companion animals has led to a surge in clinical trials in veterinary patients (Volk and Theoret, 2013a). For all the aforementioned reasons, "large" animal models are considered to be indispensable for advancing stem cell therapies for both human and veterinary clinical applications.

1.4 Isolation and characterization of adult stem/progenitor cells

In the field of veterinary medicine, different isolation methods and identification techniques have been described for obtaining different (stem) cell types. However, in contrast to human mesenchymal stem cells (MSC), no uniform characterization criteria are available for veterinary stem cells to date (De Schauwer et al., 2011b; Dominici et al., 2006). While MSC from multiple species can be identified by their ability of plastic-adherence under standard

culture condition in tissue culture flasks and tri-lineage differentiation towards adipocytes, osteoblasts and chondroblasts (Dominici et al., 2006), other stem cell types may possess substantially different properties, such as survival in suspension and differentiation towards keratinocytes and myo-epithelial cells (Broeckx et al., 2014e; Dominici et al., 2006; Dontu et al., 2003). For all these reasons, the isolation and characterization techniques are critical parameters before assessing any cell-based product in a clinical application.

1.4.1 Bone marrow-derived nucleated cells

In the center of most large bones there is a soft tissue, the so-called bone marrow (BM), which is a network of soft, sponge-like material that contains immature blood cells in an organized structure. Post-natal BM has traditionally been considered as an organ composed of two main systems or compartments rooted in distinct lineages: the hematopoietic tissue and the associated supporting stroma (Bianco et al., 2001; Jackson et al., 2001). The hematopoietic compartment provides the organism with mature blood cells of all lineages and the stromal cell compartment functions as the microenvironment for self-renewal, proliferation and differentiation of hematopoietic stem cells (HSC). In addition, the stromal cell compartment harbors vascular progenitor cells (Jackson et al., 2001) and MSC capable of differentiation into cells of most connective tissues (Bianco et al., 2001). HSC are a well-characterized population of self-renewing cells that produce progenitors that differentiate into every type of mature blood cell in a well-defined hierarchy (Kondo et al., 2003). To date, HSC and MSC are the stem cell types having received a lot of attention from the scientific community because of their potential use as a regenerative medicine (Volk and Theoret, 2013a).

Bone marrow-derived MSC (BM-MSC) have great potential in tissue engineering and clinical therapy and various methods for isolation and cultivation have been reported. However, the isolation and cultivation techniques have not yet been optimized (Zhang et al., 2014). BM tissue has been harvested/aspirated to isolate BM-MSC from bone marrow-derived nucleated cells (BMNC). The BM-MSC are then cultured, expanded and injected back into the host (autologous cell therapy) to promote osseous or soft tissue healing (Ishihara et al., 2013). Isolation of BM cells is being performed by aspiration of this substance from various sites of the animal or human. To date, BM has been harvested from humerus, iliac crest or tibia in dogs and cats, and from the sternum and tuber coxae in horses (Volk et al., 2012). In rabbits, guinea pig, mouse and chicken, BM has mainly been aspirated from the tibia (Sundberg and

Hodgson, 1949). In addition, it can also be isolated from the iliac crest (Awad et al., 2000; Sundberg and Hodgson, 1949) and femur (Powsner and Fly, 1962). In this thesis (Chapter 4.1 and 4.2), BM has been aspirated aseptically from tibia of experimental rabbits under general anesthesia. BM aspirates consist of a heterogeneous cell population, including fibroblasts, adipocytes and mononuclear cells (BMMNC) (Wu et al., 2007a). BM-MNC include HSC, BM-MSC, endothelial progenitors and cellular precursors (Taylor and Zenovich, 2008). Unfortunately, MSC constitute only a small percentage of the BM populating cells, and therefore, subsequent cultivation of BM-MSC is advisable when their specific effects are being studied.

Several methods are currently available for BM-MSC isolation and cultivation. Although separation methods that include immune-magnetic beads or flow cytometry generate BM-MSC with higher purity, the expense, procedural complications and cell damage that occur during this process, restrict their usage (Zhang et al., 2014). Alternatively, BM-MNC can be concentrated by using volume reduction protocols or gradient centrifugation techniques such as Ficoll or Percoll density gradient (Kasten et al., 2008; Zhang et al., 2014). The number of MSC isolated from different tissues may vary in terms of the yield, quality and purity, even when the cells have been obtained from the same donor (Phinney et al., 1999).

Studies reveal that BM cells, most notably MSC, play a major role in skin regeneration (Brittan et al., 2005; Crigler et al., 2007; Deng et al., 2005; Kim et al., 2013b) and vascularization (Asahara et al., 1999). BM has been used as concentrated autologous BM-MNC or as BM-MSC, which have been isolated from the BM concentrate and expanded and characterized *in vitro* before clinical use (Altaner et al., 2013). MSC are characterized based on their ability to self-renew, differentiate towards different cell types of the mesodermal germ layer and express certain cell surface markers (Dominici et al., 2006). Population doubling levels are an estimate for the growth efficiency and proliferation potential of MSC (Kang et al., 2013). A study in humans has shown that doubling time of cultivated BM-MSC is age dependent. Indeed, BM-MSC from older persons possess higher doubling times than their younger counterparts (Altaner et al., 2013). With regard to their differentiation potential, BM-MSC have to differentiate into osteoblasts, adipocytes and chondroblasts, according to the proposed minimum criteria set by International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). In this regard, a number of studies have demonstrated the *in vitro* and *in vivo* differentiation potential of MSC along multiple lineages at a preclinical and clinical

level (Kraus and Kirker-Head, 2006; Martinello et al., 2010a). These lineages consist of cell types from all germ layers of the embryo, such as mesodermal cells (skeletal muscles, cardiomyocytes), but also ecto- and endodermal cells, e.g. neuronal cells, glial cells, keratinocytes and hepatocytes (Bajek et al., 2011; Bieback et al., 2012). Particularly interesting for the present thesis is the ability of BM-MSC differentiate to different skin cells and secrete wound healing factors as reported in the following studies. In an experimental murine model, adult BM-derived stromal cells gave rise to epidermal keratinocytes, follicular epithelial cells, sebaceous gland cells, dendritic cells and endothelial cells after transplantation (Goodell et al., 2001; Ratajczak et al., 2004). Direct transdifferentiation of remotely recruited BM-MSC types into epidermal keratinocytes was also reported by a number of researchers (Borue et al., 2004a; Brittan et al., 2005). Additionally, experiments by different groups (Sasaki et al., 2008; Tamai et al., 2011) also showed that BM-MSC, rather than some hematopoietic progenitors, are able to undergo direct epidermal transdifferentiation. In addition to the wound healing effect of BM-MSC through differentiation and/or transdifferentiation into different skin cells, a number of researchers have also reported this could be possible through the release of paracrine factors by BM-MSC including growth factors, cytokines and chemokines (Chen et al., 2008b; Kim et al., 2013b). In fact there have been evidences against and for plasticity of ASC in general, which made it a subject of debate. Even though some techniques which have been used to assess *in vivo* and *in vitro* plasticity are convincing, issues of cell to cell fusion and lack of reproducibility of findings in other laboratories are the two primary controversies in BM-MSC plasticity (Grove et al., 2004; Herzog et al., 2003; Zhang and Alexanian, 2014). However, it is not yet known whether fusion is responsible for much of the plasticity data (Grove et al., 2004). While transdifferentiation of MSC is still controversial, these unique properties make MSC an ideal autologous source of easily reprogrammable cells (Zhang and Alexanian, 2014). Therefore, there is a need for accurate and sensitive technique to establish the *in vivo* and *in vitro* works to confirm plasticity/transdifferentiation of MSC and to better understand ASC plasticity that can be used for treating diseases and tissue injuries in patients. In the present thesis, an in-depth review concerning wound healing potential of MSC through their differentiation, transdifferentiation and paracrine effect can be found in Chapter 2.

MSC have been isolated from humans and a variety of animal species (Penny et al., 2012). Successful isolation and differentiation of MSC from different sources such as BM, adipose tissue, umbilical cord blood, Warton's jelly, amniotic fluid or peripheral blood (Cremonesi et

al., 2008a; Kang et al., 2013; Koch et al., 2007; Martinello et al., 2010a; Spaas et al., 2013b; Spaas et al., 2013c), shoulder tissue (Utsunomiya et al., 2013), gingival tissue (Ge et al., 2012), placenta (Wang et al., 2012), muscle and periosteum (Kisiel et al., 2012) have been reported. However, currently there is no consensus on how these cells should be identified and characterized. This is particularly due to the lack of standardized specific cell markers for MSC (De Schauwer et al., 2011b; Penny et al., 2012) and limited availability of monoclonal antibodies for animals, which are the two major factors complicating successful research on MSC (De Schauwer et al., 2011b). In human medicine, the Mesenchymal and Tissue Stem Cell Committee of the ISCT proposed three criteria to define human (h) MSC: i) plastic-adherent when maintained under standard culture conditions, ii) express specific surface antigens and iii) possess a multipotent differentiation potential (Dominici et al., 2006) (Table 1.1). These minimum criteria apply only to hMSC.

Table 1.1 Minimum criteria to define hMSC as proposed by ISCT (Dominici et al., 2006)

	Culture characteristic	Differentiation potential	Expression of surface antigen	
			Positive	Negative
hMSC	Plastic adherent in standard culture conditions (*in vitro*)	Osteoblasts, adipocyte, chondroblasts	CD73, CD90 & CD105	CD34, CD45, CD14 or CD11b, CD79α or CD19, MHC class II antigens

CD = Cluster of Differentiation; hMSC = human Mesenchymal Stem Cells; ISCT = International Society for Cellular Therapy

Although rabbit bone marrow mesenchymal stem cells (rbBM-MSC) have been used extensively, especially in translational research, these cells have neither been defined morphologically and ultra-structurally, nor been compared with their human counterparts (hBM-MSC) concerning multi-lineage differentiation ability. According to a study by Tan et al. (2013) rbBM-MSC expressed CD29, CD44, CD73, CD81, CD90 and CD166 but were negative (or dim positive) for CD34, CD45, CD117 and HLD-DR (Tan et al., 2013). A recent study by Zhang et al. (2014) also reported BM-MSC isolation from rabbits on the basis of positive expression of CD29, CD44, CD90, CD105 and no expression for hematopoietic markers and endothelial markers such as CD14, CD34, CD45 and CD184 (Zhang et al., 2014). However, even though MSC have been isolated and characterized from a variety of animal species including rodents, dogs, horses and rabbits (Penny et al., 2012), there is no consensus concerning common standards and harmonized isolation and characterization protocols, which are important to identify the used cell type and ensure safety and efficacy

(Bieback et al., 2012). Moreover, MSC of animal origins do not express all of the abovementioned surface molecules (Gronthos et al., 2001; Peister et al., 2004). When hMSC have been correctly isolated from a biopsy, the expression of some surface markers can also be attributed to the presence of different factors excreted by other mononuclear cells present at initial passages (Gronthos et al., 2001). A summary of the currently available characterization methods including marker expression on MSC of different tissues and animals is shown in Table 1.2. In addition, it is also challenging for clinicians to utilize cultured BM-MSC for the purpose of wound healing due to issues regarding the approval by the food and drug administration (FDA). Autologous whole BM transplantation is a simpler method and does not require FDA approval because it is considered as a single-step surgical procedure when immediately applied to the same patient. Accordingly, in the present thesis, whole autologous BM-derived cells have been used after volume reduction (as indicated in the materials and methods of Chapters 4.1 and 4.2) for treating full thickness skin wounds of rabbits.

1.4.2 Skin-derived epithelial-like cells

In recent years, different classes of stem cells have been investigated for their ability to regenerate organs and tissues after injury. The skin, which is the largest organ of the mammal body is a rich and easily accessible source of stem cells. So far, stem cells have been isolated from different skin compartments including the isthmus (i.e. above the bulge, where the sebaceous gland duct inserts) (Gordon and Andersen, 2011), sweat glands (Lu et al., 2012), the bulge of the hair follicle, sebaceous glands, dermis and epidermis as discussed/shown in Chapter 2 (Broeckx et al., 2014e; Draheim and Lyle, 2011; Frances and Niemann, 2012; Ito, 2005; Plikus et al., 2012; Taylor et al., 2000; Toma et al., 2001; Toma et al., 2005).

The skin is a tissue which undergoes continuous self-renewal throughout the lifetime of an organism and also has an extensive ability to repair inconsistencies (Nowak and Fuchs, 2009). It has been well documented that tissue homeostasis and wound repair are ensured by stem cells, located within a specialized microenvironment, being the skin “niche”. Each niche is tailored to accommodate the regenerative needs of its tissue. However, some tissues like skin epithelium harbor multiple stem cell niches, each with their own responsibility for maintaining cellular balance within their particular domain (Blanpain and Fuchs, 2014).

Table 1.2 Overview of frequently cited self-renewal methods, differentiation potential and marker expression of MSC derived from different tissue sources and animals.

Reference	Cell type	Source, animal	Self- renewal	Diff	Marker Positive	Negative
(De Schauwer et al., 2014)	MSC	UCB, horse	PDT	O, A, C	CD29, CD44, CD73, CD90, CD105, MHC-I	CD45, D79a, Mo/Mφ, MHC-II
		UCM, horse	PDT	A, C	”	”
		PB, horse	PDT	O, A, C	”	”
(Tan et al., 2013)	”	BM, rabbit	PDT, proliferation	”	CD29, CD44, CD73, CD81, CD90, CD117,	CD34, CD45, HLA-DR
		BM, human	”	”	CD29, CD44, CD73, CD81, CD90	CD34, CD45, CD117, HLA-DR
(Spaas et al., 2013c)	”	PB, horse	CFU, PDT	”	CD29, CD44, CD90, CD105	CD45, CD79α, MHC-II, Mo/Mφ
(Carvalho et al., 2013)	”	PB, horse	ND	A, C	CD44, CD90	CD13
(De Schauwer et al., 2011a)	”	UCB, horse	Sphere, PDT	O, A, C	CD24	ND
(Martinello et al., 2010a)	”	PB, horse	Telomerase, proliferation, PDT	O, A, M	CD44, CD90, CD117, CD13	CD34, CD45
(Kim et al., 2013b)	”	BM, dog	ND	ND	CD29, CD44	CD34, CD45
(Sasaki et al., 2008)	”	BM, mice	ND	O, A, C	CD29, CD44 , CD90	CD34, CD31
(Wu et al., 2007b)	”	BM, mice	ND	O, A, C	CD29, CD44, CD105, CD90, Sca-1	CD34, CD45, CD14, CD3, CD19
(Badiavas and Falanga, 2003)	MNC	BM, human	ND	ND	CD31	CD68, CD34
(Badiavas et al., 2007)	MNC	BM, human	CFU	A, E, F	CD146, CD133, CD14	N/D
(Kasten et al., 2008)	MNC	BM, human	CFU	O, A, C	CD13, CD44, CD73, CD90, CD105, HLA-A, B, C	CD14, CD34, CD45, CD271, HLA-DR

A = Adipocyte; BM = Bone Marrow; CD = Cluster of Differentiation; CFU = Colony Forming Unit; C= Chondroblast; Diff = Differentiation capacity; E = Endothelial cell; F = Fibroblast; HLA-DR = Human Leukocyte Antigen D-related; M = Myocyte; MHC = Major Histocompatibility Complex; MNC = Mononuclear Cell; Mo/Mφ = Monocyte/Macrophage; MSC = Mesenchymal Stem Cells; ND = Not Determined; O = Osteoblast; PB = Peripheral Blood; PDT = Population Doubling Time; UCB = Umbilical Cord Blood; UCM = Umbilical Cord Matrix

The role of EpSC during wound healing is not limited to regenerating stratified epidermis. By studying the regenerative response in large cutaneous wounds, it has been discovered that epithelial cells in the center of the wound can acquire greater morphogenetic plasticity and, together with the underlying wound dermis, can engage in an embryonic-like process of hair follicle neogenesis (Plikus et al., 2012). However, in severe pathological cases, such as burn wounds, chronic wounds, and ulcers, the endogenous skin repair mechanisms might be insufficient. For this reason, exogenous purification and multiplication of EpSC might be useful in the treatment of these skin diseases.

In correspondence with the characterization of other ASC, specific functional and immunophenotypic properties have been identified to characterize skin stem cells. These techniques include sphere formation assessment, self-renewal assays, multilineage differentiation evaluation and assessing the presence of specific cell surface or intracellular markers (Table 1.3). Sphere formation has been reported from both crude dermal suspensions cultured on adherent surfaces (Toma et al., 2005) and epidermis-derived cells seeded at clonal density under ultralow attachment conditions (Broeckx et al., 2014e). Besides the evaluation of population doubling times (Broeckx et al., 2014), there is only limited information available on the self-renewal capacity of skin stem cells. Indeed, the survival and proliferation rate of sphere-initiating cells under different culture conditions and after serial passaging remains to be elucidated.

Concerning the differentiation capacity of skin stem cells, several reports confirm their multipotent properties. In this regard, it has been described that rodent skin-derived precursors (SKP) differentiated into both neural and mesodermal cell types, including neurons, glia, smooth muscle cells, and adipocytes (Toma et al., 2001). Also human scar tissue-derived stem cells (hSTSC) were able to form neurogenic progenitor cells (Yang et al., 2010). Moreover, it has been recently reported that equine epidermal-derived stem cells differentiated towards two major skin cell types, being keratinocytes and adipocytes (Broeckx et al., 2014e)-. For immunophenotypic characterization of skin-derived stem cells several types of surface molecules have been tested so far. In one of the studies on murine dermal cell suspensions, phenotypic analysis revealed that a small population of $CD45^+$ cells and a large population of $CD45^-$ cells expressed CD34, CD117 and stem cell antigen-1 (Meindl et al., 2006). This indicates that a certain degree of cell impurities were present in these spheres. A study by Broeckx et al. characterized equine epidermis EpSC by means of positive expression for

CD29, CD44, CD49f, CD90, Casein Kinase 2β, p63 and Ki67, low expression for cytokeratin (CK)14, and negative expression for CD105, CK18, Wide CK and Pan CK (Broeckx et al., 2014e). The authors reported no cellular impurities in the three screened isolates.

Table 1.3 Summary of the currently available characterization criteria used by different researchers including self-renewal assessment method, differentiation potential and marker expressions on epithelial stem cells of different sources.

Reference	Cell type, species	Source, animal	Self-renewal	Differentiation	Marker		Other method
					Positive	Negative	
(Broeckx et al., 2014e)	EpSC, horse	Epidermis	Sphere formation, PDT	K/A	CD29, CD44, CD49f	CD105	ND
(Frances and Niemann, 2012)	HFSC, mice	HF	ND	Sebocyte	Sox 9, Lrig1	ND	LR
(Lu et al., 2012)	SGP, mice	Epidermis and dermis	CFA	ND	Sca-1, CD29, CD49f, CD24, Sox9	ND	LR
(Toma et al., 2005)	SKP, human	Dermis	Sphere, PDT	Neurons, glia, adipocytes, SMC	ND	ND	LR
(Toma et al., 2001)	SKP, mice	Dermis	Sphere, PDT	Neurons, glia, adipocytes, SMC	ND	ND	LR
(Cotsarelis et al., 1990; Taylor et al., 2000)	Bulge stem cells, mice	HF,	ND	HF	ND	ND	LR
(Jones and Watt, 1993)	Epidermal stem cells, human	Epidermis	CFA	ND	CD29	ND	ND

A = Adipocyte; C = Chondrocyte; CD = Cluster of Differentiation; CFA = Colony Forming Assay; EpSC = Epithelial-like Stem cells; HF(SC) = Hair Follicle (stem cells); K = Keratinocyte; LR = Label Retention; Lrig1 = Leucine-rich repeats and immunoglobulin-like domains protein 1; MHC = Major Histocompatibility Complex; M = Myogenic; ND = Not Determined; O = Osteocyte; PDT = Population Doubling Time; SC = Schwann cells; SKP = Skin-Derived Precursor; SMC = Smooth Muscle Cells; Sox9 = Sry-related high mobility group box; SGP = Sweat Gland Progenitor cells

1.5 Administration routes of stem cells and evaluation of wound healing

Various cell-based therapies have been clinically administered using local delivery or systemic infusion. Local application consists of topical product delivery (in the form of a gel, liquid, patch, etc.), intradermal/injecting the wound edges or intramuscular injection. For clinical transplantation, cells can be derived from the same animal (autologous), from an animal within the same species (allogenic) or from an animal of a different species (xenogenic) (Gade et al., 2012). Systemic injections on the other hand, involve injecting into the blood of the local vein or artery, or into the general circulation. More details concerning the different routes of administration of cell-based therapies to different wound types in different animal models are shown in Table 2.2 of Chapter 2 in the current thesis.

The process of wound healing is dynamic and takes place over months , during which angiogenesis, continued wound contraction and connective tissue remodeling occur (Junker et al., 2014). Evaluation of the skin wound healing following transplantation of cells can be performed by using different scoring systems as developed by different researchers (Abramov et al., 2007; Babaeijandaghi et al., 2010; Bigbie et al., 1991). This evaluation includes macroscopic wound evaluation and histologic evaluation. The macroscopic wound evaluation can include the status of swelling, the time to detection and completeness of epithelium/skin resurfacing, the wound size/contraction, the wound scores of granulation tissue formation, the vascularity and dermal thickness (Table 1.4). The histologic evaluation includes the status of inflammatory cell infiltration, the vascular proliferation, fibroplasia, the presence and depth of scar tissue, the epithelialization rate and the absence of adnexa including hair follicles, apocrine glands and smooth muscle (Table 1.4). In the present thesis, some modifications were made for the evaluation of different wound healing parameters in rabbits and horses.

Table 1.4 Scoring system for macroscopic and histologic evaluation of wound healing. Adopted from Bigbie et al. (1991), Abramov et al. (2007) and Babaeijandaghi et al. (2010) in wound models of horse, rabbit and mouse, respectively.

Parameter	**Score**			
	0	**1**	**2**	**3**
Macroscopic evaluation				
Quantity of exudate	Exudate is evident and slight pressing results in excessive exudation	Moist wound and slight exudate on pressing	Wound is most but no oozing on pressing	Apparently dry wound
Type of exudate	Purulent	Fibrinious	Slight dry cast	-
Peripheral swelling	Marked	Moderate	No/slight swelling	-
Granulation tissue	Elevated above skin edge	Proliferated to the level of skin	Depressed below skin edge	Absent
Color of granulation tissue	Pale	Pale red	Pink	-
Histologic evaluation				
EPIDERMIS				
Dermal-epidermal separation	Diffuse	Focal	None	-
Crust formation	Present	Absent	-	-
Re-epithelialization	None	Partial	Complete but immature or thin	Complete and mature
Acanthosis	Severe (> 15 layers) or moderate irregular	Moderate regular (10-15 layers	Mild (5-10 layers)	None
DERMIS				
Edema	Severe	Moderate	Mild	None
Acute inflammation	Abundant	moderate	Scant	None
Depth of inflammation	Deep myonecrosis	Superficial myonecrosis	Lower dermis	Upper dermis
Chronic inflammation	Abundant	moderate	Scant	None
Granulation tissue amount	Abundant	moderate	Scant	None
Granulation tissue fibroblast maturation	None	Mild	Moderate	Full
Collagen amount	None	Scant	Moderate	Abundant
Neovascularization (Number of capillaries per 200x field)	None	Up to five vessels	6-10 vessels	More than 10 vessels

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CHAPTER 2

Regenerative Skin Wound Healing in Mammals: State-of-the-Art on Growth Factor and Stem Cell Based Treatments

Abstract

Mammal skin plays a key role in several life-preserving processes such as hydration, protection against chemicals and pathogens, initialization of vitamin D synthesis, excretion and heat regulation. Severe damage of the skin may therefore be life-threatening. Skin wound healing is a complex, yet well-orchestrated process that involves the interaction of various cell types, growth factors and cytokines aiming at closure of the skin and preferably resulting in tissue repair. Despite a variety of therapeutic modalities proposed to accelerate the healing of acute and/or chronic wounds over the past decades, the development of novel approaches for wound treatment remains a clinical challenge. The time-consuming conservative wound management is often limited to wound maintenance rather than restitution of the tissue integrity (the so-called "restitutio ad integrum"). Therefore, more efficacious wound therapies are urgently required to reduce health care burden, provide patients with long-term relief and ultimately scar less wound healing. Recent advances in regenerative medicine provide encouraging results both from *in vitro* and *in vivo* studies of skin wound regenerative therapies in animals as a model for humans. Nevertheless, more protracted studies will have to determine whether regeneration can be achieved rather than reparation. For all the aforementioned reasons, this article reviews the emerging field of regenerative skin wound healing in mammals with particular emphasis on growth factor- and stem cell-based treatments.

Key words: Regenerative veterinary medicine; Skin wound healing

2.1 Introduction

The skin is the largest organ of vertebrates (± 10% of body mass) and is critical for defense as well as survival. Therefore, each injury may induce loss of the skins' integrity with functional imbalance accompanied by disability or even death (Theoret, 2009). Skin injury initiates mechanisms to limit damage and to subsequently induce repair. Both phenomena cover a complex cascade of temporal and spatial events that are required for tissue homeostasis. These events include induction and resolution of inflammation on the one hand, and remodeling and formation of tissue on the other hand. This way complete reconstruction of the wounded area should be achieved (Clark, 1996a). Regeneration is expected to specifically substitute the lost tissue, however, repair of adult skin lesions is often achieved by an unspecific form of wound healing by fibrosis and scar formation (Reinke and Sorg, 2012). Repaired skin in adults usually heals as a scar which is weaker than the original skin because it contains disorganized extracellular matrix (ECM) (Soo et al., 2003; Gurtner et al., 2008). This repair process differs from regeneration, as the latter tissue is almost indistinguishable from the original intact tissue (Gurtner et al., 2008, Dudas et al., 2008). In this context it should be mentioned that the mammalian fetal skin regenerates perfectly in contrast to adult skin. The underlying regulation of this switch in repair mechanism potential during mammalian development has not yet been completely resolved (Kawasumi et al., 2013)

Full-thickness skin wounds may result in extensive damage to the different skin structures. This damage compromises the homeostatic mechanisms involved in spontaneous healing and therefore clinical intervention is often needed. The primary aim of clinical skin wound treatment is to promote rapid wound repair with functional and aesthetical satisfactory scar tissue formation (Singer and Clark, 1999).

Today, skin wound therapies are categorized as either conservative/conventional or regenerative. Conservative skin wound management includes debridement of necrotized tissue, restoration of adequate tissue perfusion, limitation of pressure at the wound site, use of several types of wound dressings, control of infection and of any underlying health problem that could alter the healing process (Singer and Clark, 1999). Wounds healed by conventional therapy are characterized by scar formation with cosmetic and possible functional impairment. For example, skin scars are less resistant to ultraviolet radiation, and nor sweat glands nor hair follicles grow back within scar tissues (Kraft and Lynde, 2012). Regenerative

skin wound therapy is a novel and rapidly expanding field of biomedical research that focuses on the development of innovative therapies aiming at restoring the damaged cells and diseased skin tissue without scar formation. Since quality care is a critical requirement for wound healing (Edlich et al., 1983), regenerative strategies should not be considered as a substitute for certain indispensable conservative treatments. Selecting an appropriate therapeutic strategy depends on the condition of the wound and is crucial for its successful wound healing in order to minimize the risk of complications, enhance the speed of this process and minimize scar tissue formation. Therefore, in the present review information has been gathered on preclinical and clinical studies regarding regenerative therapies for mammal skin wound management with particular emphasis on growth factor- and stem cell-based therapies.

Literature search

The standard research databases like PubMed and Web of Science between January 1955 and April 2014 and the Google Scholar search engine were consulted for collection of full papers and abstracts.

2.2 Mammalian skin and wound healing

2.2.1 Adult mammalian skin anatomy and physiology

Mammalian skin is the largest organ of the adult body and consists of several layers: (i) the superficial epidermis, (ii) the intermediate dermis (Stashak and Theoret, 2007; Theoret, 2009) and (iii) the hypodermis, and is supported by a matrix of loose connective tissue (Stashak and Theoret, 2007) (Fig. 2.1).

Noteworthy, there are significant differences in the anatomy and physiology of each skin layer between species (Wong et al., 2011). Consequently, these differences result in a difference in wound healing (Sllivan et al., 2001). For instance, in animals with a more “loose” skin such as rats and mice, healing occurs very rapidly as a result of wound contraction due to the presence of the *panniculus carnosus* muscle in their subcutaneous tissues. On the other hand, in “tight-skinned” species such as human and porcine, which lack this muscle, wound contraction is not rapid thus heal mainly through re-epithelialization (Wilmink et al., 2001; Theoret and Wilmink, 2013). Striking differences in wound healing can

even be exhibited within the same species, which have been observed at gross, cellular and molecular level. For example more rapid onset and resolution of wound inflammation leading to more rapid wound contraction and epithelialization was detected in ponies in comparison to horses (Wilmink et al., 2001; Wilmink and van Weeren, 2005). This difference in wound healing was attributed to differences in local inflammatory responses, where leukocytes in ponies were found to produce more inflammatory mediators. The inflammatory mediators resulted in better local defense, faster cellular debridement, faster transition to the repair phase and more wound contraction (Wilmink and van Weeren, 2005).

Mammalian epidermis, which is a terminally differentiated and stratified squamous epithelium consists of 4 to 5 sublayers (Fig.2.1). The basal cell layer is the innermost cell layer of the epidermis and is a single layer consisting of basal cells including an epidermal stem cell subpopulation. This stem cell population can give rise to distinguished types of cell layers, such as prickle, granular and horny cell layers, when these cells move from the basal layer outwards and progressively differentiate. The prickle cell layer or stratum spinosum is composed of many layers interconnected by prickle-like structures, which compose of desmosomes with keratin tonofibrils that form a supportive mesh in the cytoplasm of the cells. Desmosomes in turn are composed of the structural molecules cadherins, desmogleins and desmocollins (Hopwood et al., 1978). The main component of the granular layer is released as corneum lipid into the intercellular space of the horny cell layer. The latter is the outer most layer where keratinocytes enucleate and become corneocytes as a terminal differentiation phase. Additionally, there are several more specialized cells in the epidermis, including melanocytes, Langerhans cells, dendritic cells and Merkel cells (Theoret, 2009, Kawasumi et al., 2013). Similar to a wall the cornified layer consists of hard building blocks (i.e. the individual corneocytes) stuck together with space filling mortar (i.e. the inter-corneocytal lipids). The barrier function of normal epidermis depends on the quality of its bricks and mortar (Nemes and Steinert, 1999; Zhang and Michniak-Kohn 2012). Epidermis and dermis are physically separated by the basement membrane or basal lamina, a highly specialized ECM structure, which provides a stabilizing and dynamic interface (Breitkeutz et al., 2009). Besides providing structural adhesion of the epidermis to the dermis, the basement membrane has a gate-keeping function strictly controlling diffusion of cells (Iozzo, 2005) and bioactive molecules in both directions (Iozzo, 2005; Breitkeutz et al., 2013). On the other hand, it binds a variety of cytokines and growth factors, hence, acts as a reservoir for a controlled release during physiologic remodeling and repair processes after injury (Iozzo, 2005).

The dermis is situated directly beneath the epidermis and is composed of the papillary and reticular layers (Fig.2.1). The upper papillary layer edges into the epidermis across the basement membrane and nourishes it. This layer consists of (i) cellular components, including fibroblasts, mast cells, macrophages and dermal dendrocytes and (ii) ECM components namely stromal components (e.g. collagen and elastic fibers) and matrix component (glycoproteins and proteoglycans, etc.). The lower reticular layer is characterized by an ECM-containing network of coarse collagen, elastic fibers and fibroblasts (Kawasumi et al., 2013). The dermis is vascularized, innervated and invaded by epidermal appendages. The hypodermis is the layer located beneath the dermis and contains a considerable amount of adipose tissue (Fig. 2.1). It is well vascularized and contributes both to the mechanical and thermoregulatory properties of the skin (Theoret, 2009). The epidermis of the skin and its appendages (e.g. hair follicles, sebaceous glands, sweat glands and nails) are derived embryologically from the prospective epidermal ectoderm and neural crest cells, which are also ectodermal in origin. Nevertheless, the skin appendages have their roots in the dermis or even in the hypodermis, both of which are derived from embryonic mesoderm (Kawasumi et al., 2013).

Every disruption of the normal anatomic structure of a tissue with consecutive loss of its function can be described as a wound (Lazarus et al., 1994). Wound healing is a critical survival factor for mammals (Lau et al., 2009). Skin wound healing relies on a complex dynamic process which involves interaction of multiple cell types, growth factors, cytokines and chemokines (Singer and Clark, 1999; Maxson et al., 2012). Dysregulation of this process leads to problems such as chronic, non-healing wounds or excessive healing in the form of keloids and hypertrophic scars (Ko et al., 2011). However, there are reports which indicate that even when this balance is disrupted, the deficiency of a cell type or the absence of a mediator can be compensated (Eming et al., 2007a). Adult skin wounds heal by repair and/or regeneration. There is a clear difference between both types of healing. Repair refers to the physiologic adaptation of an organ after injury in an effort to re-establish continuity without aiming at the exact replacement of lost/damaged tissue. Regeneration on the other hand, refers to the replacement of lost/damaged tissue with an ‘exact’ copy, so morphology as well as functionality of the tissue are completely restored. It should be remarked that the mammalian skin does not regenerate spontaneously, but heals with scars (a typical feature of the repair process).

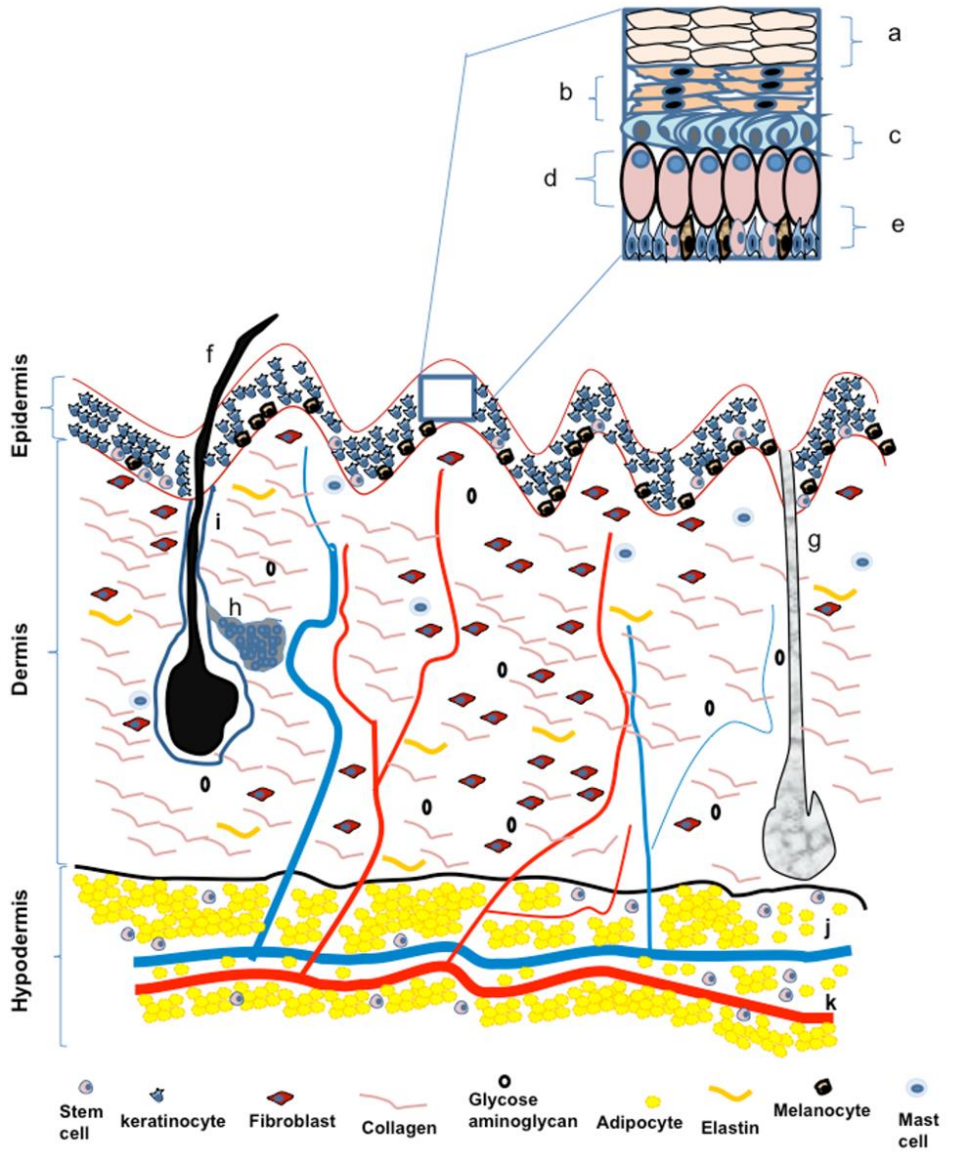

Figure 2.1 Schematic representation of mammalian skin structure and its cell populations. The skin contains three layers: epidermis, dermis and hypodermis. The epidermis is a stratified epithelium composed of 4 to 5 layers: stratum corneum (a), stratum lucidum (b), stratum granulosum (c), stratum spinosum (d) and stratum basale (e). Other structures in the skin include the hair (f), sweat glands (g), sebaceous glands (h), hair follicles (i), nerves (j) and blood vessels (k).

Wound healing occurs in three overlapping time windows: inflammation, repair and remodeling (Singer and Clark 1999; Theoret, 2004; Knox et al., 2006; Maxson et al., 2012; Warner and Grose, 2003). When there is injury and damage to cutaneous blood vessels, platelets will get exposed to ECM proteins, and immediate coagulation resulting in fibrin clot formation will occur (Clark, 1996b). Some authors consider this hemostasis as one of the time windows of wound healing, thus dividing the healing process into 4 phases (Li et al., 2007) (Fig. 2.2). This phenomenon is followed by an inflammatory phase, characterized by infiltration of neutrophils and macrophages (Fig. 2.2). Contaminants and dead tissue liberate mediators such as cytokines and growth factors from phagocytes sustaining this immune cell influx (Theoret, 2004; Guo and Dipietro, 2010). The proliferative phase of repair becomes apparent as inflammation signs subside. It is characterized by formation of granulation tissue and epithelialization (Theoret, 2009; Guo and Dipietro, 2010). In the reparative dermis, fibroblasts and endothelial cells proliferate and are the most prominent cell types present, supporting capillary growth, collagen formation and formation of granulation tissue (Guo and Dipietro, 2010). The last phase of repair is the remodeling which involves reduction in wound size by a process known as wound contraction as well as reorganization and maturation of the ECM (Theoret, 2009; Guo and Dipietro, 2010). Wound contraction is achieved by myofibroblasts, which differentiate, invade and repair injured tissue by secreting and organizing the ECM and by developing contractile forces. Different precursor cells have the ability to form myofibroblasts, but the major contribution is from local recruitment of connective tissue fibroblasts. However, when local fibroblasts are not able to satisfy the requirement for these cells during repair, myofibroblasts can also be derived from non-fibroblast sources such as local mesenchymal stem cells (MSC), bone marrow-derived MSC and cells derived from an epithelial-to-mesenchymal transition process (Henz et al., 2007; Micallef et al., 2012).

2.2.2 Mammalian embryo and amphibians as a model for scarless skin wound healing

Adults of humans and other mammals have a limited capacity to regenerate and restore their tissues and organs. This can be achieved either through the activation of somatic stem cells located in a niche or by inducing differentiated cells to proliferate (Jopling et al., 2011). Accordingly, the wound healing process in adult mammals is imperfect and less restorative than in the juvenile or in the embryo (Kawasumi et al., 2013).

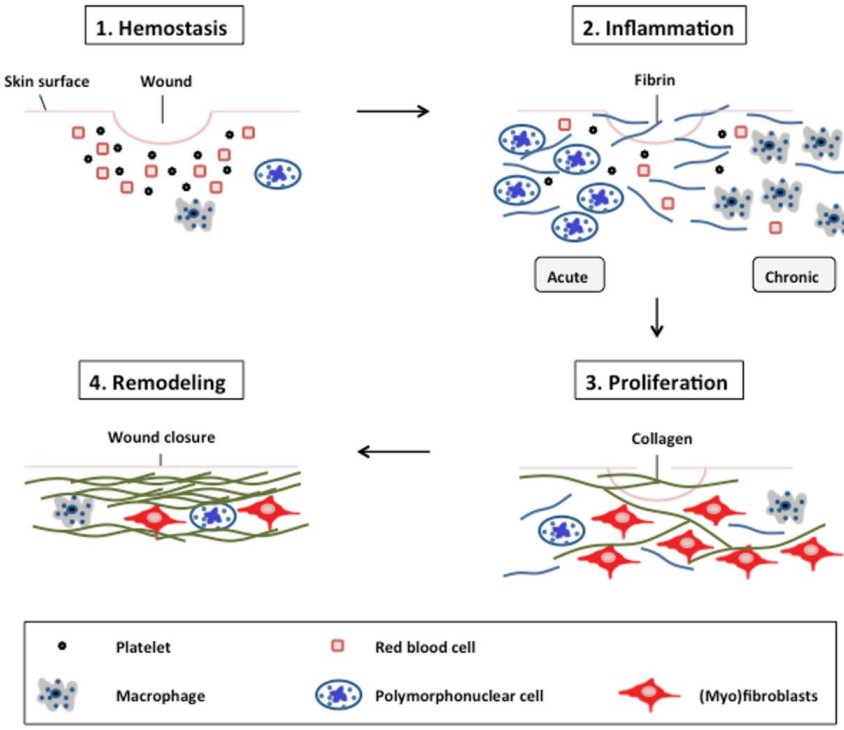

Figure 2.2 Schematic representation of the basic steps of cutaneous wound healing. Following injury wound healing proceeds in four interrelated dynamic phases which overlap in time.

In contrast, skin wounds in early mammalian embryos can heal perfectly without scar tissue formation and with complete restitution of normal skin architecture (Whitby et al., 1991). There are many differences between the healing process of embryonic and adult wounds. Although this is mainly attributed to the tissue development itself, fetal scarless wound healing mechanisms are intrinsically different. In fact, fetal wounds heal with a markedly diminished inflammatory response (Wulff et al., 2012), a faster production of ECM molecules such as fibronectin and tenascin, a high hyaluronic acid content and altered profiles of growth factor expression. In an embryonic wound site, the levels of transforming growth factor (TFG)-β1 and TGF-β2 are lower while TGF-β3 is higher than in an adult wound site (Cowin et al., 2001). A further key difference between fetal and adult wound repair relates to the mechanism of wound contraction and epithelialization. Unlike adult wound closure, fetal wounds close through an actin cable which acts like a purse string (Martin and Lewis, 1992). Adult wound closures involve active movement of connective tissue and epidermis in order to

bring two wounded edges in close proximity to allow the epidermis to migrate and cover the exposed connective tissue (Nodder and Martin, 1997).

In contrast, some other vertebrate species like amphibians have extensive regenerative capacities that in certain cases stretch as far as replacing a complete limb (Dent, 1962; Odelberg, 2004). However, many aspects of amphibian skin wound healing remain unclear, while some studies have suggested that the initial phase of wound healing shares mechanisms with that in mammals (Kawasumi et al., 2013). Despite the histological similarities between amphibian and mammalian skin and the common process of their early healing, their subsequent regenerative ability differs remarkably. Unlike mammalian wound healing, amphibian wound healing results in no scar formation indicating that skin wound healing is perfect and ends up with restoration of tissue architecture and function (Seifert et al., 2012). Complete regeneration of the limb in some amphibians occurs as a result of dedifferentiation and proliferation of cells adjacent to the wound and re-patterning by molecular mechanisms similar to those for developmental limb formation (Muneoka and Sassoon, 1992). Regeneration of amphibian skin shares cellular and molecular features of the early events of limb regeneration which depends on expression of the Prx1 gene. Thus, it has been recently proposed that Prx1 itself, through activation of tenascin-C, may contribute to scarless skin wound healing (Seifert et al., 2012). Hence, comparing the wound healing capacities of different species might lead to novel insights in the field of skin regeneration.

2.2.3 Role of stem cells in wound healing

Most epithelia self-renew through a mechanism called tissue homeostasis, in which the number of cell divisions creates a balance with the number of lost cells (Blanpain and Smona, 2013). Tissue homeostasis is guaranteed by stem cells (SC) present within specialized microenvironments, the so-called niches. Each niche is tailored to accommodate the regeneration needs of its tissue (Morrison and Spradling, 2008). The skin epidermis and its appendages harbor spatially distinct SC niches (Blanpain and Fuchs, 2014) (Fig.1). For instance, the hair follicle fated bulge stem cells only generate a transient amplifying progeny toward wound epidermis as a part of an “emergency” repair strategy (Ito et al., 2005). In marked contrast, a later experiment by Levy et al (2007) reported that there are follicular progenitors distinct from bulge SC which are able to permanently convert into interfollicular epidermis SC in response to wounding in mice.

Adult SC are considered to replace lost cells and are therefore identified as central players in tissue regeneration. They deliver daughter cells to renew the lesioned tissues by differentiation and/or by releasing paracrine factors to attract progenitor cells (Chen et al., 2008). When a SC compartment is being inflicted, other SC can be recruited to enhance lesion repair. However, the fate and differentiation potential of epithelial cells can also adapt during wound regeneration. In some cases, unipotent progenitors acquire multipotency, whereas in other cases normally committed cells revert to a SC-like state. The cellular plasticity observed in adult epithelial tissues have not been associated with "trans"differentiation into completely unrelated fates, but rather with contribution to the repair of the tissue from which the cells originated. In this regard, the plasticity seems to arise through a process of "de"- and/or "re"-differentiation (Blanpain and Fuchs, 2014).

2.3 General skin wound management

Mammalian skin has a large potential for efficient and functional repair, because of the constant natural cellular turn-over, indicating a high intrinsic regenerative capacity. The purpose of skin wound management is to restore a skin defect and regain, at least partially the lost integrity, tensile strength and barrier function (Mustoe et al, 2002). Wound healing in mammalian patients is affected by several factors including blood supply, wound size, tension and mobility of wound margins, susceptibility to infection, and the type and condition of the underlying tissue (Knox et al., 2006). The ultimate goal of wound management is to decrease the risk of opportunistic infection and to induce rapid development of granulation tissue and epithelialization with a minimum of scar tissue (Woods et al., 1997).

Improperly managed wounds can be life threatening, functionally disabling and cosmetically devastating. There are a variety of modes of wound healing/repair, which include healing by primary, secondary and tertiary intention (Bae et al., 2012). Primary intention involves re-epithelialization, in which the skin's outer layer closes over the wound. Wounds that heal by primary intention are most commonly superficial wounds that either involve only the epidermis (such as superficial burn), or show no loss of dermal tissues (such as sutured large surgical incisions). Second intention wounds heal by granulation formation, contraction and epithelialization. Wound care must be performed to prevent infection and to encourage granulation tissue formation (Han et al., 2012a). Tertiary intention wounds are initially left

open and closed after several days by approximation or by the use of tissue grafts. This type of healing may be desired in cases of contaminated wounds (You and Han, 2014).

The earliest written records of wound management date to Sumerian carvings more than 4,000 years ago. During history, a cornerstone of therapy has always been topical treatment (Murray et al., 2008). Currently conventional methods of local wound management include mechanical cleansing, disinfection with antiseptic solutions, wound debridement, wound closure, topical antibiotic treatment, dressing and use of negative pressure devices (Komarcevic, 2000; Jeffcoate and Harding, 2003; Chung et al., 2009). In general, conventional wound treatment results in wound healing by 'repair' rather than by regeneration. Moreover, each conventional treatment method has its advantages and limitations.

For readers interested in the details of conventional therapies, which are not included here because they do not fall within the scope of this review, we would like to refer to previous excellent and comprehensive review papers (Komarcevic, 2000; O'Meara et al., 2000; Leaper and Durani, 2008; Jull et al., 2013; O'Meara et al., 2013).

The development of advanced wound healing technologies has triggered the use of growth factors and cells to overcome the limitations of conventional wound treatments.

2.4 Regenerative therapies for skin wound management

Regenerative medicine is defined as a new and rapidly expanding field of biomedical research that focuses on the development of innovative therapies allowing the body to replace, restore and regenerate damaged or diseased cells, tissues and organs (Dieckmann et al., 2010). This can potentially be accomplished using the process of dedifferentiation (which involves terminally differentiated cells reverting back to a less differentiated stage within their own lineage), transdifferentiation (a processes which takes dedifferentiation a step further and cells differentiate into a cell type of another lineage) and reprogramming (which aims to induce differentiated cells into a pluripotent state) (Jopling et al., 2011). Regenerative therapy combines several technological approaches including the use of soluble molecules, gene therapy, stem cell transplantation, tissue engineering and the reprogramming of cell and tissue types (Greenwood et al., 2006). In this review we are only surveying the existing cell-based and growth factor-based *in vitro* and *in vivo* studies and therapies that are at preclinical and

clinical level. For readers interested on the remaining regenerative therapies, which are not included in this review, we would like to refer to previous review papers (Branski et al., 2007; Branski et al., 2009; Dieckmann et al., 2010; Eming et al., 2007b; Gauglitz and, Jeschke , 2011).

2.4.1 Growth factor-based therapies

Growth factors (GF's) are signaling proteins (tissue hormones) that regulate the metabolism of other cells (Fortier and Smith, 2008). GF's are released at the wound site and are required for communication between a variety of cells like fibroblasts, myofibroblast, smooth muscle cells, endothelial cells, keratinocytes and immune cells. Many experimental and clinical studies have demonstrated a variety of effects of exogenous growth factors on the healing process (Steel and Group, 1995; Warner and Grose, 2003). Accelerated healing of the wound is based on wound characteristics and the function of GF's (Fu et al., 2000). Different studies in human patients have confirmed that GF's such as platelet-derived growth factor (PDGF) play a role in shortening wound healing time from 1 to 4 days in acute wounds and even provide complete healing in chronic wounds (Robson et al., 1992a; Robson et al. 1992b; Mandracchia et al., 2001).

The main GF's involved in the wound healing process includes PDGF, epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF_1, IGF_2), vascular endothelial growth factor (VEGF), TGF-β (Koveker 2000; Grazul-Blska et al., 2003; Jeffcoate et al., 2004; Falanga 2005) and keratinocyte growth factor (KGF) (Grazul-Blska et al., 2003). To date only PDGF has been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for clinical application in patients (Papanas and Maltezos, 2007; Murphy and Evans, 2012). An overview of the most cited GF-based therapies for skin wound management in animals models are summarized in Table 2.1.

Platelet-Rich plasma (PRP)

PRP (or autologous platelet concentration) is blood plasma that has been enriched with platelets through specific centrifugation. The platelet count is often two to four times higher than normal (Fortier and Smith, 2008). Platelets are cytoplasmic fragments derived from bone marrow megakaryocytes and they contain (and release through degranulation) several

different types of growth factors and cytokines that might stimulate wound healing (Sandoval et al., 2013).

Table 2.1 Overview of most cited growth factor-based therapies for skin wound management in animals and their functional effects.

Reference	Therapy	*In vivo* model	Type of wound	Functional *in vivo* effects
Carter et al., 2003; DeRossi et al., 2009	Autologous PRP-gel	Horse	Full thickness	Rapid epithelialization and collagen organization
Monteiro et al., 2009	Autologous PRP	Horse	Full thickness	No quality improvement or healing enhancement
Iacopettietal., 2011	Autologous PRP-gel	Horse	Torn wound	No exuberant granulation and minimum scarring
Kim et al., 2009	Autologous PRP gel	Dog	Chronic	Reduced swelling and hair regrowth at wound margin
Man et al., 2001	Autologous PRP-gel	Human	Surgical flap	Improved healing with short recovery time
Galiano et al., 2007	Autologous VEGF	Diabetic mice	Full thickness	Enhanced re-epithelialization, matrix deposition and cellular proliferation
Tsuboi and Rifkin, 1990	Rec hFGF-2	Diabetic mice	Full thickness	Improved re-epithelialzation, increase of macrophages, fibroblasts, neovascularization and granulation tissue.
Shi et al., 2013	Rec hbFGF	Rabbit /rat	Full thickness	Stimulate fibroblast growth, reduce scar formation and regulate inflammatory response
Brown et al., 1993; Brown et al., 1994	Rec hPDGF and Rec hTGF-alpha	Normal and diabetic mice	Full thickness	Enhanced collagen deposition, granulation tissue formation and maturation.
Kim et al., 2010	Rec hEGF	Mice	Full thickness	Reduced scaring, suppressing inflammation, decreasing TGF-β_1 and mediating collagen formation
Yu et al., 2007	Rec hIGF	Diabetic mice	Full thickness	Increased hypoxia-inducible factor 1-α protein synthesis and function in diabetic wounds
Tsuboi et al., 1995	hIGF-I and IGF-1 binding protein	Diabetic mice	Full thickness	Increased granulation tissue formation and capillary number
		Normal rabbit		
Yoshida et al. 2003	Rabbit HGF	Mice	Full thickness	Enhanced re-epithelialization, neovascularization and granulation tissue formation
Li et al., 2013	hHGF	Diabetic rat	Full thickness	Enhanced re-epithelialization, neovascularization and granulation tissue formation

bFGF = Basic Fibroblast Growth factor; EGF = Epidermal Growth Factor; HGF = Hepatocyte Growth Factor (HGF), h = Human; Rec = Recombinant; IGF = Insulin-like Growth Factor; PDGF = Platelet Derived Growth Factor; PRP = Platelet Rich Plasma; TGF-α = transforming growth factor-alpha; VEGF = Vascular endothelial growth factor.

They activate the inflammatory process by releasing cytokines (i.e. Interleukin: IL-1α, IL-1β, IL-6 and tumor necrosis factor: TNF-β), stimulate collagen synthesis (FGF-2, IGF-1, TGF-β), activate transformation of fibroblasts to myofibroblasts (TGF-β), start angiogenesis (EGF-2. VEGF-A, TGF-β) and support the re-epitheliaization process (EGF, FGF-2, IGF-1, TGF-α) (Bennett and Schultz, 1993;Warner and Grose, 2003; Li et al., 2007). The main difficulty in assessing the therapeutic effects of PRP is to define which growth factor(s) were at the origin of the observed effects since PRP delivers a mixture of growth factors associated with natural scar healing (Fortier and Smith, 2008).

There are a number of *in vivo* studies in dogs and horses regarding the use of PRP for cutaneous wound therapy. Carter et al. (2003) and DeRossi et al. (2009) reported PRP gel promoting epithelial differentiation and regeneration during equine wound healing. In the same species, a case study reported by Iacopetti et al. (2011) indicated that topical treatment with autologous PRP enhanced healing of clinical large torn wound of dorsal elbow region. In contrast to the above findings, evaluation of the effect of PRP on wounds of the distal aspect of forelimb in horses indicated excessive granulation tissue development and a significantly slower wound healing (Monteiro et al., 2009). Recently, Broeckx et al. (2014a) also reported on the inferior regenerative effect of PRP treatment in comparison to skin-derived SC for the treatment of experimental full thickness skin wounds induced dorsally on the *musculus gluteus* medius region of horses, but this experiment lacks placebo control. On the other hand, Kim et al. (2009) studied the curative effect of PRP on a large cutaneous lesion on dorsal tail of a dog and found that the autologous PRP was beneficial in the management of large skin defects or in delayed wound healing.

In humans, PRP has been applied as a medical tool for several diseases and injuries, such as cutaneous wounds. PRP received special attention because of its use in treating sport injuries in professional athletes (Hall et al., 2009). A study by Man et al. (2001) demonstrated quantitative improvements of human skin wound healing after topically treating cutaneous flaps with autologous PRP. Other studies on human patients with chronic wounds of various etiologies treated with PRP gel also showed some degree of improvement, reflected by reduction of wound area, volume and wound closure (Mazzucco et al., 2004; Frykberg et al., 2010).

Platelet-derived growth factor (PDGF)

PDGF is one of the first factors produced in response to injury and induces cellular responses throughout all phases of the wound healing process. PDGF exists in three isoforms: PDGF-AA, PDGF-BB, and PDGF-AB (Kiritsy et al., 1993). It is mainly secreted from the α-granules of the platelet (Pananas and Maltezos, 2007), and is also produced by other cells involved in early wound healing, i.e. macrophages, keratinocytes, fibroblasts and endothelial cells (Warner and Grose, 2003; Pananas and Maltezos, 2007). The level of PDGF and PDGF receptor expression have been shown to be low in diabetic and aged mice that have a delayed response to injury (Werner and Grose, 2003). Similarly PDGF levels are depressed within non-healing human ulcers (Pierce et al, 1995) probably because of underproduction and/or excessive protease mediated degradation. Therefore, wound treatment using exogenous PDGF has been investigated and was found to be beneficial for those patients with chronic wounds (Brown et al., 1993; Leahy and Lawrence, 2007). As a result of FDA approval, PDGF-BB has been extensively used for the treatment of diabetic ulcers (Leahy and Lawrence, 2007; Demidova-Rice et al., 2012). Despite this approval, there have been reports showing a limited clinical success. This might be due to low expression of PDGF receptor by the cells residing within chronic wounds (Flanga, 2005), or could be caused by rapid degradation of the GF by proteolytic enzymes within the chronic wound bed (Tomic-Canic et al, 2008). Moreover, the complexity and persistence of the chronic wound bed suggests that delivery of a single entity may not be sufficient as a corrective therapeutic measure. Therefore, sustained GF delivery systems with a combined and/or patient specific approach to chronic wound treatment might be required to enable optimal healing and wound closure (Hinchliffe et al., 2008, Beenken and Mohammadi, 2009; Demidova-Rice et al., 2012). Recently, a combination of AMD3100 (which mobilizes marrow-derived progenitor cell) and PDGF-BB therapy on experimental full thickness skin wound on the dorsum of mice, has synergistically shown to improve progenitor mobilization and trafficking, resulting in significantly improved diabetic wound closure and neovascularization (Allen et al., 2014).

Fibroblast growth factor (FGF)

FGF-1, FGF-2, FGF-7, FGF-10 and FGF-22 are all being expressed upon dermal injury (Barrientos et al., 2008). FGF-1 and -2, also known as acidic and basic FGF, respectively, are produced by inflammatory cells, vascular endothelial cells, fibroblasts and keratinocytes and

play roles in re-epithelialization, angiogenesis and granulation tissue formation (Warner and Grose, 2003; Xie et al., 2008). In addition to their direct role in wound healing, FGF-7 and FGF-10 also stimulate the production of TGF-α by dermal keratinocytes, indirectly contributing to epithelialization (Niu et al., 2007).

Studies in animal models showed that there is abnormal expression of FGF-1, FGF-2 and FGF-7 in wounds of diabetic aged animals (Komi-Kuramochi et al., 2005). Administration of FGFs successfully improved wound healing in these animals (Bing et al., 2007). In this regard, Tsuboi and Rifkin (1990) reported that bFGF (FGF-2) accelerated wound healing of full thickness skin wounds on the dorsum in diabetic mouse model. Another study by Shi et al. (2013) reported that bFGF regulated ECM synthesis and degradation via interference in collagen distribution, α-smooth muscle actin (α-SMA) and TGF-β1 expression. Moreover, bFGF application resulted in reduced scarring and promoted wound healing by inhibiting the TGF-β1/SMAD-dependent pathway. Therefore, it has been suggested that bFGF possesses favorable therapeutic effects for hypertrophic scars *in vitro* and *in vivo (*Shi et al., 2013). Based on the aforementioned promising animal models, clinical trials in humans were performed where FGF-1 and -2 were used for treating chronic (burn) wounds and resulted in a modest improvement in the healing rates in some studies (Robson et al., 2001; Barrientos et al., 2008). In another study by Ma et al. (2007), human recombinant FGF-1 mostly for treatment of deep partial-thickness burn or skin graft donor site the treatment resulted in fast healing. Future studies may provide better insights on the beneficial effects of different types of FGFs in different patient groups.

Transforming growth factor-β (TGF-β)

The TGF-β family includes TGF-β1-3, bone morphogenic proteins (BMP) and activins. TGF-β1, TGF-β2 and TGF-β3 are the main forms found in mammals, but TGF-β1 predominates in cutaneous wound healing. They are produced by macrophages, fibroblasts, keratinocytes and platelets (Barrientos et al., 2008). TGF-β is a multifunctional growth factor that attracts new fibroblasts and macrophages to the wound site, stimulates fibroblast proliferation and collagen synthesis, decreases extracellular matrix degradation (Cromack et al, 1991) and modulates the immune system (Finnson et al., 2012).

TGF-β1, -β2, and -β3 have overlapping but distinct functions during wound healing. Both TGF-β1 and -β2 are prominent inducers of fibroblast-myofibroblast differentiation, ECM

deposition, contraction, and scar formation, whereas TGF-β3 has been shown to inhibit scarring (Barrientos et al., 2008). Much of the current knowledge on TGF-β action in wound healing has been obtained from animal studies using incisional and/or excisional wounding models (Finnson et al., 2012). Preclinical studies indicated that intradermal injection of avotermin (TGF-β3) to both margins of adult rat cutaneous wounds resulted in a significant reduction in scarring and a considerably improved dermal architecture (Saha et al., 1994; Saha et al., 1995). Moreover, phase II clinical trials showed that intradermal injections of avotermin in scar revision surgery (So et al., 2011) and bilateral leg wounds (McCollum et al., 2011) were well tolerated and resulted in significantly improved scar appearance compared to placebo. In addition, other phase II clinical trials demonstrated significant improvement in scar appearance with avotermin treatment as well (Occleston et al., 2011).

Vascular endothelial growth factor (VEGF)

During wound healing, VEGF is secreted by platelets, macrophages, fibroblasts, and keratinocytes where it acts in a paracrine way on endothelial cells, inducing and/or supporting wound angiogenesis (Warner and Grose, 2003). Topical VEGF application has shown to accelerate diabetic wound repair through increased epithelialization, angiogenesis, granulation tissue deposition and minimum scar formation in a mouse model (Galiano et al., 2004). In another study using mice, treatment with the angiogenesis inhibitor endostatin caused a delay in wound healing, and this effect was nearly completely reversed after application of topical VEGF (Delgado et al., 2005), confirming the strong angiogenic capacity of this growth factor. However, recombinant VEGF requires frequent repeated topical applications for sustained drug level in the local tissue, which may be a reason why it has not been successful in clinical trials for diabetic foot ulcers (Brem et al., 2009). Recently, Tan et al. (2014), have used collagen scaffolds with VEGF in a diabetic rat wound model and found that the treatment resulted in a higher wound healing rate, better vascularization and higher level of VEGF in the granulation tissue. A limited number of human clinical trials has been published on testing VEGF in diabetic wound healing models. A phase I trial on safety of topical recombinant human VEGF (telberim) in patients with chronic diabetic foot ulcers by Hanft et al. (2008) showed that the treatment was well tolerated and reduced time to complete ulcer healing.

Epidermal growth factor (EGF)

EGF is secreted by platelets, macrophages, fibroblasts, and bone marrow–derived mesenchymal stem cells (Barrientos et al., 2008). EGF helps maintain tissue homeostasis by regulating epithelial cell proliferation, growth, and migration. It also induces angiogenesis, which provides nutritional support for tissue and thus plays an important role in wound healing and tissue generation (Girdler et al., 1995). It has been reported that treatment of cultured epithelial cells with EGF, stimulated outwards migration of keratinocytes within colonies (Barrandon and Green, 1987). It has also been demonstrated that EGF stimulates keratinocyte division and epidermal regeneration *in vitro* and *in vivo*, respectively (Schultz et al., 1991; Nanny, 1991).

Experimental studies in animals have shown that the topical application of EGF accelerates the epidermal regeneration rate of partial-thickness wounds and second-degree burns (Brown et al., 1989). Kim et al. (2010) studied the role of EGF in the formation of cutaneous scars in mice using full thickness wounds, and concluded that local application of the EGF enhanced wound healing rate and reduced cutaneous scaring. They suggested EGF reduces cutaneous scars by suppressing inflammatory reactions, decreasing expression of TGF-β1, and mediating the formation of collagen. Recently, Lee et al. (2013) treated laser induced murine burn wounds with EGF and reported a significantly enhanced wound healing effect in the EGF treated group. Clinical studies concerning the influence of EGF treatment of acute skin wounds in humans have also shown a stimulatory function of this growth factor (Brown et al., 1989). In another study by Brown et al. (1991), the topical treatment of human chronic wounds with EGF accelerated the healing in 8 out of 9 patients of the wounds which failed to heal with conventional therapies. EGF has not only been widely used for the treatment of wounds but also for cancer therapy and vaccines, based on its cell proliferation regulatory properties (Oldooz, et al., 2013).

Insulin-like growth factor (IGF)

IGF-I and IGF-II can be found in plasma at high concentration in an inactive state and also produced in most tissues/cells, such as liver, kidneys and fibroblasts (Kiritsy et al., 1993). *In vitro* assays of wound re-epithelialization showed that both EGF and IGF stimulate migration of keratinocytes and enhance the re-epithelialization process (Haase et al., 2013). In the same study it was found that EGF and IGF have synergistic effects on wound epithelialization.

Furthermore, it has been reported that levels of IGF-1 are decreased in non-healing skin wounds of diabetic individuals (Yu et al., 2007). Accordingly there are many studies indicating that the addition of exogenous IGF-1 accelerates wound healing in diabetic mice (Tsuboi et al., 1995; Brown et al., 1997; Yu et al., 2007), non-diabetic mice (Brown et al., 1997) and rabbit (Tsuboi et al., 1995). In addition, an *in vivo* study by Greenhalgh et al.(1993) showed enhanced tissue repair in genetically diabetic mice when treated with PDGF and IGF-II, again with a synergistic effect. The aforementioned studies implicate that IGF has the strongest effect when being used in combination with other growth factors, demonstrating the necessity of finding the right combination of growth factors in order to enhance the healing process.

Hepatocyte growth factor (HGF)

HGF is secreted by mesenchymal cells and is well known to regulate cell growth, motility, and morphogenesis in various types of cells, including epithelial and endothelial cells, supporting the hypothesis that it promotes epithelial repair and neovascularization during wound healing (Matsumoto and Nakamura, 1997; Conway et al., 2006). However, there are only few reports regarding *in vivo* studies of HGF in animal models. In this regard, an *in vivo* study by Yoshida et al. (2003) showed that when normal rabbit immunoglobulin G (IgG) or neutralizing anti HGF IgG was locally and continuously delivered to full thickness excisional wounds of mice, the number of capillary vessels decreased with the neutralization of HGF and there was an associated decreased expansion of granulation tissue. Likewise, neutralization of endogenous HGF on days 4 and 7 post-wounding resulted in retardation of re-epithelization and the rate of wound closure. From these finding they have concluded that HGF is involved in enhancing cutaneous wound healing processes, including neovascularization, granulation tissue formation and re-epithelialization. Similar results were reported by Li et al. (2013) in Wistar rats.

What are the possible risks/side-effects of the above mentioned factors?

It has been demonstrated that recombinant GF's used to treat chronic wounds undergo rapid enzymatic degradation. The GF's that are provided exogenously in solution form into the wound site have low bioactivity and availability due to their relatively large size and slow tissue penetration. Repeated administration of high non-physiologically concentrations are necessary to support the healing, however, the excess concentration of GFs results in local

toxicity, adverse effects (Andree et al., 1994; Chen et al., 2010; Bodnar, 2013) and may lead to increased risk of cancer (Vasquez et al., 2004). Nevertheless, this problem might be solved by the major advances made over recent years in the construction of polymer-based/biomaterial GF delivery systems which allow the controlled release of growth factors (Lee et al., 2011a).

2.4.2 Cell-based therapies

Cell-based therapies are defined as the process of introducing new cells into a tissue in order to treat diseases or regenerate damaged tissue. Wound healing is a complex process, which requires coordinated action of multiple cell types (Martin 1997; Young and McNaught, 2011) which is in response to a variety of cytokines and micro-environmental conditions (Young and McNaught, 2011; Maxson et al., 2012). Cell-based, therapies might be considered as an alternative approach to growth factors for wound management. Cells so far studied for cutaneous wound healing effect includes stem cells, keratinocytes, fibroblasts and platelets which have been discussed under the previous section because of the growth factor secretion. An overview of the most frequently reported cell-based therapies for skin wound management in animals models are summarized and presented in Table 2.2.

Cell-based therapies can regulatory be classified as human tissues and cells (Directive 2004/23/EC) or as advanced therapy medicinal products (ATMP) (Directive 2001/83/EC and Regulation 1394/2007) (if not blood components or organs). The FDA has implemented a regulatory outline that controls cell-based products based on three topics: (i) prevention of using contaminated cells, (ii) prevention of handling that may cause contamination and (iii) clinical safety of cells used (George, 2011). There is a significant gap between promising laboratory-based research and approved stem cell based products (SCBP) in this fast emerging field (Leanne and Sarah, 2007). In order to translate SCBP from bench to bedside and ensure patient safety compliance with existing regulations and guidelines is required to ensure that the product is safe, pure and potent; and meeting good tissue practices (GTP), good manufacturing practices (GMP) and good clinical practices (GCP) requirements are indispensable (George 2011).

Table 2.2 Overview of most cited cell-based therapies for skin wound management in animals and their functional effects.

Reference	Therapy	Route of administration	*In vivo* model	Type of wound	Functional *in vivo* effects
Wu et al., 2007	Allogenic BM-MSC	Edges and topical	Normal and diabetic mice	Full thickness	Increased re-epithelialization, cellularity and angiogenesis.
Stoff et al., 2009	hMSC	Topical	Rabbit	Incisional	Increased tensile strength, granulation tissue and reduced scar formation
McFarlin et al, 2006	Allogenic BM-MSC	Systemic or local	Rat	Incisional	Increased collagen production and early histological maturation
Nakagawa et al., 2005	hMSC and Rec bFGF	Topical	Rat	Full-thickness	Transdifferentiation into skin cells
Shumakov et al., 2003	Allogenic BM-MSC	Topical	Rat	Deep burn	Decreasing infiltration of inflammatory cells, increased neovascularization and granulation tissue
Kim et al., 2013	Allogenic BM-MSC	Injected intradermally	Canine	Full thickness	Increased collagen synthesis, cellular proliferation, angiogenesis and decreased expression of pro-inflammatory cytokines
Sasaki et al., 2008	Allogenic BM-MSC	IV (tail vein)	Mice	Full thickness	MSC transdifferentiated into keratinocytes, endothelial cells and pericytes
Lee et al., 2011	hESC-EPC	Topical and subcutaneous	Rat	Full thickness	Increased tensile strength, rapid formation of granulation tissue and re-epithelialization
Pratheesh et al., 2014	CaprineWarton's jelly-derived MSC	Subcutaneous (4 edges)	Rabbit	Full thickness	Increasing wound contraction, epithelialization, vascularization and collagenization
Spaas et al., 2013	Autologous PBSC	Intradermal and IV	Horses	Chronic	More rapid wound closure and crust formation

BM = Bone Marrow; bFGF = Basic Fibroblast Growth factor; hESC = Human Embryonic Stem Cell; EPCs = Endothelial Precursor Cells; IV = Intravenous; MSC = Mesenchymal Stem Cells; PBSC = Peripheral Blood Stem cells.

Stem cells

Stem cells are defined as unspecialized cells that have two defined properties: the ability to differentiate into other cells and the ability to self-regenerate (Fortier, 2005). Stem cells may be categorized according to their potency: (i) totipotent (ability to form a complete organism), (ii) pluripotent (ability to differentiate into every cell type of the three germ layers of the embryo), (iii) multipotent (ability to differentiate to many but limited cell types or into closely related family of cells) or (iv) progenitor cells (Bongso and Richards, 2004). Stem cells differentiate through progenitor cells, to produce terminally differentiated cell types of the tissue or organ (Rodgerson and Harris, 2011). Stem cells may also be classified conferring to the sources they originate from. Broadly they are classified into 4 types: embryonic stem cells (ESC), fetal stem cells, neonatal or umbilical cord stem cells and adult stem cells (Bongso and Lee, 2005), or they can be categorized as embryonic and non-embryonic stem cells (Koch et al., 2009). Adult stem cells (ASC) could more properly be termed as tissue stem cells, post-

natal stem cells or non-embryonic stem cells. Since ASC escape the ethical issues associated with ESC, they are the prime targets for cell-based therapies (Volk and Theoret, 2012) and hence mainly reviewed in this article. ASC, which can be at different stages of the cell cycle, can be harvested from a variety of sources, such as bone marrow, peripheral blood, umbilical cord blood, adipose tissue, skin and hair follicles (Bongso and Lee, 2005; Branski et al., 2009; Gauglitz and Jeschke, 2011).

To date, especially the role of stem cells in wound healing is increasingly valued. For this reason, a rising number of regenerative therapies for wound healing are currently under development and encouraged by promising findings from both animal models and human studies. It has been suggested that the application of stem cells might be advantageous over administering single biological diffusible factors for several reasons (Lau et al., 2009). Indeed, stem cells have the ability to differentiate into multiple cell lineages, interact with their environment and release multiple wound healing enhancing factors (growth factors and cytokines) that makes them particularly valuable as a potential therapeutic tool (Lau et al., 2009; Tamama and Kerpedjieva, 2012).

Embryonic stem cells (ESC)

The development and application of ESC and induced pluripotent stem cells from large animals is at an early stage (Volk and Theoret, 2012). Nevertheless, it has been demonstrated that endothelial precursor cells (EPC) derived from ESC secrete different cytokines and chemokines such as EGF, bFGF, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-6, IL-8, PDGF-AA and VEGF, which are well known to play an important role in normal wound healing (Lee et al., 2011b). To date, there are only a few reports on skin wound regeneration capacities of ESC or their precursor cells on laboratory animal models. An *in vitro* study in mice by Lee et al. (2011b) indicated that EPC derived from hESC improve proliferation and migration of dermal fibroblasts and epidermal keratinocytes and increased ECM synthesis. In addition, Petrova et al. recently generated epidermis from ESC and induced pluripotent stem cells (iPSC) (Petrova et al., 2014). Different *in vivo* animal models showed that ESC enhanced the healing of both acute and chronic cutaneous wounds (Lee et al. 2011c&b). Lee et al. (2011b) reported that topical and subcutaneous injection of hESC-EPC into cutaneous excisional/acute wounds in rat resulted in accelerated wound healing and increased wound tensile strength, formation of granulation tissue and re-epithelization. In

another study of Lee et al. (2011c) an injection of ESC into diabetic/chronic wounds of rats resulted in enhanced wound healing effect.

Mesenchymal stem cells (MSC)

Adult MSC are a population of multipotent cells that can be found in any tissue including adipose tissue, but are abundantly present in the bone marrow, which is a typical tissue of mesodermal origin (Richardson et al., 2007; Fox et al., 2010). These cells represent a unique tool for tissue engineering. Indeed, it has been reported that MSC can differentiate into multiple tissue forming cell lineages such as osteoblasts, adipocytes, chondrocytes, tenocytes and myocytes (Krampera et al., 2006). Rather than their differentiation potential, the secretory/paracrine effects of MSC have been found to increasingly attribute to the therapeutic efficacy of MSC transplantations. In this regard, it has been demonstrated that MSC have important potential immunomodulatory effects. Indeed, MSC can directly attenuate inflammatory responses by decreasing the secretion of the pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-γ (IL-γ) (Aggarwal and Pittenger, 2005; Kim et al., 2013) while simultaneously increasing the production of anti-inflammatory cytokines including interleukin-10 (IL-10) and IL-4 (Aggarwal and Pittenger, 2005). Therefore, the anti-inflammatory properties of MSC increase their importance for chronic wound treatment. Particularly vasculogenesis and angiogenesis, which are crucial steps in wound healing are also stimulated by paracrine factors released by MSC, including IGF-1, PDGF-BB (Chen et al., 2008), VEGF, angiopoietin-1 (Wu et al., 2007; Chen et al., 2008), bFGF and matrix metalloproteinase-2 (Kim et al., 2013). In addition to enhancing wound healing, MSC are recognized to have antibacterial activity which is critical for protection against infection (Mei et al., 2010). This antimicrobial activity is mediated directly by secretion of antimicrobial factors and indirectly by secretion of immunomodulative factors which up-regulate bacterial killing and phagocytosis by immune cells (Krasnodembskaya et al. 2010; Mei et al., 2010).

A number of parameters are used to assess experimental and clinical wound healing by using MSC therapy. For assessing MSC in experimental wound healing, parameters like histological analysis, tensiometry and tracing of the transplanted cells in the wound tissue over time are currently being used. The histological analyses are used to evaluate the status of infiltration of inflammatory cells, vascular proliferation, fibroplasia, presence and depth of scar tissue,

epithelialization rate and absence of adnexa including hair follicles, apocrine glands and smooth muscle. The parameters used to assess clinical wound healing on the other hand, include measuring wound size/contraction, analysis of wound scores of granulation tissue formation, vascularity, dermal thickness and skin resurfacing.

Recent reports consider that allogenic as well as autologous MSC for the treatment of different types of wounds in humans as well as animals impact all phases of wound repair (Maxson et al, 2012). Animals are being used as a model for the assessment of innovative human regenerative therapies. In this regard, Shumakov et al. (2003) reported that MSC treatment of deep burn wounds in rats resulted in decreased inflammatory cell infiltration and accelerated the formation of new vessels and granulation tissue. It was also suggested that the cells produced bioactive substances that would accelerate the regeneration process. Moreover, Nakagawa et al. (2005) reported that MSC together with bFGF accelerated acute wound healing and showed that the human MSC transdifferentiated into epithelium in a rat skin defect model. Furthermore, a study by McFarlin et al. (2006) showed that systemic or local administration of bone marrow-derived MSC augments healing of surgical fascial or cutaneous wounds by increasing collagen production (I and III) and hence increasing wound bursting and tensile strength in rats. The latter study reported that MSC may also facilitate wound healing by trans-differentiating into myoblasts and keratinocytes after migration to the wound site. Transplantation of human MSC also resulted in promotion of incisional wound repair in rabbits and resulted in increased tensile strength, granulation tissue formation and decreased evidence of scar formation (Stoff et al., 2009). Experiments by Wu et al. (2007), Sasaki et al. (2008) and Tamai et al. (2011) demonstrated that bone marrow-derived MSC enhance wound healing by undergoing epidermal trans-differentiation and angiogenesis. Recently, Kim et al (2013) applied allogenic MSC for treating skin wounds in a canine experimental wound model and demonstrated that MSC-treated wounds showed rapid wound closure and increased collagen synthesis, cellular proliferation and angiogenesis. Apart from bone marrow, transplanted MSC isolated from Wharton's jelly of caprine umbilical cord to full-thickness skin incision of goats Azari et al. (2011) and full thickness excisional skin wounds in rabbit (Pratheesh et al., 2014) resulted in less inflammation, thinner granulation tissue formation with minimum scar in the incisional wound and significantly higher percentage of wound contraction, epithelialization and collagenization with matured vascularization in the excisional wounds. In addition, peripheral blood-derived stem cells (PBSC) have been reported to enhance different wound healing parameters of chronic

naturally occurring wounds in horses, such as granulation tissue and scar tissue formation (Spaas et al., 2013). However, the latter study contained no control groups with untreated wounds, so further research is definitely warranted in order to confirm their conclusions.

In the study by Vojtassák et al. (2006), bone marrow aspirate of a diabetic patient was applied directly to the foot wound, injected into the margins and finally covered with a prepared autobiograft. The wound was treated again on day 7 and 17 with cultured MSC. The results of their study showed that the treatment resulted in wound size decrease, dermal vascularization and thickness increase after 29 days of combined treatment. Falanga et al (2007) suggested that bone marrow-derived MSC contributed to accelerated skin resurfacing when used combined with fibrin spray applied topically up to three times as a treatment of surgical defects from excision of non-melanoma skin cancers. Dash et al (2009) indicated that simultaneous administration of cultured autologous bone marrow-derived MSC intramuscularly into the affected limb and topically directly onto the ulcer of non-healing ulcers of low extremity, accelerates the healing process and improves clinical parameters as compared to the control wounds treated by standard wound care alone. A similar result was also observed when Jain et al. (2011) used a single application (injection and topically application) of autologous bone marrow-derived cells for the treatment of chronic lower extremity wounds and compared it with whole blood (control). A double-blinded, randomized controlled trial by Lu et al. (2011), demonstrated the clinical benefits of parenteral administration of bone marrow-derived MSC for treatment of diabetic critical limb ischemia and foot ulcer by significantly increasin in ulcer healing rate.

The major differences of using adult multipotent MSC instead of pluripotent ESC for therapeutic purposes is the fact that MSC, in contrast to ESC, do not have the ability to form a teratoma *in vivo* (Fong et al., 2010; Lensch et al., 2007) and can be used autologous (of the same individual). Indeed, in practice ESC are mainly being used in an allogenic set-up (i.e. derived from a different individual than the patient), which raises the concern of immune rejection (Menendez et al., 2005). Nevertheless, it has been shown that human (allogenic) ESC 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 ESC are not very immunogenic.

Hematopoietic stem cells (HSC)

Besides MSC, the bone marrow also contains HSC. The HSC are a well-characterized population of self-renewing cells (Kondo et al., 2003), which are currently mainly being used for the treatment of acquired and inherited bone marrow and hematologic disorders in man (Volk and Theoret, 2012). Krause et al. (2001) conducted research on multi-organ, multi-lineage engraftment by a single bone marrow-derived HSC. The authors found that adult bone marrow cells gave rise to follicular epithelial cells, sebaceous gland cells, epidermal keratinocytes and even dendritic cells after their transplantation in mice. From these findings, they have suggested that HSC could possibly contribute to the clinical treatment of skin wounds. In this regard, it has also been reported that topical treatment of full thickness excisional wounds in diabetic mice with a side population of HSC resulted in a high percentage of wound closure (Chan et al., 2007). Nevertheless, more research is warranted in order to determine whether HSC alone have similar wound healing enhancing effects as other ASC types reported so far.

Epithelial stem cells (EpSC)

EpSC are adult SC which are quiescent but self-renew and differentiate into at least one type of progeny (Plikus et al., 2012). A clear distinction should be made between dermal papillae stem cells that contribute to multiple skin appendage formation and epidermal stem cells which are mainly designed to reconstitute epidermis. There are different types of EpSC which have been identified in the hair follicle (Plikus et al., 2012) or the basal layer of the epidermis (Broeckx et al., 2014a). In haired skin, progeny of bulge SC become recruited towards new epidermis during wound re-epithelialization (Ito et al., 2005). In a study by Yang et al., (2007), epidermal adult SC were cultivated on bioengineered dermis to reconstruct artificial skin and successfully transplanted those artificial tissues in goats with acute full thickness skin defects. Their results showed that epidermal adult SC reconstructed the skin (with the hair observed in the restored areas). Recently, Broeckx et al. (2014a) reported the isolation and purification of equine epidermis-derived EpSC. The authors noticed that EpSC addition to PRP treatment significantly enhanced several wound healing parameters. Indeed, the dermis was significantly thinner and exhibited more restricted granulation tissue than the PRP-treated wounds. Moreover, the latter study reported a considerable increase in vascularization, elastin content and follicle-like structures in the EpSC-treated group. Since these cells are lineage

committed and preliminary experiments demonstrated similar healing enhancing capacities as MSC, EpSC might be considered as a valuable alternative.

Adipose-derived stem cells (AdSC)

AdSC represent alternative sources of multipotent cells with characteristics similar to bone marrow-derived MSC (Zuk et al., 2002; Izadpanah et al., 2006). Compared to BM-MSC, AdSC are easier to isolate and are relatively abundant, and hence considered as a better source for wound repair and regeneration (Lee et al., 2012). An *in vitro* study by Lee et al. (2012) showed that AdSC conditioned medium promoted wound healing of HaCaT cells (i.e. immortalized human keratinocytes) by increasing proliferation of HaCaT cells and fibroblasts. Moreover, the latter study reported an increased contraction of the fibroblast-populated collagen lattice. The authors concluded that the enhancing effect of AdSC on wound healing was partially mediated by paracrine effects on co-residing skin cells. Lee et al. (2011d) investigated the effects of human AdSC on the healing of cutaneous wounds in nude mice and they found that the AdSC promoted healing of full thickness wounds. Moreover, it has been demonstrated that AdSC possess anti-inflammatory, vasculogenic and angiogenic properties, which are lacking in other cell types, such as dermal fibroblasts. Due to these paracrine advantages, AdSC can be used as a substitute for fibroblasts in grafting engineered skin (Blasi et al., 2011). Nevertheless, more protracted studies are necessary to demonstrate the working mechanism of these cells.

Differentiated cells

Adult terminally differentiated cells are also used for skin wound treatment. At present, keratinocytes, fibroblasts and adipose-derived stromal vascular fraction (SVF) cells are actively used in a clinical setting of human skin wounds. They are usually used with artificial dermis as a tissue engineered dermis to optimize wound healing (You and Han, 2014). Tissue engineered skin was developed due to limitations associated with the use of autografts. Culturing autologous cells takes time and may result in donor site pain and healing insufficiency for patients with large skin defects, scarring, infection and/or slow healing (Bello et al., 2001; Han et al., 2012a). On the other hand, when allogenic sources of adult cells are used, immune rejection may occur and compromise the treatment outcome (Han et al., 2012b). In recent years, skin tissue engineering has made significant advances, but there are still several factors that hinder its further development. Besides the critical choice of using the

correct cell type, it remains a challenge to create a precise and complex new skin comprising all the necessary cells and to arrange them in a specific 3D pattern (Michael al., 2013). Since mature cells are mostly terminally differentiated, these cells are not the first choice for tissue engineering and regenerative medicine compared to stem cells (Han et al., 2012b). For more information concerning several skin tissue engineering methods, the authors would like to refer to other review papers (Cichowski et al., 2014; Mayet et al., 2014; Nyame et al., 2014). Skin keratinocytes represent a constantly renewing cellular component, and have raised continuous interest from a scientific as well as therapeutic point of view. Keratinocyte-based wound healing therapies exist in a different forms. Chronic leg ulcers are being treated for a long time with autologous (Hefton et al., 1986; Leigh and Purkis, 1986) and allogenic epidermal keratinocytes (Leigh et al., 1987). Since then, several keratinocyte sources have been utilized in humans: own skin cells, cells from cadavers and bioengineered "immortalized" keratinocytes. Researchers have also used keratinocytes as one component in cellular constructs and reported that it contributed to improved quality of wound healing (Spiekstra et al., 2007; Woijtowicz et al., 2014). A retrospective study of 15 years by Auxenfans et al. (2013) showed that cultured allogenic keratinocytes allowed rapid healing of donor sites and deep second degree burns in extensively burned human patients.

Kazemi-Darabadi et al. (2014) used allogenous skin fibroblast transplantation for diabetic wound in sheep and found that the treatment positively affected the wound healing by increasing re-epithelialization, number of fibroblasts and blood vessels Adult bone marrow derived cells have also been studied for experimental wounds in animals and human clinical cases. Accordingly a study by Yamaguchi et al. (2005) showed that adult bone marrow cells accelerate wound healing in rats by differentiating into wound myofibroblasts. An experimental burn wound model in rabbits (Oloumi et al. 2008), surgically created wounds in rabbits (Borena et al 2009a; Borena et al., 2010) and clinical cases in dogs (Borena et al., 2009b) showed early disappearance of inflammatory reaction, significantly higher neovascularization, more fibroplasia and early maturation of collagen using an autologous bone marrow-derived cell transplantation. Apart from acute wound cases, a study conducted by Lu et al (2011) showed the clinical benefits of intramuscular injection of fresh bone marrow-derived mononuclear cells for the treatment of diabetic critical limb ischemia and foot ulcer. Badiavas and Falanga (2003) treated chronic non-healing wounds in humans by direct application of bone marrow derived cells and found that the treatment resulted in wound closure and tissue reconstitution. In this regard it has to be mentioned that both SVF

and BM-MNC are heterogeneous population, thus the above mentioned wound healing effects might be contributed by the stem cell population in the content.

2.5 Hurdles in regenerative therapies for skin wound healing and future perspectives

Due to recent advances in regenerative medicine, our understanding of skin wound healing mechanisms improved. However, there are several issues and questions that still need to be considered when administering cells or growth factors to skin wounds of patients, which undoubtedly will become the topic of future studies on skin regenerative therapies.

Growth factors are indispensable for directing cell to cell and cell to matrix interactions during normal wound repair. However, current therapies do not consider the physiological interactions between growth factors to regulate the repair process. Therefore, using only one growth factor is possibly not the way to go. In fact, during *in vivo* wound repair, protein growth factors often interact with non-protein soluble mediators (i.e. lipids). In fact, these lipids act synergistically with growth factors, stimulating their function and enhancing wound healing. For this reason, therapies combining growth factors and other bio-active, particularly lipid moieties, could be beneficial (Demidova-Rice et al., 2012). Moreover in some cases there is insufficient bioavailablity of growth factors, because of diminished synthesis and/or excessive degradation in chronic wounds (Andree et al., 1994). The therapeutic approaches for such type of skin wounds might need repeated administration of exogenous growth factors. Unfortunately, the repeated administration of high concentrations of growth factors for the treatment of chronic wounds might result in adverse effects (Andree et al., 1994). Moreover, it has been suggested that due to the complexity and persistence of chronic wound bed delivery, a single entity may not be sufficient as an optimal treatment. Thus, the use of a combination therapy and/or a patient specific approach might be required for optimal wound healing (Hinchliffe et al., 2008, Beenken and Mohammadi, 2009). With major advances in the recent years, there has been attempts to address the abovementioned problems through the construction of polymer-based (i.e biomaterial) growth factor delivery systems that allows controlled release of growth factors (Lee et al., 2011a). The main goal of drug delivery systems would be to ensure the stability of the growth factor in a protease environment, extend its function at the injury site, minimize its systemic absorption, and inhibit immune responses. In this regard, different types of systems have been reported such as proteinaceous

ECM-derived, carbohydrate-based and synthetic vehicles. Many are biodegradable or biocompatible and have been considered as safe by the FDA (Demidova-Rice et al., 2012). Still, a number of other challenges remain present and deserve more attention. These include i) enhancing stability of encapsulated growth factors in the biomaterial/construct to allow release for extended times, ii) difficulties in scaling up certain approaches and iii) determining the appropriate compartmentalization of delivery materials to allow multiple factors to be released with distinct kinetics. The interactions among growth factors in cases of multiple growth factor delivery system, their receptors and other ECM components are critical for the clinical delivery of regenerative therapies (Bielefeld et al., 2012) and needs further investigation.

Some issues that remain to be determined in cell-based therapies include age of the donor animal, optimal time of wound treatment, dose and route of administration of cells for skin wound therapy. In addition, the local micro-environment of the injured tissue should be taken into consideration as well. As shown in Table 2.2, different researchers have used different cell types and routes of administration for different types of wounds. Both the *in vivo* study where MSC from old mice inhibited (rather than promoted) wound healing of diabetic mice (Schatteman and Ma, 2006) and the *in vitro* decrease of epidermal stem cell activity of skin cultures from old humans (Jarrold et al., 2012) confirm that the age of the donor is one of the concerns for stem cell therapy. In the case of enhanced fibrosis and scar tissue, cell-based therapies might fail due to a low blood supply and/or lack of receptors and biological molecules which provide the ideal environment for enhancing cell differentiation, proliferation and functioning (Koch et al., 2009; Kuhn and Tuan, 2010).

The use of allogenic multipotent MSC without any adverse reactions has been described in both humans and horses (Broeckx et al., 2014b; 2014c; Carrade et al., 2011a; 2011b; Fang et al., 2007; Ringden et al., 2006; Riordan et al., 2009). Moreover, MSC 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, MSC inhibit both B-cell proliferation and production of IgM, IgA and IgG, (Corcione et al., 2006). Therefore, in the future studies of stem cell-based skin wound therapy, the aforementioned parameters should be evaluated for other stem cell types, such as EpSC. It has to be mentioned that many studies suggest that the main *modus operandi* of cell-based therapies is their paracrine effect. If this would be the case, it

would implement that certain cell components or growth factors might be sufficient to achieve the required clinical improvements. Although the present literature study demonstrated similar functional *in vivo* effects after certain growth factor- and cell-based therapies, it does not allow us to conclude that stem cells only exert paracrine effects. Future studies might provide more answers to this complex, yet interesting matter.

Hereby we propose that the application of cell-based and growth factor-based therapies in animals should be done critically and cautiously and all treatment outcomes should be carefully recorded and reported adequately (Koch et al, 2009).

2.6 Conclusions

Skin wound regenerative therapies are currently studied intensively. A number of *in vitro* and *in vivo* studies have been conducted on regenerative strategies for wound healing in humans and different animal species. Regardless promising animal studies demonstrating accelerated wound healing, their clinical use still remains hampered because adequate delivery methods need further development. Therefore, clinical validation of the use of most of the cellular- and growth factor-based therapies are still in an early stage. Results of fundamental studies and preclinical trials have not yet been confirmed in large scale controlled clinical trials. In addition, there is also a lack of information on long-term outcomes of skin wound treatment using such regenerative therapies. Nevertheless, for all the aforementioned reasons, researchers should be encouraged to increase the knowledge of growth factor- and cell-based regenerative therapies and future studies should focus on the development of a solid therapy for the treatment of skin wounds in mammals.

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Author disclosure statement

SYB and JHS declare competing financial interests and Pell Cell Medicals declares a patent application. The other authors declare no competing interests.

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CHAPTER 3

Objectives of the PhD Research

3.1 Background

Mammal skin plays a pivotal role in several life preserving processes and extensive damage may therefore be life threatening. Selecting an appropriate wound healing strategy is indispensable for successful skin regeneration, since it could minimize the risk of complications, enhance the speed of wound healing and minimize scar formation. In this regard, cell-based therapies have emerged as a promising tool in those cases where conventional treatments fail.

Adult stem cells can be collected from almost any tissue and are currently most frequently used as a regenerative medicine. The main function of stem cells in adult tissue is to repair and regenerate tissues in which they reside. They have the ability to self-renew and to differentiate into at least one mature cell type. Under physiological conditions, stem cells divide to produce progenitor cells that can, depending on the tissue, go through a number of subsequent cell divisions and differentiation steps to produce a complex web of mature cells (Grove et al., 2004). Bone marrow-derived nuclear cells (BMNC) and skin-derived epithelial stem cells (EpSC) represent two entirely different cell populations and require different isolation and characterization techniques. BMNC contribute to the regeneration or repair of many tissues including skin. For this reason, BMNC have been studied for wound healing enhancement in different animal species. EpSC on the other hand, are isolated from epithelial tissues including skin. Currently, only a limited number of studies are available on the *in vitro* and *in vivo* properties of these cells.

3.2 Objectives

For all the aforementioned reasons, the objectives of the PhD research are:

1. To evaluate the wound-healing potential of BMNC after topical application and injection into wound margins of full-thickness skin wounds in rabbits (Chapters 4.1 and 4.2).
2. To extend the existing knowledge of EpSC purification through sphere formation in different culture circumstances (Chapter 5).
3. To comparatively evaluate autologous and allogeneous EpSC treatment in experimentally induced wounds in horses. (Chapter 6).

CHAPTER 4

Evaluation of Cutaneous Wound Healing Potential of Autologous Bone Marrow-Derived Cells

Adapted from:

1. Borena, B.M., Pawde, A.M., Amarpal, Aithal, H.P., Kinjavdekar, P., Singh, R. and Kumar, D. Autologous bone marrow–derived cells for healing excisional dermal wounds of rabbits (2009). Vet Rec 165, 563-568.
2. Borena, B.M., Pawde, A.M., Amarpal, Aithal, H.P., Kinjavdekar, P., Singh, R., Kumar, D. (2010). Evaluation of autologous bone marrow–derived nucleated cells for healing of full thickness skin wounds in rabbits. Int Wound J 7, 249-260.

Introduction

Use of bone marrow aspirate has received increased attention because it is known to contain stem cells with the potential to repair damaged tissues. The aspirated BM needs to be reduced in volume in order to increase its cell content including stem cells. This can be done by removing some erythrocytes (non-nucleated cells) and the plasma so that only nucleated cells (BMNC) i.e. mononuclear stem cells as well as monocytes, lymphocytes and some granulocytes will be retained (Hernigou et al., 2009). BM-MSC, lymphocytes, monocytes/macrophages are categorized as BM-MNC. The use of BM-MNC as a source of BM-MSC for therapy has attracted the attention of many researchers. This is because BM-MNC can be easily obtained and isolated in short time just before transplantation without *in vitro* expansion, which minimizes the risk of contamination (Alvarez-Viejo et al., 2013). Clinical studies have focused on the use of the entire BM-MNC fraction considering that functional effects contributed by multiple cell types and stem cell precursors in the fraction (Mathieu et al., 2009). Besides, the cells and precursors of the BM-MNC produce large amount of cytokines and trophic factors which regulate tissue regeneration (Battistella et al., 2011). BM cells has long been reported to play roles in cutaneous wound healing in different animal species (Akela et al., 2013; Borue et al., 2004b; Kim et al., 2013b) as well as humans (Badiavas et al., 2003; Badiavas and Falanga, 2003).

For the aforementioned reasons, the next two consecutive subchapters in the present thesis describe an experimental study using autologous BMNC on full thickness skin wounds in rabbit model. In the first subchapter (Chapter 4.1), three treatment groups namely: injection of autologous BMNC into wound margins (BI), topical application of BMNC over the wound surface (BT) or dressing with povidone iodine solution (PI) were compared. In the second subchapter (Chapter 4.2), injection of autologous BMNC into wound margins (BI) was evaluated as compared to the topical application of sterile saline solution (normal saline, NS) as a control.

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CHAPTER 4.1

Autologous Bone Marrow–Derived Cells for Healing Excisional Dermal Wounds of Rabbits

Adapted from:

Borena, B.M., Pawde, A.M., Amarpal, Aithal, H.P., Kinjavdekar, P., Singh, R. and Kumar, D. (2009). Autologous bone marrow–derived cells for healing excisional dermal wounds of rabbits. Vet Rec *165*, 563-568.

Abstract

The wound-healing potential of autologous bone marrow-derived nucleated cells was evaluated in full-thickness skin wounds in the thoracolumbar region of 20 clinically healthy rabbits. Three wounds of 2 x 2 cm, one on the left side and two right of the midline, were created on the dorsal lumbar region of each rabbit under xylazine-ketamine anaesthesia. The wounds of each animal were randomly assigned to one of three treatments: injection of autologous bone marrow-derived cells into wound margins (BI), topical application of bone marrow-derived cells over the wound surface (BT) or 5% povidone iodine solution (PI) (control). Wounds were observed for 28 days for granulation tissue formation, wound contraction, histomorphological and histochemical evaluation, and time to complete healing. The mean (se) time to appearance of granulation tissue was significantly less in BI-treated wounds (3.22 [0.22] days) than the BT-treated (3.89 [0.40] days) and PI-treated (4.89 [0.47] days) groups. On days 14 and 21 after surgery, wound contraction was significantly ($P<0.05$) higher in BI-treated wounds (73.00 and 97.35%) than in those treated with BT (58.75 and 84.87%) and PI (54.84 and 84.60%). Histomorphological findings showed an earlier disappearance of inflammatory reaction, better epithelization, significantly more neovascularization, more fibroplasia and collagenation, and earlier histological maturation in BI- and BT-treated wounds than in control wounds.

Key words: Bone marrow-derived cells, rabbit, skin, wound healing

4.1.1 Introduction

The four stages of the wound-healing process, inflammation, debridement, epithelization, and remodeling and maturation, generally overlap (Fossum et al., 2007). Extensive research has been carried out on the use of topical medications in healing, but many of the products investigated in domestic animals do not affect wound healing and some inhibit it (Swaim and Henderson, 1990). Antiseptics such as povidone iodine are used frequently in the management of open wound healing by secondary intention because they reduce bacterial load (Leaper and Durani, 2008). An experimental study of mice suggested that adult bone marrow cells differentiate to epidermal keratinocytes, follicular epithelial cells, sebaceous gland cells, dendritic cells and endothelial cells after their transplantation (Krause et al., 2001). Wu et al. (2007a) also found that injection of bone marrow-derived mesenchymal stem cells around the wound and their application to the wound bed in an excisional wound model enhanced healing significantly in normal and diabetic mice. Clinical trials in humans (n = 6) have suggested that the direct application of bone marrow-derived cells leads to dermal rebuilding and closure of non-healing chronic wounds of more than one year's duration (Badiavas and Falanga, 2003).

The present study was designed to evaluate the wound-healing potential of bone marrow-derived nucleated cells (BMNC) injected into the wound margins and applied topically in full-thickness excisional skin wounds in rabbits.

4.1.2 Materials and methods

Animals

Twenty clinically healthy New Zealand white rabbits of both sexes, weighing between 920 and 2300 g (mean weight 1487 [377.54] g) and of between three and six months of age were used. The Institute Animal Ethics Committee of the Indian Veterinary Research Institute, Izatnagar, India, approved the study. All the animals were obtained from the Laboratory Animals Research section of the Animal Genetics Division at the Indian Veterinary Research Institute. Animals were housed individually and provided with a commercial diet and water ad libitum. They were maintained under uniform conditions and were acclimatised to people approaching and handling them for a period of 10 to 15 days before the start of the study.

Animal preparation

Wound creation and bone marrow aspiration were carried out aseptically under general anaesthesia using 6 mg/kg xylazine administered intramuscularly, followed 10 minutes later by 60 mg/kg ketamine intramuscularly. Anaesthesia was maintained by additional doses of intravenous ketamine if required. Enrofloxacin (5 mg/kg intravenously) and meloxicam (0.2 mg/kg intravenously) were administered preoperatively. The animals were restrained in lateral recumbency for collection of bone marrow aspirate and in sternal recumbency for the creation of experimental wounds. For this purpose, the anteromedial aspect of each proximal tibia and the dorsum (thoracolumbar region) were prepared for aseptic surgery.

Bone marrow aspiration and separation of nucleated cells

Aspiration of bone marrow was done using the method described by Crow and Walshaw (1997), with some modifications. An 18-gauge biopsy needle was inserted with little force through the skin and muscle of the anteromedial aspect of the proximal tibia. Once the needle (with the stylet in place) had contacted with the bone, it was advanced deeper by rotating it slowly with steady pressure until the cortical bone had been penetrated and the needle had entered the marrow cavity. A sudden change to the penetration force of the needle was usually felt at this point, which indicated that the needle was in the marrow cavity. The stylet of the biopsy needle was then removed and the needle was connected to a 10 ml syringe containing 5000 iu of heparin. A negative pressure was applied by forcefully pulling back the plunger and approximately 2.5 ml of bone marrow aspirate was collected from each tibia. BMNC were collected from the marrow aspirate using the volume reduction centrifuge ‘buffy coat’ protocol (Kasten et al., 2007). The bone marrow aspirate was transferred into a sterile tube and centrifuged at 704 *g* for 25 minutes. The supernatant plasma was discarded and the buffy coat was aspirated using a micropipette. While collecting the buffy coat, it was ensured that no trace of buffy coat was left in the tube by aspirating a small quantity of plasma with red blood cells. It was re-centrifuged for 10 minutes at 313 *g* to further reduce the volume. Finally, the concentrated nucleated cells were mixed with 0.5 ml phosphate buffered saline (PBS) for application to the wounds.

Bone marrow nucleated cell count

The number of BMNC was calculated in two random samples to give an estimate of the number of cells being transplanted to the wound site in each case. The buffy coat was collected, diluted with PBS and centrifuged at 1252 *g* for 10 minutes. The PBS was then removed by decanting and aspiration, and the pelleted cells at the bottom with some red blood cells were diluted four times and centrifuged at 1252 *g* for 10 minutes. Re-suspension of the pelleted cells and counting was carried out as as per the method described by Barker.and Knoblock, 1982). The mean number of cells counted by this method was 1.4 x 10^8 cells/ml.

Wound creation, treatment and postoperative care

Using a clean transparency sheet template and a permanent marker, the vertices of the experimental wounds, two on the right side and one on the left, were outlined. Three full-thickness skin wounds including subcutaneous tissue measuring 2 x 2 cm were excised with a #11 Bard-Parker blade. A gap of 2 cm was kept between the two wounds on the right side and wounds were created 2 cm away from the midline on either side of the dorsum. The wounds were created at the same location on the trunk of each animal regardless of the differences in the body size. Haemorrhage, if any, was controlled by applying pressure with a sterile cotton gauze. One of three treatments was assigned to each of the wounds randomly. For the wounds of the BI group, bone marrow-derived cells diluted with 0.5 ml of PBS were loaded in a 2 ml sterile syringe and the contents of the syringe were then injected subcutaneously around the wound edges. In the wounds of the BT group, bone marrow-derived cells were applied topically by gently spraying with the help of a syringe throughout the wound area as homogenously as possible. Povidone iodine 5% solution was applied topically in the wounds of the control (PI) group using a piece of sterile cotton gauze. The antibiotic enrofloxacin and the anti-inflammatory analgesic meloxicam were administered in all the animals for three consecutive days after surgery. The animals were monitored for restlessness, dullness, inappetence, panting, vocalization and elevated respiratory and heart rates.

After the initial treatment with the respective agents, dressing with povidone iodine was done for all animals daily for the first 10 days and thereafter on alternate days up to day 28 after surgery.

Observations

Granulation and epithelization: Evaluation of granulation tissue was done 3, 7, 14, 21 and 28 days after surgery, and categorized using the method of Bigbie et al. (1991), with some modifications; (1) No granulation tissue seen, (2) Granulation tissue depressed below the skin edge, (3) Granulation tissue proliferated to the level of the skin edge, (4) Granulation tissue elevated above the skin edge, (5) Granulation tissue elevated above the skin edge and projecting over the advancing border of epithelium. The time until the appearance of granulation tissue was recorded as the first day when granulation tissue was observed. Similarly, on histological examination, the time of epithelization was recorded as the first day when the epithelium was seen.

Wound contraction: Wound contraction was measured 3, 7, 14, 21 and 28 days after surgery as a % reduction in the wound area. A progressive decrease in the wound area was monitored periodically by tracing the wound margin on tracing paper and measuring the area using graph paper. This was divided by the total wound area of the initial wound tracing and multiplied by 100 to calculate the percentage of contraction (Bigbie et al., 1991).

Time to complete healing: This was recorded as the day on which the wound healed completely. Healing was considered to be complete when the epithelium covered the entire wound and the area of the remaining granulation tissue was zero (Bigbie et al., 1991).

Histomorphological and histochemical evaluation: Four representative samples of full-thickness skin tissue from healing wounds in each treatment group were collected 3, 7, 14, 21 and 28 days after surgery and fixed in 10% buffered formalin. After fixation, the cut pieces were processed by the paraffin-embedding technique to obtain 4 to 5 μm thick paraffin sections. The sections were stained with haematoxylin and eosin (H&E) as described by Luna (1968). The H&E sections were evaluated microscopically using the histological scoring system described by Parameshwaraiah and Shivakumar (1998) and Smith et al. (2008), with some modification. The histological parameters epithelization, inflammation and fibroblast were scored from 1 to 4, (1) representing the most similarity to normal skin and 4 the least similarity. Granulation tissue width was scored as (1) Narrow or (2) Wide, and neovascularization was scored as 1 Resembling normal skin (zero or one new blood vessels), (2) Mild (two to five blood vessels), (3) Moderate (six to 10 blood vessels), or (4) Severe (greater than 10 new blood vessels).

The duplicate sections from each treatment group were stained using Masson's trichrome staining (Masson, 1929) for the detection and grading of collagen fibers in the healing wound. The scoring was done as described by Ghamsari et al. (1996). Collagen fiber density scores were (1) Denser, (2) Dense and (3) Less dense, while collagen fiber thickness scores were (1) Thicker, (2) Thick and (3) Thin and collagen fiber arrangement scores were (1) Best arranged, (2) Better, (3) Worse and (4) Worst arrangement. The mean score of each parameter was calculated for each treatment group and compared.

Statistical analysis

The means of parametric observations were compared by analysis of variance (ANOVA) as described by Snedecor and Cochran (1989), while non-parametric observations were compared using a Kruskal-Wallis one-way ANOVA (Petrie and Watson, 2006). For each comparison, differences between groups were considered significant at $P<0.05$.

4.1.3 Results

Gross evaluation of wound healing

Evaluation of granulation tissue: No significant difference was seen in the level of granulation tissue at different intervals among the various treatment groups, although higher scores for granulation tissue were recorded in the BI group compared with the BT and PI groups up to day 14 after surgery. Irrespective of the treatments given, the granulation tissue proliferated to the level of the skin edge by day 21 in all of the groups (Table 4.1.1).

Table 4.1.1 Mean (se) scores for granulation tissue in the excisional wounds of rabbits following injection of bone marrow derived cells (BI), topical application of bone marrow-derived cells (BT) or 5% povidone iodine solution (PI).

	Number of days after surgery				
Treatment	**3**	**7**	**14**	**21**	**28**
PI	1.55(0.11)	2.31(0.11)	2.67(0.14)	3.00(0.00)	3.00(0.00)
BI	2.05(0.14)	2.63(0.13)	2.92(0.08)	3.00(0.00)	3.00(0.00)
BT	1.80(0.14)	2.31(0.12)	2.67(0.14)	3.00(0.00)	3.00(0.00)

Granulation tissueappeared grossly, was significantly earlier (P<0.05) in BI-treated wounds (mean 3.22 days) than in the BT-treated (mean 3.89 days) and PI-treated (mean 4.89 days) groups.

Epithelium detection: The epithelium was not clearly visible on day 3 in any of the groups. Regenerating epithelium seven days after surgery was seen in 100%, 75% and 75%, respectively in the BT, BI and PI treatment groups. By day 14, the regenerating epithelium was visible in all of the animals in each group.

Wound contraction: Although the original wound created was 2 x 2 cm, almost all of the wounds expanded to various extents, possibly due to the loose nature of the skin of the rabbit dorsum; thus, after the surgery all the wounds had an area greater than 4 cm^2 (Table 4.1.2). However, immediately after surgery, there was no significant difference in the mean wound area among each of the treatment groups.

Table 4.1.2 Mean (se) wound area (cm^2) of excisional wounds of rabbits and percentage wound contraction, following injection of bone marrow-derived cells (BI), topical application of bone marrow-derived cells (BT) or 5% povidone iodine solution (PI).

Treatment	Number of days after surgery					
	0	**3**	**7**	**14**	**21**	**28**
PI	6.08 (0.29)	6.30 (0.30)^{a}	4.64 (0.31)	3.08 (0.42)^{a}	1.03 (0.32)^{a}	0.63 (0.30)
	0.00%	-3.63%	23.68%	49.34%	83.06%	89.64%
BI	6.04 (0.40)	5.23 (0.37)^{bc}	3.81 (0.38)	1.63 (0.46)^{b}	0.16 (0.16)^{b}	0.00 (0.00)
	0.00%	13.41%	36.92%	73.00%	97.35%	100%
BT	6.74 (0.20)	6.10 (0.32)^{ab}	4.43 (0.29)	2.78 (0.45)^{a}	1.02 (0.49)^{a}	0.54 (0.54)
	0.00%	9.50%	34.27%	58.75%	84.87%	91.99%

Values with sharing the same superscripts in the same column are not significantly different (P > 0.05)

By day 3, the mean wound area was significantly smaller (P<0.05) for the wounds treated with BI (5.23 cm^2, 13.41% contraction) than those treated with PI (6.30 cm^2, -3.63%) and BT (6.10 cm^2, 9.50%) (Table 4.1.2). On days 14 and 21, the mean %contraction in BI-treated wounds was significantly higher (73.00 and 97.35%, respectively) than those treated with BT (58.75 and 84.87%) or PI (49.34 and 83.06%). By day 28, the difference in the mean wound size and % contraction between all the treatment groups was insignificant; however, 100% contraction was recorded in the BI-treated group only (Figure 4.1.1).

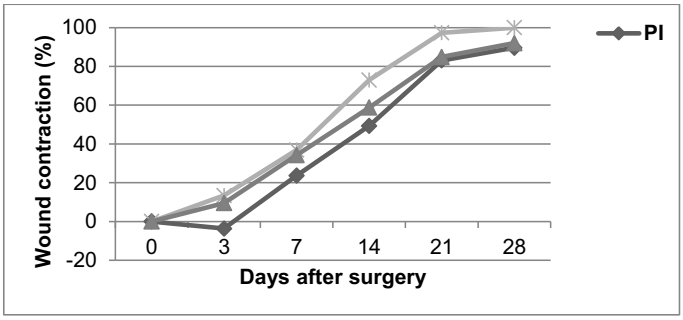

Figure 4.1.1 Wound contraction after the different treatments over time

A higher percentage of wounds healed completely between days 14 and 21, as well as between days 21 and 28 after surgery in the BI-treated group (75 and 100%, respectively) than in the BT and PI treatment groups (0.00 and 25% respectively, for both treatment groups).

Histomorphological and histochemical evaluation

Table 4.1.3 shows the mean (se) scores for histomorphological and histochemical parameters of the wounds at different intervals following each treatment.

By day 3, wider and more compact granulation tissue was seen in BT- and BI-treated wounds than in the control group (Figure 4.1.2). The mean score for inflammation was significantly higher ($P<0.05$) in the PI-treated group than in the BT group. Significantly higher scores were recorded for neovascularization in BI- and BT-treated groups than in the PI group. Although the difference was not significant, the mean scores for fibroblasts were higher in the BI- and BT-treated groups than in the control group.

By day 7, there was a significantly greater inflammatory reaction in PI-treated wounds ($P<0{\cdot}05$) than neither of the test wounds (BI and BT). Higher mean scores for fibroblasts and neovascularization, as well as denser and thicker collagen fibers, were recorded in the BI- and BT-treated groups than in the PI group (Figure 4.1.3), but the differences were not significant.

By day 14, in comparison to the PI group, BI- and BT-treated wounds had more dense and thick collagen fibers (indicated by lower scores), and showed more fibroblasts of the collagen phenotype (Figure 4.1.3).

By day 21, no significant difference was observed in the mean score of any of the parameters studied among all of the treatment groups; however, by day 28, significant differences ($P<0.05$) in the mean scores for neovascularization, and collagen fiber density and thickness were recorded. Better neovascularization, less neovascularization and thicker collagen fibers were seen in wounds treated with BI and BT than in PI-treated wounds (Figure 4.1.4).

4.1.4 Discussion

Bone marrow cells show the broadest differentiation potential among adult somatic cell populations and are considered as the most promising cell sources for clinical applications (Krause et al., 2001, Poulsom et al., 2002). Bone marrow also contains mesenchymal stem cells that secrete a large number of growth factors and cytokines, which are critical in the repair of damaged tissues (McFarlin et al., 2006; Wu et al., 2007b).

During the postoperative period in the present study, all the animals appeared comfortable and had a normal appetite. They did not show noticeable behavioural changes or alterations in heart and respiratory rates. However, the absence of signs of pain and discomfort may be attributable to the administration of analgesic. It has been reported that some topical treatments do not affect wound healing and actually inhibit it (Swaim and Henderson, 1990). However, antiseptics such as povidone iodine are frequently used in the management of open wound healing by secondary intention, owing to their ability to reduce bacterial load (Leaper and Durani, 2008). Therefore, povidone iodine was used in the control group. The time until granulation tissue appeared grossly was shorter in wounds treated with BI, followed by BT, with granulation taking longest in the control group. This was supported by the histopathological findings, which showed early deposition of relatively wider granulation tissues with more cellularity in BI- and BT-treated wounds than in control wounds. This might be attributed to the presence of BMNC in the BI- and BT-treated groups. Studies have shown that BMNC accelerate granulation tissue genesis (Badiavas et al., 2003, Badiavas and Falanga, 2003).

Full-thickness skin wound healing occurs by granulation tissue formation, contraction and epithelization (Fossum et al., 2007). Epithelization occurs by migration of undamaged epidermal cells from the wound margins across the granulation bed (Swaim and Henderson, 1990).

Table 4.1.3 Mean (se) scores for the histomorphological and histochemical parameters of excisional wounds of rabbits at different intervals following treatment with injected bone marrow-derived cells (BI), topical application of bone marrow-derived cells (BT) or 5% Povidone iodine solution (PI).

	Number of days after surgery														
	3			7			14			21			28		
	PI	BI	BT	PI	BI	BT	PI	BI	BT	PI	BI	BT	PI	BI	BT
Epithelization*	3.67 (0.33)	3.75 (0.25)	3.75 (0.25)	3.00 (0.41)	2.75 (0.48)	2.00 (0.00)	2.25 (0.25)	2.00 (0.00)	2.25 (0.25)	2.00 (0.41)	3.00 (0.00)	3.00 (0.00)	4.00 (0.00)^{a}	1.50 (0.29)^{b}	1.25 (0.25)^{b}
Inflammation†	3.33 (0.33)^{a}	3.00 (0.00)^{ab}	2.50 (0.25)^{b}	2.25 (0.25)^{b}	1.25 0.25)^{ab}	1.00 (0.00)^{b}	1.50 (0.29)	1.00 (0.00)	1.50 (0.29)	1.00 (0.00)	1.50 (0.29)	1.25 (0.25)	1.00 (0.00)	1.00 (0.00)	1.25 (0.25)
Fibroblast†	2.67 (0.33)	3.00 (0.50)	3.25 (0.25)	2.75 (0.25)	3.50 (0.29)	3.25 (0.25)	3.25 (0.25)	4.00 (0.00)	3.50 (0.29)	3.75 (0.25)	3.25 (0.25)	3.75 (0.25)	4.00 (0.00)	2.75 (0.25)	2.75 (0.25)
Neovascularization‡	1.67 (0.33)^{a}	2.50 (0.29)^{b}	2.5 (0.29)^{b}	2.75 (0.48)	3.75 (0.25)	3.00 (0.00)	3.00 (0.00)	3.00 (0.00)	2.50 (0.29)	2.25 (0.48)	2.00 (0.00)	2.00 (0.41)	2.00 (0.00)^{ab}	1.50 (0.29)^{b}	1.50 (0.29)^{b}
Collagen fiber density§	3.00 (0.00)	3.00 (0.00)	2.50 (0.29)	2.75 (0.25)	2.25 (0.25)	2.50 (0.29)	2.50 (0.29)	2.50 (0.29)	2.25 (0.25)	2.75 (0.25)	2.00 (0.00)	2.25 (0.25)	3.00 (0.00)^{a}	1.50 (0.29)^{b}	1.75 (0.25)^{ab}
Collagen fiber thickness∞	3.00 (0.00)	3.00 (0.00)	3.00 (0.00)	3.00 (0.00)	2.75 (0.25)	2.75 (0.25)	3.00 (0.00)	2.50 (0.29)	2.50 (0.29)	3.00 (0.00)	2.50 (0.29)	2.75 (0.25)	2.75 (0.25)^{a}	2.00 (0.00)^{b}	2.00 (0.00)^{b}
Collagen fiber arrangement#	3.66 0.33)	3.75 (0.25)	3.00 (0.41)	2.25 (0.75)	3.00 (0.41)	1.25 (0.25)	1.75 (0.25)	1.75 (0.48)	1.75 (0.25)	2.50 (0.29)	1.50 (0.29)	1.25 (0.25)	1.50 (0.5)	1.00 (0.00)	1.00 (0.00)

Means sharing the same superscripts within the same row during a specific number of days after surgery are not statistically significant (P>0.05)
* 1 Resembling normal skin, 2 Slightly thick to normal skin, 3 Moderately thick to normal skin, 4 Thicker than normal skin
† 1 Resembling normal skin, 2 Mild, 3 Moderate, 4 Severe
‡ 1 Resembling normal skin (0-1 new blood vessels), 2 Mild (2-5 new blood vessels), 3 Moderate (6-10 new blood vessels), 4 Severe (>10 new blood vessels)
§ 1 Denser, 2 Dense, 3 Less dense
∞ 1 Thicker, 2 Thick, 3 Thin
1 Best arranged, 2 Better, 3 Worse, 4 Worst arrangement

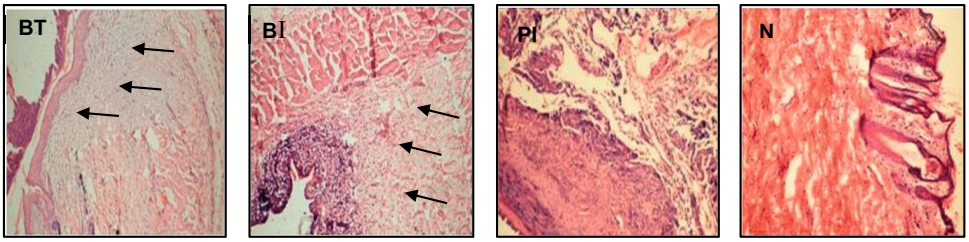

Figure 4.1.2 Photomicrographs showing the healing pattern of wounds in rabbits three days after treatment with (BT) autologous bone marrow-derived nucleated cells applied topically over the wound surface or (BI) injected into the wound margins or (PI) dressing of the wound with 5% povidone iodine solution compared with (N) normal skin. Wounds treated with bone marrow-derived cells had more compact and wider granulation tissue (arrows) than those treated with povidone iodine. Haematoxylin and eosin. X 10.

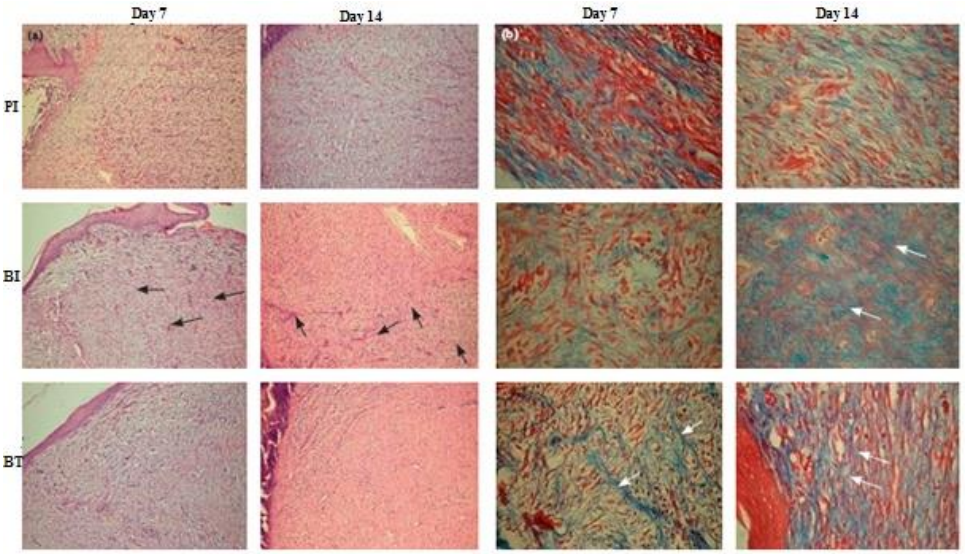

Figure 4.1.3 (a) Haematoxylin and eosin (x 10), and (b) Masson's trichrome (x 40)-stained photomicrographs of granulation tissue 7 and 14 days after surgery to create a wound, and following treatment with autologous bone marrow-derived nucleated cells either applied topically over the wound surface (BT), or injected into the wound margins (BI), or following the application of 5% povidone iodine solution (PI). Black arrows depict granulation tissue with more neovascularization and white arrows depict thick and better arranged collagen fibers in BI and BT groups.

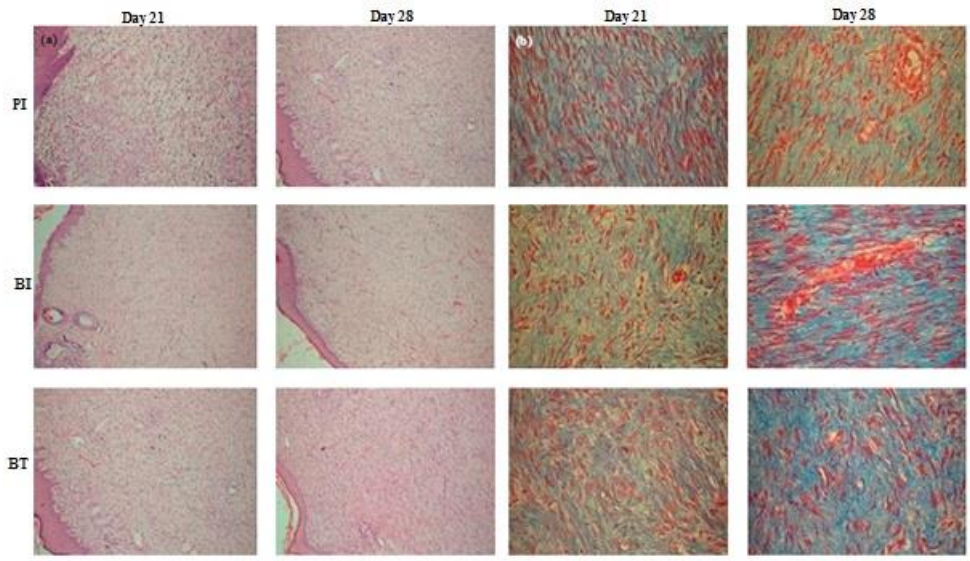

Figure 4.1.4 Haematoxylin and eosin (x 10), and Masson's trichrome (x 40)-stained photomicrographs of granulation tissue 21 and 28 days after surgery to create a wound, and following treatment with autologous bone marrow-derived nucleated cells either applied topically over the wound surface (BT), or injected into the wound margins (BI), or following the application of 5% povidone iodine solution (PI).

Recently, there has been evidence that adult bone marrow cells transplanted into the skin wounds of mice differentiate into epidermal keratinocytes, sebaceous gland cells, follicular epithelial cells, dendritic cells and endothelial cells (Kataoka et al., 2003), and fully differentiated skin (Krause et al., 2001; Badiavas et al., 2003; Kataoka et al., 2003). In the present study, early and better epithelization was observed histologically in sections from BI- and BT-treated wounds compared with those of the control group. This could be attributed to differentiation of bone marrow cells into epidermal cells. Two theories of wound contraction have been proposed in the past. The 'picture frame' theory states that myofibroblasts located in the wound margins of an open wound are responsible for the centripetal forces that lead to wound contraction (Waldon and Zimmerman- Pope, 2003), while the 'pull' theory suggests that fibroblasts distributed throughout the granulation tissue generate the forces responsible for contraction (Abercrombie and others 1956). More recently, it has been suggested that wound contraction occurs through a combination of these two processes (Swaim et al., 2001). In vivo experiments by Yamaguchi and others (2005) demonstrated that bone marrow cells differentiate into wound myofibroblasts when they enter the micro- environment of a wound. Opalenik and Davidson (2005) showed that adult bone marrow-derived cells participate in wound repair by differentiating into wound fibroblasts. In the present study, many of the bone marrow-derived

cells that were injected into the wound margins could have differentiated into myofibroblasts and fibroblasts and resulted in the rapid wound contraction and early closure of full- thickness wounds observed in the BI group compared with the other treatment groups.

It has been suggested that histomorphological assessment of healing in open wounds allows more precision than clinical examination (Abramo et al., 2004). Although inflammation is necessary for healing by fighting infection and inducing the proliferation phase, healing proceeds only after inflammation is controlled (Midwood et al., 2004). Thus, the early control of inflammation, as in the case of BI treatment in the present study, might facilitate the progress to the next phase of wound healing. Neovascularization occurs concurrently with fibroblast proliferation when endothelial cells originating from parts of uninjured blood vessels migrate to the area of the wound (Fossum et al., 2007). Liu et al. (2006) reported that, in response to the wound microenvironment, bone marrow stem cells augment wound healing through the responsive secretion of growth factors that enhance angiogenesis and promote wound repair. Wu et al. (2007a) opined that bone marrow cells enhance neovascularization and promote wound healing through differentiation and the release of proangiogenic factors. The significantly higher neovascularization in the early phase of wound healing in the BI- and BT-treated wounds than in control wounds in the present study may have been due to one or a combination of these factors. There were more fibroblasts up to day 14 after surgery and better collagenation was recorded throughout the observation period in the test wounds than the control group. These findings are in agreement with those of Fathke et al. (2004), who reported that bone marrow- derived cells were able to contract a collagen matrix and transcribe collagen types I and III. By day 28 after surgery, there were significantly denser and thicker collagen fibres in the test wounds than the control wounds. Epithelization was also more similar to the normal skin in the test wounds than the control wounds. These findings indicate earlier histo-logical maturation of the test wounds than the controls, which supports the observations of Liu et al (2006) who reported that wounds treated with BMNC were found to be histologically mature sooner than untreated wounds. The histological and histochemical findings in the BI and BT treatment groups were comparable.

Injection of bone marrow nucleated cells into the wound margins has therefore been shown to accelerate the rate of wound healing in excisional full-thickness skin wounds in rabbits. Besides, the histological findings showed that both BI and BT- treatment augmented the wound healing

activity. Therefore, topical application or injection of bone marrow-derived cells might be among the candidates to be used clinically in the management of large wounds in pet rabbits.

There have been some limitations during the in vivo study using BMNC on rabbit skin wound healing in the present thesis. i. BM aspiration: it is routinely performed and it is efficient by drilling of diaphysis of long bones. Metaphysis of long bones can be alternative sources of BM cells (Chung et al., 2008). Accordingly, due to lack of bone drill, manual aspiration of BM from medial aspect of anterior tibia (tibial metaphysis) using biopsy needle, which was relatively easy was performed in the present thesis. ii. BMNC count: Both aspiration of BM and induction of experimental wounds were done under general anesthesia, thus intraoperative manual counting of BM cell from all the 20 rabbits was difficult. For this reason counting was performed only from two samples in order to have an overview about the cells being transplanted. Therefore, future research is warranted to determine the exact number of cells at each transplant, immunophenotypic characterization of the BM-MSC after volume reduction as well as to determine survival and proliferation of the transplanted cells at wound site.

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CHAPTER 4.2

Autologous Bone Marrow–Derived Nucleated Cells for Healing of Full Thickness Skin Wounds in Rabbits

Adapted from:

Borena, B.M., Pawde, A.M., Amarpal, Aithal, H.P., Kinjavdekar, P., Singh, R., Kumar, D. (2010). Evaluation of autologous bone marrow–derived nucleated cells for healing of full thickness skin wounds in rabbits. Int Wound J 7, 249-260.

Abstract

The aim of the study was to evaluate the potential of autologous bone marrow-derived nucleated cells to enhance the rate of healing of full-thickness excisional skin wounds in rabbits. The study was conducted on 20 New Zealand white rabbits of either sex. Two 2cm × 2cm full-thickness skin (thoracolumabar region) excisional wounds were created; one on each side of the dorsal midline in each animal. The wounds were randomly assigned to either injection of autologous bone marrow-derived nucleated cells into the wound margins (BI), or topical application of sterile saline solution (normal saline, NS), which served as control. The wound healing was assessed by evaluation of granulation tissue formation, wound contraction, epithelization and histopathological and histochemical changes up to 28 days after creation of the wound. Granulation tissue appeared significantly faster in BI-treated wounds (3.22 ± 0.22 days) than in NS-treated wounds (4.56 ± 0.47 days). Better epithelization was seen histologically in BI-treated wounds than in NS-treated wounds. Wound contraction was significantly more in BI-treated wounds when compared with NS wounds on 21 days post-surgery. Histopathological examination of the healing tissue showed early disappearance of inflammatory reaction, significantly more neovascularization, and more fibroplasias and early lay down and histological maturation of collagen in BI wounds than in control wounds. It was concluded that injection of autologous bone marrow-derived nucleated cells in the wound margins induced faster and better quality healing of excisional skin wounds in rabbits when compared with normal saline. The injection of autologous bone marrow-derived nucleated cells can be used to promote healing of large full-thickness skin wounds in rabbits.

Key words: Bone marrow-derived cells, Rabbit, Skin, Wound healing

4.2.1 Introduction

There are four stages of the process of wound healing, that is, inflammation, debridement, repair and remodeling, and maturation, which are clearly differentiated from each other but overlap in time (Fossum et al., 2007). Despite appreciable advances in understanding the basic principles of wound healing, problems in wound healing continue to cause significant morbidity and suffering in animals. Bone marrow-derived stromal cells (BM-MSC) exhibit extraordinary degree of plasticity and growth factor repertoire (Dulchavsky et al., 2008). Experimental study in mice suggested that adult bone marrow cells gave rise to epidermal keratinocytes, follicular epithelial cells, sebaceous gland cells, dendritic cells, and endothelial cells after transplantation (Goodell et al., 2001; Krause et al., 2001). In addition, treatment with BMSC increased the expression of growth factors critical to proper repair and regeneration of the dam- aged tissue (Kwon et al., 2008; Salvolini et al., 2009). Researchers have also noted that bone marrow-derived mesenchymal stem cells when injected around the wound or applied to the wound bed of in excisional wounds significantly enhanced wound healing in normal and diabetic mice (Wu et al., 2007). Clinical trials in people suggested that bone marrow-derived cells (BMDC) applied directly to non-healing chronic wounds of greater than one year's duration led to dermal rebuilding and healing (Badiavas and Falanga, 2003). The purpose of the study reported here was to test the hypothesis that bone marrow-derived nucleated cells injected into the wound margins in excisional wounds of rabbits can enhance the rate of wound healing.

4.2.2 Material and methods

Animals

Twenty clinically healthy New Zealand white rabbits of either sex, weighing from 920 to 2300 g (mean weight 1487 ± 377·54 g) and between 3 and 6 months of age, were used. The Institute Animal Ethics Committee of Indian Veterinary Research Institute Izatnagar, India approved the study. All the animals were procured from Laboratory Animals Research (LAR) section, Animal Genetics Division of Indian Veterinary Research Institute, Izatnagar (UP), India. Animals were housed individually, provided with commercial diet and water ad libitum and maintained under uniform conditions. Animals were acclimatized to approaching and handling for a period of 10–15 days before the start of the study.

Animal preparation

Wound creation and bone marrow aspiration were carried out aseptically under general anaesthesia using xylazine (6mg/kg intramuscularly) followed, 10 minutes later by ketamine (60 mg/kg intramuscularly). Anaesthesia was maintained by additional dose of intravenous ketamine, if required. Enrofloxacin (5 mg/kg intravenously) and meloxicam (0.2 mg/kg intravenously) were administered preoperatively in all the animals. The animals were restrained in lateral recumbency for collection of bone marrow aspirate and in sternal recumbency for creation of experimental wounds. For the purpose of bone marrow collection and creation of wounds, the antero-medial aspect of each proximal tibia and the dorsum (thoracolumbar region), respectively, were prepared for aseptic surgery.

Bone marrow aspiration and separation of nucleated cells

Aspiration of bone marrow was done under aseptic condition from the tibial metaphysis. An 18-gauge biopsy needle was inserted with little force through the skin and muscle of anteriomedial aspect of proximal tibia. Once the needle (with stylet in place) had contacted with the bone, it was advanced further deep by rotating it slowly with steady pressure until the cortical bone was penetrated and the marrow cavity was entered. Usually a sudden change to the penetration force of the needle was felt at this point, which indicated that the needle was in the marrow cavity. The stylet of the biopsy needle was removed and it was connected to a 10-ml syringe containing 1000 IU of heparin and a negative pressure was applied by forcefully pulling back the plunger. Approximately 2.5 ml bone marrow aspirate was collected from each tibia. Bone marrow nucleated cells were collected from the marrow aspirate by volume reduction centrifuge ‘buffy coat’ protocol (Kasten et al., 2007). While collecting the buffy coat, it was ascertained that no trace of buffy coat was left in the tube and even a small quantity of plasma with red blood cells (RBC) was aspirated along with buffy coat. It was re-centrifuged for 10 minutes at 313 g to further reduce the volume. The concentrated nucleated cells were mixed with 0.5 ml phosphate buffered saline (PBS) for application to the wounds.

Bone marrow-nucleated cell count

Bone marrow-nucleated cells (BMNC) were counted randomly in two samples to have an estimate of the number of cells being transplanted to the site of wound. The buffy coat was

collected and diluted with PBS and centrifuged at 1252 g for 10 minutes. The PBS was removed by decanting and aspiration and the pelleted cells at the bottom with some RBCs were diluted four times and centrifuged at 4000 rpm for 10 minutes. Re-suspension of the pelleted cells and counting were as per the method described by earlier researchers (Barker and Knoblock, 1982). The average number of cells counted by this method was 1.4×10^8 cells/ml.

Wound creation

Using a clean transparency sheet template and a permanent marker, the vertices of the experimental wounds of 2 × 2 cm dimensions were outlined on the dorsolumbar region of the rabbits. Two full-thickness skin wounds including subcutaneous tissue, one on each side of the midline, were excised with a #11 BP blade in each animal. The wounds were created 2 cm away from the midline on the two sides of the dorsum. The wounds were created at the same location on the trunk of each animal regardless of the differences in the body size. Haemorrhage, if any, was controlled by applying pressure with sterile cotton gauze. The two treatments bone marrow-derived nucleated cells into the wound margins (BI) and normal saline (NS) were assigned to the wounds randomly. In the wounds designated as BI group, bone marrow cells diluted with 0.5 ml of PBS were loaded in a 1-ml sterile syringe and the contents of the syringe were then injected subcutaneously around the wound edges through a 26 G hypodermic needle. In the wounds designated as NS group, normal saline was applied topically using a piece of sterile cotton gauze. These wounds were taken as control. The antibiotic (enrofloxacin) and an anti-inflammatory-analgesic (meloxicam) were administered in all the animals for three consecutive days after the surgery. The animals were observed for restlessness, dullness, loss of appetite, panting, vocalization and elevated respiratory and heart rates, if any, for 1 week and for wound healing up to 28 days.

Evaluation of wound healing

Granulation tissue

Granulation tissue evaluation was performed daily up to day and then on days 14, 21 and 28 after surgery and categorized with some modifications as: (1) no granulation tissue seen, (2) granulation tissue depressed below the skin edge, (3) granulation tissue proliferated to the level of skin edges, (4) granulation tissue elevated above skin edges and (5) granulation

tissue elevated above skin edges, projecting over the advancing border of epithelium (Bigbie et al., 1991). A granulation score of 3 was considered better than 4 or 2, which were considered better than 5 or 1. Time of appearance of granulation tissue was recorded as the first day when the granulation tissue was observed.

Wound contraction

Wound contraction was calculated on 3rd, 7th, 14th, 21st and 28th postoperative days as a percentage reduction in wound area. Progressive decrease in the wound area was monitored periodically by tracing the wound margin on a tracing paper and the area assessed by using graph paper. The total open wound area at each tracing was subtracted from that of the initial tracing to determine the area of contraction and epithelization during the period since wounding. The area of contraction since wounding was divided by the total wound area of the initial wound tracing and multiplied by 100 to calculate the percentage of wound contraction (Bohling et al., 2004; Majeske, 1992). The wound area, percentage and the mean percentage of wound contraction were calculated in four animals for each interval for both groups of wounds. These four animals were then used for biopsy collection and excluded from the study.

Time of complete healing

Time of complete healing was recorded as the day on which wound healed completely. Healing was considered complete when epithelium covered the entire wound and the area of the remaining granulation tissue was zero (Bigbie et al., 1991). The number of days required for each type of wound to heal completely was recorded and mean days to complete healing were calculated for both treatment wounds.

Histomorphological and histochemical evaluation

Full-thickness skin tissue samples, including about 1cm normal skin around the healing wounds from both treatment group, were collected from four animals each on 3rd, 7th, 14th, 21st and 28th postoperative days. After collection of tissues, these wounds were repaired by suturing and the animals were excluded from the study. The tissues were fixed in 10% buffered formalin. After fixation, the tissues were processed by paraffin- embedding technique to obtain 4–5 µm thick paraffin sections. The sections were stained with

haematoxylin and eosin (H&E) stain as per the standard procedure (Bancroft and Cook, 1984). The H&E sections were evaluated microscopically by using histological scoring system with some modifications (Parameshwaraiah and Shivakumar, 1998; smith et al., 2008). The histological parameters namely: epithelization, inflammation, fibroblast were scored from 1 to 4 (1 standing for best similarity to normal skin and 4 for least similarity) and neovascularization-score: 1: resembling normal skin, 0–1 new blood vessels; 2: mild, 2–5 blood vessels; 3: moderate, 6–10 blood vessels and 4: severe, greater than 10 new blood vessels. Time of epithelization was recorded as the day when the epithelium was seen histologically for the first time. The duplicate sections of both types of wounds were stained using special staining techniques called Masson's Trichrome stain (Masson, 1929) for detection and grading of collagen fibers in the healing wound. The scoring (Chamsari et al., 1996) was done as follows: collagen fiber: density-score: 1, denser, 2, dense and 3, less dense; thickness-score: 1, thicker, 2, thick and 3, thin; arrangement-score: 1, best arranged, 2, better, 3, worse and 4, worst arrangement. The mean score of each parameter was calculated for each treatment group and compared.

Statistical analysis

The means of objective parameters were compared by analysis of variance (ANOVA), whereas data from subjective scorings were compared by Kruskal–Wallis one-way ANOVA (Snedecor and Cochran, 1989). For each comparison, differences between groups were considered significant at $P < 0.05$.

4.2.3 Results

Postoperatively, the animals appeared comfortable as evidenced by normal appetite and behavior and stable heart and respiratory rates.

Granulation tissue

The time (day) of appearance of granulation tissue was significantly ($P < 0.05$) less in BI-treated wounds (3.22 ± 0.22 days) than that in NS-treated wounds (4.56 ± 0.47 days) wounds. No significant ($P > 0.05$) difference was seen in the level of granulation tissue in the wounds of the two treatment groups at different intervals.

Epithelization

On day 3 after surgery, there was no evidence of epithelization in NS-treated wounds, whereas regenerating epithelium was detected in one of four wounds in BI wounds. Epithelium was evident histologically in the sections taken from the center of the wounds in 75% and 100% of the wounds on days 7 and 14 post-operation, respectively, in both BI-treated and NS-treated wounds.

Wound contraction

Although the original wound were created measuring 2 cm × 2 cm dimensions, all the wounds expanded to variable extents possibly because of loose nature of skin of the dorsum in rabbits; thus, after the surgery all the wounds had an area in excess of 4 cm^2 (Table 4.2.1). However, there was no significant ($P > 0.05$) difference in the mean of wound area between the groups.

Table 4.2.1 Mean ± SE value of wound area (cm^2) and percent wound contraction (in parenthesis) in excisional wounds of rabbits.

	Post-wounding days					
Treatment	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
NS	5.53 ± 0.28^a (0.00)	4.75 ± 0.27^a (14.10)	3.33 ± 0.30^a (39.78)	1.79 ± 0.37^a (67.63)	0.41 ± 0.17^a (92.59)	0.07 ± 0.04 (98.73)
BI	6.04 ± 0.40^a (0.00)	5.23 ± 0.37^a (13.41)	3.81 ± 0.38^a (36.92)	1.63 ± 0.46^a (73.01)	$0.16 + 0.16^b$ (97.35)	$0.00 + 0.00$ (100)

* Values with different superscripts in the same column are significantly different ($P < 0.05$) BI = Bone marrow-derived nucleated cells injected into wound margins; NS = Normal Saline

The wound area decreased gradually over time in both treatment groups. After day 7 post surgery, better contraction was recorded in BI-treated wounds as compared to NS group. The difference in wound area was significant ($P < 0.05$) between BI and NS on day 21 post-operation where mean percent contraction of 97.35% and 92.59%, respectively, was recorded. By day 28, difference in the mean wound size and percent contraction between the groups was not significant ($P > 0.05$), but 100% contraction was recorded in BI-treated group only (Figure 4.2.1).

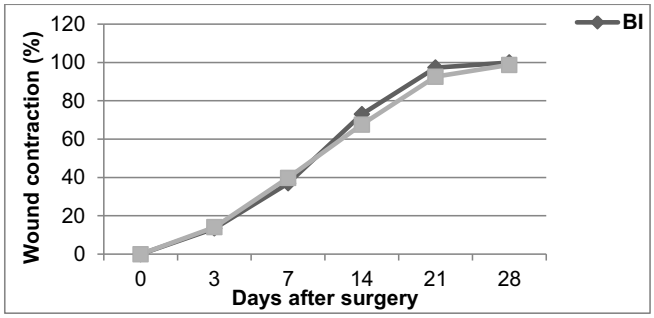

Figure 4.2.1 Wound contraction after the different treatments over time

Time of complete healing

None of the wounds healed completely by 14th day of surgery in two treatment groups. Of the eight animals observed for wound healing, on 21st day after wounding, complete healing was observed in 75% animals in BI treatment group and in 37.5% animals of NS treatment group. On 28th post-wounding day, the percentage of completely healed wounds treated by BI and NS were 100% and 75%, respectively, of the four animals observed for wound healing at this stage (Table 4.2.2).

Table 4. 2.2 Number and percentage of wound healed completely in excisional wounds of rabbits *

Treatment	Between 14 and 21 days (n = 8)	Between 21 and 28 days (n = 4)
NS	**3/8 (37.50%)**	**3/4 (75.00%)**
NS	**6/8 (75.00%)**	**4/4 (100.00%)**

BI = Bone marrow-derived nucleated cells injected into the wound margins; NS = Normal Saline.
* n = number of wounds, number in the parenthesis indicates percentage of wounds completely healed

Histomorphological and histochemical evaluation

Day 3

Granulation tissues were wider and compact in BI-treated wounds when compared with NS (Figure 4.2.2). The mean score values of inflammation were significantly ($P < 0.05$) higher (i.e. more inflammatory reaction) in the NS group than that in the BI group. With regard to neovascularization, significantly ($P < 0.05$) higher scores (i.e. more neovascularization) were recorded in BI-treated wounds than in NS-treated wounds. Although statistically insignificant, the mean score values for fibroblasts were higher in BI than in control group (Table 4.2.3).

Day 7

As on day 3 after surgery, there was significantly ($P < 0.05$) more inflammatory reaction in NS-treated wounds than the BI-treated wounds. Higher mean score values for fibroblasts and neovascularization as well as denser and thicker collagen fibers were recorded in BI-treated wounds than in NS-treated wounds, but the difference was not significant ($P > 0.05$) (Table 4.2.3; Figure 4.2.3).

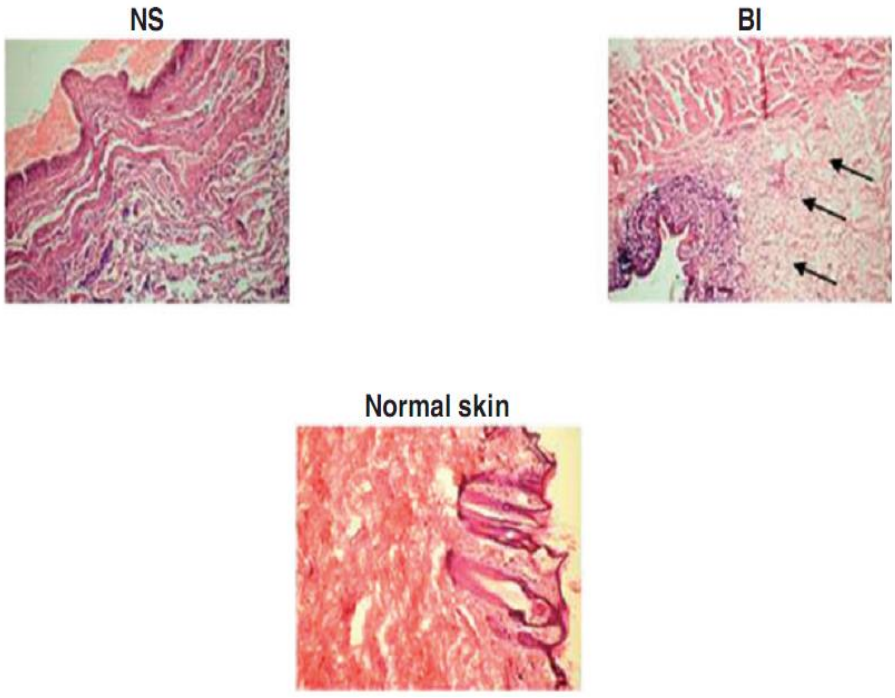

Figure 4.2.2 Photomicrographs showing healing pattern on day 3 wounds treated with normal saline (NS), and autologous bone marrow-derived cells injected to wound margins (BI). Healing tissue in BI-treated wound was better with compact and wider granulation tissue (arrows) than NS-treated wound (H&E sections 10×, day 3)

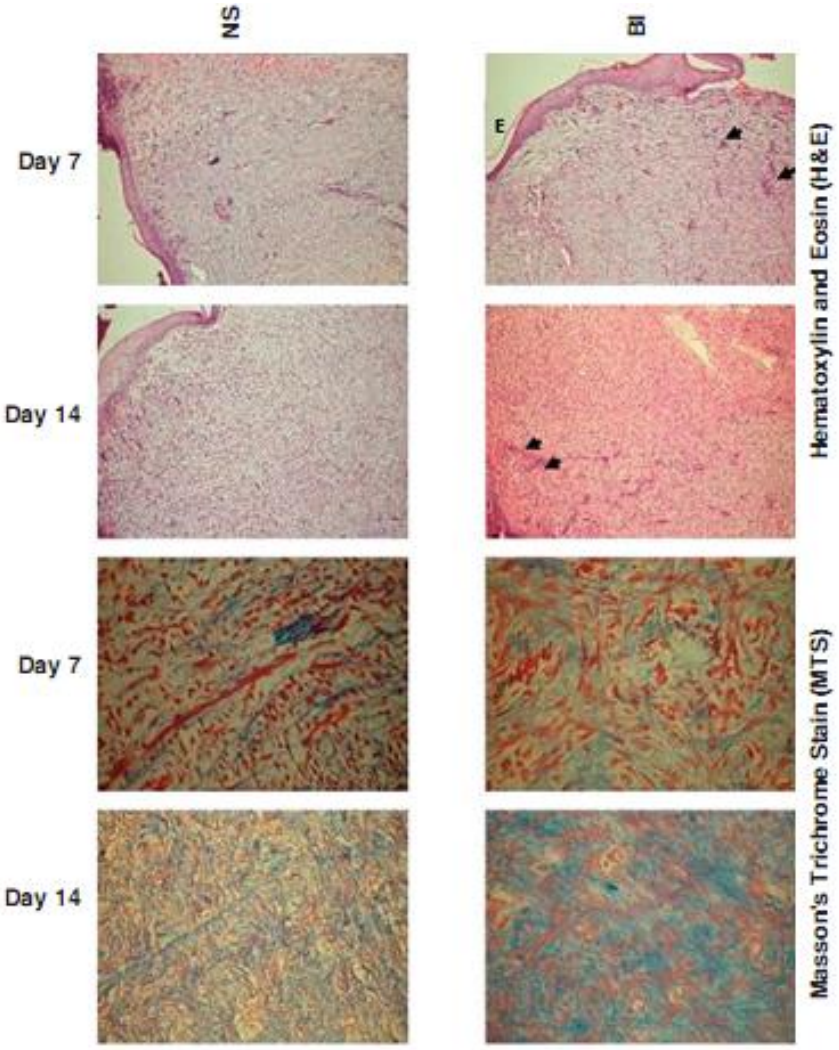

Figure 4.1.3 Photomicrographs of granulation tissue of control (normal saline, NS) and bone marrow-derived nucleated cells into the wound margins (BI) treated animals showing thick epithelium covering wider surface (E), mild to nil inflammatory reaction and more neovascularization (arrow heads) by days7 and 14 (shown in H&E) and moderate deposition of collagen fibers which are denser and thicker in the sections from BI group (arrow head) than in NS groups (shown in MTS) (HE 10× and MT Sections, 40×).

Table 4.2.3 Mean ± SE score values of histomorphological and histochemical parameters of excisional wounds of rabbits in various treatment groups at different intervals.

Parameters	Post wounding days									
	Day 3		Day 7		Day 14		Day 21		Day 28	
	NS	BI	NS	BI	NS	BI	NS	BI	NS	BI
Epithelization[‡]	4.00 ± 0.00	3.75 ± 0.25	2.50 ± 0.50	2.75 ± 0.48	2.50 ± 0.50	2.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.75 ± 0.25[a]	1.50 ± 0.29[b]
Inflammation[‡]	4.00 ± 0.00[a]	3.00 ± 0.00[b]	2.50 ± 0.29[a]	1.25 ± 0.25[ab]	2.50 ± 0.29	1.00 ± 0.00	2.25 ± 0.25	1.50 ± 0.29	1.50 ± 0.50	1.00 ± 0.00
Fibroblast[§]	2.33 ± 0.33	3.00 ± 0.50	2.50 ± 0.29	3.50 ± 0.29	3.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.08	3.25 ± 0.25	2.75 ± 0.48	2.75 ± 0.25
Neovascularization[‖]	1.00 ± 0.00[a]	2.50 ± 0.29[b]	2.50 ± 0.29	3.75 ± 0.25	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	2.00 ± 0.00	3.25 ± 0.25[a]	1.50 ± 0.29[b]
Collagen fibre density[¶]	3.00 ± 0.00	3.00 ± 0.00	2.75 ± 0.25	2.25 ± 0.25	2.50 ± 0.29	2.50 ± 0.29	2.75 ± 0.25	2.00 ± 0.00	2.75 ± 0.25[ab]	1.50 ± 0.29[b]
Collagen fibre thickness[**]	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	2.75 ± 0.25	3.00 ± 0.00	2.50 ± 0.29	3.00 ± 0.00	2.50 ± 0.29	2.50 ± 0.29[a]	2.00 ± 0.00[b]
Collagen fibre arrangement[††]	3.00 ± 0.00	3.75 ± 0.25	2.25 ± 0.25	3.00 ± 0.41	2.50 ± 0.87	1.75 ± 0.48	2.50 ± 0.29	1.50 ± 0.29	1.75 ± 0.25	1.00 ± 0.00

BI = Bone marrow-derived nucleated cells into the wound margins; NS = Normal saline.
*Values of the two groups differ significantly, if possess different superscripts within the same row at the specific day of observation ($P < 0.05$).
†Epithelialisation graded as: 1, resembling normal skin; 2, slightly thick to normal skin; 3, moderately thick to normal skin; 4, thicker than normal skin.
‡Inflammation: 1 = resembling normal skin; 2 = mild; 3 = moderate; 4 = severe.
§Fibroblast: 1 = resembling normal skin; 2 = mild; 3 = moderate; 4 = severe.
‖Neovascularization: 1 = resembling normal skin, 0–1 new blood vessels; 2 = mild, 2–5 new blood vessels; 3 = moderate, 6–10 new blood vessel; 4 = severe, >10 new blood vessels.
¶Collagen fiber density (1–3): 1 = denser; 2 = dense; 3 = less dense.
**Collagen fiber thickness (1–3): 1 = thicker; 2 = thick; 3 = thin.
††Collagen fiber arrangement (1–4): 1 = best arranged; 2 = better; 3 = worse; 4 = worst arrangement.

Day 21

The mean scores of inflammation, fibroblast, neovascularization, collagen fibre density, collagen fibre thickness and collagen fibre arrangement were lower in BI-treated wounds than in NS-treated wounds; but no significant ($P > 0.05$) difference were found in mean scores of any parameter between the treatments (Table 4.2.3; Figure 4.2.4).

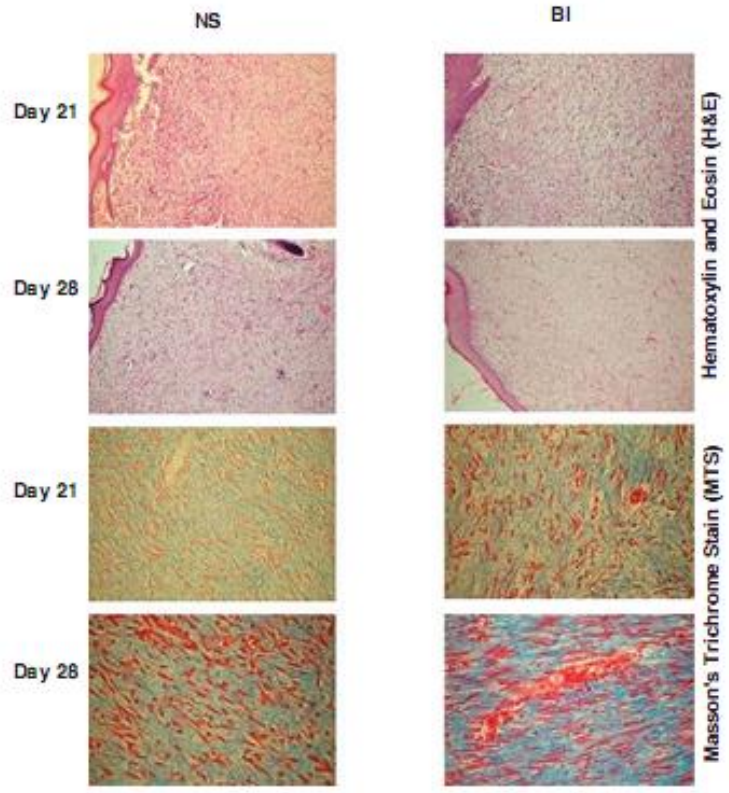

Figure 4.2.4 Photomicrographs of granulation tissue from normal saline (NS), and bone marrow derived nucleated cells into the wound margins (BI) treated wounds showing histologically more mature granulation tissue of the wounds treated by BI than by NS. Sections of granulation tissue from BI-treated wounds showed denser and thicker collagen fibres and lesser fibroblasts than that of NS on days 21 and 28 (H&E 10× and MTS 40×).

Day 14

In comparison with NS group, BI-treated wounds had more dense and thick collagen fibres and showed more fibroblasts of collagen phenotype (Figure 4.2.3).

Day 28

Significant ($P < 0.05$) difference in the mean scores values of neovascularization, collagen fibre density and thickness was recorded during this period (Table 4.2.3). Better epithelization was seen in BI-treated wounds than in NS-treated wounds.BI-treated wounds had significantly less neovascularization than the control (NS). Collagen fibres were significantly ($P < 0.05$) thicker in BI-treated wounds than in NS-treated wounds (Figure 4.2.4).

4.2.4 Discussion

Bone marrow cells show the broadest differentiation potential among adult somatic cell populations and are considered as one of the most promising cell sources for clinical applications (Goodell et al., 2001; Krause et al., 2001; Poulsom et al., 2002). Bone marrow also contains mesenchymal stem cells (BM-MSC) that secrete a large number of growth factors and cytokines, which are critical for proper repair and regeneration of damaged tissue (Kwon et al., 2008).

Considering the reported capacity of bone marrow cells to secrete growth factors and cytokines and the ability to trans-differentiate to the healing tissue components, we hypothesized that there would be quantitatively and qualitatively better healing in the wounds- treated bone marrow cells compared with the control. It was assumed that excisional wounds treated with BI would heal faster with more granulation tissue formation, epithelization, rapid wound contraction, early histological maturation and complete healing than control group.

The injection of bone marrow cells in BI wounds might have caused some trauma and caused inflammation leading to release of cytokines, which may affect wound healing. However, the injection of bone marrow cells was made slowly using a fine hypodermic needle to minimize the trauma. Furthermore, the injection trauma was considered negligible compared with the surgical trauma inflicted in creation of wounds and thus may be of little significance.

Grossly, the granulation tissue appeared significantly faster in wounds treated with BI than in control group. These findings were also supported by the histopathological findings of early deposition of relatively wider granulation tissues with more cellularity in BI-treated wounds than in control wounds. This might be attributable to the role of BMNC in BI group as studies have shown that BMNC accelerate granulation tissue genesis (Kasten et al., 2007; Badiavas et al., 2003).

Full-thickness skin wound healing occurs by granulation tissue formation, contraction and epithelization (Johnston, 1981; Lazarus et al., 1994; Waldron and Zimmerman, 2003). epithelization occurs by migration of undamaged epidermal cells from the wound margins across the granulation bed (Fossum et al., 2007; Deodhar, 1997). Recently, there is evidence that adult bone marrow cells transplanted in skin defect had differentiated into epidermal keratinocytes, sebaceous gland cells, follicular epithelial cells, dendritic cells, and endothelial cells and fully differentiated skin with hair was reconstituted within 3 weeks (Kataoka et al., 2003). Many researchers also suggested that bone marrow stem cells can produce new skin cells (Krause et al., 2001; Badiavas et al., 2003). In the present study, early and better epithelization observed histologically in sections from BI-treated wounds when compared with con-trol wounds are possibly also attributed to differentiation of bone marrow cells into epidermal cells (Krause et al., 2004; Badiavas et al., 2003; Kataoka et al., 2003) or enhanced proliferation of resident epidermal cells in the presence of epidermal growth factor, possibly produced by bone marrow cells (Kwon et al., 2008). Wound contraction plays very important role in the healing of excisional skin wounds. There are two theories of wound contraction that have been proposed in the past. The ‘picture frame’ theory of wound contraction states that myofibroblasts located in the wound margins of an open wound are responsible for the centripetal forces that lead to wound contraction (Waldron and Zimmerman, 2003; Grillo et al., 1958). On the other hand, the ‘pull theory’ suggests that fibroblasts distributed throughout the granulation tissue generate the forces responsible for contraction (Abercrombie et al., 1956). More recently, it has been suggested that wound contraction occurs through a combination of these two processes (Swaim et al., 2001). *In vivo* experiments showed that bone marrow cells differentiated into wound myofibroblasts after they entered the microenvironment of the wound (Abe et al., 2001; Yamaguchi et al., 2005). Another study showed adult BMDC participated in wound repair by differentiating into wound fibroblasts (Opalenik and Davidson, 2005). In the present study, many of the BMNC, which were injected into the wound margins, might have differentiated into myofibroblasts

and/or fibroblasts or recruited more fibroblasts from the surrounding tissues through chemotaxis (Dulchavsky et al., 2008) that resulted in significantly early and fast wound contraction and early closure of full- thickness wounds in BI group than control group.

It was suggested that histopathological assessment of mode and rate of healing in open wounds allows more precision than clinical examination (Abramo et al., 2004). Inflammation is necessary for healing as it plays a role in combating infection and inducing the proliferation phase, but healing proceeds only after inflammation is controlled (Midwood et al., 2004; Diegelmann and Evans, 2004). Thus early disappearance of inflammation in BI wounds in the present study might have facilitated the progress to the next phase of wound healing. Because the activity of fibroblasts and epithelial cells requires oxygen, angiogenesis is imperative for other stages in wound healing, such as epidermal and fibroblast migration. Neovascularization occurs concurrently with fibroblast proliferation when endothelial cells, originating from parts of uninjured blood vessels, migrate to the area of the wound (Kuwahara and Rasberry, 2007). Bone marrow stem cells are reported to release factors that recruit macrophages and endothelial lineage cells into wound (Chen et al., 2008). In another study, it was suggested that bone marrow cells enhance neovascularization and promote wound healing through differentiation and release of proangiogenic factors (Badiavas and Falanga, 2003). This might be the main reason for the significantly higher neovascularization in early phases of wound healing in BI wounds than control wounds.

The fibroblast is the connective tissue cell responsible for collagen deposition that is needed to repair the tissue injury (Fossum et al., 2007). In the present study, there were also more fibroblasts up to day 14 post-surgery, better collagen formation throughout the observation period were recorded in the BI-treated wounds than in the NS-treated wounds. These findings are in agreement with the earlier findings (Fathke et al., 2004) where BMDC were able to contract a collagen matrix and transcribe both collagen types I and III. In addition, BM-MSC secrete several growth factors critical to repair of damaged tissues (Kwon et al., 2008).

Compared with NS, BI-treated wounds showed more compact and thicker granulation tissue with less fibroblasts and better- arranged fibrocytes, and denser and thicker collagen fibres by day 21, which indicated the maturation phase of the wound healing. By day 28 post-wounding, there were significantly denser and thicker collagen fibres in the BI wounds than in the control wounds. epithelization was also more comparable with the normal skin in the BI

wounds than the control wounds. All these findings indicate early histological maturation of test wounds than the controls, which conform to the observations of earlier researchers, who reported that the wounds treated with BM-MSC were found to be histologically mature earlier when com-pared with the untreated wounds (Liu et al., 2006). Early deposition and maturation of collagen in BI wound may also be attributable to the reported interaction of BM-MSC with fibroblasts, which results in promotion of growth and chemotaxis of fibroblasts (Dulchavsky et al., 2008).

It was concluded that injection of bone marrow-derived nucleated cells to the wound margins facilitated early and better healing than the normal saline. Histological and histochemical observations showed that the bone marrow cells augmented wound healing activity significantly by increasing cellular proliferation, formation of granulation tissue, neo-vascularisation, synthesis of collagen, epithelization and early histological maturation in excisional wounds. Bone marrow-nucleated cells can be used clinically in the management of large skin wounds in rabbits.

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CHAPTER 5

Sphere-Forming Capacity as an Enrichment Strategy for Epithelial-Like Stem Cells from Equine Skin

Adapted from:

Bizunesh M. B., Meyer, E., Chiers, K., Martens, A., Demeyere, K., Broeckx, S. Y., Duchateau, L. and Spaas, J. H. (2014). Sphere-forming capacity as an enrichment strategy for epithelial-like stem cells from equine skin. Cell Physiol Biochem *34*, 1291-1303.

Abstract

Mammal skin plays a pivotal role in several life preserving processes and extensive damage may therefore be life threatening. Physiological skin regeneration is achieved through ongoing somatic stem cell differentiation within the epidermis and the hair follicle. However, in severe pathological cases, such as burn wounds, chronic wounds, and ulcers, the endogenous repair mechanisms might be insufficient. For this reason, exogenous purification and multiplication of epithelial-like stem/progenitor cells (EpSC) might be useful in the treatment of these skin diseases. However, only few reports are available on the isolation, purification and characterization of EpSC using suspension cultures. Therefore, in the present study, skin was harvested from 6 mares and EpSC were isolated and purified. In addition to their characterization based on phenotypic and functional properties, sphere formation was assessed upon isolation, i.e. at passage 0 (P_0), and at early (P_4) and late (P_{10}) passages using different culture conditions. On average 0.53 $\pm$ 0.28% of these primary skin-derived cells showed the capacity to form spheres and hence possessed stem cell properties. Moreover, significantly more spheres were observed in EpSC medium versus differentiation medium, corroborating the EpSC privileged ability to survive in suspension. Furthermore, the number of cells per sphere significantly increased over time as well as with subsequent passaging. Upon immunophenotyping, the presumed EpSC were found to co-express cytokeratin (CK) 14, Casein kinase 2 beta and Major Histocompatibility Complex (MHC) I and expressed no pan CK and wide CK. Only a few cells expressed MHC II. Their differentiation towards keratinocytes (at P_4 and P_{10}) was confirmed based on co-expression of CK 14, Casein kinase 2 beta, pan CK and wide CK. In one of six isolates, a non-EpSC cell type was noticed in adherent culture. Although morphological features and immunohistochemistry (IHC) confirmed a keratinocyte phenotype, this culture could be purified by seeding the cells in suspension at ultralow clonal densities (1 and 10 cells/cm^2), yet with a significantly lower sphere forming efficiency in comparison to pure EpSC ($P = 0.0012$). In conclusion, the present study demonstrated sphere formation as a valuable tool to purify EpSC upon their isolation and assessed its effectiveness at different clonal seeding densities for eliminating a cellular contamination.

Key words: Purification; Skin; Epithelial; Stem Cells; Horse; Sphere formation

5.1 Introduction

Adult stem cells possess a low antigenicity (Hoynowski et al., 2007) and can be isolated from a variety of sources including bone marrow (Pittenger et al., 1999; Jiang et al., 2002), adipose tissue (Zuk et al., 2002), peripheral blood (Spaas et al., 2013) and skin (Toma et al., 2001). Unlike embryonic stem cells, little or no ethical issues are related to the use of their adult counterparts. Being the body's largest organ, the skin is a highly accessible source and the largest reservoir of epithelial stem cells (EpSC), at least in humans (Barthel et al., 2005). Furthermore, their surprisingly diverse differentiation potential has also contributed to a significant recent interest in adult skin stem cells (Toma et al., 2005). Permanently residing EpSC in mammalian skin are able to differentiate into multiple lineages and have been reported in the epidermis (Broeckx et al., 2014), hair follicle (Braun et al., 2003) as well as dermis (Toma et al., 2001).

Like stem cells from other tissues, epidermal EpSC play a central role in tissue homeostasis and wound repair, but also represent a major target of tumor initiation and gene therapy (Lavaker and Sun, 2000). These EpSC can differentiate into interfollicular epidermis, hair follicles and sebaceous glands (Niemann and Watt, 2002). The extensive regenerative capacities of the epidermis is due to the presence of EpSC that continuously produce keratinocytes, which further undergo terminal differentiation towards a keratinized layer and provide the skin’s barrier properties in mammals (Chen et al., 2009). Furthermore, the epidermis contains a population of stem cells with apparently different levels of maturity (Terskikh and Vasil’ev, 2001). This heterogeneous population displays subpopulations of a different morphology than its more differentiated progeny at the homing site (Staniszewska et al., 2011). Interestingly, the bulge of the hair follicle is a major repository of skin keratinocyte stem cells and the latter have been regarded as the ultimate EpSC (Costarelis et al., 1999; Lavker and Sun, 2000; Taylor et al., 2000; Braun et al., 2003). However, it should be emphasized that lineage tracing *in vivo* demonstrated the existence of interfollicular stem cells (Ito et al., 2005; Langton et al., 2008) and indicated that epidermal and hair follicle stem cells represent distinct populations (Ito et al., 2005).

Stem cells are being isolated from different tissues, have been evaluated in numerous human clinical trials and are since recently commercially used in veterinary medicine to treat horses and dogs (Hoynowski et al., 2007). However, purification of stem cells is technically difficult

because of their scarcity in the tissue of origin and the lack of universal morphologic traits for stem cells (Blau et al., 2001). Many of the methods developed for the isolation and analysis of specific cell types cannot be used for medical experimentation on living materials because of the damaging nature of these techniques, such as isotope radiation and cell fixation (Yano et al., 2005). For isolation of EpSC, *in vitro* methods describe the use of (i) colony forming culture to localize multipotent stem cells in the hair follicle bulge region (Rochat et al., 1994a), (ii) Hoechst exclusion technique to isolate a side population in the hair follicles (Rochat et al., 1994b), (iii) cell sorting using CD71 and α6-integrin expression markers for harvesting keratinocyte stem cells (Li et al., 2004) and (v) sphere formation (Broeckx et al., 2014).

Once skin-derived precursor cells differentiate, they lose typical stem cell properties and their survival in suspension is substantially reduced (Ruetz et al., 2013). In the mammary gland, another ectodermal organ, so-called mammospheres are also intensively studied for stem/progenitor cell purification purposes (Dontu et al., 2003; Spaas et al., 2012). Unfortunately, a major disadvantage of this 3-D technique is contamination with other adult cell types surviving between the sphere-forming stem cells (Dontu et al., 2003). Moreover, no reports are available concerning sphere forming capacities of skin-derived EpSC and their differentiated progeny during long-term serial passaging. Furthermore, only limited information is available concerning cell impurities in skin-derived sphere cultures. For all these reasons, this study was designed to extend the existing knowledge of EpSC purification through sphere formation in different culture circumstances.

5.2 Materials and methods

Donor animals and skin harvesting

Six healthy French trotter mares with an age between 5 and 7 years and in good general health condition (no clinical or hematological signs of infections) were used for skin sample collection. The isolation and characterization of skin-derived EpSC was performed as previously described (Dontu et al., 2003; Broeckx et al., 2014) with some minor modifications. Briefly, horses were sedated with an intravenous injection of detomidine hydrochloride (0.01mg/kg, Medesedan®, VIRBAC, Belgium) and the analgesic butorphanol (0.01mg/kg, Dolorex®, Intervet, Belgium). After surgical preparation (clipping, scrubbing

and disinfecting) and local anesthesia of the neck region with 4% Procaine-Adrenalin, one full thickness skin sample of about 2cm^2 was excised. Samples were kept in transport medium consisting of Hank's balanced salt solution (HBSS, Gibco) with 2% of penicillin-streptomycin-amfotericine B (P/S/A, Sigma-Aldrich). The experimental procedure was approved by the ethical committee of Global Stem Cell Technology (EC_2012_002, EC_2013_003 and EC_2014_001) and the Faculty of Veterinary Medicine, Ghent University (EC_2014_020).

Isolation and culture of skin-derived epithelial-like stem cells

In the laboratory, the samples were maintained at 4°C overnight in 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco) solution with the hair side down. Afterwards, the epidermis was mechanically disconnected from the dermis by deep scraping using sterile scalpel and forceps on a sterile petri dish. Subsequently, a second enzymatic dissociation step with 1% collagenase III (Worthington Biochemical Corporation) at 37° C for 60 minutes was performed in order to remove epidermal cells. The suspension was poured through a 40µm filter (BD Falcon) in a 50ml tube containing 2ml of fetal bovine serum (FBS, Gibco) to inactivate the collagenase III and washed by centrifugation at 300G for 8 minutes at room temperature. The pellets were re-suspended in epithelial-like stem cell (EpSC) medium, consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco), 20% FBS, 2% P/S/A, 20 ng/ml human recombinant-basic fibroblast growth factor (hr-bFGF) and 20 ng/mL epidermal growth factor (EGF) (all from Sigma-Aldrich). The suspension was then filtered again and cells were counted using Trypan blue staining (1:1 dilution). Subsequently, the cells were cultured on ultralow attachment plates to induce sphere formation. After 7 days of suspension culture, spheres were centrifuged and seeded on adhesive tissue culture flasks for further characterization.

Sphere formation assay

From the cells harvested by the procedure above, an average of $(0.4\pm0.2)x10^5$ cells/cm² were planted per well of an ultralow attachment 6-well plate for all 6 skin samples (Corning, Elscolab) in EpSC medium to induce primary spheres. The spheres were counted at day 4 and 7 and the medium was refreshed by means of centrifugation at 300G for 8 min at both time points. Sphere forming efficiency was calculated by dividing the average number of spheres counted in a well by the number of cells planted and multiplied by 100 to express as a

percentage. After the first sphere cycle (7 day culture on ultralow attachment plates), all spheres were collected and planted onto adherent tissue culture flasks in EpSC medium. Upon 80% confluency, the adherent cells were trypsinized with 0.25% trypsin-EDTA and cultured in fresh medium from passage 1 (P_1) to P_{10}. At P_4 and P_{10} cells were seeded in duplicate at a clonal density of 100cells/cm^2 on 6-well ultralow attachment plates to initiate a second and third sphere cycle for all 6 skin samples, respectively. The second and third sphere cycle were performed in both EpSC and differentiation medium. Differentiation medium consisted of DMEM/F12 (Gibco), 20% FBS (Gibco), 5µg/ml insulin, 1µg/ml hydrocortisone and 0.1mM beta-mercaptoethanol (all from Sigma-Aldrich). The medium was refreshed every two days and the following parameters were recorded in 10 fields on day 1, 4 and 7 post-seeding: number of cell units/field (a separate cell or a sphere is counted as 1 cell unit at each time point), number of spheres/field and number of cells/sphere (all at 200x magnification).

Purification of EpSC

For purification of an impure EpSC culture, adherent cells from P_{10} of this culture and a pure culture as a positive control were cultured in suspension on 6-well ultralow attachment plates at different densities: 1 cell/cm² (=10 cells/6-well), 10 cells/cm² (=100 cells/6-well) and 100 cells/cm² (=1000 cells/6-well) using EpSC medium. The experiment was performed three times in triplicate for each seeding density and the number of spheres and number of cells/sphere were compared on day 1, 4 and 7 post-seeding with a pure culture. On day 7, the spheres were centrifuged and reseeded on adherent tissue culture plates and grown for 7 days to assess morphological differences and perform IHC.

Population doubling time (PDT)

After culturing the spheres in adherent tissue culture flasks, EpSC were trypsinized at 80% confluency using 0.25% trypsin-EDTA. Cells were counted using trypan blue staining and subcultured at $1x10^4$/cm² until P_{10}. The proliferation rate was quantified using the population doubling time (PDT), which was calculated for each passage from P_1 to P_{10} as previously described by Martinello et al. (2010) and Spaas et al. (2013), using the formula: $T/[Log(N_f/N_i)/Log2]$, where T is the time in days, N_f the final number of cells and N_i the initial number of cells.

Flow cytometry assay

A flow cytometric technique was used to characterize sphere-derived cells for the expression of major histocompatibility complex (MHC) I and MHC II. Adherent cells from P_4 and P_{10} were first detached using 0.25% trypsin-EDTA (Gibco) and frozen at 400,000 cells per vial using 10% DMSO (Sigma) as a cryoprotectant (for analysis of the different time points at the same time). Per series, 400,000 cells were thawed and labeled with the following primary antibodies: mouse anti-horse MHC class I IgG_{2a} (Washington State University, 1:50) and mouse anti-horse MHC class II IgG_1 (Abd Serotec, 1:50). Cells were incubated with the primary antibodies for 15 minutes on ice in the dark and washed twice in washing buffer, consisting of DMEM with 1% bovine serum albumin (BSA). Rabbit anti-mouse-FITC (Dako, 1:40) antibodies 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 at least 10,000 cells were evaluated using the FACSCanto™ flow cytometer (Becton Dickinson). 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 and used at the same protein concentration as the corresponding antibodies. To evaluate cell viability the cell impermeable DNA dye propidium iodide (Sigma-Aldrich, 1µg/ml) was added just before measurement.

Induction of differentiation

Differentiation of the isolated cells towards keratinocytes was induced using a distinct differentiation protocol for EpSC developed by Broeckx et al. (2014). Briefly, adherent cells from P_4 and P_{10} were trypsinized using 0.25% trypsin-EDTA and EpSC were seeded into 24-well plates at a density of $2.5x10^3$ per cm^2. The cells were induced in differentiation medium for 7 days and the medium was refreshed every 2 days. In parallel to this, EpSC were also cultured in similar plates at the same density using EpSC medium as a control.

Immunohistochemistry (IHC)

To identify whether the skin-derived cells expressed previously reported EpSC markers (Broeckx et al., 2014) and to qualitatively assess the success of keratinocyte differentiation and EpSC purification, IHC at P_4, P_{10} and P_{11} was performed. Adherent cells were washed with HBSS, fixed for 10 minutes with 4% paraformaldehyde and permeabilized for 2 minutes

with 0.1% Triton X, both at room temperature. Cells were then incubated with hydrogen peroxide for 5 minutes followed by washing with HBSS before incubation for 2 hours at room temperature with the following primary mouse IgG_1 monoclonal antibodies: anti-human cytokeratin 14 (CK 14) (Abcam, clone LL002, 1:50) and anti-human Pan CK (Dako, clone, AE1/AE3, 1:50) and the following rabbit antibodies: anti-human Wide CK (Abcam, 1:50) and anti-human IgG Ab casein kinase 2β (CK-2β) (Abcam, clone EP1995Y, 1:50). After washing with HBSS, secondary ready to use either goat anti-mouse or anti-rabbit peroxidase (PO)-linked antibodies (Dako) -depending on the primary antibody used- were added and incubated for 30 minutes at room temperature. Finally, 3, 3'-diaminobenzidine (DAB) was added for 2-10 minutes and a counter staining with hematoxylin was performed to visualize the surrounding cells. Identical staining performed on undifferentiated EpSC was used as a control for the differentiation experiment 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 antibodies. The staining was then observed using an inverted light microscope and pictures were taken using a digital microscope camera.

Statistical analysis

The statistical analysis for the sphere formation assay and the population doubling time was based on the mixed model with horse as random effect and medium, time, sphere cycle and the two-way interactions as fixed effects. For testing the difference in sphere forming efficiency between a pure and impure EpSC isolate, a fixed effects model was fitted with the number of seeded cells (10, 100 and 1000), isolate (pure vs impure) and their interaction as fixed effects factors. SAS Version 9.3 was used for the analyses (SAS/STAT Software, Version 9.3, SAS Institute Inc.). The relationship between the number of cell units/field and the number of spheres/field was determined by the Pearson correlation coefficient. Values are given as mean ± standard error and a global significance level of 5% was used.

5.3 Results

Isolation and culture of skin-derived epithelial-like stem cells

Presumed EpSC were isolated from all 6 equine skin samples following mechanical and enzymatic digestion. First, the isolated primary skin-derived epithelial cell suspensions were

grown in non-adherent (spherical) culture conditions for stem cell purification. Secondly, these spheres were further expanded in adhesive culture circumstances. The impurities detected in one of the six horses were thirdly effectively purified as reported in detail in the "Purification of EpSC" section.

The first and thus very small "spheres" consisting of on average 2 cells/sphere were observed in all cultures as early as 1day post seeding. With time the size of these initial spheres significantly increased at all passages as illustrated in Figure 5.1. At P_0 other cell structures such as floating fragments, keratinocytes and melanocytes were visible and no adherent cells could be noticed in all suspension cultures (Figure 5.1). Upon subsequent seeding on adherent tissue culture flasks, the cells in the spheres dispersed and reached 70-80% confluence at approximately 4-5 days post-seeding. Moreover, trypsinization and reseeding from adherent plates on ultralow attachment plates resulted in the formation of subsequent generations of spheres (Figure 5.1).

Sphere formation assay

To test the self-renewal capacity of the presumed EpSC, the sphere-initiating capacity of serially passaged cells cultured as spheres was assessed (Figures 5.1 and 5.2). A similar assay has been previously used for the characterization of human mammospheres (Dontu et al., 2003). An average sphere-forming efficiency of 0.53 $\pm$ 0.28% (means $\pm$ SD, n=6; Table 5.1) was recorded from the primary skin-derived epithelial cells seeded on the non-adherent plates at an average density of $0.4x10^5$ cells/cm².

Sphere formation was successful at P_4 (second sphere cycle) and P_{10} (third sphere cycle) from seeding 100 cells/cm² (Figure 5.1), which indicated that these cultured cells were capable of self-renewal, proliferation and clonal expansion in suspension. The number of cell units/field increased over time in EpSC medium, but decreased over time in the differentiation medium ($P < 0.0001$). By day 7, a significantly ($P < 0.0001$) higher number of cell units/field was observed in EpSC medium versus differentiation medium (Figure 5.2A). Regardless the medium used, the number of cell units/field was significantly ($P < 0.0001$) higher at the second cycle (P_4) than at the third cycle (P_{10}) (Figure 5.2A). The number of cells/sphere significantly increased from day 4 to day 7 post-seeding ($P < 0.0001$) and with sphere cycle ($P < 0.0001$ and Figure 5.2B), and the increase was larger in the third cycle ($P < 0.0001$).

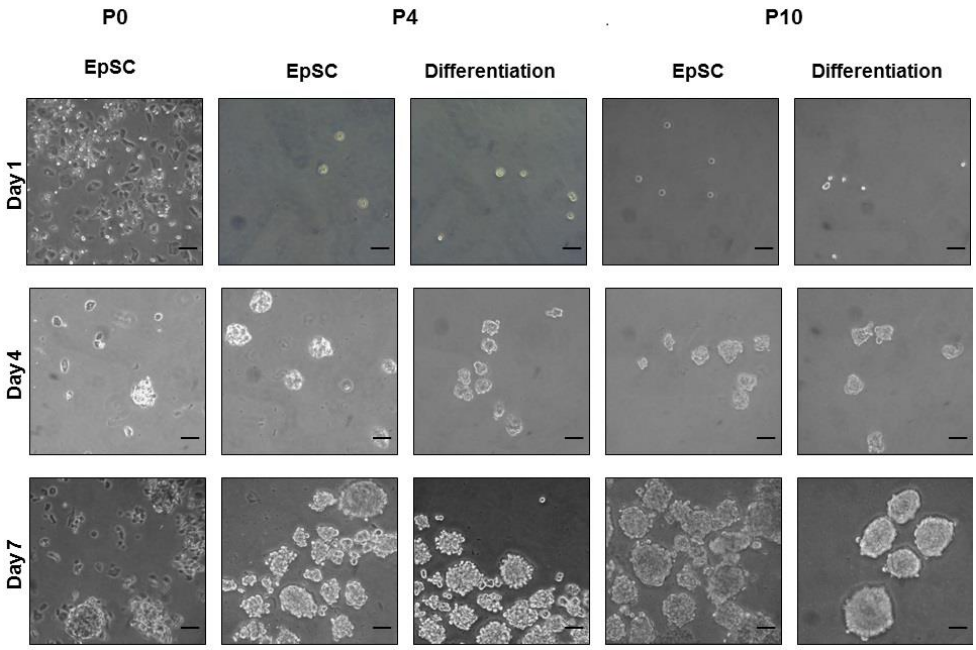

Figure 5.1 Sphere formation in epithelial stem cell (EpSC) and differentiation medium at different passages cultured on ultralow attachment plates for 7 days. Primary spheres in EpSC medium at Passage (P) 0, second cycle sphere formation in EpSC and differentiation medium at P4 and third cycle sphere formation in both media at P10. The picture indicates the increase in number of cells/sphere over time (200x magnification, scale bar = 50µm).

Regarding the number of spheres/field on both day 4 and day 7, this parameter was significantly higher in EpSC than in differentiation medium ($P = 0.008$, $P < 0.0001$, respectively and Figure 5.2C). A strong correlation ($R^2 = 0.9$) between the number of cell units/field and of the number of spheres/field existed in both media. However, unlike the number of cell units/field, the number of spheres/field was not significantly affected by sphere cycle ($P = 0.203$, data not shown). Overall, these data indicated that presumed EpSC clonally expanded in suspension, however, they experienced difficulties in sphere formation when going into the differentiation process.

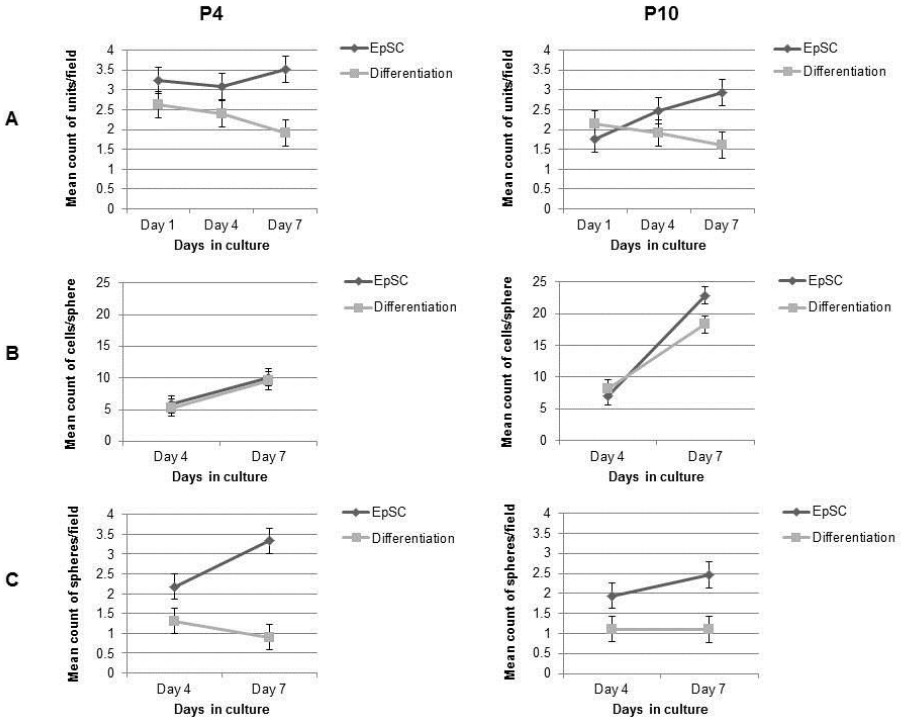

Figure 5.2 Epithelial-like stem cell (EpSC) proliferation assays. Sphere generating cells from adherent culture of passage 4 and 10 were cultured in suspension at a density of 100cells/cm² in ultralow attachment plates using EpSC and differentiation medium to generate 2nd (P4) and 3rd (P10) cycle of spheres. Number of units/field (A), number of cells/sphere (B) and number of spheres/field (C) were counted per 200x magnification field and compared between both media.

Table 5.1 Sphere forming efficiency (SFE) from the primary skin-derived epithelial cells planted on non-adherent plates. The SFE (in %) was calculated by dividing the average number of spheres counted per well by the seeded cells multiplied by 100.

Horse number	Seeding density	No. of spheres	SFE (%)
1	0.15×10^6	1089.17	0.73
2	0.75×10^6	968.15	0.13
3	0.54×10^6	2299.36	0.43
4	0.35×10^6	1452.23	0.42
5	0.26×10^6	2420.38	0.95
6	0.45×10^6	2299.36	0.51
Average	0.42×10^6	1754.78	0.53
Standard deviation	0.21×10^6	661.74	0.28

Population doubling time of EpSC

The population doubling time (PDT) of isolated skin-derived epithelial cells from fresh adherent cultures (i.e. not cryopreserved) was used as an indicator of self-renewal and proliferation potential and was calculated from P_1 up to P_{10} (Table 5.2). Our results indicate that there was no statistically significant (P = 0.319) difference in the population doubling time of the different passages. The PDT in the adherent cultures for all the passages was positive and varied between 0.89 $\pm$ 0.13 days and 1.39 $\pm$ 0.35 days (means $\pm$ SD, n = 6; Table 5.2).

Immunophenotyping of the isolated and differentiated cells

Flow cytometric analysis was performed to immunophenotypically characterize the isolated skin-derived epithelial cells and to evaluate their expression of typical immunological proteins over time and after differentiation towards keratinocytes. Compared to the relevant isotype controls, most of the presumed EpSC and differentiated cells expressed MHC I concomitant with only a limited number of cells expressing MHC II at P_4 as well as at P_{10} (Figure 5.3). No significant differences in number of cells expressing MHC I and II could be noticed between the two cell types at different time points (Figure 5.3).

Table 5.2 Population doubling time (PDT) in days of 6 adherent EpSC cultures calculated from passage (P) 1 to 10.

Horse	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	1.28	0.68	1.11	1.19	1.12	1.24	1.11	1.01	2.02	0.70
2	0.93	0.99	1.20	1.17	1.30	1.48	1.48	1.30	1.50	0.94
3	1.12	1.03	1.02	1.11	1.11	1.12	1.38	1.16	1.31	1.04
4	1.14	0.78	1.09	1.08	1.14	1.13	1.23	1.03	1.14	0.90
5	1.51	0.96	1.01	0.97	1.20	1.04	1.25	1.08	1.04	0.87
6	1.10	0.88	1.22	1.12	1.27	1.13	1.38	1.13	1.31	0.92
Average	1.18	0.89	1.11	1.11	1.19	1.19	1.31	1.12	1.39	0.89
STD	0.20	0.13	0.09	0.08	0.08	0.15	0.13	0.10	0.35	0.11

STD = standard deviation

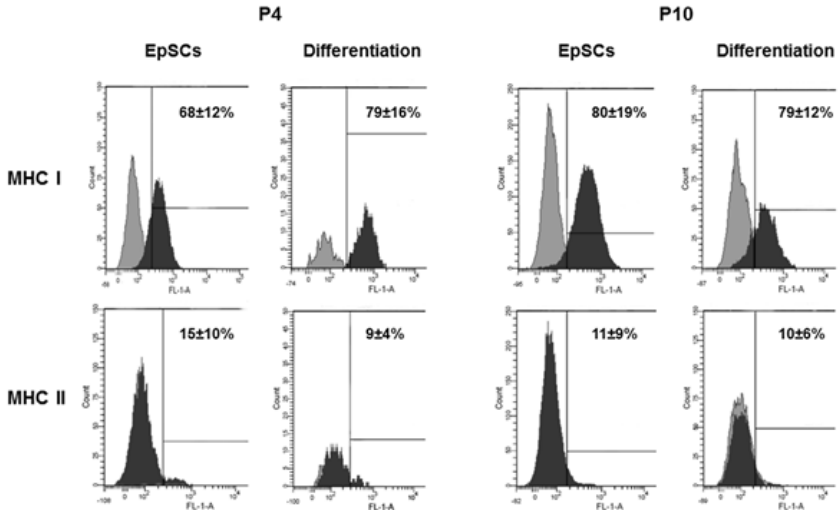

Figure 5.3 Immunophenotypic characterization of skin-derived epithelial-like stem cells (EpSC) at passage (P) 4 and 10. Flow cytometery was performed to assess the number of undifferentiated EpSC and differentiated cells (Diff) expressing major histocompatibility complex (MHC) I and MHC II. The histograms show relative number of live cells versus fluorescence intensity with isotype control staining (light gray) and marker antibody staining (dark). Data represent the mean percentage of six samples + standard deviation.

Immunohistochemistry of EpSC and cells differentiated towards keratinocytes

The immunophenotypic characterization of sphere-derived cells and their differentiation potential was evaluated using immunohistochemistry analysis. Accordingly, the isolated undifferentiated skin-derived epithelial cells, which were used as control for the differentiation experiments, were negative for typical keratinocyte markers, namely Pan CK and Wide CK (Papini et al., 2003) while staining positive for the epithelial markers Casein kinase 2β (Deshiere et al., 2013) and CK 14 (Figure 5.4). Differentiated cells on the other hand, were positive for all three cytokeratines tested i.e. CK 14, Pan CK and Wide CK as well as for the epithelial marker Casein kinase 2β. No positive staining cells were detected in the undifferentiated as well as differentiated cell culture upon relevant isotype control staining. Morphologically, the undifferentiated cells were elongated or typically spindle-shaped, whereas the differentiated cells were more stretched, stellate-shaped and formed cobblestone-like cells (Figure 5.4).

Purification of EpSC

Cellular contamination was detected in one out of the six skin samples. The impurity was detected at P_4 on adherent plates and remained present over time (Figure 5.5). Both the average number of spheres/well and cells/sphere from seeding at clonal densities i.e.at 10 cells/6-well and 100 cells/6-well increased from day 4 to day 7 post-planting in pure and impure cultures (Figure 6A and B). However, compared to pure EpSC, the sphere forming efficiencies were significantly lower ($P = 0.0012$) in impure cultures (Figure 5.6C). In marked contrast to the ultralow seeding densities, higher seeding densities (i.e.at 1000 cells/6-well) resulted in unsuccessful purification (Figure 5.7B) and a decrease in the number of spheres over time (data not shown), yet with an increase in sphere size (Figure 5.6D). The keratinocyte impurity was confirmed by IHC as shown in Figure 7A based on positive expression of a panel of selected appropriate markers as described (CK 14, Pan CK, Wide CK and Casein kinase 2β). During the process of purification by planting at different clonal densities, the impurity was still detected in several wells derived from the 1000 cells/6-well purification (Figure 5.7B). A successful purification of the cell culture was achieved by planting at a density of 10 cells/cm² (Figure 5.7C) and 1 cell/cm² (Figure 5.7D). This was confirmed by the spindle shaped cell morphology and negative expression of Pan CK and Wide CK.

5.4 Discussion

Being the body's largest organ, skin has been extensively used to study adult/somatic stem cells (Li et al., 2013). In particular, it has been suggested as the main source of epithelial-like stem cells (EpSC) (Grandi et al., 2012; Staniszewska et al., 2011). Besides the presence of somatic stem cells in hair follicles and dermis, the epidermis also contains a compartment of stem cells (Broeckx et al., 2014; Terskikh and Vasil'ev, 2001) which are of different maturity levels. However, there is no common criterion that allows to recognize individual stem cells with confidence (Terskikh and Vasil'ev, 2001). In a human skin study, it was identified that the latter stem cells are firmly adherent to the basal lamina and account for 10% of the cells in the basal layer at the dermal-epidermal junction (Papini et al., 2003). So far EpSC have been obtained either by dedifferentiating adult epidermal cells (Li et al., 2004; Zhao et al., 2012), or by inducing pluripotent stem cells (Li et al., 2012), or by their direct harvest from the epidermis (Yang et al., 2007; Broeckx et al., 2014). Isolation of sphere-forming skin-derived

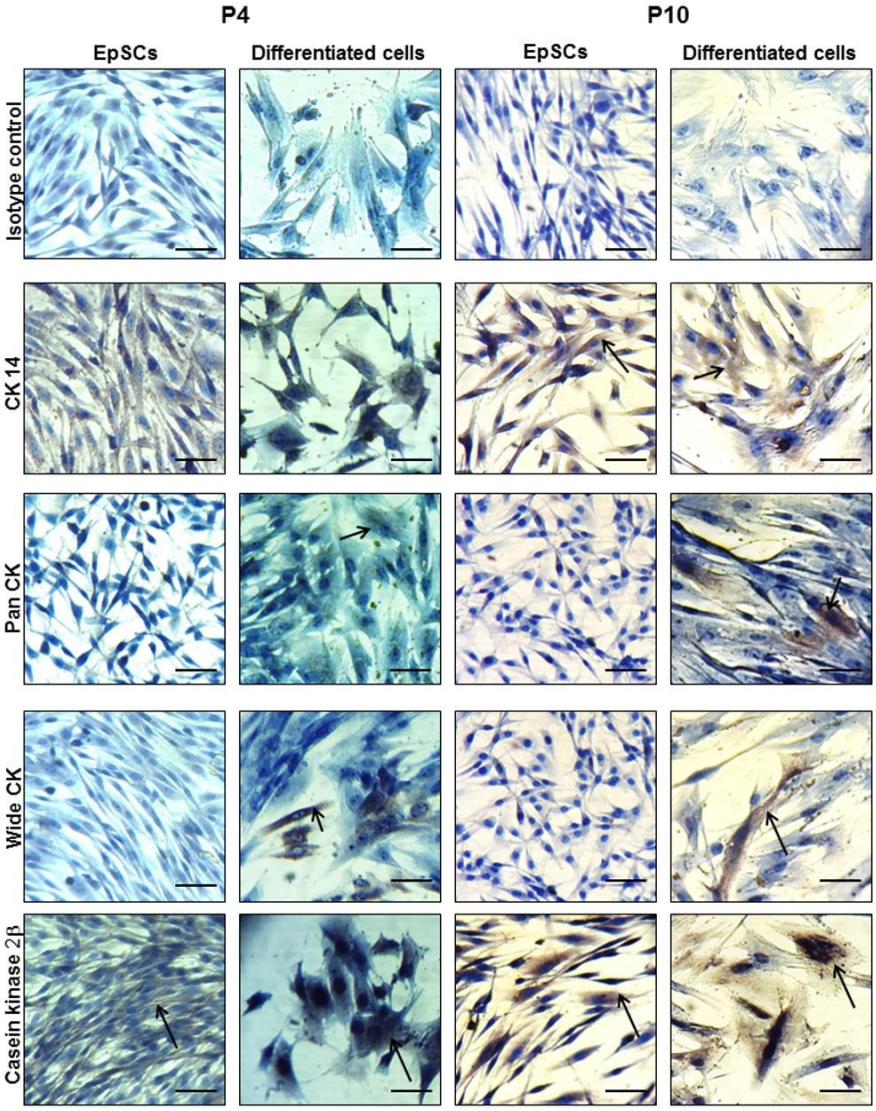

Figure 5.4 Differentiation of epithelial-like stem cells (EpSC) into keratinocytes at passage (P) 4 and 10. Immunohistochemistry was performed on adherent undifferentiated and differentiated cells using markers including, CK14, Pan CK, Wide CK and casein kinase 2β. Relevant isotype controls were also included. After Hematoxylin counterstaining, pictures were taken using a digital microscope camera, at 400x magnification. Arrows indicate positive staining and bars represent 50μm.

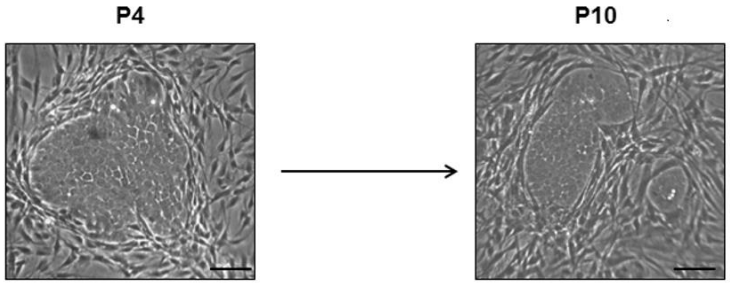

Figure 5.5 Morphology of adherent keratinocyte impurities (colonies of cobblestone shaped cells) in the epithelial-like stem (EpSC) isolate at passage (P) 4 and 10.

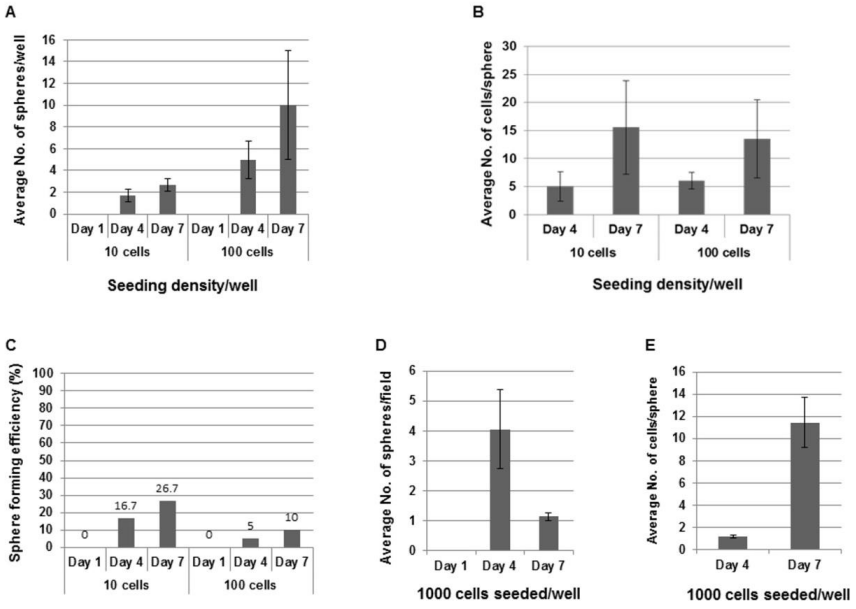

Figure 5.6 Sphere forming assay for purification of the impure samples (1000 cells seeded) in comparison to a pure isolate (10 cells and 100 cells seeded). Average number of spheres/well from planting 10 cells/6-well and 100 cells/6-well (A), average number of cells/sphere from planting of 10 and 100 cells/6-well (B), sphere forming efficiency in percentage of cells that were able to form a sphere (C) and average number of cells/sphere from planting of 1000 cells/6-well (D) were compared between pure and impure epithelial-like stem cells (EpSC). Systematic counts were done at 200x magnification. Data represent the average values of 3 wells per experiment + standard deviation.

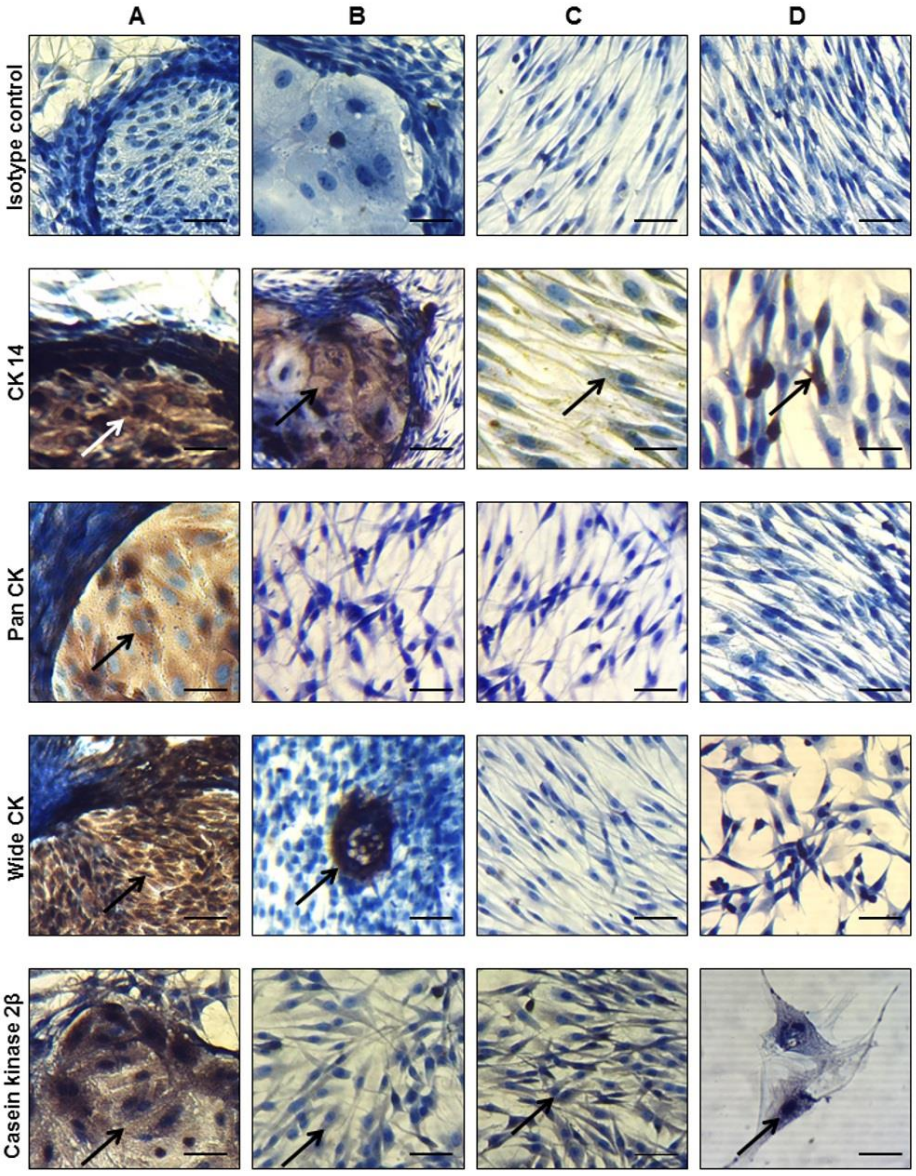

Figure 5.7 Immunohistochemistry of adherent epithelial-like stem cells (EpSC) with keratinocyte impurity (A) before purification, keratinocyte detected in two of the 100 cells/cm² purification (B), purified EpSC derived from seeding 10 EpSC/cm² (C) and from 1 cell/cm² (D). Pictures in rows show Isotype control and markers used as CK 14, Pan CK, Wide CK and Casein kinase 2β. Arrows indicate positive reaction in the cytoplasm at 400x magnification. Scale bars represent 50μm.

epithelial cells from suspension culture has been described already in 1986 (Soule and McGrath, 1986). Such a suspension commonly consists of stem cells and their transit-amplifying progeny (Pastrana et al., 2011). A potential disadvantage of this isolation technique is the likely contamination with other cell types due to adult cells surviving between the sphere-forming stem cells (Dontu et al., 2003). So far, sphere formation was successful both from bulk skin tissue-derived cell suspension (Toma et al., 2001) and from culture of epidermis-derived cells at a clonal density (Broeckx et al., 2014) on ultralow attachment plates.

In the current study, skin was harvested from six horses and an average sphere-forming efficiency of 0.53 ± 0.28% of these seeded cells was demonstrated. As is the case for primary mammary cells (Dontu et al., 2003), most of the primary skin-derived epithelial cells died in suspension culture. It has to be mentioned, that although the starting material from all donors had the same size, the initial cell seeding density varied 0.21 $x10^6$ cells between donors and might have caused a variation in sphere forming efficiency at the first cycle. In agreement with our results, it has been described that 0.4% of the seeded mammary cells were able to form mammospheres in humans (Dontu et al., 2003) a percentage which was higher in horses ranging from 0.8 to 3.2% depending on the lactation stage (Spaas et al., 2012). Moreover, subsequent generations of spheres could be generated from P4 and P10 with a relatively constant sphere-forming efficiency of almost all purified cells (data not shown). This finding corroborates a previous study (Broeckx et al., 2014) and is indicative for the fact that each sphere contained approximately one sphere-initiating cell. Thus, the self-renewal and clonal expansion capacities of the isolated cells being main characteristics of stem cells, was clearly demonstrated (Pastrana et al., 2011). Visual examination of suspension culture revealed that, although spheres could also grow in the differentiation medium, their number was significantly lower compared to that observed in the EpSC medium during both second and third sphere cycles. This clearly indicated an overall lower cell survival of the more differentiated EpSC progeny or of the undifferentiated EpSC in differentiation medium. Based on the first hypothesis, this difference could be attributed to the fact that once skin-derived precursor cells are differentiated, they lose the typical stem cell properties and their survival in suspension is substantially reduced (Ruetz et al., 2013). The strong correlation between the numbers of cell units/field and of spheres/field was an indicator that most of the skin-derived purified EpSC resulted in sphere formation. Additionally, the number of

cells/sphere increased from day 4 to day 7 post seeding which indicated that clonal expansion of cells within the sphere occurred up to P10.

Cellular contamination was noticed in one of the six horse skin isolates. Cell contamination is usually difficult to detect, and consequently is a potentially major culture problem than the problems caused by other types of biological contamination (Fogh et al., 1971). In the present study, cellular impurities were clearly visible microscopically based on morphology and on IHC staining for keratinocytes. This contamination could be noticed from P4 on and the level of impurity increased during passaging. Nevertheless, it has been reported that serial passaging would rather eliminate more committed progenitors and select for self-renewing stem cells (Pastrana et al., 2011). For that reason, cells were seeded at ultralow clonal densities varying from 1 cell to 100 cells per cm^2 on 6-well ultralow attachment plates followed by adherent culture. Here, IHC staining demonstrated that only the lowest densities allowed EpSC to survive and removed the cellular impurities from culture. Although the purification in the present study consisted of cells from only one donor, the experiment was performed three times (in triplicate for each time point) and generated consistent results. Nevertheless, future studies should confirm the reported purification technique in different isolates with different degrees of impurities.

Another key finding of the present study pertains to the immunophenotypic characterization of EpSC by flow cytometry. Expression of Major Histocompatibility Complex (MHC) I and MCH II was evaluated on equine EpSC and their differentiated progeny. These data showed that most EpSC and differentiated cells expressed MHC I and only a few cells expressed MHC II. This result is in contrast with a previous report using equine EpSC (Broeckx et al., 2014), where only few cells expressed MCH I. It has to be mentioned though, that in the present study only live cells were gated, because a rather high death rate was present during measurement, whereas in the previous study all cells were measured including the dead ones. Nevertheless, the high number of cells expressing MHC I in this study is also in contrast with a previous study on differentiated keratinocytes, where a low number of cells expressing this marker was reported in a sub-population of basal human keratinocytes (Matic et al., 2005). It is known that the rejection response to grafted tissues/cells is caused by cell surface molecules that induce antigenic stimulus. MHC molecules are one of the families within the highly heterogeneous group of transplantation antigens that have been described so far (Ayala et al., 2012). While MHC I antigens are present on all nucleated cells and are involved in antigen

presenting activities (King et al., 2000), MHC II are expressed on activated B-lymphocytes, macrophages, dendritic cells and epithelial cells (Klein et al., 2000) and involved in graft rejection (Natarajan et al., 2002). Therefore, the low number of undifferentiated as well as differentiated EpSC expressing MHC II suggest that these cells might be attractive candidate cells for allogenic transplantation in horses. Nevertheless, future studies will have to confirm this statement and should focus on the relative expression levels (per cell) of these and other immunomodulating molecules as well.

In conclusion, the present study reports sphere formation as a valuable tool to purify stem cells in skin cell cultures upon isolation and after serial passaging. Further research should focus on the optimization and validation of this purification strategy of stem cells cultured from skin as well as from other tissues.

Acknowledgement

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CHAPTER 6

Comparison of Autologous versus Allogenic Epithelial-Like Stem Cell Treatment in an In Vivo Equine Skin Wound Model.

Abstract

Several studies report beneficial effects of stem cells on wound healing, however, to date no comparison between autologous versus allogenic epithelial-like stem cells (EpSC) has been made in a large animal wound induction model. Therefore, 12 full-thickness skin wounds were created in six horses. Each group was subjected to: (i) autologous EpSC, (ii) allogenic EpSC, (iii) vehicle treatment or (iv) untreated control. Double-blinded macroscopic and histological evaluation of wound healing was performed at week 1, 2 and 5 through biopsies.

In contrast with depth and volume, the circumference and surface of autologous EpSC-treated wounds were significantly smaller than vehicle treated wounds. Compared to vehicle treatment, a significantly lower amount of total granulation tissue was observed after autologous and allogenic EpSC treatment.

Together, these results suggest that wounds treated with autologous EpSC heal significantly faster, but the observed differences in wound healing parameters were rather small, indicating a limited clinical relevance in the used model.

Key words: Epidermis; Stem Cells; Wound; Autologous; Allogenic

6.1 Introduction

The skin is the largest organ in mammalian vertebrates and plays a key role in several life-preserving processes. Loss of skin integrity and function may therefore induce dysfunction or even death (Theoret, 2009a). For superficial wounds, the endogenous healing mechanisms in combination with traditional wound care should be sufficient to achieve functional repair. However, in larger, more complex wounds impaired wound healing can occur and result in fibrosis and scar formation (Reinke and Sorg, 2012). Such scar tissue is weaker than intact skin due to a disorganized extracellular matrix (Gurtner et al., 2008; Soo et al., 2003). Since it has been reported in different animal species that fetal wounds have the ability to heal without scar formation and with complete restitution of the skin architecture (Goss, 1977; Hallock, 1985; Hallock et al., 1987; Soo et al., 2003), the scientific community has been focusing on regenerative strategies for many years.

Most of the research on harvesting and characterizing adult equine MSC use bone marrow (Berg et al., 2009; Ranera et al., 2012; Violini et al., 2009), adipose tissue, peripheral blood (Martinello et al., 2010b; Spaas et al., 2013d) or umbilical cord (blood) (Cremonesi et al., 2008b; Koch et al., 2007). Conversely, ongoing regeneration of the skin is achieved through differentiation of stem cells within the epidermis and the hair follicle. Currently, these skin stem cells have been isolated from dermis (McKenzie et al., 2006; Shim et al., 2013; Toma et al., 2005), hair follicles (Yu et al., 2006) and epidermis from humans, mice and horses (Broeckx et al., 2014d; Fujimori et al., 2009; Nowak and Fuchs, 2009). Beneficial effects of mesenchymal stem cells (MSC) for skin wound healing in laboratory animals have been described by several groups (Akela et al., 2012a; Borena et al., 2014; Borena et al., 2010). MSC also improved the healing capacity of persistent leg wounds in human diabetic patients (Jain et al., 2011b) and demonstrated enhanced wound closure, collagen synthesis, cellular proliferation and angiogenesis in dogs (Kim et al., 2013a). In horses, it has been recently reported that a combination of equine epithelial-like stem cells (EpSC) and platelet-rich plasma (PRP) significantly restricted granulation tissue and increased the vascularisation in comparison to PRP control wounds within the same horse (Broeckx et al., 2014d). Other authors described reconstruction of goat skin with hair formation and cellular repopulation using adult EpSC (Yang et al., 2007).

Both the EpSC harvesting and culture expansion are time consuming processes usually taking more than 3 weeks, thus excluding autologous EpSC treatment of wounds during the acute inflammatory peak. Therefore, allogenic EpSC treatment using cells obtained from a fully characterized master cell bank from only a few donors would substantially standardize the treatment modality. In this regard, it has been reported that MSC are immune-privileged (Bartholomew et al., 2002; Beyth et al., 2005; Carrade et al., 2014), allowing allogenic transplantations in horses (Broeckx et al., 2014a; Broeckx et al., 2014b; Broeckx et al., 2014c; Carrade et al., 2011a; Carrade et al., 2011b; Guest et al., 2008). Since both humans and horses are the only mammals reported to develop excessive granulation tissue during natural wound healing (i.e. keloid versus hypergranulation tissue), the horse can be considered as a robust animal model for evaluating novel therapies for human applications (Theoret et al., 2013).

The aim of this study was to comparatively evaluate autologous and allogenic EpSC treatment in experimentally induced wounds in horses. Both macroscopic and histologic evaluation of relevant regenerative parameters at different time points were performed.

6.2 Materials and methods

Experimental horses and isolation of skin-derived epithelial-like stem cells

The experimental procedure was approved by the ethical committee of Global Stem cell Technology (EC_2012_002, EC_2013_003 and EC_2014_001) and the Faculty of Veterinary Medicine, Ghent University (EC_2014_020). Six healthy French trotter mares aged between 5 and 7 years and in good general health condition were included in this study. Twelve weeks before the start of the experimental wound model, a 1 cm^2 skin sample was retrieved from the neck region of each horse for isolation of epithelial-like stem cells (EpSC) as previously described (Borena et al., 2014). In the laboratory, the samples were processed and stem cells were characterized as previously reported (Borena et al., 2014; Broeckx et al., 2014d).

Equine cutaneous wound model

The horses were sedated with Detomidine (0.04 mg/kg IV; Medesedan®), and pain relief was established by administering Butorphanol (0.1 mg/kg IV; Dolorex®). Thereafter, a local subcutaneous anesthesia was administered with Procaine 4% plus adrenalin. In each horse, twelve full-thickness square shaped wounds (four groups of three wounds) were created in the

gluteus region. The wounds measured 2 cm by 3 cm and were at least 2 cm apart from each other (Figure 6.1). The day on which the wounds were created was designated as day 0 (T0). The removed skin was fixed in 10% buffered formalin and stored for subsequent histopathologic analysis.

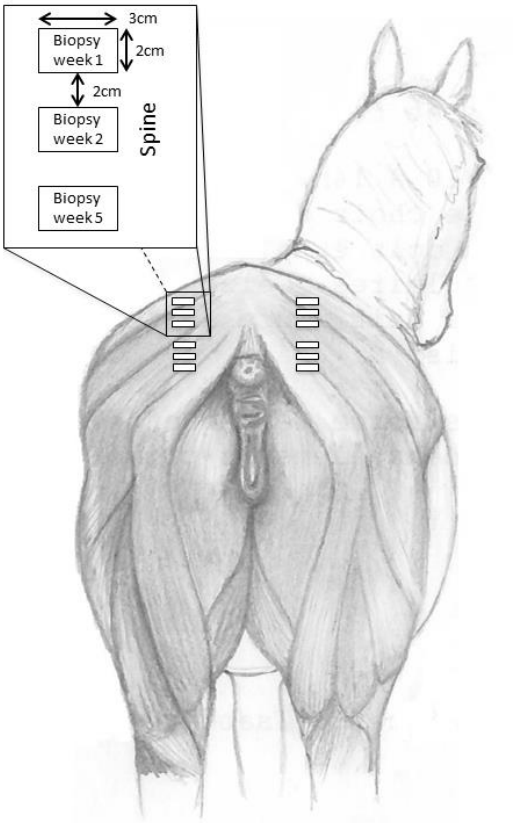

Figure 6.1 The dorsal area of the gluteus region was surgically prepared to induce 12 wounds per horse to cover 4 treatment groups (☐) which were biopsied at 3 time points: week 1, 2 and 5.

Topical and subcutaneous epithelial-like stem cell treatment and control groups

Each group of three wounds was randomly assigned to one of the four treatments: (i) autologous EpSC treatment, (ii) allogenic EpSC treatment of a randomly chosen donor within the same group of horses (three recipients received EpSC from one and the same donor and the other three from one other donor), (iii) DMEM as a vehicle control and (iv) untreated controls. Twenty minutes after wound induction, when hemostasis was obtained, $4x10^6$ cells (in 2 ml DMEM) were injected subcutaneously in the wound margins and $4x10^6$ cells (in 1 ml

DMEM) were applied topically in the wound for the autologous and allogenic treatment. Two out of the six horses received half the doses in both the autologous and allogenic treated group, because the obtained number of cells for autologous treatment in these horses was insufficient. In the vehicle control wounds 2 ml DMEM was injected subcutaneously and 1 ml DMEM was applied topically. The remaining group of wounds was left untreated to monitor the normal healing process. Fresh wounds were covered with a sterile plastic foil (Oper Film©), which was replaced after the second topical application of EpSC in the stem cell treated or DMEM in the vehicle treated wounds at day 1. From day 3 onwards, a local non-adherent dressing (Zorbopad) was used to cover the wounds and was changed at each macroscopic evaluation until day 16, after which no more wound protection was used. The horses did not receive any other medication and their general condition and wound covers were monitored daily.

Macroscopic wound evaluation

At days 0, 1, 3, 7, 14, 21, 28 and 35 a macroscopic evaluation and measurement of the wound dimensions were performed for those wounds that were not biopsied. Before each macroscopic evaluation, crusts (if present) were carefully removed and the adjacent skin was cleansed with a sterile 0.9% saline solution, in order to enhance visibility of the wound boundaries. Next, a subjective macroscopic evaluation of the wounds for presence of (exuberant) granulation tissue and signs of infection was performed. Subsequently, the wound dimensions were measured with a laser beam camera (SilhouetteStar, ARANZ Medical Ltd., Christchurch, New Zealand). The camera was held perpendicular to the wound surface, with the three laser lines projected by the camera crossing in a star shape and the ends of the lines lying on the adjacent skin. The central focus point of the star was positioned at or near the centre of the wound. Image analysis with the accompanying software (SilhouetteConnect, ARANZ Medical Ltd., Christchurch, New Zealand) was then used to calculate the circumference, surface, maximum depth and volume of the wounds. All measurements were made by the same assessor.

Skin biopsy and histological analysis

At 1, 2 and 5 weeks after treatment, the horses were sedated with a combination of Detomidine (0.04 mg/kg IV; Medesedan®), and Butorphanol (0.1 mg/kg IV; Dolorex®), and a local subcutaneous anesthesia was administered with Procaine 4% without adrenaline. One

wound per group was sacrificed. For each wound three punch biopsies were taken: one central (8mm) and two at the wound edges (8 and 3mm). The 3mm tissue samples were liquid frozen for future PCR analysis. The 8mm tissue samples were fixed in neutral buffered 10% formalin, embedded in paraffin, sectioned at 4 µm thickness and stained with Hematoxylin & Eosin according to standard protocols. All samples were blindly analyzed by an ECVP certified pathologist (SM) using a modified scoring system, as previously reported (Broeckx et al., 2014d).

Briefly, the epidermis was scored for thickness, crust formation, dermo-epidermal separation and completeness of re-epithelization. The dermis was evaluated using following parameters: amount and morphology of dermal stroma, edema, thickness and morphology of the total granulation tissue and young granulation tissue and relative difference with the original thickness (= total granulation tissue/ dermal granulation thickness of removed intact skin x 100). The skin removed when creating the wounds at T0 was used as positive control sample to compare the aforementioned parameters wherever applicable. All measurements were performed with a computer-based program (LAS V4.1, Leica Microsystems).

Statistical analysis

The statistical analysis for continuous measurements was based on the mixed model with horse as random effect and time, treatment and their interaction as categorical fixed effects. Histological scores were analyzed based on a mixed logistic regression model with horse as random effect and treatment as categorical fixed effect. SAS Version 9.3 was used for the analyses (SAS/STAT Software, Version 9.3, SAS Institute Inc.). A global significance level of 5% was used and Tukey's adjustment technique was used for multiple comparisons with adjusted P-values reported.

6.3 Results

Harvesting of skin-derived epithelial-like stem cell

Skin-derived cells were able to form spheres, multiply in adhesion and possessed all properties to be immunophenotyped as EpSC as previously described (Borena et al., 2014; Broeckx et al., 2014d).

Wound induction model

In 5 out of 6 horses wounds could be induced in a standing position as reported in the materials and methods section (Figure 6.1). For safety reasons, one horse had to be positioned in lateral decubitus and the topical treatments were compromised. This horse was therefore excluded from the study. Daily inspection of all horses showed no signs of either clinical discomfort or fever.

Macroscopic analyses of the wound healing process

All measured macroscopic wound healing parameters significantly decreased over time ($P < 0.0001$). Significant differences between treatments were observed for wound circumference ($P < 0.0001$), surface ($P = 0.0283$) and depth ($P = 0.0100$). The circumference of autologous EpSC-treated wounds was significantly smaller in comparison to vehicle treatment ($P = 0.0060$) and untreated controls ($P < 0.0001$, Figure 6.2A). Compared to untreated controls, allogenic EpSC-treated wounds also displayed a smaller but not significantly different ($P = 0.0549$) circumference. Also a significantly smaller surface was noticed in autologous EpSC-treated wounds in comparison to vehicle treatment ($P = 0.0284$, Figure 6.2B). The untreated control wounds were deeper than autologous ($P = 0.0720$) and allogenic ($P = 0.0919$) EpSC-treated wounds, however, statistical significance was only observed in comparison with vehicle treatment ($P = 0.0077$, Figure 6.2C). No significant treatment effect ($P = 0.3198$) could be observed for the volume (Figure 6.2D). Although in only 2 of the autologous (n=18) and 1 of the allogenic EpSC-treated (n=18) wounds hypergranulation tissue was noticed in comparison to 6 in vehicle treated group (n=18) and 4 in the untreated control group (n=18), no significant difference ($P = 0.2035$) could be demonstrated between the different treatment groups.

Histological analysis of the skin biopsies

No significant differences in the scores for epidermal thickness, crust formation, dermo-epidermal separation and completeness of re-epithelization could be observed between the 4 treatment groups. Epithelialization was completed in almost all wounds at 5 weeks after wound induction.

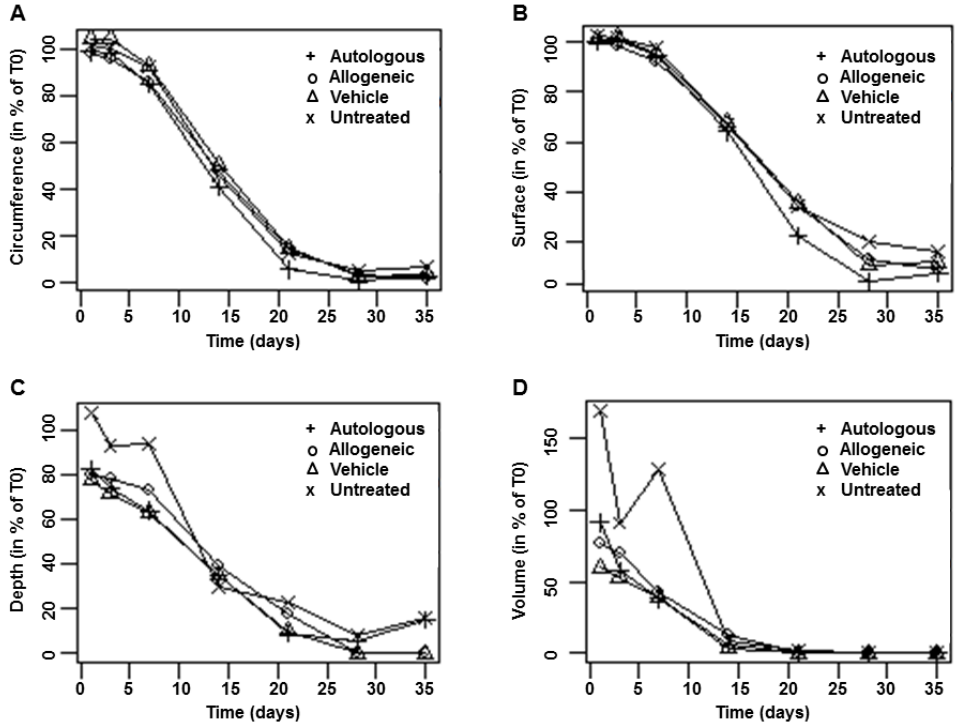

Figure 6.2. A laser camera measured the reduction in wound circumference (A), surface (B), depth (C) and volume (D) of all wounds over time (in days) in comparison to day 0.

Also for the parameters edema, collagen amount and morphology, fibroblast maturation and granulation tissue morphology, no considerable differences could be noticed between the 4 treatment groups. In contrast, total granulation thickness significantly increased over time ($P < 0.0001$) with a significant treatment effect ($P = 0.0047$). A significantly lower amount of total granulation tissue was observed in the autologous (4000 µm, $P = 0.0077$) and allogenic EpSC-treated wounds (4147 µm, $P = 0.0199$) compared to the vehicle treated group (5404 µm) but not compared to the untreated control (4843µm). There was also a significant treatment effect ($P = 0.0105$) on the formation of young granulation tissue, yet without a significant difference over time ($P = 0.1269$). Significantly less young granulation tissue was found in the autologous (1594µm, $P = 0.0121$) and allogenic (1746µm, $P = 0.0371$) EpSC-treated wounds in comparison with the vehicle treated group (2729µm) but not in comparison with the untreated control (2190µm). Comparing the formed granulation tissue thickness with the original dermal thickness, a significant treatment effect ($P = 0.0189$) and time effect ($P < 0.0001$) was observed. The autologous (86.5%, $P = 0.0171$) but not the allogenic (92.2%, P =

0.0662) EpSC-treated wounds were significantly closer to the original normal skin thickness as compared to the vehicle treated wounds (118.5%) whereas no significant differences were found for the untreated control (101.8%). Five weeks after wound induction, the untreated wounds scored considerably better than the vehicle treated wounds and approached the allogenic treatment in terms of total granulation tissue formation and difference to original thickness.

6.4 Discussion

To date, several independent studies in different animal species reported beneficial effects of autologous and allogenic bone marrow-derived (stem) cell treatments on skin wound healing (Akela et al., 2012a; Borena et al., 2010; Jain et al., 2011b; Kim et al., 2013a). However, so far only one study described the clinical use of lineage committed skin-derived stem cells in an allogenic set-up in one patient (Broeckx et al., 2014d). The goal of this study was therefore to explore this treatment further in a large group of horses and compare wound treatment with allogenic EpSC's to autologous EpSC's, vehicle treatment and a negative control. Relevant macroscopic and histologic parameters were evaluated at different time points after wound induction.

Standardized wound size measurements revealed a significant decrease in circumference, surface, depth and volume of all wounds over time for all treatment groups. Because the time effect was more pronounced than the treatment effect, the graphs of different treatments were closely approaching (Figure 6.2). Nevertheless, a statistically significant treatment effect could be noticed for wound circumference, surface and depth. In our hands, autologous EpSC-treated wounds displayed the smallest circumference and surface and allogenic EpSC did not significantly enhance any macroscopic wound healing parameter in comparison to both control groups. The largest and deepest wounds were found in the untreated controls. Although not significant, less formation of hypergranulation tissue was macroscopically noticed in both EpSC-treated groups compared to vehicle treatment. The non-significant difference ($P = 0.7514$) between allogenic EpSC-treated and vehicle treated wounds for all evaluated macroscopic parameters is in contrast with a previous equine study where a statistically significant improvement in manually measured wound circumference (referred to as "wound filling") was noticed after treatment with allogenic EpSC in combination with platelet-rich plasma (PRP) compared to PRP treatment alone (Broeckx et al., 2014d). In this

regard, it might be postulated that PRP addition would have stimulated the pro-angiogenic effect of EpSC after platelet degranulation, as reported in epithelial cancer cell lines (Egan et al., 2011). Although others also observed macroscopic wound healing enhancement after both autologous (Jain et al., 2011b; Spaas et al., 2013a) and allogenic cell treatments (Auxenfans et al., 2014; Leigh et al., 1987), it has to be mentioned that the latter studies used visual clinical assessment, whereas our results are based on a more objective device, a 3D-laser camera. Nevertheless, further research is warranted to fully unravel the efficacy of allogenic cell therapies as a regenerative strategy for enhancing wound healing.

In our study, total granulation thickness significantly increased over time ($P < 0.0001$), whereas young granulation tissue did not significantly alter over time ($P = 0.4226$). In addition, an overall significant decrease in total granulation thickness (more than 1000 µm) could be noticed in both EpSC treatment groups in comparison to control groups. This is in accordance with the histological data of a previous equine study where allogenic EpSC/PRP-treated wounds contained approximately 1300 µm less granulation tissue than PRP-treated wounds at 30 days after treatment (Broeckx et al., 2014d). Moreover, this study demonstrated a significant overall decrease of young granulation tissue in both allogenic and autologous treated wounds compared to vehicle treated wounds. Furthermore, the dermal thickness was considerably closer to the intact skin after autologous ($P = 0.0144$) and allogenic EpSC treatment ($P = 0.0501$) compared to the vehicle treatment. Since horses easily develop excessive granulation tissue during natural wound healing (hypergranulation tissue) (Theoret et al., 2013), the reduction in young granulation tissue and enhanced dermis normalization are promising findings. However, in our study, the untreated wounds scored considerably better than the vehicle treated wounds and approached the allogenic treatment concerning the total granulation tissue and comparison to original dermal thickness at 5 weeks after wound induction. Whether EpSC counteract possible negative effects caused by vehicle treatment remains to be proven.

Two horses in the present study received a dose of 0.67×10^6 EpSC/cm² (=4×10^6/6cm² total wound surface), which was half the dose of the other horses (1.33×10^6 EpSC/cm²). One of the low-dose horses was omitted from the study due to peroperative complications. Therefore, a comparison of the clinical efficacy of a low or high dose is hardly possible. This is in agreement with a previous study in dogs where no significant differences were noticed in wound healing parameters after using doses ranging from 0.01 to 1×10^6 MSC/0.28cm² and the

cell number also decreased at day 14 (Kim et al., 2013a). In contrast, a human study reported that at least $1x10^6$ MSC/cm² were needed in order to obtain the required therapeutic effect in diabetic foot ulcer healing (Falanga, 2005a). This might be explained by the fact that diabetic microangiopathy might cause a lower response to pro-angiogenic effects of EpSC, which has been reported to be one of the healing mechanisms induced by EpSC (Broeckx et al., 2014d). Nevertheless, all the aforementioned strongly suggest that stem cells exert a paracrine effect, as reported by others (Wu et al., 2010a), and their *modus operandi* is probably not affected by the dose of cells above a certain minimum. Comparable to the observed effect of EpSC in our study, the use of growth factors has also demonstrated to increase vascularization and several other wound healing parameters in mouse models (Chen et al., 2008a; Galiano et al., 2004). Whether growth factors are an equal substitute for stem cells, remains to be determined.

Conclusion

In conclusion, these results suggest that wounds treated with autologous EpSC heal significantly faster, but the observed differences in wound healing parameters were rather small, indicating a limited clinical relevance in the used model.

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CHAPTER 7

General Discussion

7.1 Overview

The present thesis describes the isolation of bone marrow-derived nuclear cells (BMNC) in rabbits and skin-derived epithelial-like stem cells (EpSC) in horses. In adult mammals, BMNC can be obtained from bone marrow (BM) aspirate while EpSC are obtained from skin biopsies. Both isolates contain adult stem cells, which preferentially generate differentiated cells of the same lineage as the tissue of origin, but also have the capacity to transdifferentiate into cell types different from their lineage due to their plasticity (Jopling et al., 2011; Sasaki et al., 2008; Toma et al., 2001). Although BMNC are derived from the mesodermal germ layer (Woodbury et al., 2002), it has been demonstrated that besides the resident skin cells, BMNC also contribute to skin healing in physiological and pathological conditions (Higashiyama et al., 2011). Since BM contains hematopoietic (HSC) and mesenchymal stem cells (MSC), long term reconstitution of the healed dermis and collagen type I and III production could be supported by both stem cell populations (Fathke et al., 2004). Moreover, BMNC such as MSC have the ability to regulate the immune response and inflammation and possess powerful tissue protective and reparative mechanisms (Abbas and Mahalingam, 2009; Chen et al., 2008; Kim et al., 2013). On the other hand, skin-derived EpSC originate from the ectodermal germ layer and are physiologically designed to regenerate local skin defects (Grapin-Botton and Melton, 2000). For all these reasons, both cell types possess a potential for enhancing wound regeneration in mammals. Different aspects of the use of these two cell types in stem cell based therapy are discussed in the following subsections.

7.2 In vitro studies

7.2.1 BMNC harvesting

BM cells have demonstrated the broadest differentiation potential among adult somatic cell populations and are therefore one of the most promising cell sources for clinical applications (Krause et al., 2001; Poulsom et al., 2002). BM has been used by different researchers either as a source of autologous BMNC or BM-MSC after isolation and characterization. The contribution of autologous BMNC to cutaneous wound healing in experimental wounds in rabbit (Akela et al., 2012; Akela et al., 2013) and clinical cases particularly for chronic wounds in human have been reported in many studies (Badiavas and Falanga, 2003; Dabiri et al., 2013; Jain et al., 2011). Although the BM-derived cells described in the present thesis

have not been further characterized, quantitative and qualitative enhanced healing effects could be demonstrated after clinical application in skin wounds in rabbits.

There are different techniques for concentrating and/or isolating MSC from BM aspirate. Separation methods that include immune-magnetic beads or flow cytometry generate BM-MSC with higher purity, however, the expense, procedural complications and cell damages that occur during cell processing restrict their usage (Zhang et al., 2014). Routinely, the mononuclear cell (MNC) fraction containing MSC is being isolated using a Ficoll density gradient separation method (Beeres et al., 2007; Van Tongeren et al., 2008). After isolation, the MSC can be multiplied to obtain high cell numbers, but this comes at a high cost. Omission of such expansion step would dramatically lower the cost and facilitate the use of MSC for clinical cases where such facilities are not available (Dozza et al., 2014). Moreover, it is also difficult for clinicians to utilize cultured BM-MSC for the purpose of wound healing due to issues regarding the health authorities that consider the product as an advanced therapy medicinal product (ATMP) which requires cleanroom circumstances and good manufacturing production (GMP) guidelines from the moment cell culture techniques are involved. Moreover, human ATMP require authorization by the European Medicines Agency (EMA) for clinical application in Europe or Food and Drug Administration (FDA) covering the USA. In addition, rigorous and expensive quality tests are a requirement from both agencies before batches of cells are allowed to be released for clinical application. On the other hand, uncultered BM aspirates, such as the BMNC fraction as reported in the present thesis, are considered as a one-step surgical procedure and do not require the aforementioned tests. This has a substantial implication on the costs, feasibility and time frame in which the product is being prepared. On the other hand, BM aspiration requires a certain expertise and uncultured cells are less uniform than cultured MSC produced under GMP conditions. Further research should definitely focus on the comparison between cultured or purified cells and unpurified BMNC or BM-MNC and demonstrate is superior for which applications.

7.2.2 EpSC harvesting

For our research, EpSC were isolated from 1 cm^2 full thickness skin biopsies collected from 6 horses. Mechanical deep scraping of the epidermis followed by enzymatic digestion and serial centrifugations in combination with filter processes were employed to harvest epithelial cells from the equine skin. An average of 0.4 x 10^6 cells were obtained after this procedure, yet

with a standard deviation of 0.2 x 10^6 cells, indicating a large variation in initial cell number. This might influence the efficacy of the purification protocol and should be further investigated in future studies. The cells were subsequently cultured in suspension on ultralow attachment plates for 7 days to induce primary sphere formation. Sphere formation was successful in all the samples with only two medium changes. So far, the isolation of sphere forming stem cells have been successfully performed from bulk skin tissue cultured on adherent plates (Toma et al., 2001) or from epidermal-derived cells seeded at a clonal density on ultralow attachment plates (Broeckx et al., 2014). In the latter experiment, 1 cm^2 full thickness skin biopsies were used and EpSC were harvested by peeling the epidermis from the dermis followed by enzymatic digestion. Both studies reported no impurities in their cultures, so the fact that more material was used at the start in the present thesis might have been at the origin of the impurity in one culture. Nevertheless, more protracted studies should confirm this hypothesis.

It has been found that stem cells in mammalian skin are firmly adherent to the basal lamina and account for 10% of the cells in the basal layer at the dermo-epidermal junction (Blanpain et al., 2007; Papini et al., 2003). Thus, separation of this skin layer from the underlying dermis might avoid contamination by a large number of unwanted non-epithelial cells. Therefore, manual detachment of the epidermis from the dermis by using a sterile forceps (Ruetze et al., 2013), peeling (Broeckx et al., 2014) or deep scraping technique (Borena et al., 2014) followed by enzymatic digestion could be other alternatives for isolating EpSC. There has been an assumption that ultralow attachment culture conditions purify EpSC, since only these cells have the ability to clonally expand in suspension (a stem cell-specific property) (Ruetze et al., 2013), thereby substantially reducing the survival of differentiated cell clusters. However, in the present thesis it has been demonstrated that if impurities initially exist, they can still survive and proliferate within the clonally expanding EpSC seeded at high density of 100 cells/cm^2 or more. In this regard, the present thesis reports that a seeding density of 10 cells/cm^2 or less on ultralow attachment plates is necessary to purify cellular impurities in EpSC cultures after harvesting equine skin cells. Nevertheless, further studies are required to demonstrate whether this technique is suitable for stem cell purification of other origins and sources and whether 10 cells/cm^2 is sufficient in those cases as well.

Even though the existence of adult stem cells has been demonstrated in many tissues, their unexpected plasticity and potential in cell based therapy have created a lot of excitement

(Weissman, 2000). Nevertheless, the functional and phenotypic characterization of these cells is not completely addressed to date. Failure to characterize the isolated cells might result in contamination with other cell lines which might cause unexpected or even unsatisfactory results in stem cells studies. In contrast with MSC, where the International Society for Cellular Therapy (Dominici et al., 2006) have set clear criteria, no guidelines and minimal standards have been set for equine EpSC so far, which makes the comparison of different studies difficult. Therefore, this thesis focused on expanding the existing knowledge of functional characterization of EpSC through sphere formation in different culture circumstances and seeding at ultralow clonal densities on ultralow attachment plates.

Functional characterization

Different methods have been investigated to study the fate, self-renewal and differentiation potential of EpSC (Blanpain and Fuchs, 2014). The first functional demonstration of EpSC was made when methods were identified to culture human epidermal keratinocytes under conditions where they could be maintained and propagated for hundreds of generations without losing stem cell properties (Rheinwald and Green, 1975). Besides immunophenotypic properties, the following functional characteristics of the isolated cells were assessed in the present thesis: (i) proliferation potential, (ii) self-renewal rate as spheres and (iii) differentiation capacity into keratinocytes.

(i) Proliferation potential

Population doubling time (PDT) experiments were performed to determine the cell proliferation potential and rate of EpSC under adherent culture conditions (Eslaminejad et al., 2010) using 6 samples from different horses. Adherent EpSC from all 6 samples were found to have the capacity to proliferate consistently over the 10 passages studied as indicated by PDT values that varied between 0.89 and 1.37 days. The overall average PDT was 1.14 ± 0.16 and there were no considerable differences in the PDT at different passages. Indeed, the PDT remained relatively constant with passaging, indicating a stable proliferation capacity of the cells in culture over time. This is in agreement with a previous study where the PDT varied between 0.78 and 1.16 for multiple passages of 3 skin samples from the same species (Broeckx et al., 2014). Also for equine peripheral blood-derived MSC (PB-MSC) a rather consistent PDT has been reported (Spaas et al., 2013). This is in contrast with another report concerning equine PB-MSC where an increased PDT (in hours, hr) could be noticed with

increasing passage number: P1 ~ 30.57 ± 1.3; P4~ 40.37 ± 2.5 and P8~ 47.35 ± 4.3 (Martinello et al., 2010). Other studies considering equine MSC also recorded a similar PDT (days) as reported in the present thesis of 1.24 (± 0.28) for umbilical cord blood-derived MSC (UCB-MSC) and 1.64 (± 0.69) for umbilical cord matrix-derived MSC (UCM-MSC) (De Schauwer et al., 2014). In general, the PDT of MSC from different sources was slightly higher than the PDT of EpSC as described in this thesis and reported by Broeckx (Broeckx et al., 2014). Nevertheless, the PDT of EpSC was considerably higher than reported for rbBM-MSC (6.4 ± 1.3 hr) and hBM-MSC (7.6 ± 1.7 hr) (Tan et al., 2013). Since the latter PDTs relate to rabbit and human MSC, a difference in animal species is expected to be at the origin of the noticed discrepancy.

It has to be mentioned though, that a study in humans demonstrated that the doubling time of cultivated hBM-MSC was age dependent (Altaner et al., 2013). The hBM-MSC from old donors had an average doubling time of 56.3 hr, which was considerably higher than their younger counterparts (48.7 hr). In addition, there was a great individual variation in the values of cell doubling time, reflecting an influence of other physiological parameters, such as life style and co-morbidity (Altaner et al., 2013). From these findings it can be concluded that more research is warranted before any conclusion can be drawn concerning the causes of a difference in PDT and whether this is related to the stem cell type or not. Nevertheless, the consistent and rather rapid proliferation of the EpSC in the present thesis corresponds with one of the major criteria of stem cells, being a high proliferative potential (Roh and Lyle, 2006).

(ii) Sphere cell-renewal rate

Sphere forming assays are being used in stem cell biology for assessing the self-renewal and differentiation capacity at a single cell level (Pastrana et al., 2011). Stem cells from diverse tissues are typically cultured under adherent conditions, but can also be maintained as spheres under non-adherent conditions (Pastrana et al., 2011). In the present thesis, sphere formation capacity was used as a tool for isolating, enriching and purifying EpSC from equine skin biopsies. Sphere formation was successful from all 6 biopsies and an average of 0.53% of the ± $0.4x10^6$ seeded cells were able to form a sphere (sphere forming efficiency or SFE). The SFE recorded in this thesis was in agreement with the 0.4% reported for human

mammospheres (Dontu et al., 2003) and a bit lower than the 0.8 - 3.2% (depending on the lactation stage) described in equine mammospheres (Spaas et al., 2012).

The sphere formation assay has been proposed as a functional assay for uniquely detecting stem cells *in vivo* and as a valuable *in vitro* assay to study stem cells during different physiological phases (Spaas et al., 2012). However, spheres do not only consist of stem cells, but also of a mixture of progenitor cells, their progeny and non-stem progenitor cells (Broeckx et al., 2014; Pastrana et al., 2011; Stingl, 2009). In line with this finding, an assessment of the sphere formation capacity and subsequent growth of pure EpSC in maintenance versus differentiation medium in the present thesis resulted in significantly less number of spheres during differentiation. However, the sphere formation assay still evaluates the potential of cells to behave as stem cells when removed from their original *in vivo* niche (Pastrana et al., 2011). Moreover, the subsequent generation of spheres in the present thesis, demonstrated a relatively constant SFE, a strong correlation between the number of cell units/field and spheres/field and an increase in the number of cells/sphere over time. The aforementioned properties clearly confirm self-renewal and clonal expansion capacities of the isolated cells which are main functional characteristics of stem cells (Blanpain et al., 2007; Moore and Lemischka, 2006; Nadig, 2009).

Contamination or impurity of cell culture is a common problem and sometimes ends up with serious consequences (Stacey, 2011). Contaminations in the cell culture can be classified as chemical contaminants and biological contaminants such as bacteria, mycoplasma yeast, molds, viruses and contamination by other cell lines (Lincoln and Gabridge, 1998). Cell line contamination is usually difficult to detect and a potentially more serious culture problem than biological contamination (Fogh et al., 1971). Cellular impurities may result in invalid research outcomes (Stacey, 2000). Therefore, regular checking of cellular morphology and growth characteristics during subsequent passages and comparison with the reference cell/sample might assist in early detection of impurities (Dirks et al., 2010).

(iii) Keratinocyte differentiation capacity

There have been several studies demonstrating that skin adult stem cells show a surprisingly diverse differentiation repertoire (Toma et al., 2005). Differentiation of rodent skin derived precursors (SKP) into both neural and mesodermal cell types, including neurons, glia, smooth muscle cells, and adipocytes has been reported (Toma et al., 2001). Furthermore, human scar

tissue-derived stem cells (hSTSC) differentiated into neurogenic progenitor cells (Yang et al., 2010) and bilineage differentiation of equine EpSC towards two major skin cell types, keratinocytes and adipocytes, has been demonstrated (Broeckx et al., 2014). In our experiment we have successfully differentiated equine EpSC into keratinocytes in adherent culture conditions. Although IHC was performed more rapidly (7 days) in the present thesis than in the report by Broeckx et al. (10 days), this does not allow to conclude that the differentiation process was completed earlier in our study. Differentiated cells (i.e. keratinocytes) were detected visually based on their cobblestone-like shaped morphology and IHC confirmed that the cells became positive for Pan cytokeratin (CK), Wide CK, CK14 and Casein kinase 2β. It should be mentioned though, that the typical keratinocyte morphology was not reached yet after 7 days of differentiation, whereas this was the case after 15 days of differentiation (data not shown). Based on both criteria, it has been concluded that induction towards keratinocytes was successful. Further research should focus on differentiating EpSC towards multiple cell types. In order to assess the plasticity of the EpSC, differentiation towards cell types of another germ layer would be an interesting addition to the currently available data. I*n vitro* differentiation capacity towards keratinocytes does not implicate, however, in vivo keratinocyte reconstitution. Further studies should confirm whether or not EpSC have the capacity to actually regenerate functional keratinocytes *in vivo*.

Immunophenotypic characterization

It has been suggested that phenotypic characterization using stem cell markers will provide novel insights to better understand stem cell biology and behavior (Abbas and Mahalingam, 2009). Therefore, in addition to uncovering EpSC from equine skin based on their proliferation and differentiation potential, the expression of several markers has been evaluated to enhance the phenotypic characterization of this cell type. There are specific products, such as antibodies and substrates that assist the immunophenotypic characterization of stem cells making use of different methods such as flow cytometry and IHC staining (Tan et al., 2013). Even though different markers are available for stem cell localization in mammalian skin, such experiments are difficult to interpret because these markers lack cellular specificity. In this regard, stem cell factor (SCF) has been reported as a marker for skin stem cells, although it is also expressed by mature melanocytes (Takahashi et al., 1995). Also CK19 (Li et al., 2012) and ß-catenin are expressed in skin stem cells (Ridgway et al., 2012), however, both are also expressed in adult epidermal cells (Zhao et al., 2012). More

recently purified cells from epidermis were characterized as EpSC by means of positive expression for a set of markers: CD29, CD44, CD49f, CD90, Casein Kinase 2β, p63, and Ki67 (Broeckx et al., 2014). In addition, a low expression for cytokeratin (CK)14 and negative expression for CD105, CK18, Wide CK, and Pan CK was previously reported (Broeckx et al., 2014). In our study, the isolated EpSC were positive for CK 14 and Casein Kinase 2β and negative for Pan CK and Wide CK. Flow cytometric analysis on the other hand, has shown that both EpSC and the differentiated cells expressed high levels of MHC-I but low levels of MHC-II. Except for MHC-I, all the results of the IHC and flow cytometric analysis in this study were in agreement with the previous studies by Broeckx et al. (2014). Further research should determine the cause of different MHC-I expression and its clinical implication. Stem cells are functionally defined by their ability to self-renew and differentiate into the cell lineages of their tissue of origin (Abbas and Mahalingam, 2009; Moore and Lemischka, 2006). Therefore, it can be concluded that the isolated cells showed the main characteristics of stem cells. Absence of minimum set criterion to define EpSC like that of hMSC, makes comparison between different reports more difficult. Nevertheless, also in the present thesis no single protein could be identified as the ideal "EpSC marker".. Future studies should therefore focus on analyzing specific markers for the identification and purification of EpSC.

7.3 *In vivo* studies

In vivo evaluation of the wound healing effects of BMNC and EpSC on full thickness skin wounds in rabbits and horses, respectively, were performed in the experiments described in Chapters 4, 5 and 6.

Macroscopic evaluation of the wounds treated by BMNC or EpSC was performed in our study by adjusting wound evaluation protocols developed by other researchers (Abramov et al., 2007; Babaeijandaghi et al., 2010; Bigbie et al., 1991). For subjective evaluation in the clinical BMNC studies, the analysis was based on the average score of a pathologist who was blinded to the treatment assignment. In the present thesis, rabbit full thickness excisional wounds treated by injecting autologous BMNC into wound margins (BI) showed earlier appearance of granulation tissue and significantly more contraction than those wounds treated by topical application of autologous BMNC (BT) and control groups (wound dressed by normal saline solution: NS and wound dressed by povidone iodine solution: PI). Even though

injection of autologous BMNC into the wound margin was superior than the topical application in the overall wound healing effect, both routes showed beneficial and promising wound healing potential for treating full thickness skin excisional wounds in rabbits. On the other hand, equine full thickness excisional skin wounds treated with autologous EpSC also showed significant improvement compared to both control groups for different macroscopic parameters, which was not the case for allogenic EpSC.

Whether the macroscopic improvement after autologous EpSC treatment has any clinical relevance, remains to be demonstrated. In addition, the inferior macroscopic improvements of allogenic EpSC deserve more attention. Indeed, several aspects might be at the basis of the noticed discrepancy. First of all, an enhanced cellular immune response or graft rejection might have counteracted beneficial effects of EpSC. Secondly, one might postulate that donor EpSC could have been of inferior quality in comparison to the autologous EpSC. However, in the present thesis the same donor horses were used to harvest both autologous and allogenic EpSC. A third hypothesis might be that paracrine effects of autologous cells are not/less inhibited, and therefore, might influence local leukocytes and even fibroblasts and/or myofibroblasts in order to enhance the healing process. Probably the first and third hypotheses were at the basis of the noticed difference between both treatments, yet further analyses of the biopsies might clarify certain issues. More research is also warranted to provide new answers concerning the plasticity of skin-derived EpSC and the regenerative pathways they use in order to assist wound healing.

Healing of cavity wounds such as full thickness excisional wounds can be monitored by measuring the circumference of the wound, which is directly related to both volume and area (Flanagan, 2003). In previous studies, improved macroscopic wound healing using whole BM aspirate (Lee et al., 2008; Nishimoto et al., 2013; Nishimoto et al., 2009), BM derived cells (Badiavas and Falanga, 2003; Jain et al., 2011), BM-MSC (Akela et al., 2012; Akela et al., 2013; Chen et al., 2008; Kim et al., 2013; Wu et al., 2007b) and EpSC (Broeckx et al., 2014) have been reported. Most of the macroscopic findings in this section were also supported and explained by the histological findings detailed below. The discrepancy between treated and untreated wounds can be attributed to the above reported (trans)differentiation capacity of stem cells, however, releasing paracrine factors such as growth factors and cytokines might also be at the basis of the noticed clinical effects, as demonstrated by several researchers (Kim et al., 2013; Maxson et al., 2012; Wu et al., 2010).

Histomorphologically, the autologous BM treated wounds of rabbits showed an earlier disappearance of inflammatory reaction, better epithelialization, significantly more neovascularization and more fibroplasia than the control wounds. In addition, the BM treatment also resulted in earlier lay down and histological maturation of collagen. Several studies indicate that BM contains stem cells with a potential for differentiation into a variety of tissues including skin (Huttmann et al., 2003; Jones and McGonagle, 2008; Kang et al., 2013). Therefore, it may be plausible that the wound healing effect of BMNC in this thesis is due to its progenitor and/or stem cell content. Nevertheless, further studies should confirm whether or not these cells actually integrate and regenerate the damaged tissue.

Inflammation is essential to establish cutaneous homeostasis following injury and to recruit inflammatory cells, which act as a source of cytokines that affect the wound healing process (Singer and Clark, 1999; Theoret, 2009). However, massive or prolonged inflammation can delay wound healing, thus local suppression of the inflammatory process is required for the wound healing to proceed. The treatment of canine wounds with BM-MSC resulted in the reduction of pro-inflammatory cytokines (interleukin-2 and interferon-γ) from which it has been concluded that MSC exhibit paracrine effects by decreasing inflammation post-injury (Kim et al., 2013). In addition to attenuating inflammation in the wound site, BM-MSC are also involved in reprogramming the resident immune and wound healing cells to favor tissue regeneration and inhibit fibrotic tissue formation. As a result, these cells have been considered and tested as likely candidates for a cellular therapy to promote scarless wound healing (Jackson et al., 2012). Therefore, the earlier disappearance of inflammatory reaction in the BI and BT group observed in our studies can be attributed to this anti-inflammatory effect of BM-MSC that were present within the cell mixture. The disappearance of inflammatory reaction will assist wound progression to the next phase of the healing process, which is the proliferative stage (Broughton et al., 2006; Janis and Harrison, 2014).

Autologous cell therapy (ACT) is a novel therapeutic intervention which minimizes the risk of systemic immunological reactions (bio-incompatibility) and of disease transmission associated with grafts or cells not cultivated from the individual (Kazmi et al., 2009). However it has been found that adult MSC also possess a remarkably diverse array of immunosuppressive characteristics and could thus also suppress an allogenic rejection (English and Mahon, 2011; Fu and Li, 2009). In vitro studies in the present thesis showed that EpSC expressed a very low level of major histocompatibility complex-II (MHC-II), which is

involved in graft rejection (Natarajan et al., 2002). In this regard, it has been reported that allogenic MSC, which were also negative for MHC II did not induce an immune response after intravenous and intradermal injections in horses (Broeckx et al., 2013; Carrade et al., 2011). Allogenic MSC have also been used to treat graft-versus-host rejection diseases in humans (Le Blanc et al., 2008; Ringden et al., 2006). Therefore, absence of any adverse effects after allogenic EpSC administration in the present thesis could have been due to low level expression of MHC-II or other immunomodulative effects. Nevertheless, the allogenic treatment groups demonstrated lower macroscopic and histologic therapeutic effects. Concerning the difference between autologous and allogenic EpSC, more studies are definitely warranted. In this regard, it has been reported that no differences between autologous and allogeneic umbilical cord tissue-derived MSC were noticed concerning adverse local or systemic responses after two intradermal injections. Indeed, both physical and histomorphologic alterations did not significantly differ between both MSC treatments (Carrade et al., 2011). Although our macroscopic and histological results indicate a superior effect of autologous EpSC, more in depth research will have to confirm this hypothesis and postulate possible reasons.

BM-MSC promote the proliferation of fibroblasts, which are responsible for deposition of collagen and granulation tissue formation (Branski et al., 2009; Chen et al., 2008; Jeon et al., 2010). Fibroblasts will initially synthesize proteoglycans and fibronectin to create the extracellular matrix, which contains transforming growth factor-beta (TGF-β). Following this, fibroblasts together with macrophages release proteases that activate TGF-β, which stimulates further fibroblast proliferation and collagen synthesis (Broughton et al., 2006). Therefore, earlier lay down of collagen and more fibroplasia in autologous BM treated wounds of rabbits in the present thesis might be due to this effect of the BM-MSC. Nevertheless, BM supernatant contains other regenerative factors, such as growth factors and cytokines as well and this might have caused a similar effect on wound healing. In addition, the other cells in the BMNC namely white blood cells and progenitor cells might have interacted among themselves and with other wound cells through paracrine mechanism and contributed for the healing effect recorded. Studies show that such type interaction with vascular endothelial cells and immunomodulation has been found to play significant role in accelerating wounds (Sorrell and Caplan, 2010).

To date, a large number of studies have been using mice, rabbits and other laboratory animals for investigating wound repair and scarring (Aksoy et al., 2002; Dorsett-Martin, 2004; Morris et al., 1997). However, the wound healing process in these laboratory animals is significantly different when compared with humans (Greenhalgh, 2005) and horses (Theoret and Wilmink, 2013). Unfortunately, the skin healing process in horses is often being hampered by complications, leading to excessive scarring and granulation tissue formation (exuberant granulation tissue), which are detrimental to functional and esthetic outcomes (Theoret and Wilmink, 2013). In a previous studies, allogenic EpSC in combination with autologous PRP resulted in thinner and more normal granulation tissue than PRP alone (Broeckx et al., 2014). In agreement with this finding, a significant inhibition of granulation tissue formation was also recorded using both autologous and allogenic EpSC without PRP in the present thesis. However, the reduction in granulation thickness was higher after using autologous EpSC than after allogenic EpSC and comparison with the untreated negative controls were not significant anymore. Also young granulation tissue formation was affected by the treatment with most reduction in the autologous EpSC treatment group. Young granulation tissue has been described as poorly oriented/disorganized granulation tissue, which is deficient in contractile activity (Theoret and Wilmink, 2013). Besides unravelling how EpSC improve granulation tissue formation and maturation in comparison to vehicle treated wounds, more protracted studies are required to confirm a superior granulation tissue in comparison to untreated controls. In addition to epithelialization and granulation tissue formation, neovascularization is also a crucial parameter in wound healing (Falanga, 2005; Singer and Clark, 1999) because it is required to sustain the newly formed granulation tissue and the survival of keratinocytes (Wu et al., 2007a; Wu et al., 2007b). Neovascularization occurs concurrently with fibroblast proliferation when endothelial cells, originating from parts of uninjured blood vessels, migrate to the area of the wound (Cerqueira et al., 2014). Differentiation of MSC into endothelial cells has been reported in several *in vitro* (Liu et al., 2008; Pittenger and Martin, 2004) and *in vivo* studies (Tang et al., 2006). Moreover, enhanced capillary density in wounds due to promotion of angiogenesis by BM-MSC through releasing angiogenic factors has been reported by many researchers (Badiavas et al., 2007; Chen et al., 2008; Nakagawa et al., 2005; Wu et al., 2007b). In the present thesis, only a subjective vascularization score was given in the rabbit biopsies, and therefore, no binding conclusions can be made concerning the effect of BMNC or EpSC on wound vascularization.

Researchers have been using different routes of administration of stem cells to the experimental animal or human. Indeed, besides stem cell injection into the wound edge or bed and/or topical application over the wound, intramuscular injection to the affected site and intra-arterial infusion have been reported for skin wound application. An enhanced wound healing rate was reported in rabbit incisional wounds by topical application of autologous BMNC (2×10^6) with placental extracts (Akela et al., 2013) and in excisional wounds by injecting autologous BMNC in plasma into the wound margin (Akela et al., 2012) by administering the cells only once on the day the wounds created . For the treatment of different types of human chronic wounds with size ranging from 15.4 cm^2 to 44 cm^2, topical application and injection into the wound bed of BM aspirate plus three additional topical treatments with cultured BM cells (ranging from 4.5×10^6 to 1.1×10^7 cells/wound) resulted in engraftment of the applied cells in combination with clinical and histological evidence of reduced scaring (Badiavas and Falanga, 2003). Kim et al. also reported positive results from intradermal injection of 10^4 to 10^7 BM-MSC in full thickness excisional circular punch wounds of 6mm diameter in dogs (Kim et al., 2013). In this thesis, autologous BMNC application (1.4×10^8 cell) to excisional full thickness wounds of 4 cm^2 resulted in improved wound healing in rabbits for both treatment groups (i.e. subcutaneous injection around wound margin and topically spraying over the wound).

Besides the variations in the route of administration, other differences between the currently reported clinical studies are definitely worth mentioning. The type of cell vehicle used in wound healing studies differs greatly between the different experiments. The following substances have been reported as a carrier for applying stem cells in experimentally induced skin wounds in different animal species and clinical wounds in human: autologous plasma, autologous platelet rich plasma (PRP), phosphate buffered saline, normal saline, placental extracts, Hanks balanced saline solution (HBSS), amongst others (Akela et al., 2012; Akela et al., 2013; Badiavas et al., 2003; Badillo et al., 2007; Broeckx et al., 2014; Castilla et al., 2012; Wu et al., 2007b) . Another important variation between the different studies is the time of cell application to the wound. Most studies use cellular transplantation immediately after wound creation with or without a repetition after 24 hr (Akela et al., 2012; Akela et al., 2013; Wu et al., 2007b), where others apply the cell-based therapy only after 24 hr or even later (Kim et al., 2013). It was suggested that an injection of at least 1×10^6 MSC cells/cm^2 into the wound bed is needed for a significant therapeutic effect (Kim et al., 2013) and that the appropriate number of cells required for treatment may differ according to the route of

delivery. No scientific information is available on the optimal frequency of the administration of stem/progenitor cells for wound therapy. Some have reported successful chronic wound treatments just by single administration (Akela et al., 2012; Akela et al., 2013; Jain et al., 2011; Kim et al., 2013; Klepanec et al., 2012), while others have reported at least two (multiple) administrations (Badiavas and Falanga, 2003; Broeckx et al., 2014). Even though promising clinical outcomes are reported by a number of studies using BM-MSC or BMNC, comparison between the different results is difficult due to number of variations in the techniques of preparation of the cells and administration mentioned above. Therefore, the appropriate route of administration, number of cells and frequency of administration for efficient wound healing should be further investigated. Based on these results a guideline for using BMNC or EpSC for cutaneous wound therapy should be developed.

7.4 Conclusions

A variety of therapeutic modalities has been proposed to accelerate acute and/or chronic wound healing in the past decades. The development of novel approaches for wound treatment remains a clinical challenge. In line with the results from the present thesis, recent advances in regenerative medicine are providing encouraging data from both *in vitro* and *in vivo* studies.

Autologous bone marrow-derived cells, promoted healing of full thickness excisional skin wounds in rabbits when injected into wound margin or applied topically over the wound (Chapters 4.1 and 4.2).

Isolation and purification of EpSC was successful from all the horse (n = 6) skin samples using sphere formation as a valuable tool for EpSC enrichment. Indeed, sphere formation purified EpSC upon their isolation and successfully eliminated cellular contaminations (Chapter 5).

In comparison with the vehicle treated groups, significantly enhanced macroscopic and histological wound healing was demonstrated in both autologous and allogenic EpSC-treated groups. Compared with the untreated control wounds no significant differences with EpSC treatments could be demonstrated (Chapter 6). Further research will have to determine why the untreated wounds scored considerably better than the vehicle treatments and how EpSC counteracted this effect.

7.5 Future research perspectives

Even though BMNC cell mixture, adult stem cells including BM-MSC and EpSC offer great therapeutic promise for a diverse range of medical applications, optimization of cell-based therapies is required at different levels, including defining donor cell characteristics (such as age of donor or site of harvest), pre-delivery strategies (isolation, enrichment, the length of time for the cells to be maintained after harvesting and still retain viability, pre-delivery conditioning and vehicle used for the cells), and delivery (ideal number of cells to be transplanted, time and frequency of transplantation, route of delivery, fate of transplanted cells and precise in vivo localization of the stem cells and recipient characteristics).

There are several animal studies and many human pilot studies demonstrating an enhancing effect on skin wound healing of BMNC. However, the primary contribution of the administered cells to cutaneous regeneration and long term systemic effects need to be investigated. In addition, it needs to be determined if there are other types of stem/progenitor cells which might be more effective than BMNC.

Although seeding EpSC at lower densities in suspension cultures could successfully remove the cellular impurities, further studies should be conducted to optimize and validate this purification method with skin stem cells as well as with stem cells from other tissues. Also minimal criteria for the characterization of EpSC should be set, as reported for human MSC by the international society for cellular therapy (ISCT). In addition, there is a need to conduct further *in vitro* and *in vivo* research in order to investigate the exact mechanism how EpSC treatment resulted in reduced macroscopic wound healing parameters and in normalized granulation tissue formation in comparison with vehicle treated wounds. Such future studies might need to consider the paracrine and immunomodulatory role of the transplanted EpSC in wound healing.

The use of stem cell-based therapies has potential for advancing treatment options for companion animals, however, further evidence-based studies in clinical patients are warranted to substantiate their efficacy. When appropriate and clinically relevant veterinary models have been identified, these clinical trials will also be critical in predicting efficacy and optimizing therapies for human patients. To establish cell therapy as a standard treatment, more randomized clinical trials with a larger number of patients are required. Future research

should also focus on improving wound bed preparation and infection control to maximize cell engraftment, expediting cell culturing and looking at long term patient follow-ups at a functional and aesthetic level.

7.6 References

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Summary

Summary

Veterinary patients are frequently affected by complex skin wounds. Skin injury may induce loss of skins' integrity with functional imbalance accompanied by disability or even death. Despite a variety of therapeutic modalities proposed to accelerate the healing of acute and/or chronic wounds over the past decades, it remains important to develop novel and more successful approaches for wound treatment. Recently, various wound healing technologies that promote cell activity or minimize scar formation have been developed and some of them are being actively used at present. Regenerative medicine is among those new technologies. It aims to restore, maintain and improve body function. Regenerative wound therapy might prove to be one of the best alternatives where current conventional therapies fail or lead to inadequate results.

Cell therapy is a part of the broad field of regenerative medicine and it is defined as a set of strategies which use live cells with therapeutic purposes. Cells used in cell therapy can be broadly categorized as stem cells and adult/differentiated cells. Stem cells are undifferentiated cells, which have the capacity to differentiate into more specialized daughter /adult cells and have the ability to self-regenerate. Traditionally, they are divided into embryonic stem cells (ESC) and adult stem cells (ASC). Since ASC escape the ethical issues associated with ESC, they are the prime source for cell-based therapies and thus find their way through the regenerative landscape for treatment of several diseases in mammals.

This dissertation contains a literature overview and own experimental work. The general introduction (Chapter 1) consists of a literature review on different aspects of stem cell based therapies for cutaneous wounds. The isolation and characterization of adult stem/progenitor cells from bone marrow and skin is reviewed, followed by a description of the different routes of administration of stem cells and the evaluation of wound healing. This chapter introduces background knowledge on techniques used in the experimental work of the dissertation. Chapter 2 is devoted to an overview on regenerative skin wound healing in mammalian species. It highlights the adult mammalian wound healing as compared to the scarless wound healing in the mammalian embryos and amphibians and further discusses wound management in a general way. It gives an account of the current knowledge on cutaneous regenerative wound healing techniques with special emphasis on growth factors and cell based therapies. The most commonly cited growth factors and stem cells with positive effects on skin wound

healing of mammals are discussed, particularly for rabbit and horse, the two animals species studied in this thesis.

The experimental work focuses on two different types of cells in two different animal models: (1) Autologous bone marrow-derived nucleated cells (BMNC) and their potential use in cutaneous regenerative therapy in rabbit full thickness excisional (thoracolumbar region) wounds (Chapters 4.1 and 4.2) and (2) Epithelial-like stem cells (EpSC) and their in vivo regenerative capacity in full thickness excisional wound in horses (Chapter 6). New isolation and purification techniques for EpSC in horse are also developed in this work (Chapter 5)

The objective of Chapters 4.1 and 4.2 was thus to investigate the use of BMNC in rabbits (n = 20) for wound healing. Wounds were treated by either (i) injection of bone marrow-derived cells into wound margin (BI), (ii) topical application of bone marrow-derived cells over the surface of the wound (BT), (iii) wound dressing with povidone iodine (PI) or (iv) with the normal saline (NS). The latter two constituted the two control treatments. The wounds were followed for 28 days after wound induction for evaluating the status of granulation tissue formation and the wound contraction, and for assessing the wound histomorphologically and determining the time to complete wound healing. Granulation tissue appeared significantly faster in BI (3.22 $\pm$ 0.22) than in BT (3.89 $\pm$ 0.40), PI (4.89 $\pm$ 0.47) and NS (4.56 $\pm$ 0.47). On day 21 wound contraction was significantly higher in BI (97.35%) than in BT (84.87%), PI (84.6%) and NS (92.59%) treated wounds. Histological examination of the healing wound indicated earlier disappearance of inflammatory reaction, significantly more neovascularization and fibroplasias, earlier laydown and histological maturation of collagen and better epithelialization were in BI and BT than in control wounds.

Before applying EpSC in a clinical setting in horse skin wounds, the production of an EpSC dose was optimized in Chapter 5. The sphere-forming capacity was used as an enrichment strategy in EpSC from equine skin. Skin samples were harvested from horses (n = 6, mares) and EpSC were isolated and purified from all the samples. In addition to their characterization based on immunophenotypic and functional properties, subsequent sphere formation was assessed upon isolation at passage 0 (P0) and at early (P4) and late (P10) passages in suspension culture using different culture media. An average of 0.53% ($\pm$ 0.28%) of the primary skin-derived cells could form spheres and hence showed stem cell properties. Significantly more sphere formation occurred in EpSC medium than in differentiation

medium verifying the EpSC' privileged ability to survive in suspension. Immunophenotypic characterization showed that EpSC express cytokeratin (CK) 14, Casein kinase 2 beta and Major Histocompatibility Complex (MHC) I but not Pan CK and wide CK, while only few cells expressed MHC II. Keratinocyte differentiation of EpSC at early and late passages was successful for all six isolates (as confirmed by co-expression of CK 14, Casein kinase 2 beta, pan CK and wide CK). A non-EpSC cell type, which was confirmed to be a keratinocyte by immunohistochemistry, was detected in one of the six isolates in adherent culture. The isolate with keratinocyte impurity was purified by seeding the cells in suspension culture at ultralow clonal densities of 1 and 10 cells/cm^2 yet with a significantly lower sphere forming efficiency in comparison to pure EpSC.

Different studies in animal and human models have demonstrated beneficial effects of autologous and allogenic mesenchymal stem cells on skin wound healing. Only a few reports are available on the difference between autologous and allogenic EpSC for the treatment of skin wounds. Therefore, Chapter 6 deals with the *in vivo* assessment of autologous and allogenic EpSC-treated skin wounds in horses (n = 6, mares). In the gluteus region, 12 full thickness skin wounds of 2 x 3 cm were created and four treatment groups were considered: (i) autologous EpSC, (ii) allogenic EpSC, (iii) vehicle treatment (placebo) and (iv) sham operated. Each treatment group contained 3 wounds for double-blind macroscopic and histologic examination at week 1, 2 and 5. The results revealed significantly reduced circumference in wounds treated by autologous EpSC compared allogenic EpSC and placebo. The total granulation tissue thickness was 1257 µm smaller in allogenic EpSC treated wounds ($p = 0.0103$) and 1403 µm smaller in the autologous EpSC treated wounds ($p = 0.004$) as compared to vehicle treated wounds. Moreover young granulation tissue was 983 µm smaller in allogenic EpSC treated wounds ($p = 0.0247$) and 1135 µm smaller in the autologous EpSC treated wounds ($p = 0.0084$) as compared to vehicle treated wounds. Finally, In comparison to the vehicle treated wounds, the allogenic and autologous treated wounds were 26% ($P = 0.0501$) and 32% ($P = 0.0144$) closer to the intact skin thickness, respectively.

In conclusion, it has been demonstrated that regenerative skin wound treatment and more particularly cell-based therapies have a lot of potential, both from *in vitro* and *in vivo* studies of skin wounds in different animal models. In line with this, the experimental work in the present thesis indicates that full thickness skin excisional wound healing can be promoted

using autologous bone marrow-derived cells and autologous and allogenic EpSC in rabbits and horses, respectively.

Samenvatting

Samenvatting

Huidwonden met een complex karakter komen vaak voor in de diergeneeskundige praktijk. Huidwonden kunnen leiden tot verlies van de integriteit van de huidbarrière en een functioneel onevenwicht, met als mogelijk gevolg een infectie of zelfs de dood. Hoewel er de afgelopen jaren verschillende nieuwe behandelingsmodaliteiten zijn voorgesteld om de genezing van zowel acute als chronische huidwonden te bespoedigen, blijft het belangrijk om innovatieve en meer succesvolle therapieën voor huidwonden te ontwikkelen. Recent zijn verschillende nieuwe technieken ontwikkeld die gebaseerd zijn op het verhogen van de cel activiteit binnen de wonde om de vorming van littekenweefsel zoveel mogelijk te minimaliseren. Deze technieken worden momenteel al toegepast in de diergeneeskundige praktijk. De regeneratieve geneeskunde is één van die nieuwe technologieën. Ze beoogt het herstel, behoud en verbetering van de lichaamsfunctie. Regeneratieve wondheling zou een goede alternatieve therapie kunnen zijn waar de conventionele therapieën falen of tot een weinig bevredigend resultaat leiden.

Celtherapie maakt deel uit van de bredere discipline van regeneratieve geneeskunde en wordt gedefinieerd als een set van behandelingsstrategieën die levende cellen gebruiken voor therapeutische doeleinden. De cellen gebruikt in celtherapie kunnen opgedeeld worden in stamcellen en volwassen/gedifferentieerde cellen. Stamcellen zijn ongedifferentieerde cellen, die de capaciteit hebben om te differentiëren in meer gespecialiseerde dochter /volwassen cellen maar ook om zichzelf te vermenigvuldigen. Traditioneel worden stamcellen opgedeeld in embryonale stamcellen (ESC) en volwassen stamcellen . Omdat er met volwassen stamcellen veel minder ethische problemen zijn dan met ESC worden ze meest ingezet in cel-gebaseerde therapie; aldus vinden ze hun weg in de regeneratieve geneeskunde voor de behandeling van verschillende ziekten, vooral dan bij zoogdieren.

Deze dissertatie bevat een literatuuroverzicht en eigen experimenteel werk. De algemene introductie (Hoofdstuk 1) bestaat uit een literatuuroverzicht met focus op stamcel-gerelateerde therapieën voor huidwonden. De isolatie en karakterisatie van volwassen stam/progenitor cellen van beenmerg en huid wordt besproken, gevolgd door een beschrijving van de verschillende wijzen van toedienen van stamcellen en van de evaluatiewijzen voor wondheling. In dit hoofdstuk wordt ook een overzicht gegeven van de huidige kennis over de

verschillende technieken die in het experimentele onderzoek van deze dissertatie worden gebruikt. Hoofdstuk 2 geeft een beschrijving van het begrip regeneratieve huidwondheling bij zoogdieren. Het vergelijkt de wondheling bij volwassen zoogdieren met die bij amfibieën en embryo's van zoogdieren en bespreekt ook het management van wonden in het algemeen. Het geeft een overzicht van de bestaande kennis over regeneratieve huidwondheling technieken met speciale aandacht voor therapieën gebaseerd op groeifactoren en stamcellen. De meest geciteerde groeifactoren en stamcellen met positieve effecten op huidwondheling bij zoogdieren wordt besproken, voornamelijk voor paard en konijn, de twee diersoorten die in deze dissertatie gebruikt worden voor het experimentele werk.

Het experimentele werk maakt gebruik van twee verschillende celtypen in twee verschillende diermodellen: (1) Autologe gekernde cellen van beenmerg (BMNC) voor het gebruik van regeneratieve therapie in 'full thickness' excisiewonden (thoracolumbale regio) bij konijn (Hoofdstukken 4.1 en 4.2) en (2) Epithelieel-gelijkende stamcellen, (EpSC) en hun in vivo regeneratieve capaciteit in 'full thickness' excisiewonden bij het paard (Hoofdstuk 6). Voor de EpSC bij het paard worden in dit werk ook nieuwe isolatie- en purificatie technieken ontwikkeld (Hoofdstuk 5).

Hoofdstukken 4.1 en 4.2 bespreken de resultaten van de experimenten uitgevoerd bij konijnen. Ze geven een antwoord op de vraag of BMNC geschikt zijn als therapie bij wondheling van het konijnen (n = 20). Wonden werden behandeld met ofwel (i) een injectie in de aflijning van de wonde met cellen afkomstig van het beenmerg (BI), (ii) lokale toediening van cellen afkomstig van het beenmerg over de volledige oppervlakte van de wonde (BT), (iii) povidone iodine (PI) of (iv) een normale zoutoplossing (NS). De laatste twee behandelingen zijn de controlebehandelingen. De wonden werden gedurende 28 dagen opgevolgd en geëvalueerd voor (1) de vorming van granulatieweefsel, (2) de contractiegraad van de wonde, (3) de evolutie van de wonde over de tijd op gebied van histomorfologie en (4) de tijd nodig voor volledig wondgenezing. Granulatieweefsel ontstond significant sneller in BI (3.22 ± 0.22) dan in BT (3.89 ± 0.40), PI (4.89 ± 0.47) en NS (4.56 ± 0.47). Op dag 21 was er significant meer wondcontractie bij BI (97.35%) dan bij BT (84.87%), PI (84.6%) en NS (92.59%) behandelde wonden. Op histologisch niveau verdween de inflammatoire reactie sneller en was er significant meer neovascularisatie en fibroplasia bij BI en BT in vergelijking

met de controlewonden en was er een snellere histologische maturatie van collageen en een betere epithelialisatie.

Voor EpSC werden gebruikt in de klinische proef bij paarden, werd de productie van de EpSC geoptimaliseerd zoals beschreven in Hoofdstuk 5. De sfeer-vormende capaciteit werd gebruikt als een verrijkingsstrategie bij EpSC van paardenhuid. Huidstalen werden geoogst bij paarden (n = 6, merries) en EpSC werden geïsoleerd en gezuiverd voor alle stalen. Eerst werden de cellen gekarakteriseerd op basis van immunofenotypische en functionele eigenschappen. Vervolgens werd sfeerformatie geëvalueerd in passage 0 (P0) en tijdens vroege (P4) en late (P10) passages in suspensiecultuur waarbij verschillende cultuurmedia werden gebruikt. Een gemiddelde van 0,53% (± 0,28%) van de oorspronkelijke huidcellen kon sferen vormen en vertoonde aldus stamceleigenschappen. Significant meer sfeervorming trad op in EpSC medium in vergelijking met differentiatiemedium; dit komt overeen met de capaciteit van EpSC om te overleven in suspensie. Immunofenotypische karakterisatie toonde aan dat EpSC cytokeratin (CK) 14, Casein kinase 2 beta en Major Histocompatibility Complex (MHC) I tot expressie brengen, maar niet Pan CK en 'wide CK'. Slechts bij een beperkt aantal cellen werd MHC II tot expressie gebracht. Keratinocyt differentiatie van EpSC bij vroege en late passages was succesvol voor alle zes isolaten (bevestigd door co-expressie van CK 14, Casein kinase 2 beta, pan CK en 'wide CK'). Een non-EpSC celtype (geïdentificeerd als keratinocyt door immunohistochemie) was aanwezig in één van de zes isolaten. Het isolaat met de keratinocyt werd opgezuiverd door het uitzetten van de cellen in suspensiecultuur met ultra lage klonale dichtheden van 1 en 10 cellen/cm^2 met bijgevolg een significant lagere efficiëntie van sfeervorming in vergelijking met een zuivere EpSC cultuur.

Verschillende studies bij zoogdieren en bij de mens hebben aangetoond dat de toepassing van autologe en heterologe mesenchymale stamcellen een positief effect kan hebben op de wondheling. Een beperkt aantal studies bestuderen het verschil tussen autologe en heterologe EpSC voor de behandeling van huidwonden. Hoofdstuk 6 evalueert de *in vivo* behandeling van huidwonden bij het paard met autologe en heterologe EpSC (n = 6, merries). In het gebied van de m. gluteus werden 12 'full thickness' huidwonden van 2 x 3 cm aangebracht en vier verschillende behandelingen werden toegepast: (i) autologe EpSC, (ii) heterologe EpSC, (iii) behandeling met enkel oplossingsvloeistof (OV) en (iv) onbehandeld (OB). Aan elke behandelingsgroep werden per paard 3 wonden toegewezen voor dubbelblinde

macroscopische en histologische evaluatie op week 1, 2 en 5. De resultaten toonden aan dat de omtrek van de wonde significant kleiner was voor wonden behandeld met autologe EpSC in vergelijking met heterologe EpSC en OV. De totale granulatieweefsellaag was 1,257 mm dunner bij heterologe EpSC behandelde wonden ($p = 0.0103$) en 1,403 mm dunner bij autologe EpSC behandelde wonden ($p = 0.004$) in vergelijking met OV. Bijkomend was de jonge granulatieweefsellaag 0,983 mm dunner bij heterologe EpSC behandelde wonden ($p = 0.0247$) en 1,135 mm dunner bij autologe EpSC behandelde wonden ($p = 0.0084$) in vergelijking met OV. Tenslotte, in vergelijking met OV behandelde wonden was de dikte van heterologe en autologe behandelde wonden 26% ($p = 0.0501$) en 32% ($p = 0.0144$) dichter bij de dikte van intacte huid, respectievelijk.

Er werd tot besluit in deze dissertatie aangetoond dat regeneratieve huidwondbehandelingen en meer specifiek de cel-gebaseerde therapieën, een potentieel hebben, zoals blijkt uit zowel de *in vitro* als de *in vivo* studies van huidwonden uitgevoerd in verschillende diermodellen. Ook het experimentele werk voorgesteld in deze dissertatie duidt aan dat de genezing van 'full thickness' excisiewonden van de huid kan bespoedigd worden door het toedienen van autologe cellen afkomstig van beenmerg en autologe en heterologe EpSC respectievelijk bij konijn en paard.

Curriculum Vitae

Curriculum Vitae

Bizunesh Mideksa Borena was born in 1977 in Adama, Ethiopia. She followed her primary and secondary schools at Adama Number 4 primary school and Adama Coprhensive high school, respectively. She passed Ethiopian School Leaving Certificate Examination (ESLCE) with distinction and joined Addis Ababa University, Ethiopia, in 1996 and graduated in Doctor of Veterinary Medicine (DVM) in 2002. She worked as assistant researcher I for 10 months (01/04/2003 to 01/01/2004) at Bako Agricultural Research Center, Oromia Regional Research Institute. From 01/01/2004 to 01/09/2006 she worked at the department of clinical science, school of veterinary medicine of Jimma Univeristy, Ethiopia, at various capacities. In 2006/2007 she joined graduate program at Indian Veterinary Research Institute (IVRI) and obtained Masters of Veterinary Science in Veterinary Surgery and Radiology. From 19/07/2008 to date she has been working as assistant professor at Ambo University, Ethiopia at different positions including, department head and education quality and quality audit team leader.

In 2011, she obtained a scholarship from NUFFIC-NICHE ET 019 for PhD training in veterinary science, Faculty of Veterinary Medicine, Ghent University (Ghent), Belgium.

She authored and co-authored 9 scientific papers in international peer-reviewed journals.

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