Forsan et haec olim meminisse juvabit

(Vergilius)

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INTEGRATION OF BLOOD OUTGROWTH ENDOTHELIAL CELLS IN DERMAL FIBROBLAST SHEETS PROMOTES FULL THICKNESS WOUND HEALING

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*Think where man's glory most begins and ends
And say my glory was I had such friends.*

(William B. Yeats)

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*Vivez, si m'en croyez, n'attendez à demain
Cueillez dès aujourd'hui les roses de la vie.*

(Pierre de Ronsard)

*Les découvertes récentes ont anéanti toutes
nos illusions sur la simplicité de la vie.*

(Gustave Le Bon)

List of abbreviations

- α SMA : α smooth muscle actin
- Ang : angiopoietin
- bFGF: basic fibroblast growth factor
- BOEC : blood outgrowth endothelial cells
- CD : cluster of differentiation
- Dil-AcLDL : dioctadecyl tetramethyl indocarbocyanide-acetylated low density lipoprotein
- EC : endothelial cells
- ECM : extracellular matrix
- ELISA : enzyme-linked immuno-sorbent assay
- eNOS : endothelial nitric oxide synthase
- EPC: endothelial progenitor cells
- FACS : fluorescence activated cell sorting
- flt-1 : FMS-like tyrosine kinase-1
- GF: growth factors
- GM-CSF : granulocyte/monocyte-colony stimulating factor
- H&E : hematoxylin and eosin
- h(X) : human (X)
- hDFS: human dermal fibroblast sheets
- IL : interleukin
- KDR : kinase insert domain receptor
- KGF: keratinocyte growth factor
- m(X) : murine (X)
- MCP-1 : monocyte/macrophage chemoattractant protein 1
- MMP : matrix metalloproteases
- mRNA : messenger ribonucleic acid
- p(X) : porcine (X)
- PDGF : platelet-derived growth factor
- PIGF : placental growth factor
- PIGFKD: PIGF knockdown
- qRT-PCR : quantitative real time polymerase chain reaction
- shRNA : short hairpin ribonucleic acid
- SMC : smooth muscle cells
- STE: skin tissue engineering
- TEM: transmission electron microscopy
- Tie-1 : tyrosine kinase with immunoglobulin-like and EGF-like domains 1
- UEA-lectin : Ulex Europaeus Agglutininlectin
- VE-Cadherin : vascular endothelial cadherin
- VEGF : vascular endothelial growth factor
- vWF : von Willebrand factor

*La science ne sert qu'à vérifier
les découvertes de l'instinct.*

(Jean Cocteau)

Introduction

1. Skin Tissue Engineering: time for something new

The basis of wound healing is to restore the epidermal layer. However, skin is so much more than just an epidermal barrier. The dermal layer underneath the epidermis provides elasticity and aesthetic appearance to skin, whereas the subcutaneous fat layer accounts for volume and thermoregulation. Normally, wounds tend to heal spontaneously, resulting in scar tissue. The larger the area left to heal, the longer it takes for secondary healing to complete and the worse the outcome in terms of wound contraction and aesthetic appearance. Therefore, even smaller defects in the face need to be reconstructed whereas larger wounds at less visible locations (e.g. the back) can be left to secondary healing. Large skin defects may result from trauma, infections, vascular disease, burns or oncologic surgery. These wounds do not heal properly and need to be reconstructed. Ultimately, reconstructive plastic surgery aims at restoring defects with similar tissue, without creating a burden at the donor site.

Small to very large superficial defects can adequately be covered with skin grafts. Split thickness skin grafts can be harvested in large quantities and expanded by meshing the graft, create minimal donor site morbidity and are quickly re-vascularised. However, the resulting tissue is brittle, unaesthetic and prone to excessive contraction (Fig. 1).

Figure 1. Excessive skin contraction of full thickness burn wounds reconstructed with split thickness skin grafts. **A**, 'Postburn syndactyly' due to contraction of the web spaces of the fingers. The normal position of the web space is indicated with a needle. **B-C**, Severe contraction of burns in the axillary region reconstructed with split thickness skin grafts. Contractures of the anterior axillary fold (indicated with arrows in **B** and **C**) that impair shoulder abduction. The resulting skin is hypertrophic (arrowhead in **B**) or brittle, leading to superficial erosions (arrowheads in **C**). **D**, Hypertrophic scarring and wound contraction in the face and anterior neck region, with unsightly appearance and functional impairment of neck mobility. The skin of the neck is especially prone to flexion contracture upon burn wounds. In all cases, scar revision using multiple Z-plasties, full thickness skin grafts and/or flap reconstruction will be mandatory to restore mobility and minimal cosmesis.

Full thickness skin grafts include dermis and epidermis and result therefore in thicker, more elastic scars. However, these grafts initially survive through imbibition, and it takes up to one week before a decent connection is restored between the blood vessels of the wound bed and the vessels in the graft by inosculation ¹. In addition, the donor site generally needs to be closed primarily, limiting the size of those grafts. Moreover, if the wound bed is poorly vascularised or if there is hematoma or seroma between a skin graft and the wound bed, inosculation is prevented, with subsequent graft loss. In an attempt to restore like-with-like tissue, abundant adjacent tissue is often used under the form of local or locoregional flaps (Fig. 2).

Figure 2. Reconstruction of a full thickness skin defect using a local flap. A spinocellular carcinoma on the left nasal wall and cheek is removed by Moh's surgery, resulting in a large full thickness defect central in the face (A). Merely grafting this defect would result in an unaesthetic, fragile scar with distortion of the lower eyelid and nose. Therefore, a local forehead flap (B) is used to cover the defect and the donor site is closed primarily (C). Colour and texture of the flap match with the surrounding skin (D) and the defect is adequately covered without traction on surrounding structures (E). One month postoperatively, the flap has excellently healed, with acceptable appearance of the scar at the donor site. However, the flap is rather bulky, crosses several aesthetic units and will need surgical correction (F).

Of course, defect size and availability of local tissue are limiting factors for reconstruction with local flaps. If no skin graft can be used and there is no local option for covering the defect, distant tissue needs to be advocated. In that case, distant tissue is harvested on a vascular pedicle, transferred into the defect and the vascular pedicle is connected to the recipient vasculature by microsurgical techniques (Fig. 3).

Figure 3. Reconstruction of a defect of the mandibula and oral floor using a free fibula flap. A preoperative drawing indicates the position of the skin paddle and the fibula (A). The osteocutaneous flap is dissected on its vascular pedicle (B) and harvested (C) prior to ex-vivo shaping with osteotomies. Titanium plate and screw fixation reshapes the mandibula (D) and microvascular anastomoses are made on neck vessels to maintain flap viability (E). The skin paddle is used to cover the mucosal defect (F) and the donorsite of the flap is grafted (G).

Essentially, all plastic surgery reconstruction techniques create to some extent nuisance at the site where tissue is removed, ranging from an initially painful and later hyperemic donor site of split thickness skin grafts, to the potential functional impairment at the donor site of composite free flaps such as in the osteocutaneous free fibula flap ². To reduce this donor site morbidity, biotechnological advances have been made to introduce artificial components to optimise wound healing with minimal requirements of donor tissue. Already in the '80s, Burke and Yannas applied a matrix consisting of bovine collagen I and chondroitin-6 sulfate to restore the dermis in full thickness burn wounds ³, resulting in more elastic scars that tended to contract less (Fig. 4 and 5).

Figure 4. Reconstruction of a full thickness skin defect using a dermal substitute. Full thickness burn wound of the hand, debrided and covered with a commercially available dermal substitute (A) prior to skin grafting (B). The resulting skin is elastic with little tendency to contraction (C).

A few years later, the Green and colleagues described cultures of autologous keratinocytes for coverage of large burn wounds ⁴ (Fig. 5). These landmark papers undoubtedly heralded the era of skin tissue engineering (STE), as for the first time successful efforts were made to use biological scaffolds and cultured cell constructs for skin restoration. STE aims at isolating -with minimal donor site morbidity- skin cells from a patient, ex vivo expansion, integration in a matrix and subsequent transplantation to the wound, so that this skin substitute mimics natural skin in terms of appearance, elasticity, thermoregulation and barrier function. The initial euphoria ebbed away when it became clear that cultured keratinocyte grafts lead to unstable scars. Moreover, it takes weeks before blood vessels grow into artificial dermal substitutes. During this avascular period, the dermal substitute is prone to infection and subsequent loss. More recently, combined approaches tried to restore both dermis and epidermis at once ⁵. However, adding layers to engineered skin increases the distance between the host vascular network and the upper layers of the skin construct which results in hypoxia of these upper layers ⁵. Cells more than 200 μm away from blood vessels become oxygen-deprived ⁶, making STE essentially a matter of rapid and accurate vascularisation of skin substitutes to prevent hypoxia-mediated cell death.

Figure 5. Landmark papers relevant to this project. The first attempts to restore dermis and epidermis were made in the 80's. The major impediment remained however the need for vascularisation. Angiogenic growth factors, discovered in the early 90's, were assumed to solve this problem, but cell-based strategies count for a more stable solution. The identification of different types of EPC in the past decade raises hope for successful vascularisation in skin tissue engineering.

2. Vascularisation: breathing life into engineered skin

Several approaches have been tested to vascularise skin substitutes. Apart from surgical prefabrication ⁷, skin substitutes have been seeded with angiogenic growth factors ⁸, with microspheres loaded with growth factors ⁹ or with plasmids encoding growth factors ¹⁰. Since these approaches often require repetitive administration, skin substitutes seeded with cells producing these growth factors on a continuous basis

provide an attractive alternative. For instance, seeding fibroblasts or keratinocytes results in faster vascularisation, due to the secretion of growth factors ¹¹. We have used vascular endothelial growth factor (VEGF) over-expression in keratinocytes to augment vascularisation in a porcine wound healing model ¹². However, aside from growth factors supplementation, incorporating cells of endothelial origin in the graft remains the most appealing option, as these cells are predestined to directly contribute to vessel formation. The skin substitute can either be seeded with endothelial cells (EC) to form a vascular network in vitro, prior to application to the wound – the so-called ‘pre-vascularisation’ technique – or be combined with the skin substitute directly before transfer to the wound. In vitro pre-vascularisation results in faster inosculation to the host vascular network, but raises the problem of unequal cell distribution in the skin substitute, leading to incomplete vascular networks ⁵. It therefore seems more feasible to opt for ‘in situ’ assembly of vascular networks within skin substitutes that have been seeded in vitro with endothelial (precursor) cells that organise themselves in cooperation with endogenous EC into vascular tubes upon implantation. Furthermore, stable and functional vessel networks depend on the recruitment of pericytes (in small vessels) and vascular smooth muscle cells (SMC; in large vessels) around the endothelium, a process that is easier to accomplish in situ ¹³.

Formation of an endothelial network can either happen through *angiogenesis*, i.e. sprouting from existing endothelial tubes, or through *vasculogenesis*, i.e. the *de novo* formation and in situ assembly of endothelial-lined vessels. In the context of STE, angiogenesis means ingrowth and sprouting of the host’s vascular network into the skin substitute, whereas vasculogenesis is the formation of a tubular network by endothelial (precursor) cells recruited into or exogenously added to the skin substitute and, subsequent connection to and incorporation into the host’s vascular network (Fig. 6).

Figure 6. Angiogenesis versus vasculogenesis in STE. Ex vivo expanded cells of different origins are applied to the wound bed and stimulate vessel formation either by secretion of angiogenic factors that communicate with endogenous vascular cells (angiogenesis) or by direct incorporation into nascent vessels (vasculogenesis). Moreover, these cells secrete trophic factors that can stimulate dermal and epidermal healing by communication with fibroblasts and keratinocytes, respectively.

When endothelial (precursor) cells are exogenously added, these can either be allogeneic or autologous (host-derived). Allogeneic cells can be isolated, expanded to obtain the desired amounts, and stored in liquid nitrogen until required in an '*off-the-shelf*' setting. However, they imply the risk of immunologic rejection¹⁴⁻¹⁶. While autologous cells are not subject to immunologic rejection, they are often available in only very small amounts, necessitating the need for expansion of these cells to therapeutic amounts. This expansion can either be accomplished endogenously, by stimulating mobilisation of endothelial (precursor) cells by chemokines, and/or exogenously, by in vitro expansion of harvested cells.

Many cell types may contribute to vessel formation in STE through angiogenesis, vasculogenesis or both. Preferably, in addition to stimulating the formation of an endothelial network, the cells may also support vessel stabilisation by pericytes/

SMC (Fig. 6). Furthermore, the ideal candidate to allow this in a clinical setting should: (i) be easy to harvest with minimal discomfort to the donor, (ii) be easily and rapidly expandable to amounts sufficient for vascularisation of large skin substitutes, without risk of malignant transformation, (iii) be non-immunogenic (autologous) and (iv) have a trophic effect on wound healing beyond vascularisation, i.e. by communication with other cell types present in the wound bed such as fibroblasts and keratinocytes that together orchestrate the sequential phases of the wound healing process (Fig. 6). In the next chapters we present an overview of a number of candidate cell types for wound vascularisation, discuss their use in STE in vitro and in vivo and their mechanism of action, and score them according to the above-mentioned clinical criteria (Table 1).

3. Cell sources for vascularisation strategies

Different (stem/progenitor) cells can be considered for vascularisation of skin substitutes. Hereafter, we give an overview of these thereby using the (endothelial) differentiation potential/status of the cells as a way to categorise them in a hierarchical system (Fig. 7). The zygote on top of this hierarchy has the broadest differentiation potential (i.e. '*totipotent*', giving rise to all cell types in an organism, including EC). In the middle, we find '*multipotent* progenitor cells' which have the capacity to be coaxed into multiple cell types. For some of these progenitors the differentiation spectrum may contain cells from different embryonic germ layer origin (e.g. EC from the mesoderm and neuronal cells from the ectoderm). A step lower, we find the '*unipotent*' progenitors that are committed to a single lineage. Finally, cells at the bottom are terminally differentiated mature EC that cannot give rise to another cell lineage ('*nullipotent*'). The degree of differentiation is inversely correlated with the expansion capacity of the cells (Fig. 7).

Figure 7. Hierarchy of cell sources that can be used for vascularisation in STE. Candidate cell populations for vascularization can be categorized according to their differentiation potential that inversely correlates with proliferation capacity. Cells with the broadest differentiation potential are on top of the pyramid, while fully differentiated, mature endothelial cells are at the base of the pyramid. ESC: embryonic stem cells; MSC: mesenchymal stem cells; MAPC: multipotent adult progenitor cells; EPC: endothelial progenitor cells; BOEC: blood outgrowth endothelial cells; HDMEC: human dermal microvascular endothelial cells; HUVEC: human umbilical vein endothelial cells.

3.1. Mature endothelial cells

3.1.1. Human dermal microvascular endothelial cells (HDMEC)

Microvascular EC can be isolated from skin samples ¹⁷. Further refinement of the culture protocol has led to the development of serum-free culture techniques, making these cells more acceptable for clinical use in STE ¹⁸. HDMEC are commercially available as a standardised EC line that was tested in in vitro wound assays ¹⁹, as well as in in vivo blood vessel formation assays ²⁰. Even though Nör *et al.* succeeded in creating functional vessels in a subcutaneous pocket when combining HDMEC with matrigel ²⁰, Supp *et al.* were not able to demonstrate the functional connection to the host vasculature in a skin substitute ²¹. Commercial HDMEC could theoretically be used for clinical purposes, however, it seems more appealing to use them in an autologous setting ²¹. Unfortunately, only relatively small amounts of HDMEC can be harvested and therefore large pieces of donor skin are needed ²¹, with increasing donor site morbidity. Combining HDMEC, fibroblasts and keratinocytes in an autologous fashion has been used in a clinical setting, unfortunately with delayed vascularisation and subsequent loss of the constructs in most of the cases ²². Human fibroblast sheets have been combined with both human umbilical vein endothelial cells (HUVEC) and HDMEC, giving rise to endothelial-lined tube-like structures with apparent lumina in vitro. These endothelialised fibroblast sheets have however not been used in vivo so far ²³. To the best of our knowledge, no reports have been published about the ability of HDMEC to support vessel stabilisation or trophic communication with wound cells (Table 1).

Cell type	origin	Autologous approach possible?	harvest	vasculogenic	angiogenic	Effect on vessel stability	Genetic stability	Number of cells after expansion	Trophic effect on wound healing
HDMEC	Patient's skin Neonatal foreskin	Y N	Skin sample needed Discarded tissue	+ +	? ?	? ?	+ +	(+) ²¹ +	? ?
HUVEC	umbilical cord	N	Discarded tissue	+	?	-/+ ²⁴ (Bcl-2)	+/- ²⁵ (Bcl-2)	++	? ²⁶
Early EPC	AB	Y	Peripheral blood sample	- ²⁷	+ ²⁸	?	+	+	+ ²⁹
OEC	CB AB	N Y	Discarded tissue Peripheral blood sample	+ +	? ?	? ?	+/- ³⁰ + ³⁰	++++ +++	? ?
MSC	BM	Y	BM puncture	+ ³¹ / _{-32, 33}	+ ³⁴	+ ^{31, 33}	- ³⁵	++++	+ ^{36, 37}
	Fat	Y	Lipoaspiration	+ ^{38, 39} / _{-40, 41}	+ ⁴²	+ ⁴²	- ⁴³	+++	+ ⁴²
	CB	N	Discarded tissue	- ⁴⁴	- ⁴⁴	? ?	? ?	++	? ?
	AB	Y	Peripheral blood sample after pretreatment	-	+ ⁴⁵	? ?	? ?	++++ (immortalised)	? ?
MAPC	BM	N	BM puncture	+ ⁴⁶	+ ⁴⁶	+ ⁴⁶	+	+++++	+*
ESC	Embryo	N	Sacrifice of human embryo's	+ ⁴⁷	+ ⁴⁸	+ ^{47, 48}	--- ⁴⁹	++++++ ⁴⁹	? ?
iPSC	e.g. skin	Y	Skin sample needed	? ?	? ?	? ?	--	+++++	? ?

Table 1. Advantages and disadvantages of different cell types for vascularisation in STE. Increased differentiation potential is linked to decreased genetic stability. Little is known on the trophic effect on wound healing of multiple cell types, apart from their angiogenic and/or vasculogenic potential. AB: adult blood; CB: cord blood; Y: yes; N: no. BM: bone marrow; ? : no literature evidence; +/-: confounding data in literature; (Bcl-2): after Bcl-2 transduction; *: unpublished results.

3.1.2. Human umbilical vein endothelial cells (HUVEC)

When derived from umbilical cord veins, human EC can be expanded relatively well ⁵⁰. HUVEC are the most widely used standardised EC line and serve as a reference in many vascularisation studies (e.g. refs. 34-35). Unfortunately, in the absence of cell banking, these cells can only be used in an allogeneic setting (Table 1). Combination of fibroblasts and HUVEC in a hyaluronic acid scaffold resulted in only very few vascular structures in vitro. ⁵¹ The same combination of cells in a collagen/glycosaminoglycan matrix in vitro led to the formation of a capillary-like network that could firmly be increased in density by adding VEGF or fibroblast growth factor (FGF) ⁵². In combination with fibroblasts and keratinocytes in a collagen matrix in vitro, HUVEC were able to form an organised vascular network. Notwithstanding the presence of collagen, fibroblasts were required to produce extracellular matrix proteins as a guidance grid for migration of HUVEC ⁵³. Seven years later, the same team was able to demonstrate quick inosculation of this in vitro pre-vascularised skin substitute, resulting in functional hybrid vessels, 4 days after transplantation ⁵. To prolong survival, HUVEC have been transduced with a virus encoding the anti-apoptotic protein Bcl-2, leading to a dense and mature (SMC-coated) vascular network in subcutaneously implanted collagen plugs ²⁴ and in a decellularised dermal matrix ⁵⁴. Even though Bcl-2 over-expression on itself did not cause malignant transformation of EC in vitro ⁵⁵ or in vivo ²⁴, the use of retroviral vectors for clinical use has to be considered with caution because of the potential hazards of random viral integration in the genome ^{25, 56}.

3.2. Unipotent progenitor cells: Endothelial Progenitor Cells (EPC)

It has long been accepted that vasculogenesis involving endothelial precursors only occurred in utero and that post-natal vessel formation was merely the result of angiogenesis. Since the initial description of circulating endothelial progenitors ⁵⁷ in 1997 (Fig. 5), there is clear evidence that circulating EPC contribute to adult vasculogenesis. However, different EPC isolation protocols and phenotypes have been described since then, causing a lot of confusion in the field ^{58, 59}. Currently, two major types of EPC are acknowledged, regarding their isolation kinetics and functional characteristics: 'early outgrowth EPC' and 'late outgrowth EPC', of which the latter have the capacity to incorporate into growing vessels.

3.2.1. Early outgrowth EPC

Early (outgrowth) EPC are CD34⁺, VEGF-receptor 2⁺ and CD133⁺ and are obtained by adherence depletion on fibronectin of the mononuclear fraction of cord or adult blood (Fig. 8). After 4-9 days, colonies of cells with limited proliferation capacity appear that lose CD133 expression but gain mature endothelial marker expression (for review,

see: ref. 46). Also referred to as 'Colony Forming Unit-EC', early EPC are commonly derived from CD14⁺ (monocytic) cells and form clusters of spindle-shaped cells⁶⁰. They regulate the angiogenic response through secretion of growth factors^{28, 61}, without becoming part of the endothelial intima²⁷ (Table 1). In addition to their trophic effect on angiogenesis, early EPC may facilitate vessel formation by actively drilling tunnels into sites of ischemia, that become lined with true EC⁶². Application of early EPC has proven to increase angiogenesis and epidermal wound healing^{29, 63}. Overall, regarding their ease of harvest, defined angiogenic potential and trophic effect on wound healing, early EPC can safely be used for STE, even though their limited proliferation capacities may limit their impact. Using cord blood increases yield of cells⁶⁴, but as these cells are allogeneic, they are at risk for immunologic rejection (Table 1).

3.2.2. Late outgrowth EPC

The groups of Hebbel and Yoder characterised late outgrowing endothelial-like colonies of collagen-adherent cells that had a much higher proliferation capacity than early EPC. These cells have also been called 'Endothelial Colony Forming Cells' (ECFC) or blood outgrowth endothelial cells (BOEC)^{60, 65, 66}. In contrast to early EPC, BOEC actively incorporate into intimal endothelium and can be expanded to high numbers in vitro. Whether they originate from bone marrow (BM) or from the vessel wall^{58, 65}, remains a matter of debate. Besides direct incorporation into vessels, BOEC indirectly stimulate host angiogenesis by secreting growth factors^{67, 68}. Interestingly, early EPC (or the factors they secrete) have a stimulatory effect on tube formation by BOEC⁶⁹, which makes a combination therapy of both cell types attractive. Co-transplantation of early EPC and BOEC had indeed a synergistic effect on revascularisation in an ischemic limb model⁷⁰, but there are no reports so far of this combined approach in STE. BOEC can either be derived from cord or adult blood, with the former showing higher proliferation potential³⁰.

3.2.2.1. Cord blood BOEC

Many studies highlight the excellent outgrowth of BOEC from cord blood (CB-BOEC) and suggest them, due to their high expansion and vasculogenic potential, as ideal candidates for (S)TE purposes^{60, 67, 71, 72}. Au et al. examined the in vivo vessel formation capacity of CB-BOEC in combination with pericyte-like cells and noted firmly increased survival, vessel formation and vessel stability in comparison with adult blood-derived BOEC (AB-BOEC)⁷¹. CB-BOEC formed stable vessels in an artificial dermal matrix when co-transplanted with SMC⁷³. They also efficiently formed a vascular network when seeded in a decellularised dermal matrix, to a comparable extent as the Bcl-2 transduced HUVEC⁶⁷. However, in contrast to AB-BOEC, CB-

BOEC are prone to karyotypic aberrations, even though no tumor formation has been reported so far. Therefore, the CB-BOEC may not be free of risk for clinical application³⁰. Moreover, autologous CB-BOEC are not available and, as allogeneic cell source, are at risk for immune rejection (Table 1).

3.2.2.2. Adult blood BOEC

Although adult blood samples yield fewer BOEC, there is evidence that this discrepancy can be overcome by VEGF stimulation during culture expansion⁷⁴. Unlike BM-derived stem cells, their harvest creates no donor site morbidity at all. AB-BOEC have successfully been used in endothelialisation of a decellularised aorta in sheep⁷⁵ or in vitro TE vessel constructs⁷⁶, blood vessel growth in tissue-engineered bone scaffolds⁷⁷ and for vascularisation of a decellularised dermal skin substitute, in which they remained traceable for more than 4 weeks in vivo, without Bcl-2 transduction⁷⁸. Recently, Reinisch *et al.* have described a large scale, animal protein-free, humanised expansion strategy for AB-BOEC that preserves proliferation and functional characteristics, even after cryopreservation⁷⁹. As serum-free culture protocols for human dermal fibroblasts and keratinocytes are also available⁸⁰, a fully autologous, serum-free, vascularised skin substitute seems to be within reach⁶⁹.

3.3. Multipotent progenitor cells

3.3.1. Mesenchymal stem cells (MSC)

MSC represent a heterogeneous group of multipotent progenitor cells that can be harvested from several tissues, including BM, skeletal muscle, fat, (umbilical cord) blood, amniotic fluid and different fetal tissues⁸¹. They act on wound healing through transdifferentiation or cell fusion to wound healing cells⁸², production of cytokines and growth factors^{83, 84}, maintenance of the extracellular matrix, modulation of the immune system and wound contraction⁸¹.

There is evidence that adipose-derived MSC and BM-MSC kept in culture for long time become immortalised, undergo spontaneous transformation and form tumours in vivo, which might jeopardize clinical application^{35, 43} (Table 1). As the incidence of malignant transformation increases with passage number, it seems safer to consider only cells from initial passages for clinical application. On the other hand, MSC display immunosuppressive characteristics through interactions with Natural Killer cells, B and T lymphocytes and monocytes⁸⁵. Until recently, MSC were considered immunoprivileged and protected against normal alloresponses⁸⁶, making them favourable candidates for allogeneic transplantation. Recent reports however indicate that MSC can engraft in immunocompromised hosts or at

immunocompromised sites, but may trigger an immune response in hosts with an intact immune system⁸⁵. Nevertheless, taking into account the immunosuppressive features of MSC, Bartholomew et al. found that survival of allogeneic skin transplants was prolonged when combined with BM-MSC, which raises possibilities for STE with allogeneic cells.⁸⁷ Even though most recent TE approaches have chosen to work with MSC, their direct contribution to vessel formation has been controversial. In addition, when considering clinical use in STE, only few sources are possible.

3.3.1.1. BM-MSC

BM-derived MSC are known to differentiate into α -smooth muscle cell actin⁺ cells, thereby enhancing wound contraction, even in the presence of a dermal scaffold⁸⁸. Markowicz et al. noted increased vascularisation when BM-derived MSC were seeded in a collagen sponge in vivo, as well as enhanced collagen production⁸⁹. There are only few reports of endothelial differentiation of BM-MSC in vivo³¹ and their contribution to neo-vascularisation is most likely related to their trophic effect on host vessels³⁴. BM-MSC also stimulate vessel maturation, through differentiation into perivascular cells³¹ and increased angiopoietin (Ang)1/Ang2 secretion ratio³². Combination of BM-MSC with BM-derived hematopoietic cells with EPC-like characteristics seems to have a synergistic effect on vascularisation in vivo⁹⁰. There is also evidence that BM-MSC can differentiate into epidermal cells, albeit in very small amounts³¹. BM-MSC were shown to augment vascularisation in a dermal scaffold in a pig model⁹¹ and to induce healing of burns and chronic wounds when combined with an artificial dermis in preliminary clinical trials^{36, 92} or acute wounds when sprayed in a fibrin glue⁹³. None of these clinical trials, however, used a control population, making the results difficult to interpret. Standard MSC culture protocols include the use of foetal calf serum, possibly leading to zoonoses or immune reactions in the host. Platelet lysate has been advocated as a surrogate for foetal calf serum in isolation of BM-MSC, but it is not clear yet whether this isolation procedure gives rise to cells with identical properties as BM-MSC obtained through the standard isolation protocol^{94, 95}.

3.3.1.2. Adipose-derived MSC

In contrast to BM-MSC, there is more evidence of differentiation of adipose-derived MSC into EC^{38, 39, 96}, even though some authors are unable to prove direct incorporation^{40, 41}. Most authors use (a modification of) the isolation protocol as initially described by Zuk and coworkers⁹⁷, that consists of enzymatic digestion of a lipo-aspirate and centrifugation to obtain the stromal vascular fraction (SVF). Adherence depletion on plastic sorts out the cell fraction that is referred to as processed lipo-aspirate (PLA) cells⁹⁸, adipose derived stromal cells⁹⁹, adipose derived stem cells⁴², or adipose-derived MSC⁴⁰, and that consists of a heterogeneous

population that contains vascular (pericytes and endothelial progenitor) cells, besides multipotent mesenchymal stem cells ¹⁰⁰. However, as adipose tissue is highly vascular ¹⁰¹ and other authors have shown the presence of EC with high proliferative capacity in the vessel wall ¹⁰², it might be possible that EC contamination accounts for the vasculogenic effect of adipose-derived MSC. Adipose-derived MSC can also differentiate towards perivascular cells, thereby increasing vessel stability ^{99, 103}. They can easily be harvested and applied in an autologous manner. Moreover, they appear to have a high expansion capacity in vitro ¹⁰⁴ and are successfully used in STE, leading to increased vascularisation and wound closure ⁴².

3.3.1.3. CB-MSC

MSC can also be grown from cord blood and can be differentiated in vitro towards cells of all three germ layers ¹⁰⁵, including cells with endothelial characteristics ¹⁰⁶. Application in a hind limb ischemia model did not result in increased angiogenesis, but rather myogenesis ⁴⁴. They have therefore been used in a clinical setting where myogenesis might be beneficial, such as in dilated cardiomyopathy ¹⁰⁷ and Buerger's disease ¹⁰⁸, but not yet for STE vascularisation purposes.

3.3.1.4. PB-MSC

After BM stimulation, MSC can even be isolated from peripheral blood ¹⁰⁹. These cells have been used to induce angiogenesis in a dermal scaffold, but there is no evidence of direct incorporation of these cells in a vascular network ⁴⁵.

Concerning the choice of tissue source for clinical use of MSC for STE, harvesting BM represents a rather invasive and painful procedure that gives rise to a limited number of MSC ¹⁰⁴. It would therefore be preferable to opt for adipose-derived MSC, as they can easily be derived from even rather skinny patients, are easy to expand and contribute both directly and indirectly to vascularisation ^{39, 98, 104}. Moreover, BM-MSC are believed to undergo quicker senescence than adipose-MSC, which makes the former somehow less favourable candidates for STE ¹⁰⁴. However, for this comparative study, BM samples were taken from patients undergoing hip surgery, a much older population than in the adipose-derived MSC group that consisted of younger patients undergoing liposuction ¹⁰⁴.

3.3.2. Multipotent adult progenitor cells (MAPC)

Other investigators report the isolation of a different BM-derived multipotent cell type that displays a vast expansion capacity combined with a broad differentiation potential that encompasses cells from the three embryonic germ layers ¹¹⁰. These MAPC are morphologically, molecularly and functionally different from BM-MSC and can in

vitro differentiate into EC that contribute to vasculogenesis in vivo^{46, 111-113} (Table 1). While MAPC represent an almost inexhaustible source of cells that can be used in STE vascularisation, the long-term procedure required to derive MAPC makes an autologous approach not feasible. Nevertheless, similar to MSC, MAPC also seem to have immunosuppressive capacity, which may be favourable in an allogeneic setting^{114, 115}. Despite their large expansion capacity, no karyotypic instabilities have been reported for human MAPC. Our recent data reveal that MAPC transplantation improves wound vascularisation as well as re-epithelialisation in a full thickness wound model in immunodeficient mice (unpublished results) (Table 1).

3.4. Pluripotent progenitor cells

3.4.1. Embryonic Stem Cells (ESC)

ESC are derived from the inner cell mass of the blastocyst-stage embryo which puts some ethical restraints on their (clinical) use. Addition of VEGF can differentiate these cells towards EC that have been used for therapeutic re-vascularisation of ischemic limbs where they were shown to directly contribute to vessels⁴⁸. ESC have a self-renewal capacity and represent therefore an inexhaustible source of EC (Table 1). They are however, due to this vast proliferative potential, prone to teratoma formation⁴⁹ (Table 1). Transplantation of selected endothelial-predifferentiated ESC can prevent teratoma, however the presence of residual undifferentiated ESC remains a concern. Immortalised ESC-derived EC have been used for tissue-engineering of blood vessels¹¹⁶, but we have no record of ESC studies on STE so far.

3.4.2. Induced pluripotent stem cells (iPSC)

As first described by Takahashi *et al.* in mice, these ESC-like cells are obtained through reprogramming of skin fibroblasts (or other somatic cells) by transduction with a combination of only 4 genes (Oct4, Sox2, c-Myc and Klf4)¹¹⁷. In 2007, iPSC were also successfully derived from human fibroblasts, by using a combination of different genes (Oct3/4, Lin28, nanog and Sox2)¹¹⁸. As this avoids the destruction of embryos, the use of iPSC is not linked to ethical issues. Moreover, unlike ESC, they can be derived from the patient, making an autologous approach feasible (Table 1). The CD34⁺, CD31⁺, CD43⁻ fraction gives rise to vascular structures in vitro¹¹⁹⁻¹²¹, but has never been used for vasculogenic purposes in vivo so far. While iPSC are not entirely similar to ESC, they also carry the risk of teratoma formation and genetic instability^{122, 123}. Moreover, the need for genetic manipulation through viral integration renders an additional risk for transformation. Therefore, clinical use of iPSC awaits the design of new selection procedures and reprogramming protocols to avoid tumorigenicity.

4. In between safety and ambition: BOEC, the ideal son-in-law?

Mature EC are no doubt safe from tumour formation and are ready to form vessels *in vivo*. They are however difficult to obtain in an autologous fashion from a patient and hard to expand in culture. On the other side of the spectrum (Fig. 7), pluripotent stem cells have high expansion capacity but are at risk for tumour formation and therefore expelled from clinical application in wound healing. Within the multipotent group, fat-derived MSC appear to be very promising, regarding their ease of harvest, effect on vascularisation and expansion capacity. They carry however the theoretical risk of malignant transformation, which implicates they should be applied with caution. In our research, we focused on BOEC because they can be harvested with minimal donor site morbidity from any patient and can constitutively be expanded without risk of tumour formation. Whether they are able to intervene in other processes of wound healing is still largely unknown (Table 1).

5. Growth factor cross-talk in wound healing: a conference call between wound healing cells

How do wounds heal exactly? What makes fibroblasts produce organised collagen matrices and who tells keratinocytes to migrate over wound edges? There is no clear neuronal axis that tells the brain that tissue is damaged somewhere and that sends healing signals back from the brain to the wound. However, the wound healing process needs to be orchestrated and therefore, communication between cells in the healing wound is essential. Wound cells communicate with each other through special signalling peptides, cytokines, that exert their effects on other cells that have receptors for these cytokines ¹²⁴. Growth factors are an interesting group of cytokines. Besides acting as mitogens, they modulate cellular behaviour, matrix production and degradation, migration and attraction of cells. Many growth factors and cytokines are involved in wound healing, where they act as signals to coordinate the healing process. Here, we focus on some of these factors that are of particular interest in wound healing in general and in this project in particular.

The Fibroblast Growth Factor (FGF) family consists of over 20 members. *FGF-2* or basic FGF stimulates angiogenesis, granulation tissue formation, fibroblast migration and proliferation, re-epithelialisation and synthesis of various extracellular matrix proteins ¹²⁵. Another member of this family, *FGF-7* or *Keratinocyte Growth Factor (KGF)* specifically stimulates migration and proliferation of keratinocytes ¹²⁶.

Macrophage/monocyte Chemo-attractant Protein-1 (MCP-1) is a chemokine that attracts monocytes and macrophages. Even though exogenous addition of MCP-1 yielded only moderate improvements in wound healing, mice lacking MCP-1

had delayed wound healing with reduced re-epithelialisation, angiogenesis and hydroxylated collagen fibres ^{127 128}.

Hepatocyte Growth Factor (HGF), also known as Scatter Factor, stimulates the motility, migration, and morphological changes of a number of cell types, including EC and epithelial cells. HGF appears to be a potent stimulator of angiogenesis and modulator of proteolytic enzymes. Keratinocytes are known to highly express the HGF receptor and have been shown, in response to HGF, to rapidly increase their migration speed, which indicates its potential role in re-epithelialisation during the healing process ¹²⁹.

Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and inflammatory cytokines as Interleukin-6 (IL-6) both induce keratinocyte proliferation. It has been suggested that GM-CSF works directly on keratinocytes, but also indirectly by upregulating IL-6. A role of GM-CSF in angiogenesis has also been suggested ¹²⁸.

Platelet Derived Growth Factor (PDGF) is one of the earlier growth factors described in wound healing ¹³⁰. It stimulates proliferation of fibroblasts and production of dermal matrix as well as matrix remodelling by secretion of matrix metalloproteinases (MMP). Although less potent than VEGF or FGF, PDGF stimulates angiogenesis but has a particular role in vessel maturation by recruitment of pericytes. PDGF also plays a role in re-epithelialisation by upregulating the production of insulin growth factor-1 (IGF-1) and thrombospondin-1 ¹²⁸. Being involved in each stage of healing, being decreased in chronic wounds and being effective in clinical wound healing trials, it is the only growth factor currently approved by FDA in chronic wound treatment ¹³¹.

Other factors act more on vessel formation and stability. Both Angiopoietins-1 and -2 (Ang-1 and Ang-2) bind the Tie-2 receptor, Ang-1 being agonistic and Ang-2 being agonistic or antagonistic, depending on the context. It has been shown that Ang-1 can also activate Tie-1 and its signal is amplified through heterodimeric interaction with Tie-2. Whereas Ang-1 is involved in vessel maturation, attraction of pericytes and adhesion of mural cells to the endothelium, Ang-2 seems to be involved in vascular remodelling ¹³².

Vascular Endothelial Growth Factor-A (VEGF-A) is a factor that is very well known for its effects on angiogenesis. It acts mainly through two receptors, VEGFR1 or fms-like tyrosine kinase-1 (Flt-1) and VEGFR2 or kinase domain receptor (KDR; fetal-liver kinase-1 or Flk-1 in mice). Whereas activation of Flt-1 leads to induced vascular permeability, stimulation of MMP-expression by vascular SMC and the induction of anti-apoptotic proteins, activation of Flk1 /KDR results in chemotaxis and proliferation of EC¹³³. The angiogenic effect of VEGF-A is mediated through increased vascular permeability, degradation of the basement membrane by MMP-

1, MMP-2 and stimulation of vascular SMC to express MMP-1, MMP-3 and MMP-9, and EC migration and proliferation^{12, 133}. We¹² and others¹²⁸ have previously shown that VEGF-A expression enhances besides angiogenesis also granulation tissue formation and re-epithelialisation.

Another member of the VEGF family, *Placental Growth Factor (PlGF)*, is a mediator of angiogenesis, by binding selectively to Flt-1. Interestingly, this factor is specifically involved in pathological angiogenesis by amplifying the effect of VEGF, in part by cross-talk between Flt-1 and Flk-1^{134, 135}. PlGF overexpression in mouse skin was shown to firmly increase density, branching and SMC coating of blood vessels¹³⁶, whereas PlGF-null mice display decreased SMC coating of blood vessels and delayed wound healing¹³⁴. During wound healing, PlGF is expressed by migrating keratinocytes and EC, acting in a paracrine and autocrine way as both these cell types express Flt-1. PlGF is however in some cases also suggested as an angiogenesis inhibitor by forming PlGF/VEGF heterodimers which are up to 50 times less potent than VEGF homodimers¹³⁷.

How these growth factors and cytokines exert their effects on wound healing is still largely unknown, but little by little the communication code of wound healing is unravelled. As growth factors are so important in wound healing, many attempts to improve wound healing are based on growth factor supplementation.

6. Oxygen: the key to wound healing?

Even though the importance of appropriate delivery of nutrients is obvious, the role of oxygen in wound healing remains controversial. Studies on wound healing¹³⁸ demonstrate that oxygen tension in the wound centre is as low as 1%¹³⁹, which is consistent with our current measurements by electron paramagnetic resonance ($2.1 \pm 0.5\%$ at day 5) in our mouse model of wound healing. This hypoxic situation is not compatible with the high metabolic demands of the cells involved in the repair process¹⁴⁰. Although inflammatory cells tend to use little oxygen as they produce energy for respiration via the hexose-monophosphate-shunt, oxidant production (for bacterial killing), collagen synthesis, angiogenesis and re-epithelialisation require oxygen in a high concentration¹⁴¹.

On the other hand, acute hypoxia is a necessary trigger to initiate many aspects of the healing process. Tissue hypoxia activates the HIF1 α -SDF-1 cascade in attraction of angiogenic cells^{140, 142}. The constitutive (aerobic) production of lactate by inflammatory cells and fibroblasts is an additional and sufficient angiogenic trigger¹⁴¹. The initial wound bed hypoxia further triggers keratinocytes to migrate over the wound edges¹⁴³. On the contrary, sustained hypoxia on the contrary leads to tissue loss¹⁴⁰ and therefore restoration of oxygen levels is mandatory for proper wound healing.

The importance of oxygen in collagen synthesis has been well documented. An important step in the development of the final helical structure of collagen is the hydroxylation of the proline residues, which is oxygen dependent ¹⁴⁴. Hypoxia leads to the formation of an unstable triple helix that cannot be used for the assembly of neatly organised collagen fibrils ¹⁴⁵. The underlying molecular mechanisms by which oxygen tension regulates matrix organisation are incompletely understood. Siddiqui *et al.* ¹⁴⁶ noted an initially increased fibroblast replication and adequately hydroxylated collagen synthesis in response to hypoxia, likely through an autocrine mechanism via hypoxia-induced up-regulation of TGFβ1. This effect disappeared promptly after 3 days, leading to reduced fibroblast proliferation and collagen synthesis. Accordingly, Steinbrech *et al.* ¹⁴⁷ described increased collagen type I mRNA expression by fibroblasts upon hypoxia in vitro, an effect that is however abruptly down-regulated after 48 hours. Unfortunately, the latter authors only measured collagen-mRNA expression and did not look for protein expression.

7. Fibroblasts: more than just a dermal matrix factory

Dermal substitutes have been used since decades to obtain a more elastic, natural skin that is less prone to contraction ^{148, 149} (Fig. 4). Whereas acellular dermal substitutes have been used experimentally and clinically to reduce wound contraction, true regeneration of the dermis was never achieved ¹⁵⁰. Dermal substitutes seeded with autologous dermal fibroblasts have proven to be far superior in restoring a properly organised dermal matrix, compared to acellular matrices ¹⁵¹. Several fibroblast-containing dermal substitutes, with or without keratinocytes, have been approved for clinical use (e.g. Dermagraft®, Apligraf®, OrCel®) ¹⁵² and all of them rely on an artificial matrix to deliver the fibroblasts. These cellular matrices are dependent on quick and decent vascularisation for the survival of the dermal and epidermal cells ¹⁵³, as cells more than 200 µm away from blood vessels become oxygen-deprived ⁶ and in skin grafts thicker than 400 µm, blood vessels cannot penetrate quickly enough to feed the epidermal layer ¹⁵⁴. It has been shown that inclusion of fibroblasts in a decellularised dermal matrix also reduces wound contraction ^{155, 156}. Indeed, administration of dermal fibroblasts to the wound decreases fibroblast invasion from the wound edges ¹⁵¹ and these invading fibroblasts are prone to differentiate into protomyofibroblasts that become myofibroblasts, probably upon TGFβ1 stimulation. Besides, fibroblasts produce numerous growth factors and other cytokines that are involved in wound healing ¹²⁸. It is beyond doubt that fibroblasts play an essential role in wound healing, hence their popularity in STE.

8. BOEC and fibroblasts: do they go together like a horse and carriage?

Fibroblasts are indispensable in dermal healing and are valuable candidates for implementation in STE. To function properly in a dermal substitute, fibroblasts need oxygen and nutrient delivery for proliferation and decent matrix production. Thus, survival of cell-seeded constructs depends on quick vascularisation. It therefore seems logical to combine fibroblasts with endothelial-like cells. Indeed, previous publications report on the fruitful combination of fibroblasts with HUVEC⁵ or HDMEC²¹. As BOEC can be harvested with minimal donor site morbidity, can be expanded *ex vivo* and are readily predestined to form vessels *in vivo*, a combined approach of BOEC and fibroblasts was most appealing to us. As the role of fibroblasts in wound healing is rather well described, we especially focused our work here on the role of BOEC in wound healing and in STE, and investigated the strategy of combining them with a dermal substitute consisting of multilayered dermal fibroblast sheets.

9. Keratinocytes: just the topping of engineered skin?

Wound closure essentially means restoration of the epidermal layer. In large defects, this is achieved by skin grafting or application of cultured keratinocytes, whereas small defects tend to re-epithelialise spontaneously. Since re-epithelialisation is impaired in chronic wounds¹⁵⁷, many wound healing strategies focus on improved keratinocyte migration, proliferation and differentiation. Importantly, keratinocytes do not act on their own, but closely interact with the other cells involved in the healing process. They produce IL-1, a factor that stimulates fibroblasts to produce important keratinocyte mitogens in return¹⁵⁸, such as IL-6, GM-CSF and KGF. A decently formed basement membrane is important for dermal-epidermal adhesion. Laminin 5 is one of the major components of the basement membrane and is exclusively produced by keratinocytes¹⁵⁹, whereas other components are both fibroblast- and keratinocyte-derived. Also, keratinocytes produce growth factors that modulate fibroblast behaviour, such as TGF- α , EGF and PDGF, and proteinases that modulate the newly formed dermal matrix to expose appropriate epitopes for their own migration¹⁶⁰. Keratinocyte-derived TGF- β is a trigger for fibroblasts to differentiate towards myofibroblasts¹⁵⁷, in an attempt to reduce the non-epithelialised wound surface by wound contraction. On the other hand, fibroblasts provide a matrix that serves as a guidance grid for migration of keratinocytes and produce cytokines that modulate keratinocyte behaviour¹²⁸. Keratinocytes should thus not be regarded as merely coverage of wounds, but as active mediators of the healing process. Therefore, they are included in many engineered skin constructs. Already in the 1980's, fibroblast-seeded dermal matrices were used clinically in combination with keratinocytes for coverage of burn wounds (Fig. 5). Besides, both cells produce angiogenic growth factors¹²⁸ to insure appropriate delivery of oxygen and nutrients. Considering the principal role both fibroblasts and keratinocytes play in wound healing, we intended to examine the possible interaction of BOEC with both key players.

*La clef de toutes les sciences est sans contredit
le point d'interrogation ;
nous devons la plupart des grandes découvertes
au comment ?*

*Et la sagesse dans la vie consiste peut-être à se demander,
à tout propos, pourquoi ?*

(Honoré de Balzac)

Research objectives

In the introduction we made the argument that vascularisation as a route to deliver oxygen is the cornerstone of the wound healing process and that BOEC have very promising features for re-vascularisation strategies. We further emphasised the importance of growth factors to orchestrate cellular communication during wound healing and the essential role of fibroblasts in STE approaches. Therefore, in this doctoral thesis the **overall aim** was to explore in a preclinical mouse model the potential of BOEC for improving wound healing, singly or in combination with a self-assembled dermal substitute, consisting of human dermal fibroblast sheets (hDFS).

The **specific objectives** of this work were to:

1. obtain a *reproducible isolation protocol* for BOEC from humans (hBOEC; to be tested in immunodeficient mice) and pigs (pBOEC; for future testing in an immunocompetent larger animal model prior to clinical use);
2. *fully characterise BOEC* in terms of gene/protein expression, in vitro functionality and growth factor expression and compare these characteristics with other cells used for vascularisation purposes such as HUVEC and early EPC in order to accurately position them in the endothelial (progenitor) cell hierarchy;
3. apply hBOEC, hDFS or a combination of both in a full thickness wound healing model, to *analyse the effects on vascularisation/oxygenation, dermal and epidermal healing and wound contraction*;
4. *compare* the latter effects on wound healing with that of *mature EC* that have previously been used in STE, such as HUVEC;
5. *dissect out the mechanisms* by which hBOEC and hDFS exert their effect on dermal and epidermal wound healing.

The main **clinical finality** of our current work was to explore the feasibility of using a *fully autologous approach* to make a vascularised dermal equivalent for reconstruction of large skin defects, such as in patients with burns or large surgical wounds.

*If a man will begin with certainties, he shall end in doubts,
but if he will be content to begin with doubts, he shall end in certainties.*

(Francis Bacon)

Peu lire, beaucoup voir, beaucoup faire.

(Baron Guillaume Dupuytren)

Materials & Methods

1. Cell derivation, culture freezing/thawing and lentiviral transduction

1.1. Human BOEC (hBOEC)

Venous blood samples (40ml) were obtained from healthy human donors volunteers age 28-35 and anticoagulated with 100 IU/ml heparin. The mononuclear cell fraction was isolated by density centrifugation using a slightly modified protocol as described by Yoder et al.⁶⁶. Briefly, blood was diluted 1:1 in PBS, containing 2% foetal bovine serum (FBS) and 1 % penicillin/streptomycin/amphotericin (PSA). This mixture was layered onto 25 ml Ficoll-Paque (GE Healthcare, Diegem, Belgium) and centrifuged at 750 g for 30 minutes. The buffy coat was aspirated, washed 3 times in PBS and cells were resuspended in EBM-2 supplemented with bullet kit (Lonza, Verviers, Belgium) and 1 % additional PSA, and plated on collagen type I-coated dishes (Greiner Bio One GmbH, Frickenhausen, Germany). During the first week, media was changed daily and afterwards every other day. Colonies with typical cobble-stone morphology started to appear from day 11 onwards (Fig. 8). Mycoplasma contamination was excluded, using the MycoalertMycoplasma Detection kit (Cambrex, Milan, Italy), following the manufacturer's instructions. hBOEC from passage 2 to 4 were used for animal experiments.

Figure 8. Isolation of hBOEC and early EPC. Mononuclear cells were obtained from peripheral blood after density centrifugation and the buffy coat was plated onto collagen type I-coated dishes (for BOEC isolation) or fibronectin-coated dishes (for early EPC isolation). Medium was changed regularly in collagen-coated plates, to remove non-adherent cells. BOEC colonies appeared after 2-3 weeks and could be expanded prior to use in our wound healing model. After 48 hours, non-adherent cells from the fibronectin-coated dishes were replated and colonies of early EPC were visible after 4-7 days.

1.2. Human early EPC

Mononuclear cell fractions were obtained from peripheral blood of healthy volunteers by density centrifugation as described above and plated at 5×10^6 cells/6-well onto fibronectin-coated dishes in Dulbecco's Modified Eagle Medium (DMEM; high glucose) supplemented with 10 % FBS and antibiotics. Forty-eight hours later, the non-adherent cells were transferred to fibronectin-coated 24-well plates and seeded at 1×10^6 cells per well. Colonies with rounded cells in the centre and larger flat cells radiating from the centre appeared after 4-7 days (Fig. 8).

1.3. Porcine BOEC (pBOEC)

One year old female Yorkshire pigs were anaesthetised with 5 ml i.m. zolazepam/tiletamine (Zoletil 100; Vibrac, Carros, France) and 2ml xylazine (Xyl-M 2%; VMD, Arendonk, Belgium). Pigs were intubated with a 7.5mm endotracheal tube and anesthesia was maintained with 0.7-1.5 % isoflurane (ISOBA, Abott Laboratories, Shering-Plough, Heist-op-den-Berg, Belgium). If possible, venous blood samples were obtained by catheterisation of the ear veins; otherwise, a transverse incision was made in the groin region, the femoral vein was dissected and catheterised. Fifty ml venous blood was aspirated, supplemented with 600mg clindamycin (Dalacin Pharmacia, Puurs, Belgium), and anticoagulated with 100 IU heparin/ml (Leo Pharma NV, Wilrijk, Belgium). Blood samples were diluted 1:1 using PBS containing 2 % FBS and 1 % penicillin/streptomycin/amphotericin (PSA). This mixture was layered onto 25 ml Histopaque 1083 (Sigma Aldrich, Bornem, Belgium) and centrifuged at 550 g for 20 minutes. The buffy coat was aspirated, washed 3 times in PBS and cells were resuspended in EBM-2 supplemented with bullet kit without hydrocortisone (Lonza, Verviers, Belgium) and 1 % additional PSA, and plated on collagen type I-coated dishes (Greiner Bio One GmbH, Frickenhausen, Germany). During the first week, media was changed daily and afterwards every other day. Colonies appeared as soon as after 5-6 days. All animals received human care in compliance with the principles of laboratory animal care (Royal Decree of 14 November 1993, Belgium) and the European Directive 86/609/EEC.

1.4. HUVEC

Umbilical cords were stored in isotonic buffer supplemented with antibiotics until the start of the isolation procedure. To obtain HUVEC, the umbilical vein was cannulated, rinsed with PBS to get rid of blood clots, injected with collagenase type I (7.5 mg/ml; Gibco) and incubated for 20 minutes at 37°C. Detached HUVEC were collected, washed with PBS and plated on gelatin-coated flasks (0.1 % gelatin) in IMDM with 20 % foetal calf serum and 1 % penicillin/streptomycin. After 24 hours cells were washed

twice with PBS to remove non-adherent hematopoietic cells. Cells were maintained in culture until 75-80 % confluence and were replated to a confluence around 20-25 %. From passage 1, HUVEC were cultured in EBM-2 supplemented with EGM-2-MV bulletkit (Lonza, Verviers, Belgium). Cells used for in vitro and in vivo experiments were between passage 5 and 7.

1.5. BOEC freezing and thawing

hBOEC or pBOEC were divided up in aliquots of 2×10^6 cells and resuspended in 1 ml freezing medium containing 70% EGM-2, 20% FBS and 10% DMSO. Samples were stored in isopropanol-buffered freezing boxes in -80°C for 48 hours, before being transferred to liquid nitrogen storage tanks. Cells were thawed rapidly by putting the freezing vials at 37°C in a water bath. The content of each freezing vial was aspirated into 9 ml of fresh growth media, taking care to gently mix the DMSO-containing freezing media with the fresh media by air-aspiration. DMSO was removed by centrifugation and cells were plated in collagen type I-coated 10 cm dishes (Greiner Bio One GmbH, Frickenhausen, Germany). The day after, medium was replaced to remove dead cells.

1.6. hBOEC and HUVEC lentiviral transduction

hBOEC at passage 1 or HUVEC at passage 5 were plated at 75-80 % confluence in collagen type I-coated plates in complete growth media. Cells were incubated with lentivirus (Multiplicity of infection or MOI 100) overnight, after which media was changed to complete growth media. Cells were expanded as described above. Transduction efficiency was monitored by FACS and was ~100% for all viruses (data not shown). Virus constructs used were: PGK-GFP (hBOEC; Fig. 9A), pTripMR1-CMVDsRed-scrambled_shRNA (hBOEC and HUVEC; control virus; Fig. 9B) and pTripMR1-CMVDsRed-shPIGF1 (hBOEC; Fig. 9C). PIGF^{KD} virus-knockdown efficiency by the latter virus was monitored by ELISA (R&DSYSTEMS) on supernatant and by qRT-PCR on RNA from the transduced cells compared to cells that were treated with control virus. Target sequences used in the pTripMR1-CMVDsRed-scrambled_shRNA virus and the pTripMR1-CMVDsRed-shPIGF1 virus were 5' GGTATCTACTAGATGTACT 3' and 5' AAGCCAGATTCTCTGAAT 3', respectively.

Figure 9. Vector maps of the lentiviral constructs for hBOEC transduction. A, PGK-GFP vector, used for scoring of hBOEC engraftment. **B,C,** PIGF transcription was knocked down using a short hairpin construct against PIGF (C). A scrambled shRNA vector (B) served as negative control. Note the presence of CMV-promoted DsRed in both vectors, providing an additional tool to trace transduced cells in vivo.

1.7. Human DFS

Dermal fibroblasts were isolated out of human skin samples, incubated with dispase II (Roche Diagnostics, Vilvoorde, Belgium) overnight at 4°C and afterwards for 3 hours at 37°C to remove the epidermis. The dermal component was finely minced and then incubated at 37°C for 3 hours with collagenase type IA (Sigma Aldrich, Bornem, Belgium). The digested dermal fragments were then gently scraped using a skin scraper, and the resulting suspension filtered through a 100 µm cell strainer (BD Biosciences, Erembodegem, Belgium). The filtrate was spun at 400 g for 7 minutes, the pellet resuspended and the cells plated on plastic, non-coated Petri dishes in media consisting of 3:1 DMEM:Ham's F12, supplemented with 1 % PSA, 10 % FBS, 4.2x10⁻⁴ mg/ml hydrocortisone, 0.021 mg/ml adenine, 2.1x10⁻⁵ mg/ml epidermal growth factor (EGF), 5x10⁻³ mg/ml transferrin, 1.4x10⁻⁶ mg/ml tri-iodothyronine, 5.3x10⁻³ mg/ml insulin and 5.3x10⁻⁵ mg/ml ascorbate. The fibroblasts were expanded up to the third passage and then grown into multilayered lawns, changing the media every third day. After 2-3 weeks, the fibroblast layers were thick enough to be manipulated using fine forceps for engineering them into a three-dimensional fibroblast sheet with the use of a carrier membrane (N-Terface, Delasco, Council Bluffs, USA) (Fig. 10).

Figure 10. Dermal fibroblast sheets serve as a dermal substitute. Dermal fibroblasts were grown in multilayered sheets that could be handled with a forceps (A). A commercially available carrier membrane facilitated DFS application in our wound healing model (B).

1.8. Human (h)Keratinocytes

Primary keratinocytes were isolated and pooled from foreskins of five different donors (less than 6 years old) as described ¹⁶². Third to fifth passage cells were used in experiments. Keratinocytes were cultured in serum-free media (Keratinocyte-SFM; Invitrogen, Belgium), which harbours several growth factors (5 mg/ml insulin, 74 ng/ml hydrocortisone, 6.7 ng/ml triiodo-L-thyronine, 50 mg/ml bovine pituitary extract, and 5 ng/ml human recombinant EGF).

2. Cell characterisation

hBOEC, early EPC, HUVEC, hDFS and hKeratinocyte cultures were analysed under various conditions for their expression profile by quantitative (q)RT-PCR. hBOEC and early EPC were phenotyped by FACS and/or immunofluorescence (a list of FACS and immunofluorescence antibodies is provided in Table 2) and were functionally evaluated by the Dil-acetylated LDL (Dil-AcLDL) uptake assay, UEA-lectin binding and by tube formation onto matrigel, as described ¹¹¹. hDFS incubated either in normoxic (20 % O₂) or hypoxic (1 % O₂) conditions, were characterised following fixation, paraffin embedding and sectioning. Sirius red-stained 7 µm sections were used for analysing matrix organisation (polarised light). Some of the hDFS were fixed in 2.5 % glutaraldehyde for transmission electron microscopy (TEM). All experiments involving human cell/tissue donors were performed with approval of the Ethical Committee of the University Hospital, UZLeuven.

Antigen	Target species	Supplier, catalogN°	Dilution	Label/ Application
vWF	Human	Santa Cruz, Sc-14014	1:20	None/IF
CD31	Human	DAKO M0823	1:25	None/IF
CD31	Human	BD Pharmingen 555445	1:20	FITC/FACS
α-SMA	human/mouse	Sigma C-6198	1:200	Cy-3/IF
CD31	Mouse	BD Pharmingen 557355	1:500	None/IF or IHC
CD45	Human	BD Pharmingen 345808	1:20	FITC/FACS
UEA lectin	Human	Sigma L9006	1:50	FITC/IF
KDR	Human	ReliatechGmBH 101-MBi32	1:10	biotin/FACS
Tie-2	Human	ReliatechGmBH 101-MBi54	1:10	biotin/FACS
VE-cadherin	Human	Cayman 160840	1:40	None/IF in vitro
AcLDL		Biomedical Technical Inc.	1:20	Dil/FACS
Pancytokeratin	Mouse	Sigma C2562	1:1000	None/IHC
Connexin43	human/mouse	Santa Cruz sc-9059	1:50	None/IF
Hypoxyprobe-1- Mab	Mouse	Chemicon clone 4.3.11.3	1:50	None/IHC
IgG1k		BD Pharmingen 555909	1:20	FITC/FACS isotype
IgG1k		BD Pharmingen 555747	1:10	biotin/FACS isotype
Flt-1 (VEGFR1)	Human	Sigma V4262	1:500	Western blot
α-tubulin	Human	Sigma T6199	1:1000	Western blot

Table 2. Antibodies used for FACS, immunohistology and Western blot.

3. Mice, surgery, transplantation and follow-up

In this study, we chose to use male mice in order to eliminate the confounding effect of female hormones on wound healing¹⁶³. Athymic nude Foxn1 males (age 9-12 weeks) were anaesthetised with an i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Atropine (0.01 mg/kg) was administered i.p. as premedication. Under sterile and temperature-controlled (37°C) conditions, standardised full thickness wounds were made with a 0.5 cm biopsy puncher (Stiefel Laboratories, Offenbach am Main, Germany) on the back of the mouse in the mid-dorsal region. A silicone ring was fixed (using Histoacryl tissue adhesive, Braun, Diegem, Belgium) and sutured around the wound (Fig. 11) and wounds were treated with saline, 5×10^5 hBOEC, 5×10^5 HUVEC, hDFS or hDFS seeded with 5×10^5 hBOEC. For dose-response studies, some mice were transplanted with 1×10^6 or 1.5×10^6 hBOEC. An occlusive dressing (Tegaderm®, 3M, Diegem, Belgium) was used to keep the wound moist.

Figure 11. Wound healing model. Using a skin biopsy puncher (A), full thickness wounds (B) were made on the back of athymic nude mice. A silicone ring was glued and sutured to splint the wound (C) and after application of HUVEC, hBOEC and/or hDFS, the wound was covered with a semi-occlusive dressing (D).

In addition, all wounded mice were housed individually to avoid fighting and to prevent removal of the occlusive wound dressing. Postoperative analgesia was achieved by a subcutaneous injection of buprenorphin (0.1 mg/kg). Every other day, digital pictures were taken (using a NikonD1 camera and Camera Control Pro software, Nikon, Brussels, Belgium) of the wounds, under isofluorane anaesthesia, at which time the occlusive dressing was renewed. In some mice, wound blood flow was monitored every other day by scanning the area including the entire ring with a Lisca PIM II (Gambro, Breda, The Netherlands; Fig. 12) under temperature-controlled conditions, using the normal skin region under the processus xyphoideus for normalisation. Vascularisation was peaking at day 4 and day 8 in hBOEC-treated wounds, which made us decide to examine the wounds histologically at time points following the two peaks, i.e. at day 5 and day 10 (Fig. 12).

Electron paramagnetic resonance (EPR) oximetry was performed under isoflurane anaesthesia and temperature-controlled conditions (37°C) using an EPR spectrometer (Magnettech, Berlin, Germany) operating at 1.2 GHz with an extended loop resonator placed over the head of the mice, 24 hours after implantation of Lithium-Phthalocyanin crystals in the wound bed¹⁶⁴. Mice were housed in specific pathogen-free conditions and procedures involving animals were performed according to the guidelines of the Institutional Animal Care and Use Committee of KULeuven.

Figure 12. Laser doppler analysis. Representative laser doppler scans of a wound treated with PBS (A) or hBOEC (B) at day 4, revealing more perfusional activity in the latter. When flow patterns were followed-up every other day after wound surgery, two peaks of increased blood flow could be detected in the hBOEC-treated wounds (black line in C), which were not observed in PBS-treated wounds (red line in C). Data in C are expressed as arbitrary units (AU) representing an average of 5 scans for each wound relative to an average of 5 scans of normal skin. Scale bars: 3mm in A,B.

4. Live imaging and wound morphometry

Live imaging of cell engraftment was performed under anaesthesia (100 mg/kg ketamine + 10 mg/kg xylazine) using a Zeiss Lumar dissection microscope (Zeiss, Zaventem, Belgium). Wound contraction was evaluated by comparing relative wound area (RWA) over time. On a two-daily basis, digital pictures were taken of the wound and RWA was calculated using ImageJ software (NIH, Baltimore, Maryland) by dividing the healing wound area by the fixed reference area inside the silicone ring and expressing it as a percentage. To account for small inter-animal variations, for each time point, relative wound area of each individual animal was expressed as percentage compared to the relative wound area at day 0

$$[RWA_{corrected} = (RWA_{dayx} / RWA_{day0}) \times 100].$$

Wound contraction % was calculated as the complement of relative wound area

$$[100 - RWA_{corrected}].$$

5. Tissue processing, histology and microscopy

At two time points (day 5 and 10) after wounding, mice were sacrificed and square skin fragments including the circular wound area and a rim of normal skin were dissected out, rinsed in PBS and post-fixed overnight at 4°C using zinc-paraformaldehyde. Following fixation, the skin fragments were separated in two equal pieces at the midline of the wound and processed for paraffin or optimal cutting temperature (OCT) embedding.

Figure 13. Chronologic presentation of the wound healing experiment. Cells were transplanted immediately after wounding. On a two-daily basis, dressings were changed and pictures were taken. According to the blood flow peaks on laser doppler examination, mice were sacrificed at day (D)5 and D10 for histological evaluation.

In some mice, half of the tissue was fixed with glutaraldehyde (2.5 % in 0.1M Na-cacodylate buffer, pH 7.3) followed by post-fixation with 2 % osmiumtetroxide and embedding in EPON 812 resin. From the polymerised resin blocks, semi-thin sections of 0.5-1 μm were made and stained with 1 % toluidin blue in 1 % borax for light microscopic observation. Ultra-thin sections (70 nm) were placed on copper grids and contrasted with 4 % uranyl acetate. For TEM, a JEM2100 microscope (JEOL, Zaventem, Belgium) at 200 kV was used to record images of collagen bundles. For all stainings and analyses, 7 or 8 μm sections were used spanning a representative area of ~ 0.7 -1.4 mm wide. Hematoxylin&eosin (H&E) and Sirius red stainings were performed as described¹⁶⁵. Sirius red is an azo-dye that interacts with positively charged groups on collagen and the resulting parallel orientation with the long axes of the collagen fibres results in enhanced birefringency under polarised light¹⁶⁶. The birefringency colour further changes as a function of collagen organisation, more specifically, the diameter and packaging of the collagen fibres¹⁶⁷: thick, closely packed collagen appears as red-orange birefringent, whereas loosely packed, thin collagen is yellow-greenish birefringent.

Detection of hypoxic cells was done using Hypoxyprobe™ (pimonidazole hydrochloride, which specifically binds to cells that have O₂ concentrations of less than 14 µM and are therefore hypoxic), according to the manufacturer's instructions (Millipore, Brussels, Belgium), as described¹⁶⁸. Briefly, 60 minutes before sacrifice at day 5 after wounding, mice were i.p. injected with 60 µg/g Hypoxyprobe™. After perfusion-fixation, wounds were dissected out, post-fixed, dehydrated and embedded in paraffin. Seven µm sections were stained with a mAb against Hypoxyprobe™, provided in the kit. Serial sections were stained for mouse and human-cell-derived blood vessels to allow for correlation studies between re-vascularisation and hypoxia. For immunohistochemistry and immunofluorescence, a list of primary antibodies and corresponding working dilutions is provided in Table 2. To rule out cross-reactivity of human- or mouse-specific antibodies or lectins, they were tested on sections of both human and mouse skin samples (Fig. 14). Pictures for morphometric analysis were taken using a Zeiss Axio Imager connected to an Axiocam MRc5 camera (Zeiss) and analysis/image recording was performed using Axiovision (Zeiss), Image J (NIH) or KS300 (Leica) software.

Figure 14. Species-selectivity of antibodies. **A,B**, Cross-sections of human skin (Hskin in **A**) and mouse skin (Mskin in **B**) with Ulex Europaeus Agglutinin (UEA)-lectin in green revealing the specific binding of this lectin to human endothelium. **C-F**, Cross-sections of human skin (Hskin in **C,D**) and mouse skin (Mskin in **E,F**) double-stained with human-specific CD31 in red and mouse-specific CD31 in green (**C,D**) or double-stained with human-specific CD31 in green and mouse-specific CD31 in red (**E,F**) revealing the species specificity of both antibodies. DAPI (blue) was used as nuclear counterstain in **A-F**. Scale bars: 100µm in **A,B**, 150µm in **C-F**.

6. Visualisation of BOEC and HUVEC engraftment

To visualise the cells using fluorescence microscopy, some animals were transplanted with hBOEC labelled with Dil-AcLDL (Fig. 23B-D) immediately before transplantation or transduced with a lentiviral construct expressing GFP (PGK-GFP; Fig. 15A,B). For some animals, HUVEC were labelled with a lentiviral construct expressing DsRed (CMV-DsRed; Fig. 15C,D and Fig. 23H). Briefly, hBOEC were incubated for 4 hours with 10 µg/ml Dil-AcLDL in EGM-2 at 37°C, and washed 3 times with PBS, prior to transplantation in the wounds. Lentiviral transduction of hBOEC or HUVEC was performed as described above (see 1.6.).

Figure 15. Engraftment of hBOEC and HUVEC. *A,B*, GFP-transduced hBOEC are visible upon transplantation in vivo (*A*) and located in the vicinity of capillaries, organised in a vessel-like structure (indicated by white arrowheads in *B*) after 10 days. *C,D*, DsRed-transduced HUVEC are detected in the wound at day 5 (*C*) in a more scattered pattern (*D*; represents inset indicated in *C*). Scale bars: 60 μ m in *D* and 200 μ m in *A-C*.

7. RNA isolation and qRT-PCR

7.1. RNA isolation and qRT-PCR

Total RNA from cell lysates was extracted using the RNeasy minikit (Qiagen, Valencia, CA). mRNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and cDNA underwent 50 amplification rounds (ABI PRISM 7700, Perkin Elmer/Applied Biosystems, Foster City, CA) as follows: 50 cycles of a 2 step PCR (95°C for 15 seconds, 60°C for 45 seconds) after initial denaturation (95°C for 10 minutes) with 1ml of cDNA template, 1ml of primer mix and 1x TaqMan SYBR Green Universal Mix PCR reaction buffer (Applied Biosystems, Lennik, Belgium). Primers used for amplification are shown in Table 3A-D. mRNA levels were normalised using GAPDH or b-actin as housekeeping gene. For comparative qRT-PCR on samples in 1 % or 20 % O₂, hypoxanthine-phosphoribosyl-transferase-1 (HPRT1) was used as housekeeping gene (see 7.2.).

hBOEC CHARACTERISATION		
Gene/protein name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Flt-1 (VEGFR1)	GGACTGACAGCAAACCCAAG	CAGCCCCGACTCCTTACTTT
PECAM (CD31)	ACTGCACAGCCTTCAACAGA	TTTCTTCCATGGGGCAAG
VE-cadherin	GTTACAGCATCGGTTGTTC	TCTGCATCCACTGCTGTCA
KDR (VEGFR2)	TCCTGTATGGAGGAGGAGGA	CGGCTCTTTCGCTTACTGTT
Tie-1	AGTGTACGAGCTGATGCGTCA	AATGCCCGCGTAAGTGAAGT
Tie-2	TGCCCAGATATTGGTGTCTT	CTCATAAAGCGTGGTATTACAGTA
vWF	GTCGAGCTGCACAGTGACAT	CCACGTAAGGAACAGAGACCA
CD34	TGCAACATCTCCCACTAAACCC	CTTAAACTCCGCACAGCTGGA
AC133	TCCTGGGGCTGCTGTTTATT	ACGCCTTGTCTTGGTAGTGTT
eNOS	GTGGACGCACAGTAACATGG	ACCTCCAGTTCTTCACACG
CXCR4	TGAGAAGCATGACGGACAAG	GACGCCAACATAGACCACCT
CD45	TGATAAGACAACAGTGAGAAAGGA	GTGTTGGGCTTTGCCCTG
CD18	TTCGCGGTGACCAGTAGGAT	CCAGGAAGACCCTGGAGGAG
BMP-2	CCCAGCGTGAAAAGAGAGAC	GGAAGCAGCAACGCTAGAAG
BMP-4	GCTTCCACCACGAAGAACAT	TCACATTGTGGTGACCACT
MMP-2	CACCTTCTGGGCAACAAAT	TGATGTCATCCTGGGACAGA
MMP-9	TGGATCCAAACTACTCGGAAGA	ACACCAAACCTGGATGACGATGC

Table 3A. PCR primers for hBOEC characterisation.

GROWTH FACTOR PROFILE ANALYSIS		
Gene/protein name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Ang-1	TCCAAAGAGGCTGGAAGGAA	CTGCCTCTGACTGGTAATGGC
Ang-2	ACACACCACAAATGGCATCTACA	TAATTGTCCACCCGCCTCC
VEGF-A	ACCAAGGCCAGCACATAGGA	AGGCCACAGGGATTTTCTT
PIGF	GCGATGAGAATCTGCACTGTGT	GAACGTGAGCTCCACGTAGGA
PDGF-B	CATTCCCAGGAGCTTTATGAG	CTCCAGCTCGCCTCCAGAG
MCP-1	GCCTCCAGCATGAAAGTCTC	CAGATCTCCTTGGCCACAAT
TGF-β1	TGAGTGGCTGTCTTTTGACG	TCTCTGTGGAGCTGAAGCAA
bFGF	GCGCATCCACCCCGA	CCAGTAATCTTC-CATCTTCCTTCATAG
KGF	ATGAACACCCGGAGCACTAC	GGGCTGGAACAGTTCACATT
IL-6	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCCTTTT
HGF	ACACCCGCTGGGAGTACTGT	TCCTTGACCTTGGATGCATTC
GM-CSF	ATGTGAATGCCATCCAGGAG	AATCTGGGTTGCACAGGAAG
MMP-14	GAGCTCAGGGCAGTGATAG	GGTAGCCCGTTCTACCTTC
MMP-1	CACAACTGCCAAATGGGCTT	CCCTTTGAAAAACCGACTTC

Table 3B. PCR primers for analysis of hBOEC and hDFS growth factor profile.

HOUSE KEEPING GENES/KERATINOCYTE MIGRATION		
Gene/protein name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC
β-actin	TGGCACCACACCTTCTACAAT	TAGCAACGTACATGGCTGGG
HPRT1	ACCCACGAAGTGTGGATA	AAGCAGATGGCCACAGAACT
B2M	TCTCTCTTTCTGGCCTGGAG	TCTTTTTCAGTGGGGGTGAA
GUSB	AGCCTGTGACCTTTGTGACC	AAGCGTTTCCACTCCATACG
YWHAZ	TGAAGCCATTGCTGAACCTTG	TATTTGTGGGACAGCATGGA
IPO8	AACCTATGCAGTGGGCATTC	GGATCTTCTTGCCACAGCTC
POLR2A	CATCATCCGAGACAATGGTG	CATCTCATCCCCGTCAAAGT
RPLPO	TCGACAATGGCAGCATCTAC	GCCTTGACCTTTTCAGCAAG
TBP	CGGCTGTTTAACTTCGCTTC	CAGGCTGTTGTCTGATCCA
Connexin43	GGCGTGAGGAAAGTACCAA	GCAGGATTCGGAAATGAAA

Table 3C. PCR primers for housekeeping gene screening and keratinocyte migration.

pBOEC CHARACTERISATION		
Gene/protein name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
PECAM (CD31)	AGAAGACGACTGCGGAGTACA	ACGACTCCACCTTCGATCAC
VE-cadherin	CAGCAACGGCTACTCACAAA	ATTCTTCCCCATGAGGCTCT
KDR (VEGFR-2)	CTCAATGTGTCCCTTTGTGC	GCTGTCCCAGGAAATTCTGT
Tie-2	TGAGCCTTACTTTGGGGATG	TGCACGCAGAGCTCATATTC
vWF	CACCCCTCAAAACAACGAGT	CTGGACCATCGTCTTCCAGT
CD34	GGAAACCACACCAGATGCTT	AGGTCTGAGGCTGGACAGAA
eNOS	CCAGCTCGACCATCTCTACC	CTCAGGATGTCTGCACGTA
CD18	GGAGGTCTCCAGGACATCAA	CCTTGCCCAGAGAAGTTCAG
CD11b	AGAAGGAGACACCCAGAGCA	GTAGGACAATGGGCGTCACT
MMP-2	AGGGCATCCAGGAGCTCTAT	GATGAACCGGTCTTGAAGA
MMP-9	GCCGACATCGTTATCCAGTT	CCTTTTGCGTTTCCGAAGTA
MMP-14	TGCAGCAGTATGGCTACCTG	TAGCGCTTCTTCGAACATT
KGF	TGACCTAGGAGCAACGATCA	GGGCTGGAACAGTTCACATT
PIGF	GGCTCTTCACTTGCTTCCTG	CACACAGTGCAGGTTCTCGT
Ang1	ATCTTAATGGGGGAGGTTGG	GCTCTGTTTTCCTGCTGTCC
Ang2	AACACTGGCTGGGAAATGAG	AGCCTCCTGTGAGCATCTGT
MCP-1	CCGAAGCTTGAATCCTCATC	TCCAGGTGGCTTATGGAGTC
TGFβ1	TGCTGTTCAACACGTCGGAG	CTGAGGTAGCGCCAGGAATC
GAPDH	ACCCAGAAGACTGTGGATGG	AGATCCACAACCGACACGTT

Table 3D. PCR primers for pBOEC characterisation.

7.2. Housekeeping gene screening

We intended to perform comparative qRT-PCR under different oxygen conditions *in vitro*. Since variation in oxygen levels may change the expression of routinely used housekeeping genes (e.g. GAPDH or β -actin) and the appropriate housekeeping gene may be different for different cell types^{169, 170}, the choice of an appropriate alternative housekeeping gene is necessary. Therefore, we performed a separate screening of a panel of housekeeping genes (Table 3C) in order to find an appropriate candidate for hKeratinocytes and hDFS (Fig. 16) by measuring the expression of these genes in high (20 %) and low (1 %) oxygen conditions using samples with identical amounts of RNA. For an ideal housekeeping gene, the ΔC_T between high and low oxygen should be close to 0 and there should be the lowest possible variation between replicates of the same oxygen condition (estimated by calculating the standard error of the mean: SEM). We indeed detected that GAPDH and β -actin expression was altered by oxygen conditions, and that HPRT1 was the most stable gene under variable oxygen conditions for hKeratinocytes and hDFS. We therefore chose for HPRT1 as housekeeping gene in PCR-experiments with varying oxygen levels (Fig. 16).

Figure 16. Housekeeping gene screening. A,B, Diagram showing the difference in threshold cycle (ΔC_T) between hypoxia (1% O₂) and normoxia (20% O₂) for each of the genes tested by qRT-PCR on RNA isolated from keratinocytes (A) or hDFS (B), revealing that only two genes (indicated with arrows) are suitable as housekeeping gene (i.e. having a very low ΔC_T) for keratinocytes (A) and only one gene (indicated with arrow) in hDFS (B). C-F, To choose between the two housekeeping genes for keratinocytes, the standard error of the mean (SEM) for each of the O₂ conditions was determined. The diagrams in C-F show the SEM for triplicates run for each gene under hypoxic (C,D) or normoxic (E,F) conditions, revealing that SEM were low and that there were no significant differences between the two genes (indicated with arrows) for keratinocytes, meaning that both genes were equally suited as housekeeping gene.

8. Cytokine production, cell proliferation/migration assays

8.1. Cytokine production, zymography and Western blotting

To assess cytokine production, hBOEC, HUVEC, or hDFS were plated/incubated at high confluence in triplicate or quadruplicate in the appropriate media and supernatant was collected, in some cases concentrated 10x using Microcon filter units (Millipore, Brussels, Belgium), and frozen. Protein concentration was determined by the Bradford assay. ELISA kits were from R&D Systems and the procedure was performed according to the manufacturer's recommendations.

Zymography was performed as previously described ¹⁶⁵. Briefly, 75mg of total protein was diluted in sampling buffer (0.04M Tris-HCl, pH 6.8, 5 % v/v glycerol, 16.6 mg/ml SDS en 0.02 mg/ml bromophenol blue) and loaded on a Novex 10 % Zymogram gelatin gel (Invitrogen, Carlsbad, CA). The gel was run in electrophoresis buffer (24 mM Tris, pH 8.3, 192 mM glycine and 0.1 % SDS) for about two hours at 126 V and 40 mA/gel.

After a 30 minute incubation in renaturing buffer (2.5 % v/v Triton X-100) and overnight incubation in developing buffer (10 mMTris, 40 mMTris.HCl, 0.2 mMNaCl, 6.67 mM CaCl₂, 0.01 % v/v Triton X-100), gels were stained for 3 hours in 0.5 % wt/v Coomassie Blue R250 in 45 % EtOH v/v, 10 % v/v acetic acid and destained by repeated stirring in destaining buffer (45 % EtOH v/v and 10 % acetic acid). Gels were transferred to storage buffer (7 % acetic acidv/v, 25 % MeOH v/v and 2 % glycerol v/v) and dried between cellofan membranes. Gelatinase activity was visible as clear white bands in the blue gel.

For Western blot analysis, samples were collected in RIPA buffer (150 mM NaCl, 1.0 % Igepal CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris pH 8.0; Sigma, Bornem, Belgium) and protein concentration was measured by the Bradford assay. 10-40 µg of protein was loaded and run at 200 V in running buffer (MOPS SDS NuPAGE running buffer; Invitrogen, Carlsbad, CA) on a 10 % Bis-Tris-NuPAGE gel (Invitrogen, Carlsbad, CA). Protein bands were transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare) by electroblotting in transfer-buffer (NuPAGE transfer buffer + 10 % methanol; Invitrogen, Carlsbad, CA). Membranes were blocked with 5 % fat-free milk in T-TBS (0.01 % Tween 80 in 20 mM Tris.HCl, pH 7.5, 0.5M NaCl) and incubated with primary antibody against Flt-1 (Table 2) and secondary antibody (horse radish peroxidase (HRP)-conjugated donkey-anti-mouse IgG from Santa-Cruz, SC2314; dilution 1:2000). Protein bands were visualised by chemoluminescence (using the ECL-kit, a system based on peroxidase-driven oxidation of luminal yielding black bands; Thermo Scientific, Eremodegem, Belgium). α -tubulin was analysed as loading control, after stripping

the membrane (2 % SDS, 0.063 M Tris pH 6.8, + mercapto-ethanol) and incubation with mouse anti-human α -tubulin (Table 2) and secondary antibody (horse radish peroxidase (HRP)-conjugated donkey-anti-mouse IgG from Santa-Cruz, SC2314; dilution 1:2000).

8.2. Cell proliferation/migration

For hKeratinocyte proliferation assays, cells were plated on collagen type I-coated plates at low confluence in regular growth media in normoxic (20 % O₂) or hypoxic (1 % O₂) conditions in quadruplicate or in the presence of 90 hours conditioned media collected from hBOEC or hDFS (see 8.1.) or growth media incubated for 90 hours in the absence of cells (non-conditioned media or NCM). Ten days after plating, cells were harvested by and counted with a NucleoCounter (ChemoMetec, Vilvoorde, Belgium). hKeratinocyte migration was evaluated in collagen type I-coated plates by measuring the area taken up by the cells in a fixed 1 mm window, 6 or 24 hours after applying a standardised scratch wound in confluent cultures of keratinocytes grown in 20 % or 1 % O₂ or in the presence of (non)conditioned media. For studying the effect of hypoxia on keratinocyte migration, we pre-conditioned the cells for 10 days in 1 % or 20 % oxygen, in order to assess the effects of *long-term* exposure to certain oxygen levels, as this is what happens during wound healing.

9. Statistical analysis

Comparison of data, expressed as mean $\pm$ SEM, was performed by using the unpaired Student's *t*-test. A Kolmogorov-Smirnov test was used to verify that the data had a Gaussian distribution, which justified the use of a parametric test. Instat3.0a software was used for statistical analyses and differences were considered significant when $P < 0.05$.

*On fait la science avec des faits, comme on fait
une maison avec des pierres ; mais une accumulation
de faits n'est pas plus une science qu'un tas de pierres
n'est une maison.*

(Henri Poincaré)

Results

1. hBOEC, early EPC, pBOEC and hDFS characterisation

1.1. hBOEC phenotype and function

Colonies with cobble-stone morphology (Fig. 17A) grew out from the collagen-adherent mononuclear fraction of peripheral blood after $\pm$ 15 days (Table 5). qRT-PCR revealed robust EC marker expression (*vWF*, *Tie-1*, *Tie-2*, *Flt-1*, *KDR*, *VE-Cadherin*, *eNOS*, *CXCR4*, *CD31* and *CD34*) which remained stable for at least 5 passages, except for *CD34* expression which decreased in later passages (Fig. 17B; Table 4). hBOEC uniformly expressed vWF, VE-cadherin, KDR, Tie-2 and CD31 protein (Fig. 17C-G). hBOEC expressed little AC133, an EPC marker, and were distinguished from terminally differentiated EC by their higher expansion capacity and a distinct expression pattern of growth factors (Table 7). hBOEC expressed no CD45 (Fig. 17H; Table 4). hBOEC functionally behaved as EC, demonstrated by uptake of acetylated LDL, tube formation on matrigel and UEA-lectin binding (Fig. 17I-K).

Figure 17. Phenotypic and functional characterisation of hBOEC. **A**, Phase-contrast picture showing a cobble-stone hBOEC colony. **B**, Diagram revealing stable expression of endothelial markers (vWF, KDR, Flt-1, Tie-2, VE-cadherin, CD31) and a slight down-regulation of CD34 (in yellow) during 5 passages (P). Data were normalised to GAPDH and expressed as % versus HUVEC (dotted line indicates expression level in HUVEC). **C,D**, hBOEC expressed VE-cadherin (red in C) and vWF (von Willebrand factor; red in D) in a typical pattern. **E-H**, FACS histograms revealing that hBOEC expressed endothelial markers (KDR, green in E; Tie2, green in F; CD31, green in G) but not the

panleukocytic marker CD45 (green in *H*). Isotypes are shown in red. **I-K**, hBOEC functionally behaved like EC, as shown by acetylated LDL uptake (AcLDL; red in *I*), tube formation on matrigel (*J*) and Ulex Europaeus Agglutinin (UEA)-lectin binding (green in *K*). DAPI (blue) was used as nuclear counterstain in *C,D,K*. Scale bars: 100 μ m in *C,D,I,K*, 200 μ m in *J* and 500 μ m in *A*.

1.2. Early EPC phenotype and function

Colonies with rounded cells in the centre and larger flat cells radiating from the centre grew out from the replated non-adherent fraction of peripheral blood within 7 days after replating (Fig. 18A). qRT-PCR revealed EC marker expression (*vWF*, *Tie-1*, *Tie-2*, *Flt-1*, *KDR*, *VE-Cadherin*, *eNOS*, *CD31* and *CD34*) that was however significantly lower compared to hBOEC (Table 4). Consistently, protein expression levels for KDR, Tie-2 and CD31 were lower as compared to hBOEC (compare Fig. 18B-D and Fig. 17E-G). Compared to hBOEC, early EPC expressed higher levels of *CXCR4*, β_2 -*integrin* (*CD18*) and *MMP-9* (Table 4), yet lower levels of *BMP-2* and *-4* and *eNOS* (Table 4). Unlike hBOEC, early EPC uniformly expressed CD45 (Fig. 18E; Table 4), suggesting their hematopoietic origin. Early EPC took up AcLDL (Fig. 18F), however did not efficiently form tubular structures on matrigel (Fig. 18G), nor did they incorporate into the endothelial layer of blood vessels in vivo (Fig. 18H), suggesting that these cells lacked proper EC functions.

Figure 18. Phenotypic and functional characterisation of early EPC. **A**, Phase-contrast picture showing an early EPC colony consisting of small round cells in the centre and flattened cells around. **C-E**, FACS histograms revealing that early EPC expressed endothelial markers (KDR, green in **B**; Tie2, green in **C**; CD31, green in **D**), besides panleukocytic marker CD45 (green in **E**). Isotypes are shown in red. **F-H**, Although early EPC take up acetylated LDL (AcLDL; red in **F**), they did not form tubes on matrigel (**G**) and did not incorporate into murine host vessels (stained with CD31 in red in **H**) in vivo but were located along the vessels, as shown by Ulex Europaeus Agglutinin (UEA)-lectin staining (green in **H**). DAPI (blue) was used as nuclear counterstain in **H**). Scale bars: 25 μ m in **F**, 50 μ m in **G**, 100 μ m in **H** and 250 μ m in **A**.

	HUVEC			hBOEC			Early EPC
	P4	P1	P2	P3	P4	P5	P0
vWF	100	165±44	144±40	223±91	141±59	203±96	8±6 ^a
Tie1	100	161±27	120±14	146±37	172±49	116±13	12±8 ^a
Tie2	100	483±127	481±101	661±165	621±206	638±106	100±66 ^a
VEc	100	86±13	72±11	84±10	91±16	117±19	7±4 ^a
CD31	100	403±67	338±55	521±115	482±100	597±110	294±113
Flt-1	100	68±9	55±11	67±9	85±21	88±15	58±32
KDR	100	298±31	291±31	345±62	266±53	338±86	51±28 ^a
eNOS	100	2990±905	4041±1270	4679±1914	4312±1839	3884±1661	56±36 ^a
CXCR4	100	103±23	86±29	72±28	100±41	86±48	1750±991 ^a
CD34	100	95±38	40±16	59±30	59±19	28±9	7±6
CD18	100	3039±961	221±101	153±63	97±29	75±37	6x105±3x105 ^a
BMP-2	100	48±14	55±34	101±60	25±11	25±5	1±1 ^a
BMP-4	100	161±35	104±30	140±55	111±28	ND	3±1 ^a
MMP-2^b	ND	70±51	162±74	173±127	225±135	ND	136±35
MMP-9^b	ND	1±1	0.26±0.26	0.03±0.02	0.04±0.03	ND	118±52 ^c
CD45^b	ND	0	0	0	0	0	6±1 ^a

Table 4. Gene expression profile of hBOEC and early EPC. Data represent mean (± SEM) of 4-6 independent isolations, determined by qRT-PCR, expressed as % versus HUVEC and normalised to GAPDH. ^a: $P < 0.05$ versus hBOEC-passage (P)1; ^b: MMP-2, -9 and CD45 expression levels were expressed as % versus universal RNA, the mononuclear fraction of peripheral blood or bone marrow, respectively, and normalised to GAPDH; ^c: $P = 0.06$ versus hBOEC-P1; ND: not determined.

1.3. hDFS structure and function

Human dermal fibroblasts were obtained from skin samples derived from healthy donors and tissue-engineered into a multilayered construct (Fig. 19A,B). The N-terface carrier allowed us to superimpose multiple sheets, thereby increasing the thickness of the construct, and to easily apply it to the wound bed (Fig. 10). After 10 days of culture, fibroblasts deposited ECM components, including fibrillar collagens as demonstrated by Sirius Red staining (Fig. 19C,D), thus fulfilling the principal role of dermal fibroblasts in situ.

Figure 19. Characterisation of hDFS. A. hDFS in vitro, as a thin sheet that can be manipulated with forceps. B-D, hDFS revealed on cross-sections by H&E (B), Sirius red (SRed in C) and on toluidin-blue-stained semi-thin sections (D). Arrowheads in D indicate extracellular matrix. Scale bars: 10 μ m in D, 100 μ m in B,C.

1.4. pBOEC phenotype and function

Porcine blood generally yielded higher numbers of mononuclear cells (MNC) and outgrowth of BOEC colonies, but the number of colonies per 10^9 MNC remained quite comparable to hBOEC (Table 5). The expression of surface markers and functional behaviour was very similar to that of hBOEC (Fig. 20; Table 6).

N° of Donors	Donor age (years)	N° of MNC (N°x10 ⁶)	N° of colonies	Day appearance of 1 st colony	N°colonies/10 ⁹ MNC
hBOEC					
6 males	29±2	97±11	5±1	15±1	52±7
2 females	22±2	89±4	6±1	13±2	68±19
pBOEC					
8 females	1±0	511±1	39±8	6±0	65±19

Table 5. hBOEC and pBOEC derivation parameters. In total, for hBOEC, 28 isolations were done for the current and other studies from 8 different donors (6 males; 2 females). Isolations were successful in 7/8 of these donors and in 26/28 of the isolations. From the 26 successful isolations, on average 54 hBOEC colonies/10⁹ mononuclear cells (MNC) (range: 11-100/10⁹) grew out beyond day 15 (range: 11-26 days). The average data are given in the table for each gender separately. For pBOEC, 8 isolations were performed from 1 year old Yorkshire females. In comparison to hBOEC, a firmly increased MNC fraction yielded much more colonies that appeared much earlier, but the number of colonies per 10⁹ MNC stayed remarkably similar to that of hBOEC.

Endothelial markers *vWF*, *Tie-2*, *KDR*, *CD31* and *eNOS* were stably expressed, whereas *CD18* expression firmly decreased upon passaging. pBOEC expressed little to no monocytic marker *CD11b* (Table 6). Unlike hBOEC isolation protocols, pBOEC isolation protocols are rather poorly documented. We therefore explored two coatings, i.e. collagen type I-coated and fibronectin-coated dishes but did not notice any difference in expression of *vWF*, *KDR*, *PECAM*, *eNOS*, or *CD34* (data not shown). Liquid nitrogen storage of therapeutic cells is important for proper planning of experiments. We therefore examined stability of endothelial marker expression after freezing and thawing and found no significant differences in endothelial marker expression before freezing and after thawing in 3 different isolations (Fig. 21; all *P*-values are > 0.05).

Figure 20. Characterisation of pBOEC. **A**, Phase-contrast picture showing a cobble-stone pBOEC colony. **B**, Diagram revealing stable expression of endothelial markers (vWF, KDR, VE-cadherin, CD31, CD34 and eNOS) during 5 passages (P). **C**, pBOEC expressed VEC (VE-cadherin) in a typical pattern. **D-G**, FACS histograms revealing that pBOEC expressed endothelial markers (KDR, green in **E**; Tie2, green in **F**; CD31, green in **G**) but not the panleukocytic marker CD45 (green in **H**). Isotypes are shown in red. **H-J**, pBOEC functionally behaved like EC, as shown by acetylated-LDL uptake (AcLDL; red in **H**), tube formation on matrigel (**I**) and lectin binding (Bandeiraea simplicifolia-I or BS-I; green in **J**). DAPI (blue) was used as nuclear counterstain in **C, J**. Scale bars: 50 µm in **C**, 100 µm in **H-J**, 200 µm in **J** and 500 µm in **A**.

	Pig aorta EC		pBOEC		
	P3	P1	P2	P3	P4-P5
vWF	100	64±44	70±27	50±27	23±5
Tie-2	100	130±26	94±22	128±65	97±19
Vec	100	40901±1508	5803±1619	5130±1343	9046±2155
CD31	100	190±59	173±52	212±67	325±84
KDR	100	679±480	1034±616	3174±1957	944± 546
eNOS	100	209±65	219±78	267±90	262±58
CD34	100	1213±353	984±327	1076±328	883±183
CD18	100	885±597	183±164	129±89	7±4
CD11b^a	100	3±2	5±2	2±1	0±0

Table 6. Gene expression profile of pBOEC. Data represent mean ($\pm$ SEM) of 4-6 independent isolations, determined by qRT-PCR, expressed as % versus Pig aorta EC and normalised to GAPDH. *: CD11b expression levels were expressed as % versus the mononuclear fraction of peripheral blood and normalised to GAPDH.

Figure 21. Comparison of endothelial marker expression before freezing and after thawing. No significant differences were noticed in endothelial marker expression (vWF: $P=0.06$; VE-Cadherin: $P=0.08$; KDR: $P=0.34$; PECAM: $P=0.1$; eNOS: $P=0.43$; CD34: $P=0.09$) before freezing (blue bars) or after thawing (red bars).

1.5. hBOEC, hDFS and pBOEC growth factor production

1.5.1. hBOEC and hDFS

We hypothesised that hBOEC exert trophic effects on wound healing by producing factors communicating with endogenous vascular and other cell types. As hDFS are thought to synthesise similar factors, we compared hBOEC and hDFS expression levels with those found in mature EC (HUVEC). hBOEC expressed higher levels of *VEGF-A*, *PIGF*, *PDGF-B*, *Ang-2*, *MCP-1* and *GM-CSF*, allowing cross-talk with endogenous EC, SMC and inflammatory cells (Table 7). hBOEC also expressed several *MMP* (*MMP-1*, *-9* and *-14*) that may modulate collagen metabolism, and, to a comparable extent as HUVEC, *bFGF*. hBOEC expressed factors that stimulate keratinocyte proliferation and migration, including *KGF*, *IL-6*, *HGF* and *GM-CSF* (Table 7).

Most strikingly, *KGF* mRNA expression was 280-fold higher in hBOEC compared to HUVEC. Interestingly, hDFS had a distinctly different growth factor expression profile from hBOEC: hDFS did not express *PDGF-B*, *Ang-2*, *GM-CSF* and only minimally *MCP-1* and *PIGF*, but did produce readily detectable levels of *Ang-1* (Table 7).

Compared to hBOEC, hDFS expressed ~250-fold and ~19-fold higher levels of *HGF* and *KGF*, respectively (Table 7). While *MMP-14* expression was similar in hDFS and hBOEC, the latter secreted slightly more active MMP-9 (involved in vessel formation) than hDFS (Fig. 22). On the other hand, hDFS expressed significantly more mRNA for MMP-1, important for fibrillar collagen remodelling (Table 7).

	HUVEC (P4) ^a		hBOEC (P4) ^a		hDFS ^a	
	RNA	Protein	RNA (%)	Protein	RNA (%)	Protein
Communication with vascular/inflammatory cells						
-VEGF-A	100	ND	204±13 ^b	ND	199±78 ^c	ND
-PIGF	100	1800±160	300±37 ^b	3300±160 ^b	3±1 ^{b,d}	7±2 ^d
-PDGF-BB	100	< 1.4	259±14 ^b	46±18	0±0 ^{b,d}	0±0 ^d
-Angiopoietin-1	100	ND	0±0 ^b	ND	855±50 ^{b,d}	ND
-Angiopoietin-2	100	ND	861±107 ^b	ND	0±0 ^{b,d}	ND
-MCP-1	100	ND	293±59 ^b	ND	4±1 ^{b,d}	ND
-bFGF	100	ND	169±81	ND	65±3 ^b	ND
Communication with fibroblasts (matrix)						
-MMP-9	100	ND	ND	Fig.22	ND	Fig.22
-MMP-14	100	ND	73±9	ND	85±6	ND
-MMP-1	100	ND	218±18 ^b	ND	7684±560 ^{b,d}	ND
Communication with keratinocytes (proliferation/migration)						
-KGF	100	0±0	28000±3000 ^b	2±1 ^b	525000±88000 ^{b,d}	23±3 ^{b,d}
-Interleukin-6	100	ND	63±10	ND	26±17 ^b	ND
-HGF	100	ND	132±43	ND	34000±7000 ^{b,d}	ND
-GM-CSF	100	ND	6000±900 ^b	ND	0±0 ^{b,d}	ND

Table 7. Growth factor expression profile of hBOEC and hDFS. RNA data represent mean (± SEM) of 3-5 independent isolations, determined by qRT-PCR and expressed as % versus HUVEC and normalised to β -actin. Protein data represent mean (± SEM) of 3 independent supernatant samples, determined by ELISA and expressed as pg/mg protein; Statistical comparisons were performed on the absolute (not relative to HUVEC) expression data; ^a: Gene/protein levels were determined on cells incubated in normoxia (20% O₂); ^b: *P*<0.05 and ^c: *P*=0.08 versus HUVEC; ^d: *P*<0.05 versus hBOEC; ND: not determined

Figure 22. Zymography on hBOEC and hDFS supernatants. Zymogram on conditioned media of hBOEC (BCM; lane 3) or hDFS (FCM; lane 4) and on non-conditioned media (NCM; lane 5) compared to APMA-activated MMP-9 control (lane 2). The most prominent upper bands (indicated by a dashed rectangle) correspond to active MMP-9 (83 kDa), revealing more active MMP-9 in BCM. Values are arbitrary units determined by densitometry, using Image J software. Lower bands correspond to active MMP-2 (62 kDa). Lane 1 is a molecular weight ladder.

1.5.2. pBOEC

Comparable to hBOEC, pBOEC abundantly produced PIGF, but they seemed to produce less angiogenic factors and factors that communicate with fibroblasts or keratinocytes (Table 8). Unfortunately, the porcine genome is incompletely sequenced yet and therefore we were unable to perform the same extensive qRT-PCR analysis as for hBOEC.

Pig testis		pBOEC		
Communication with vascular/inflammatory cells				
	PIGF	Ang-1	Ang-2	MCP-1
100	8596±1984	0±0	54±14	29±7
Communication with fibroblasts (matrix)				
	MMP-2	MMP-9	MMP-14	TGFβ1
100	103±14	7±1	8±1	134±10
Communication with keratinocytes				
	KGF			
100	1±0			

Table 8. Growth factor expression profile of pBOEC. RNA data represent mean ($\pm$ SEM) of 3 separate sets of pBOEC, determined by qRT-PCR and expressed as % versus pig testis RNA and normalised to pGAPDH.

2. hBOEC, unlike HUVEC, induce robust wound vascularisation and re-oxygenation

2.1. hBOEC and HUVEC engraftment

When acetylated LDL-labelled hBOEC were applied onto the full thickness wounds, they were evenly distributed over the wound bed two days later (Fig. 23A,B) and detected until day 10, sometimes clearly co-localised with blood-filled vessels (Fig. 23C,D). Given the potential leakage of acetylated-LDL and subsequent uptake by host cells, the engraftment pattern as shown by acetylated-LDL was confirmed using a different method, i.e. by transplanting hBOEC transduced with a lentiviral construct harbouring a GFP expression cassette (Fig. 15A,B). Since UEA-lectin binds hBOEC (Fig. 14A) but not murine EC (Fig. 14B), we used UEA-lectin to quantify hBOEC engraftment. At day 5, 138 ± 23 UEA-lectin⁺ cells/mm² ($N=4$) were detected in the wound bed, some of which actively participated in the formation of large vessels at the wound borders (Fig. 23F), while others were scattered as single cells in the wound centre. At day 10, only few hBOEC were traceable. Using species-specific CD31 antibodies or human-specific UEA-lectin, we observed formation of functional hybrid vessels containing hBOEC and adjacent murine EC (Fig. 23E).

Figure 23. hBOEC and HUVEC engraftment. **A**, Wound with silicone ring. **B-D**, AcLDL-carrying hBOEC were homogenously distributed (white arrowheads in **B**) in the wound at day 2, were located in vicinity to vessels (dashed white lines in **C**) and sometimes formed a sleeve around blood-filled channels (white arrowheads; **D**). Green autofluorescence was used as background in **D**. **E-G**, hBOEC (revealed by green human-specific CD31) incorporated into mouse vessels (revealed by red mouse-specific CD31) and formed hybrid vessels (incorporated hBOEC indicated by white arrowheads and lumen indicated by asterisk in **E**). hBOEC incorporated in vessels in the border (day 5) and in the centre (day 10) of wounds (hBOEC-derived vessels in green and mouse-derived vessels in red in **E-G**). **H-J**, In some animals, HUVEC could be traced by their expression of dsRed in the wound edges at day 5 (**H**). HUVEC (stained with UEA-lectin in green in **I,J**) also incorporated into murine vessels (mCD31 in red in **I,J**) in the wound edge at day 5, however some cord-like structures in the centre of the wound were not connected to the host vasculature (**J**). DAPI was used as nuclear counterstain in **E-J**. Scale bars: 3 μ m in **E**, 20 μ m in **D,F,G**, 25 μ m in **H-J**, 150 μ m in **C**, 1 mm in **B**, and 4 mm in **A**.

HUVEC were found in the wound edges at day 5, some of them making hybrid vessels with mouse cells (Fig. 23*H,I*). Cord-like structures of HUVEC were also found in the wound centre at day 5, however these did not connect with the host vasculature and were therefore not functional (Fig. 23*J*). On the contrary, when human early EPC were applied in this wound healing model, we were unable to find cells of human origin incorporated into blood vessels (Fig. 18*H*).

2.2. Effect of hBOEC and HUVEC on vascularisation and re-oxygenation

Two vascularisation peaks were identified by Laser Doppler - at day 4 and 8 - in hBOEC but not in saline-treated wounds (Fig. 12C). At day 5, vessels were predominantly found at the wound borders, $8 \pm 1\%$ of which contained hBOEC (Fig. 23F), indicating a significant direct contribution to wound vascularisation. HUVEC also incorporated in host vessels, although much less frequently. At day 10, vessels were observed throughout the wound, however hBOEC were only occasionally found in these (Fig. 23E,G). The presence of erythrocytes in hBOEC-derived vessels indicated their functional connection to the host vascular network (Fig. 23E-G). In contrast, many HUVEC structures did not contain blood cells, perhaps indicating their failure to connect to the host vasculature (Fig. 23J). Based on their expression of angiogenic factors (Table 7), we assumed hBOEC to stimulate host angiogenesis. Indeed, wound edges of hBOEC-treated mice contained twice as much mouse (m)CD31⁺vessels than those of the saline-treated mice (Fig. 24A,B; Table 9). At day 10, the entire wound contained more mCD31⁺vessels when seeded with hBOEC, compared to the saline group (Fig. 24C,D; In contrast, HUVEC treated wounds showed no increased host angiogenic response (Table 9).

Interestingly, dose-response studies revealed that upon increasing the hBOEC dose from 0.5×10^6 to 1×10^6 or 1.5×10^6 , there was no increase in mCD31⁺ area fraction (data not shown), however, the number of vessels per area increased significantly when using higher hBOEC doses (Fig. 24I-J; number of mCD31⁺ vessels/mm²: 376 ± 14 for 1×10^6 hBOEC; 376 ± 39 for 1.5×10^6 hBOEC and 271 ± 27 for 0.5×10^6 hBOEC; $N=4-6$; $P<0.05$ and $P<0.05$ versus 0.5×10^6 hBOEC). Interestingly, despite their engraftment in wounds, HUVEC transplantation did not increase the mCD31-positive area fraction compared to saline-treated wounds (Table 9).

Since hBOEC secreted PIGF and PDGF-BB, factors that have a chemo-attractive and/or proliferative effect on SMC, we determined whether hBOEC increased SMC-coated vessels. At day 5, hBOEC-treated wounds contained twice the amount of SMC-actin (SMA)-coated vessels compared to saline-treated or HUVEC-treated wounds (Fig. 24E,F; Table 9). hBOEC-treated wounds also contained more SMA⁺ vessels than the saline-infused wounds at day 10 (265 ± 22 versus 179 ± 16 ; $N=5$; $P<0.05$; Fig. 24G,H).

Wound revascularisation and closure in early phase (day 5)					
	Saline	HUVEC	hBOEC	hDFS	hDFS +hBOEC
Wound revascularisation; N=6-12					
-mCD31 ⁺ area fraction (%) ^a	6±1	5±1	12±1 ^{b,d}	10±1 ^b	13±1 ^{b,c}
-N° α -SMA ⁺ vessels/mm ²	97±11	87±11	208±29 ^b	205±26 ^b	180±15 ^b
Wound closure; N=6-12					
-% contraction (day4)	22±3	25±4	21±7	17±4	21±4
-% myofibroblast area fraction (%)	3.8±0.4	5.0±2.0	3.3±1.0	0.7±0.3 ^b	0.4±0.1 ^b
-epidermal closure					
-% epithelial coverage ^f	14±3	19±3	34±7 ^{b,e}	60±14 ^b	43±10 ^b
-N° of keratinocytes/mm ^g	410±40	ND	410±30	440±30	590±40 ^{b,c}
-% connexin43 ^{low} keratinocytes	59±3	57±2	77±4 ^{b,d}	ND	ND
Wound revascularisation, closure and matrix organisation in late phase (day 10)					
	Saline	HUVEC	hBOEC	hDFS	hDFS +hBOEC
Wound revascularisation; N=8-11					
-mCD31 ⁺ area fraction (%) ^h	8±2	5±1	25±5 ^b	10±3	24±5 ^{b,c}
Wound closure					
-% contraction; N=6-12	81±3	84±3	79±4	67±5 ⁱ	59±8 ^j
-epidermal closure					
-% epithelial coverage ^f ; N=6-23	87±4	94±3	99±1 ^b	98±4 ^b	100±0 ^b
-epithelial thickness (μ m) ^k ; N=7-11	66±6	ND	53±6	47±8	98±13 ^{b,c}
Wound matrix organisation; N=6-15					
-% fibrillar collagen	58±3	62±3	63±3	67±3 ^b	71±2 ^{b,i}
-% red-birefringent collagen	28±3	28±3	47±2 ^{b,d}	32±3	48±5 ^c

Table 9. Early and late effects of HUVEC, hBOEC, hDFS or hDFS+hBOEC on wound healing. Data represent mean ($\pm$ SEM). ^a: % of total area in wound borders; ^b: $P < 0.05$ versus saline; ^c: $P < 0.05$ versus hDFS; ^d: $P < 0.05$ versus HUVEC; ^e: $P = 0.06$ versus HUVEC; ^f: % of total wound length, defined as the distance between the two first appearing hair follicles; ^g: counted in the advancing epithelial edges; ^h: % of total area in entire wound bed; ⁱ: $P = 0.07$ versus saline; ^j: $P < 0.05$ versus hBOEC; ^k: average thickness determined over the entire wound length; ^l: $P = 0.05$ versus saline hBOEC; SMA: smooth muscle cell actin; ND: not determined.

Figure 24. hBOEC induce angiogenesis and vessel maturation in murine wounds. A-D, Cross-sections of wound borders at day 5 (A,B) and wound centres at day 10 (C,D); stained with green mouse-specific CD31) revealing more vessels in hBOEC-treated (B,D) compared to PBS-treated wounds (A,C). E-H, Wound cross-sections at day 5 (E,F) and day 10 (G,H) double-stained for EC in green (revealed by a combination of mouse-specific CD31 and human-specific Ulex Europaeus Agglutinin (UEA)-lectin) and SMC(revealed by red SMC- α -actin) showing more SMC-coated vessels in hBOEC-treated (F,H) than in PBS-treated (E,G) wounds. I-K, In comparison to wounds treated with regular dose of 5×10^5 hBOEC (I), doubling or tripling (J) BOEC doses resulted in significantly increased vessel number (K). Scale bars: 30 μ m in I,J, 150 μ m in E,F, and 200 μ m in A-D,G,H.

Since wound revascularisation is supposed to relieve hypoxia, we tested whether hBOEC-induced vascular expansion decreased the amount of hypoxic cells at 5 days. Therefore, a separate set of animals was injected with Hypoxyprobe™ and hypoxic cells were traced by immunohistochemistry. hBOEC treatment significantly reduced the hypoxic cell fraction by ~40% (24 ± 1 in saline-treated versus 15 ± 1 in hBOEC-treated wounds; $N=5$; $P<0.05$; Fig. 25 AC). Moreover, the decrease in hypoxic cell fraction inversely correlated with the area taken up by blood vessels ($R^2=0.545$; $P<0.05$; Fig. 25F) and hypoxic cells mainly resided in avascular areas (Fig. 25D,E).

Figure 25. Increased wound bed vascularisation by BOEC correlates with decreased hypoxia. **A-C**, Wound cross-sections at day 5 stained with a mAb against HypoxyprobeTM, revealing more hypoxic cells in saline-treated (A; white bar in C; expressed as % of total cell number) versus hBOEC-treated wounds (B; black bar in C). **D-F**, Cross-sections (D,E serial to A,B) of wounds at day 5 double-stained for mouse/human EC (revealed by red mouse-specific CD31 and green UEA-lectin, respectively) revealing more vessels in hBOEC-treated (E) compared to saline-treated wounds (D). Note that hypoxic cells (indicated by white arrowheads in A,B) resided in avascular areas. Panel F reveals that the extent of hypoxia (expressed as % hypoxic cells) inversely correlated with the vascular area (expressed as % of the total area). Scale bars: 20 μ m in A,B,D and E.

2.3. hBOEC, unlike HUVEC, improve dermal matrix organisation and accelerate re-epithelialisation

Next, we evaluated epidermal and dermal healing in terms of re-epithelialisation and collagen deposition/organisation. Compared to saline and HUVEC, hBOEC transplantation increased re-epithelialisation at day 5 (Fig. 26A,B; Table 9). The fraction of migrating keratinocytes, detected by down-regulation of connexin43, was higher in the presence of hBOEC (Fig. 26C,D; Table 9), while HUVEC did not affect connexin43 expression (Table 9). At day 10, all hBOEC-treated wounds were re-epithelialised completely, while re-epithelialisation was only $87 \pm 4\%$ in the saline group and $94 \pm 3\%$ in the HUVEC group (Table 9).

Figure 26. hBOEC induce re-epithelialisation in vivo. *A,B*, The epidermal front (revealed by pancytokeratin (PCK)) moved faster over the wound in hBOEC-treated (*B*) compared to PBS-treated wounds (*A*). Hair follicles marking wound boundaries and the length covered by the epidermis are indicated by white arrowheads and black lines, respectively. *C,D*, Cross-sections stained with connexin43 in red showing more migrating (i.e. cells that have down-regulated connexin43) keratinocytes in the epidermal tongue (indicated by white dashed lines) of hBOEC-treated (*D*) versus PBS-treated (*C*) wounds. Scale bars: 75 μm in *C,D*, and 500 μm in *A,B*.

To evaluate fibrillar collagen deposition and organisation, we analysed Sirius red-stained sections under bright (deposition) and polarised (organisation) light. The presence of neither hBOEC nor HUVEC increased collagen deposition (% fibrillar collagen; Table 9) but unlike HUVEC, hBOEC improved organisation at day 10 (% red birefringent collagen; Fig. 27A,D; Table 9). This improvement in organisation was confirmed on semi-thin sections (Fig. 27B,E) and TEM images that clearly visualise thick aligned collagen bundles in BOEC treated wounds (Fig. 27C,F). Intuigingly, tripling the hBOEC dose to 1.5×10^6 nearly doubled the percentage of organized collagen (not shown).

Figure 27. hBOEC improve dermal matrix organisation in vivo. Collagen organisation was analysed by Sirius red polarisation microscopy (A,D), on semi-thin sections (B,E) and by TEM(C,F), revealing that collagen was more aligned in thick bundles (shown by the red birefringence, the thicker blue matrix structures (indicated by black arrowheads) on semi-thin sections and the parallel positioning of the collagen fibres on TEM) in hBOEC-treated (D-F) compared to PBS-treated (A-C) wounds. Blood vessels are indicated by white arrowheads in B,E. Scale bars: 0.5 μm in C,F, 25 μm in B,E and 50 μm in A,D.

3. hBOEC stimulate healing by improving oxygenation and by growth factor cross-talk

3.1. Effect of oxygenation

As increased vascularisation by hBOEC improved wound bed oxygenation (Fig. 25), we studied the effect of increased O_2 levels on cultured hDFS and keratinocytes. hDFS or keratinocytes were cultured in hypoxic (1% O_2) or normoxic (20% O_2) conditions for 10 days (corresponding to the duration of our in vivo experiment). A review of the literature reveals that O_2 levels in healing human wounds are estimated to be < 1.5% O_2 in the wound centre^{138, 171}. The use of occlusive dressings even further decreases O_2 tension in wounds¹⁷². Measurement of O_2 tension by electron paramagnetic resonance (EPR) oximetry¹⁶⁴ revealed that the oxygen levels in the wound centre of mice were in a similar low range ($2.7 \pm 0.6\%$ and $2.1 \pm 0.5\%$ 1 and 5 days after wounding, respectively ($N=3-6$)).

3.1.1. Oxygen and collagen organisation

High O₂ levels resulted in increased organisation of fibrillar collagen in hDFS (% red birefringent collagen: 13 ± 3 at 1% O₂ vs. 32 ± 2 at 20% O₂; $N=4$; $P<0.005$; Fig. 28A,B), further confirmed by semi-thin sections (Fig. 28E,F). Interestingly, qRT-PCR analysis revealed higher MMP-1 expression in hDFS in hypoxic conditions, perhaps causing increased collagen destruction (Fig. 28H).

Figure 28. Collagen organisation in hDFS in vitro is stimulated by O₂ and BOEC-CM. A-C, Sirius red-stained cross-sections (viewed under polarised light) of hDFS in 1% O₂ without hBOEC-conditioned media (BCM; A, red bar in D) or 20% O₂ in the absence (B, black bar in D) or presence (C, grey and blue bar in D) of BCM, revealing more organised (i.e. red-birefringent collagen; red BF collag.; % versus total area in D) collagen in 20% O₂, further increased by BCM. E-G, Semi-thin sections revealing thicker collagen bundles (black arrowheads in E-G) in normoxia (F) compared to hypoxia (E) and even more thick bundles in the presence of BCM (G). qRT-PCR analysis revealed higher MMP-1 expression in hDFS in hypoxic conditions (H) Scale bars: 10 μ m in E-G and 50 μ m in A-C.

3.1.2. Oxygen and keratinocyte behaviour

Keratinocytes migrated more slowly on a collagen-coated surface after long-term exposure to hypoxia in a scratch-wounding assay (area covered by keratinocytes in a 1 mm window 24 hours after wounding (μ m² $\times 10^5$): 2.8 ± 0.4 at 1% O₂ versus 5.1 ± 0.3 at 20% O₂; $N=4$; $P<0.05$; Fig. 29A,B). In agreement, qRT-PCR revealed that 24 hours after wounding, keratinocytes expressed 3-fold more connexin43 in hypoxia ($N=3$; $P<0.05$; Fig. 30), corresponding to a less migratory phenotype. In addition, lowering O₂ levels significantly decreased keratinocyte proliferation by a factor of 1.5 ($N=4-6$; $P<0.005$; Fig. 29F,G).

Figure 29. Keratinocyte migration and proliferation in vitro is stimulated by O_2 and BOEC-CM.

A-E, Wounding assay revealing more migrating keratinocytes (KC; area in μm^2 occupied by migrating KC in a 1 mm window in **M**) at 24 hours in normoxia (**B**; black bar in **E**) versus hypoxia (**A**, white bar in **E**). Migration was also increased with BCM (**D**; 'B' and grey bar in **E**) compared to NCM (**C**; 'N' and red bar in **E**). Moreover, hDFS-CM (**F** and blue bar in **E**) also increased KC migration. Original wound is indicated by dashed white lines and the moving KC front by wavy black lines. **F-J**, Proliferation assay of KC grown in normoxia (**G**; black bar in **J**) or hypoxia (**F**; white bar in **J**) revealing more cell expansion in normoxia (number of cells/ cm^2 in **J**). In addition, BCM (**I**; 'B' and grey bar in **J**), and even more so, hDFS-CM (**F** and blue bar in **J**) increased KC proliferation compared to NCM (**H**; 'N' and red bar in **J**). *: $P < 0.05$ versus hypoxia or NCM. Scale bars in all panels: 300 μm .

Figure 30. Connexin43 expression by keratinocytes is down-regulated due to low O_2 and hBOEC-CM in vitro. Diagram showing the expression of *connexin43* (Cx43), expressed as % ($\pm$ SEM) versus universal RNA, in keratinocytes grown under hypoxic (1% O_2) and normoxic (20% O_2) conditions or with hBOEC-CM (BCM), hDFS-CM (FCM) or non-conditioned media (NCM), using *HPRT1* as a housekeeping gene. Keratinocytes had significantly less expression of Cx43 (corresponding to a more migrating phenotype) under normoxic conditions and in the presence of BCM or FCM. *: $P < 0.05$ versus 1% O_2 ; #: $P < 0.05$ versus NCM.

3.2. Effect of growth factor cross-talk

hBOEC communicate with fibroblasts and keratinocytes via factors that influence dermal organisation or keratinocyte migration/proliferation (Table 7). Collagen produced by hDFS cultured in hBOEC-conditioned media was significantly more organised compared to non-conditioned media (% red birefringent collagen: 32 ± 2 with non-conditioned media versus 51 ± 2 with hBOEC-conditioned media; $N=4$; $P<0.005$; Fig. 28B,C; confirmed on semi-thin sections; Fig. 28F,G) suggesting a paracrine effect of hBOEC on hDFS.

No significant differences in collagen organisation were seen when 50% or 75% BOEC-conditioned medium was used, suggesting a saturation of the effect induced by hBOEC (Fig. 28D). On the other hand, hBOEC-conditioned media contained factors that boosted keratinocyte migration (area covered by keratinocytes in a 1 mm window 6 hours after wounding ($\mu\text{m}^2 \times 10^5$): 2.8 ± 0.3 with hBOEC-conditioned media versus 1.9 ± 0.3 with non-conditioned media; $N=4$; $P<0.05$; Fig. 29C,D) and proliferation (number of keratinocytes $\times 10^3/\text{cm}^2$ after 10 days: 74 ± 6 with hBOEC-conditioned media vs. 51 ± 6 with non-conditioned media; $N=4$; $P<0.05$; Fig. 29H,I). In accordance, keratinocytes had a more migratory phenotype in hBOEC-conditioned media, shown by their 2.3-fold lower expression of *connexin43* compared to non-conditioned media ($N=4$; $P<0.05$; Fig. 30).

4. hBOEC improve matrix organisation and re-epithelialisation of hDFS in wounds

4.1. Effect of hDFS (+/- hBOEC) on wound contraction and re-epithelialisation

While hBOEC alone did not reduce contraction, the presence of hDFS –singly or in combination with hBOEC– significantly decreased contraction near the end of the healing period (Fig. 31A,B; Table 9). Reduced contraction in the presence of hDFS was accompanied by a decrease in the area taken up by SMA-expressing myofibroblasts at the wound edges 5 days after wounding (Fig. 31C-F; Table 9). Like hBOEC alone, HUVEC did not affect wound contraction (Table 9).

In addition, the presence of hDFS –singly or in combination with hBOEC– significantly increased collagen deposition in the wounds at 10 days, compared to the corresponding conditions without hDFS (% fibrillar collagen; Table 9). Finally, filling the wound gap with hDFS –singly or in combination with hBOEC– had markedly increased the epithelialisation rate at 5 days compared to saline (Fig. 26A,B and 32A,B; Table 9). At 10 days, wounds containing hDFS and hDFS+hBOEC were significantly more re-epithelialised than those in the saline group (Table 9). Regarding the (nearly) complete epithelial coverage of wounds treated with hBOEC or hDFS, no additional effect was noticed in the hDFS+hBOEC group.

Figure 31. hDFS reduce wound contraction. **A,B**, Size (**A**) and contraction (%) relative to original wound size in **B**) in wounds treated with PBS (red bars in **B**), hBOEC (black bars in **B**) or hBOEC+hDFS (blue bars in **B**) revealing reduced contraction in conditions with hDFS. **C-F**, Cross-sections of PBS (**C**), hBOEC (**D**), hDFS (**E**) or hBOEC (hB) +hDFS (**F**) treated wounds double-stained for EC (mouse and human, using mouse-specific CD31 and human-specific Ulex Europaeus Agglutinin (UEA)-lectin in green) and SMC (revealed by red SMC- α -actin) showing less myofibroblasts (corresponding to SMC- α -actin⁺ signal not associated with green EC) at the wound edges (lined by dashed white lines) in conditions with hDFS. DAPI (blue) was used as nuclear counterstaining in **C-F**. Scale bars: 75 μ m in **C-F** and 3 mm in **A**.

However, in the latter group, thickness of the epithelium was significantly increased, with less nucleated keratinocytes, suggesting improved differentiation (Fig. 32C,D). Growth factor analysis (Table 7) and keratinocyte proliferation/migration assays in the presence of hDFSconditioned media (Fig. 29E,J) further support the notion that the improved epithelial coverage is related to cross-talk of fibroblasts and keratinocytes. In accordance, keratinocytes had a more migratory phenotype in the presence of hDFS-conditioned media, as shown by their 1.3-fold lower expression of *connexin43* compared to non-conditioned media ($N=4$; $P<0.05$; Fig. 30).

Figure 32. hDFS stimulate wound re-epithelialisation. *A,B*, Cross-sections of wounds treated with hDFS alone (*A*) or combined with hBOEC (hB; *B*) stained with pancytokeratin (PCK) revealing faster epithelial coverage in conditions with hDFS compared to PBS-treated wounds (see Fig. 26*A,B*). Hair follicles marking wound boundaries and the length covered by epidermis are indicated by white arrowheads and black lines, respectively. *C,D*, Cross-sections stained with H&E revealing that the epithelial tongue has a larger thickness (indicated by white lines) in hBOEC containing hDFS (*D*) than in hDFS alone (*C*). Scale bars: 75 μ m in *C,D* and 500 μ m in *A,B*.

4.2. Effect of hBOEC on vascularisation of hDFS

Next, we assessed whether hBOEC were able to increase vascularisation of hDFS. At day 5, compared to saline, the presence of hDFS alone was sufficient to increase the number of mCD31⁺ vessels in the wound borders, likely by a trophic effect (Table 7) on angiogenesis. Addition of hBOEC further increased this trophic effect on the host vasculature (Fig. 33*A,B*; Table 9). The presence of hDFS alone also proved to be sufficient to increase the number of SMA-coated vessels, an effect that was not further enhanced by hBOEC (Table 9). At 10 days, vessels occupied the entire wound, including the hDFS. While hDFS alone did not increase vascularisation compared to saline, the presence of hBOEC did enhance vascularisation of the entire wound (mCD31⁺ area fraction; Fig. 33*C,D* and 24*C,D*; Table 9).

Figure 33. BOEC enhance angiogenesis in hDFS. Cross-sections of wound borders at day 5 (stained with green mouse-specific CD31 in *A,B*) and wound centres at day 10 (stained with mouse-specific CD31 in *C,D*) revealing more vessels in hDFS combined (*B*) or not (*A*) with hBOEC as compared to PBS-treated wounds at day 5 (see Fig. 24*A*). At day 10, hBOEC increased vascularisation of hDFS in the wound centre (*D* versus *C*). DAPI (blue) was used as nuclear counterstaining in *A,B*. Scale bars: 200 μ m.

4.3. Effect of hBOEC on matrix organisation in and re-epithelialisation of hDFS

Finally, we investigated whether hBOEC-mediated neo-vascularisation of hDFS was consistent with our *in vitro* findings at increased O₂ levels. Although hBOEC did not affect collagen deposition (% fibrillar collagen; Table 9), they did improve collagen organisation within hDFS implanted in wounds (% red birefringent collagen; Fig. 34A,D; Table 9).

This increased organisation was confirmed by TEM (Fig. 34C,F) and on semi-thin sections (Fig. 34B,E). Consistent with the observed *in vitro* effect of increased O₂ levels on keratinocyte proliferation, hBOEC increased the number of keratinocyte layers in the advancing epithelial tongues at 5 days on top of the implanted hDFS (number of keratinocytes/mm: 590 ± 40 in hDFS+hBOEC versus 440 ± 30 in hDFS; $N=6$; $P<0.05$; Fig. 32C,D). Moreover, at day 10, the epithelium covering the hDFS was thicker in the presence of hBOEC (epithelial thickness; Table 9). Interestingly, under low O₂ conditions, hDFS secreted significantly less KGF as compared to normoxic conditions (pg/mg protein: 14 ± 1 in hypoxia versus 23 ± 3 in normoxia; $N=4$; $P<0.05$).

Figure 34. hBOEC improve dermal matrix organisation in hDFS *in vivo*. Collagen, analysed by Sirius red polarisation microscopy (A,D), semithin sections (B,E) and TEM (C,F), was more aligned in thick bundles in hDFS+hBOEC(hB)- (D-F) compared to hDFS-treated (A-C) wounds. Scale bars: 0.5 μ m in C,F, 20 μ m in B,E and 75 μ m in A,D.

5. Effect of PIGF on keratinocytes and hDFS

As we found PIGF to be abundantly produced by hBOEC, and given its documented role in wound healing^{134, 173, 167}, we hypothesised that at least some of the beneficial effects of hBOEC on wound healing were mediated by PIGF. Therefore, we used a number of 'gain-of-function' (by using recombinant PIGF) and 'loss-of-function' (by knocking-down the *PIGF* gene in hBOEC and thereby generating PIGF^{KD}-hBOEC conditioned media, i.e. hBOEC-conditioned media without PIGF) strategies to test the effect of PIGF on hDFS, keratinocytes and hBOEC in vitro. First, we determined whether dermal fibroblasts and keratinocytes, like hBOEC (Table 4), readily expressed Flt-1, the receptor for PIGF. We confirmed previous findings¹⁶⁶ that both keratinocytes and dermal fibroblasts express Flt-1 protein (Fig. 35A). Using an shRNA-encoding lentivirus (Fig. 9C), PIGF was successfully knocked-down as compared to hBOEC transduced with a virus encoding a scrambled shRNA (Fig. 9B), as shown by a dramatic decrease in PIGF protein secretion in their growth media (Fig. 35B). While knocking-down PIGF in hBOEC did not significantly change their expression levels of endothelial markers (i.e. *Tie-1*, *Tie-2*, *Flt-1*, *KDR*, *VE-cadherin*, *CD31*, *eNOS*; data not shown), absence of PIGF expression in hBOEC significantly decreased their proliferation, suggesting a role for PIGF as an autocrine mitogen for hBOEC (Fig. 35C). Importantly, except for *PDGF-BB* that was slightly lower in PIGF^{KD}-hBOEC (% expression relative to universal human RNA: 930 ± 93 in hBOEC transduced with scrambled shRNA versus 685 ± 49 in PIGF^{KD}-hBOEC; $N=3$; $P<0.05$), PIGF knock-down in hBOEC did not alter the expression of other growth factors (i.e. *VEGF-A*, *KGF*, *GM-CSF*, *Ang2*) or MMP (*MMP-1*, *-2*, *-9*). This means that the observed biological effects were a direct consequence of the absence of PIGF.

Figure 35. PIGF knock-down in hBOEC decreases their proliferation and effect on keratinocytes. **A**, Western blot revealing the expression of FIt-1 protein on dermal fibroblasts (Fb) and keratinocytes (KC). α -Tubulin was used as loading control. **B,C**, ELISA on supernatants (**B**) and proliferation rate (**C**) of hBOEC cultures transduced with PIGF shRNA (white bars) or scrambled shRNA (black bars) revealing efficient PIGF knock-down and reduced hBOEC proliferation upon PIGF knock-down. *: $P < 0.05$; $N = 3-4$. **D**, Proliferation assay of KC grown in the presence of 50% conditioned media from hBOEC treated with scrambled shRNA (black bar), or shPIGF (white bar); normal KC growth media (red bar), KC growth media supplemented with 10 ng/ml PIGF (grey bar) or 100 ng/ml PIGF (blue bar) revealing more cell expansion (number of cells/cm²) upon addition of PIGF and decreased cell expansion upon PIGF knock-down. *: $P < 0.05$; $N = 4$. **E-I**, Wounding assay revealing less migrating keratinocytes (KC; area in μ m² occupied by migrating KC in a 1 mm window at 6 hours in **I** or at 24 hours in **E-H**) in the presence of 50% conditioned media from hBOEC treated with shPIGF (**F**; white bar in **I**) as compared to 50% conditioned media from hBOEC treated with scrambled shRNA (**E**, black bar in **I**). Migration was however increased in KC growth media supplemented with 10 ng/ml PIGF (**H**, blue bar in **I**) as compared to without supplemented PIGF (**G**, grey bar in **I**); *: $P < 0.05$; $N = 4$.

Despite the expression of FIt-1 on dermal fibroblasts (and hence their ability respond to PIGF), increasing or lowering PIGF exposure to hDFS did not alter collagen organisation (data not shown). However, PIGF^{KD}-BOEC conditioned media applied to keratinocytes significantly decreased keratinocyte proliferation (Fig. 35D) and migration (Fig. 35E,F,I) in a scratch wound assay, as compared to conditioned media derived from hBOEC transduced with a scrambled shRNA. Accordingly, adding recombinant PIGF to keratinocytes cultures increased keratinocyte proliferation (Fig. 35D) and migration (Fig. 35G-I) in vitro. Thus, PIGF secreted by hBOEC could – at least in part – be responsible for the effects of hBOEC on vascularisation and re-epithelialisation, but perhaps not on dermal matrix organisation.

*Truth is a constant variable.
We seek it, we find it, our viewpoint changes,
and the truth changes to meet it.*

(William J. Mayo; Annals of Surgery 94: 799 (1931))

Discussion

1. BOEC, an all-round player in wound healing

Impaired wound healing finds its origin in many deficits. Nevertheless, in cases where wounds tend not to heal properly (e.g. diabetic wounds, pressure sores and vascular ulcers), impaired vascularisation seems a common denominator ¹⁶⁸. Especially in the early inflammation and granulation phases of wound healing, new capillary formation is essential to re-establish the supply of oxygen and nutrients to the wounded tissue ¹⁶⁹. Therefore, our principal objective was to find a suitable strategy to increase wound vascularisation. Besides, when using dermal substitutes to restore the dermal component in large cutaneous defects, quick and stable vascularization is quintessential. We explored the effect of BOEC singly and in combination with an engineered human dermal fibroblast construct on vascularisation in full thickness wound healing. BOEC improved wound vascularisation by direct incorporation into the host vasculature, and via trophic expansion of the host vessel network. The ultimate result was faster re-epithelialisation and better wound matrix organisation (Fig. 36). Most current cell-related wound vascularisation strategies focus on the use of MSC ^{177, 171}. However, they may cause excessive contraction by differentiating into myofibroblasts ⁷². Moreover, direct vascular contribution of MSC is debated ^{32, 79}. Early EPC have also proven effects on angiogenesis in vivo, albeit that they do not actively incorporate in vessels. Likewise, in our wound model, we did not find vascular incorporation of early EPC. In addition, it remains questionable whether early EPC can be obtained in sufficient numbers to be used clinically in humans. We opted for peripheral blood-derived BOEC as a source of new vessels. While they may have the molecular signature and functional behaviour of mature EC ¹⁷³, we demonstrated that BOEC show several distinctly differential characteristics. Indeed, a thorough characterisation of BOEC involves documentation of their differential behaviour not only from early EPC but also mature EC (Fig. 37). We determined for the first time that the latter difference concerns their proliferative behaviour, as well as other functional aspects, including, for example, secretion of MMP and growth factors. Although others have suggested that BOEC do not contribute to vessel growth by secreting growth factors, these investigators restricted their assays to an in vitro transwell system ²⁰. We demonstrated an important trophic role for BOEC in vessel growth in vivo where cell-cell contact is critical for optimising this cross-talk (Fig. 36). Importantly, BOEC also secreted significant quantities of factors that facilitated communication with non-EC cells (e.g. keratinocytes and fibroblasts), as shown in our in vitro assays. We believe that these interactions add to the observed effects on re-epithelialisation and matrix organisation in vivo. The difference between mature EC and BOEC is further underscored by the fact that, unlike BOEC, mature EC (e.g. HUVEC) did not efficiently contribute to neo-vascularisation in ischemic hind limbs ^{179, 174} or in wound healing (this thesis).

Figure 36. Role of BOEC in wound healing. BOEC induce vascularisation both by direct incorporation and by secretion of angiogenic factors, with subsequent increased wound bed oxygenation, leading to improved epidermal and dermal healing. Besides, BOEC produce factors that stimulate keratinocyte and fibroblast behaviour.

Integration of BOEC in a multilayered fibroblast construct resulted in quick vascularisation and further promoted epithelial coverage and improved matrix organisation. Our in vitro studies suggested that the effects of BOEC on re-epithelialisation and matrix organisation were at least partly due to the improved vascularisation of the wound bed, in addition to the growth factor cross-talk with surrounding wound cells, i.e. keratinocytes and fibroblasts (Fig. 36). All of these cells can be harvested from the host and thus offer the possibility of an exclusively autologous and safe approach to tissue engineering for wound repair.

2. Will the 'true EPC' please stand up?

One of the objectives of this thesis was to extensively characterise BOEC, a subset of the EPC family, with the intention to better position them in the endothelial (progenitor) cell (EPC) family tree (Fig. 37). Indeed, since the initial description of circulating cells contributing to neo-vascularisation in the seminal paper of Asahara *et al.* in 1997 (ref. 41), much confusion has arisen on the exact nature and function of EPC. In this milestone article (Fig. 5), they used minimally enriched populations of CD34⁺ and Flk-1⁺ cells to culture a fibronectin-adherent cell population that homed to sites of neo-angiogenesis in vivo and these cells were named 'progenitor endothelial cells'. Since then, multiple investigators have used a myriad of isolation protocols to obtain endothelial progenitors. All protocols start from the mononuclear cell fraction

and obtain cells with endothelial characteristics through culture adherence, surface marker selection or a combination of both.

To illustrate this briefly here, we only discuss the most authoritative publications. Ito *et al.*¹⁷⁵ and Hill *et al.*¹⁷⁶ used a respectively 24 and 48 hour adherence depletion step on fibronectin-coated dishes to obtain clusters of hematopoietic cells that were surrounded by spindle-shaped cells that expressed endothelial markers but did however not incorporate in vessels *in vivo*. Vasa *et al.*¹⁷⁷ described a different isolation method by plating mononuclear cells on fibronectin- and gelatin-coated dishes and discarding non-adherent cells after 4 days. These cells readily ingested India ink, a functional characteristic consistent with macrophage activity¹⁷⁸. Adherent cells were stained with AcLDL and UEA-1-lectin and double-positive cells were considered as EPC. In another landmark paper, Lin *et al.* described delayed massive outgrowth of cells with endothelial characteristics after plating mononuclear cells on collagen-coated dishes and named these cells 'Outgrowth Endothelial Cells (OEC)'⁵¹ (Fig. 5). OEC behave *in vivo* as real EC, actively integrating in functional vessel networks, unlike the previously mentioned EPC. It is beyond doubt that these different isolation protocols yield entirely different populations of supposed EPC⁴⁷.

Regarding the obvious abundance and variety of cell populations in the EPC family, it is quintessential to find a consensus on and clearly define what EPC really are. As the term is commonly used today, in the broadest interpretation of the publications in the field, EPC are "circulating cells that promote neo-vascularisation in sites of hypoxia". This broad definition encloses multiple cell types that could play important roles in different stages of vascularisation, and this is the cradle of the controversies currently affecting the field⁴⁷. Reaching back to the initial description of EPC as circulating cells that *in vivo* participate in vasculogenesis⁴¹, we prefer to *narrow* the definition of 'true EPC' to "circulating cells that produce endothelial progeny and spontaneously form endothelial tubes *in vivo*"⁴⁷. Only the OEC described by Lin *et al.* and ECFC described by Ingram *et al.*⁴⁴, meet these requirements. To obtain BOEC for our studies, we used a slightly modified isolation protocol as described by Ingram *et al.*⁴⁴ and demonstrated endothelial characteristics and – most importantly – active incorporation into the host vasculature. We therefore consider BOEC to be progeny of 'true EPC' (Fig. 37). The numerous other types of circulating cells involved in angiogenesis are however beyond this definition. Considering their isolation protocols, we prefer to group them under the common denominator 'early EPC', as they appear much earlier in culture compared to (B)OEC. To obtain early EPC for our comparative studies with BOEC, we used the method described by Hill *et al.*¹⁷⁶ and confirmed that these cells display endothelial surface markers, bind UEA-1-lectin, take up AcLDL, but do not form vascular tube-like structures on matrigel *in vitro* nor do they actively incorporate in the host vasculature. Derived from the hematopoietic lineage, we confirmed that they display hematopoietic markers such as CD45 and CD14 (Fig. 18; Table 4). In some reports, early EPC are described to be able to ingest

bacteria⁵², thus combining endothelial with monocyte markers and functions. They play an important role in vascular homeostasis by stimulating angiogenesis through the secretion of paracrine factors^{28, 179}, by degrading the extracellular matrix to allow for angiogenesis or vasculogenesis^{70, 180} and possibly by temporarily lining of the vessel wall where the vascular integrity is disrupted^{27, 181}. There is also evidence that early EPC act in synergy with BOEC during neo-vascularisation⁵⁵.

Thus, according to our comparative studies and our resulting narrow definition, we distinguish two branches in the EPC family tree (Fig. 37), one called 'early EPC' which combines endothelial characteristics with monocyte features, another that we call 'true EPC' given their potential to functionally behave like an EC, that is, becoming part of the endothelial lining of blood vessels. A perhaps ironic consequence of this narrow definition of 'true EPC' is that the original EPC described by Asahara *et al.* may not be considered as 'true EPC' after all.

3. On the origin of 'true EPC'

Apart from the confusion on the identity of EPC, the exact origin of these cells remains also uncertain. Knowing the correct ancestor of EPC is important not only because this may help to better define these cells, but also because it may teach us how to better isolate these cells for therapeutic use and, moreover, the origin of EPC likely predetermines their functional behaviour. A first question is about which cell lineage gives rise to EPC. It has been put forward that the endothelial and hematopoietic lineage share a common progenitor, the so-called 'hemangioblast' (Fig. 37). The idea of a common progenitor of both hematopoietic cells and EC has been suggested as early as in the 1920's¹⁸². More recently, evidence has arisen on the existence of hemogenic endothelium during embryogenesis. Jaffredo *et al.*¹⁸³ described the emergence of clusters of hematopoietic cells from the CD45⁻ VEGFR2⁺ EC lining of the aortic lumen during embryogenesis. When these hematopoietic cells bulged into the aortic lumen, all cells became CD45⁺. Besides, hematopoietic cells express surface markers common with EC, like CD34, CD31, VEGFR2 and VE-Cadherin¹⁸⁴. As indirect evidence for the existence of hemangioblast-like cells during adult life, hematopoietic stem cell transplantation in irradiated mice was shown to contribute to vasculogenesis. Indeed, when single HSC were transplanted in animals who subsequently underwent retinal injury, newly formed vessels were of donor origin, suggesting the endothelial progeny of the transplanted HSC¹⁸⁵. Cogle *et al.*¹⁸⁶ used human umbilical cord blood-derived CD34⁺ HSC in irradiated mice. These cells effectively repopulated the murine hematopoietic system and were found in newly formed vessels upon retinal injury. These findings strongly suggest the existence of a CD34⁺ cell with both 'true' endothelial and hematopoietic progeny¹⁸⁷, even though there is still a chance that 'true EPC contamination' in the hematopoietic stem cell transplant can explain the vasculogenic potential of these repopulated bone marrows.

How then do the two EPC types defined in the previous chapter relate to such a common precursor? In patients with polycythemia vera, known to bear the JAK2 V617F mutation, Oppliger Leibundgut *et al.*¹⁸⁸ succeeded in isolating CD34+CD133+KDR⁺ early EPC bearing the mutation, once more suggesting that early EPC originate from the hematopoietic arm derived from hemangioblasts. On the other hand, Yoder *et al.*⁵² were unable to demonstrate the JAK2 V617F mutation in BOEC derived from patients with this mutation, suggesting that the mutation is only committed to the hematopoietic lineage and that BOEC are not derived from this lineage, but perhaps from a more distant mesodermal progenitor (Fig. 37). Nevertheless, it is still possible that 'true EPC' or BOEC derive directly from hemangioblasts and have lost hematopoietic characteristics and gained EC characteristics instead (Fig. 37). While our extensive characterisation of BOEC also excluded a hematopoietic origin for these cells, we did not resolve the issue concerning a hemangioblast or other mesodermal precursor origin of BOEC.

Figure 37. On the origin of EPC. This schematic representation outlines what is known and still uncertain in the confusing field of EPC biology. Only 'true EPC' can give rise to BOEC and subsequently EC, whereas early EPC are derived from the hematopoietic lineage, which is reflected in the expression of cell surface markers. Both BOEC and early EPC are probably derived from a common progenitor, the 'hemangioblast', even though another more distant mesodermal precursor may give rise to 'true EPC' and BOEC without going through a hemangioblast intermediate. Furthermore, in addition to bone marrow (BM) as a source for BOEC, there is evidence for vessel wall-derived EC with high proliferative capacity, suggesting that BOEC may have a vessel wall origin. Our contribution to this schematic outline is that our comparative characterisation studies have firmly established the position of BOEC as being clearly distinct from mature EC and being in the non-hematopoietic branch of the EPC family tree.

In addition to the question about cell lineage, a second question is related to the anatomical location of the EPC ancestors. In the seminal paper by Lin *et al.* in which (B)OEC were initially described, they were identified as circulating bone marrow-derived cells with high proliferation capacity that contributed to vasculogenesis, as opposed to circulating EC that were of vessel wall origin and had low proliferation capacity⁵¹. There is however mounting evidence for vessel wall-derived EC with a high proliferative capacity. Using single cell assays, Ingram *et al.* further defined this vessel wall-derived OEC population and described an entire proliferative hierarchy ranging from non-replicating to very highly proliferative EC¹⁹⁶. Cord blood-derived BOEC¹⁸⁹ and peripheral blood-derived BOEC¹⁹⁰ are however superior to vessel-wall derived EC in terms of vasculogenesis. In summary, there is evidence for a vessel wall-derived EC with high proliferative potential but its relevance for vasculogenesis needs to be determined. Notwithstanding the elaborate identification we performed on BOEC, we were unable to demonstrate whether the BOEC we used for our experiments are vessel wall-derived cells or bone marrow-derived cells, as actually no surface marker can be used to distinguish OEC by their anatomic origin (Fig. 37). Nevertheless, the evidence in the paper by Lin *et al.*⁵¹ strongly suggests a bone marrow origin for the BOEC we used.

4. The quest for the ultimate EPC marker

A unique surface marker – or a panel of markers – for EPC would allow to directly select the ‘true’ EPC and – if so desired – expand them *ex vivo* to BOEC quantities sufficient for clinical application, thereby bypassing the time consuming culture selection step as we described in the Materials & Methods section. Initially, CD34 and VEGFR2 were used to select EPC⁴¹. The rationale for using these markers lies in the abovementioned hypothesis that both endothelial and hematopoietic cells originated from a common precursor, the so-called hemangioblast^{188, 185}. CD34 is widely recognised as the principal marker to isolate hematopoietic stem cells¹⁹¹ and is expressed on a range of cells of mesodermal origin, varying from fibroblasts to blood and endothelial cells. VEGFR2 (KDR in humans, Flk-1 in mice) is typically expressed by EC, but also occurs on other cells of the mesodermal lineage, even on hematopoietic stem cells¹⁹² (Fig. 37). Enrichment for both markers gave rise to cells that homed to and induced angiogenesis in sites of neo-vascularisation⁴¹. Peichev *et al.* expanded this panel of markers with a third marker, AC133 (CD133 in humans, prominin-1 in mice), a surface marker on epithelial, hematopoietic and neuronal precursors and cancer cells¹⁸¹. As both CD34 and CD133 are down-regulated during hematopoietic cell differentiation, they considered the CD133⁺CD34⁺KDR⁺ cells to be the endothelial progenitor fraction. Indeed, these cells expressed endothelial markers *in vitro* and were found on cells on the luminal surfaces of implanted left ventricular assist devices in humans. As the combination of these markers gave rise to cells that

undoubtedly contributed to adult neo-vascularisation, CD34, KDR and CD133 were used in a variety of experiments to identify EPC¹⁸⁷.

It later appeared however that more than 99 % of the cells expressing these markers were also positive for CD45, a common leukocyte marker, and were unable to give rise to EC that actively contributed to vessel formation *in vivo*⁵⁰. This elucidates that the progeny of cells expressing hematopoietic markers CD34, KDR and CD133 are monocytic cells with endothelial characteristics that contribute to angiogenesis without active vessel formation and are therefore to be considered 'early EPC' (Fig. 37).

Thus, until now there is no (panel of) surface marker(s) that identifies 'true EPC' and the isolation of BOEC therefore still depends on time consuming culture adherence⁴⁷. When searching for specific EPC-markers, it should also be taken into account that the expression profile of surface markers may change upon *ex vivo* culture¹⁸⁷. When we examined the expression of multiple markers over different passages in BOEC, we found no major differences over time, except for CD34 expression that was decreased with senescence, in agreement with findings of others²¹. However by the time of our first RNA sampling (i.e. the first passage), the majority of population doublings is already beyond, assuming that each colony stems from one 'true EPC'. In the absence of a reliable surface marker for isolation of true EPC, it is impossible to judge whether the expression pattern really changes during the transition from true EPC to BOEC. Also, BOEC colonies are derived through adherence on type I collagen in specific endothelial growth medium. It might therefore be possible that BOEC do not really exist as such *in vivo*, but are an *in vitro* generated population derived from 'true EPC'. Even though no surface marker for 'true EPC' is identified yet, progress is made in the field of peptide ligands that selectively bind BOEC and have no affinity for HUVEC, lymphocytes or neutrophils¹⁹³. Further research is necessary to distinguish whether these ligands are also able to select the 'true EPC' and have no affinity for 'early EPC'.

Thus, despite more than a decade of EPC research, several issues concerning EPC described in chapter 2-4 of this discussion are still not resolved. Therefore, the main conclusion from all of this is that when working with EPC, it remains of utmost importance to thoroughly identify the cells that are used in an experimental setting, so that other investigators would have a benchmark for comparison. Therefore, we combined extensive expressional, phenotypic and functional characteristics *in vitro*, together with *in vivo* vasculogenesis analysis to characterise BOEC and prove the true endothelial identity of BOEC. Based on this extensive characterisation, we propose to position BOEC as direct descendants of 'true EPC', distinct from mature EC that are a step lower on the endothelial lineage hierarchy (Fig. 37).

5. Back to the basics of wound healing: vascularisation and oxygenation

While many revascularisation therapies have merely suggested a link between revascularisation and re-oxygenation, here we directly demonstrated, by means of hypoxy-probe™ staining, that BOEC-induced revascularisation inversely correlated with the extent of hypoxia. The deleterious effect of long-term hypoxia on wound cell behaviour was further tested by exposing the cells to high or low oxygen for 10 days. Keratinocyte migration and proliferation were compromised by sustained low oxygen levels. These results appear to be in conflict with earlier studies. O'Toole *et al.*¹⁴¹ described increased keratinocyte migration in an in vitro scratch wound assay, whereas we found keratinocyte migration to be decreased in hypoxia. However, this study evaluated the effect of acute hypoxia on keratinocytes, whereas in our experiments, keratinocytes were cultured in low oxygen conditions for 10 days, which is the duration of our in vivo experiment, and were then subjected to the in vitro scratch wound assay. On the other hand, in support of a positive role for oxygen in keratinocyte behaviour, hyperbaric oxygen treatment is a well-known strategy to improve epithelialisation^{200, 295}.

In accordance with the well-known effect of oxygen on collagen hydroxylation^{144, 143}, we noticed decreased collagen matrix remodelling when DFS were cultured for 10 days in low oxygen conditions. The expression and activity levels of the key matrix-modulating factors, the MMP, may be affected by changes in oxygen levels. Indeed, we (this thesis) and others²⁹⁶ have shown in a three-dimensional fibroblast culture that MMP-1 levels are up-regulated by hypoxia. Based on the notion that improved oxygenation enhances healing, several approaches to deliver oxygen to wounds may be considered. While 'exogenous' oxygen delivery can be performed by hyperbaric oxygen therapy²⁹⁴, oxygen can also be provided 'from within' by forming new vessels. We have proven BOEC to be effective in wound vascularisation and subsequent oxygenation and we suggest that this in part explains the improved re-epithelialisation and matrix organisation in BOEC-treated wounds.

6. BOEC: conductor of the wound healing cytokine orchestra?

Unlike previously assumed²⁰, we demonstrated for the first time that BOEC produce a broad range of growth factors and cytokines that are known to be involved in wound healing (Table 7). We specifically analysed BOEC for production of cytokines involved in angiogenesis, re-epithelialisation and matrix production/remodelling. We showed VEGF-A, PIGF, PDGF-BB, Ang-2, MCP-1, bFGF, GM-CSF and HGF to be produced/expressed by BOEC, all factors that induce angiogenesis (Table 10). Combined action of these factors at least in part explained the angiogenic effect of BOEC in wound healing.

Interestingly, the expression profile was often complementary which supports the strategy of combining BOEC and DSF in a tissue engineering construct. For instance, we found that Ang-2 and Ang-1 were complementarily expressed by BOEC and DFS, respectively (Table 7), suggesting an autocrine role for BOEC in vascular remodelling through Ang-2 and a supportive role for DFS in stabilising the vessel network through Ang-1. Moreover, it appears that BOEC are much more responsive to the effects of Ang-2 than mature EC, as in the latter the effect of Ang-2 is attenuated due to formation of heterocomplexes between Tie-1 and Tie-2 (ref ²⁹⁷)

Cytokine	Effect on vascularisation	Effect on epidermal healing	Effect on dermal healing
VEGF-A	-vascularisation $\uparrow$ ¹³³ -migration and proliferation of EC $\uparrow$ ¹³³ -survival of BOEC $\uparrow$ ²⁰⁴	-re-epithelialisation $\uparrow$ ¹²	-granulation tissue formation $\uparrow$ ¹³³
PIGF	-angiogenesis $\uparrow$ ¹³⁶ -vessel maturation $\uparrow$ -BOEC proliferation $\uparrow$ ⁶⁸	-KC migration $\uparrow$ -KC proliferation $\uparrow$ ⁶⁸	
PDGF-BB	-angiogenesis $\uparrow$ -vessel maturation $\uparrow$ ¹²⁸	-re-epithelialisation $\uparrow$ ¹²⁸	-production of matrix $\uparrow$ -matrix remodelling $\uparrow$ ¹²⁸
Ang-2	-vascular remodelling $\uparrow$ ¹³²		
MCP-1	-angiogenesis $\uparrow$ ¹²⁷	-re-epithelialisation $\uparrow$ ¹²⁷	
bFGF	-angiogenesis $\uparrow$ ¹²⁵	-re-epithelialisation $\uparrow$ ¹²⁵	-FB proliferation and migration $\uparrow$ -matrix protein synthesis $\uparrow$ ¹²⁵
KGF		-KC proliferation and migration $\uparrow$ ^{125, 126}	
IL-6		-KC proliferation $\uparrow$ ⁹	
GM-CSF	-angiogenesis $\uparrow$ ¹²⁸	-KC proliferation $\uparrow$ ¹²⁸	
HGF	-angiogenesis $\uparrow$ ¹²⁹	-KC migration $\uparrow$ ¹²⁹	-regulation of MMP ¹²⁹

Table 10. BOEC-derived cytokines that exert trophic effects on wound healing.

Also, BOEC are more sensitive to the anti-apoptotic and proliferative stimuli of VEGF than HUVEC ²⁰⁴. It furthermore appears that other factors produced by BOEC may enhance VEGF expression in a paracrine way, such as KGF, bFGF and PDGF ¹²⁸.

Of particular interest is PIGF, which is produced in high amounts by BOEC and acts as a strong autocrine mitogen. Moreover, BOEC in which the expression of PIGF is silenced by shRNA partially lose their angiogenic effect on wound healing in vivo (not shown), in comparison with BOEC transduced with a scrambled-shRNA vector.

Considering epidermal healing, nearly all cytokines we looked for in BOEC, are somehow involved in keratinocyte migration and/or proliferation (Table 10). Accordingly, BOEC conditioned medium stimulated keratinocyte migration and proliferation in vitro. Interestingly, the effect of PIGF on keratinocyte behaviour had not been documented before. We showed both by 'loss-of-function' and 'gain-of-function' experiments that this factor strongly activates keratinocyte migration and proliferation in vitro and re-epithelialisation in vivo (data not shown). Consistent with a keratinocyte response to PIGF, we and others ²⁰⁵ (Print have demonstrated the expression of the PIGF receptor Flt-1 on keratinocytes. Besides, numerous cytokines made by BOEC are involved in dermal matrix deposition and/or remodelling (Table 10).

While others documented an effect of PIGF on dermal fibroblast migration ¹⁷³, we did not see a significant effect of recombinant PIGF on collagen organisation in DFS in vitro, nor did we see organisational changes upon exposure of DFS to conditioned media of BOEC in which PIGF expression was knocked-down (data not shown). Therefore, other factors produced by BOEC may be responsible for their favourable effect on dermal matrix organisation. We hypothesise that a favourable MMP expression profile of BOEC (see 7.) may correlate with better matrix organisation.

It is important to emphasise that the role of cytokines in wound healing depends on a delicate balance of those factors and that distortion of this balance in either direction, by loss or over-expression of a specific growth factor, might lead to an impaired healing response. On the other hand, as many growth factors act on multiple components of the wound healing cascade, loss of a specific growth factor might be compensated for by other factors, thus concealing the expected effect. In addition, while a certain factor can be provided by different cell sources in the wound bed, the relative contribution of each of these cell sources can be different. Consistent herewith we found that the presence of DFS in the wound was the rate-limiting factor for re-epithelialisation, likely because some factors (e.g. KGF and HGF) acting on keratinocyte migration and proliferation were more abundantly expressed by DFS compared to BOEC.

7. Dermal matrix organisation as a benchmark for dermal maturation

The exact mechanisms by which the initially formed fibrin cloth is transformed, first in a fibronectin and hyaluronic acid matrix, later in a collagen-proteoglycan matrix and afterwards in an organised network of collagen fibres which is called a scar, is still largely unknown. Collagen deposition starts as early as after 24 hours, but this initially deposited collagen is merely collagen type III and is poorly organised. This is no more than a temporary filling of the defect, as this jumbled matrix is replaced by neatly organised collagen fibres in a basket weave-pattern ²⁰⁶. In uninjured skin, the dermis consists of neatly woven, densely packed collagen fibres (Fig. 38), which results in skin pliability and elasticity. We found that BOEC had a favourable effect on collagen remodelling, which was even more pronounced in the presence of DFS, resulting in collagen fibres that started to resemble those found in uninjured skin (Fig. 38). Collagen remodelling consists of a delicate balance between destruction and production. In molecular terms, collagen remodelling is mediated through enzymatic digestion by proteinases (mainly MMP), through inhibitors of these MMP (Tissue Inhibitors of MMP or TIMP) and through the production and orderly deposition of collagen fibrils. As we noticed a considerable effect of BOEC on dermal matrix organisation, we considered some of the molecular pathways that can explain this effect.

We suggest that the BOEC-mediated increased matrix organisation is both due to increased tissue oxygenation (and hence the improved function of fibroblasts involved in matrix deposition and remodelling) and the secretion of trophic factors (Fig. 36 and 39). Amongst these factors, some may affect matrix production by fibroblasts (e.g. PDGF-BB and bFGF), while others may affect collagen remodelling directly (such as MMP) or indirectly by controlling MMP expression (e.g. PDGF, KGF, bFGF, MCP-1 or VEGF-A) (Fig. 39).

Figure 38. Matrix organisation on semi-thin sections. In the presence of hDFS alone (A), collagen is deposited in between the cells, however the alignment is poor. In contrast, when hDFS are combined with hBOEC (B), collagen alignment is much better and the overall organisation is more close to that of normal skin (C), in which collagen is aligned in very thick, basket-weave patterned collagen bundles. Scale bars: 20 μm in all panels.

While TGF- β 1 was suggested as a potential 'organiser' ²⁰⁷, BOEC did not synthesise this factor in measurable amounts (data not shown). They did however produce PDGF-BB, although there is no consensus on whether this factor improves collagen organisation ^{207, 208}. Perhaps, MMP-9 expression by BOEC contributed to collagen organisation, since a lack of this MMP results in disordered fibrillogenesis ²⁰⁹. Furthermore, BOEC produce MMP-2, an enzyme that is presumably involved in matrix remodelling, as it is expressed in the early remodelling phases of wound healing ²¹⁰. Moreover, synthesis of MMP is stimulated by FGF, MCP-1, VEGF-A, KGF and PDGF ²¹¹, expressed by BOEC, providing an alternative pathway of increased collagen remodelling by BOEC. BOEC abundantly produce PIGF. However, as mentioned above (see 6.), PIGF did not affect collagen organisation of DFS in vitro.

Figure 39. Suggested mechanisms of action of BOEC on matrix remodelling in wound healing.

8. DFS-BOEC marriage: will it stand the test of time?

We already briefly touched upon the remarkably complementary growth factor expression profiles of BOEC and DFS as a strong argument for combining them in a tissue-engineered dermal substitute (see 6.). Importantly, this complementarity was manifested within each category of factors, i.e. those related to vessel growth/inflammation, those involved in matrix remodelling/deposition and those mediating keratinocyte migration/proliferation (Table 11; Fig. 40).

Figure 40: Synergism between cytokines produced by BOEC and DFS.

Below we demonstrate that this complementarity is also reflected in the different processes of wound healing, i.e. wound contraction, re-vascularisation, dermal matrix homeostasis and re-epithelialisation and give some suggestions on how this may relate to differential growth factor expression (Table 11).

8.1. Wound contraction

Mice differ from humans during skin wound healing since they are loose-skinned animals. Therefore, unlike in humans, murine wounds heal mostly by contraction, instead of re-epithelialisation^{212, 213} and the contraction rate is strain-dependent²¹⁴. In order to mimic human wound healing and to allow evaluation of certain treatments

on re-epithelialisation, contraction can be limited by physically restraining the skin ('splinting'). This is done by suturing a ring around the wound, which reduces contraction ²¹⁵. Nevertheless, there was still a significant degree of contraction in our wound model to also allow an evaluation of the effect of our cell treatment strategies on contraction. While wound contraction may be less pronounced in humans, it does represent a significant problem when it occurs at certain anatomical locations, e.g. the back of the ear ²¹⁶. Contraction also occurs after skin grafting ²¹⁷ (Fig. 1), which gives rise to detrimental functional/aesthetic results, as in joint regions or in the face. Besides increasing elasticity and augmenting the aesthetic appearance of the skin, reducing wound contraction should therefore be the principal goal of using dermal substitutes.

	BOEC	DFS
Wound contraction	no effect	Inhibition
Re-vascularisation	Vasculogenesis	no effect
	Angiogenesis: early and late	Angiogenesis: early
	Vessel stabilisation	Vessel stabilisation
Re-epithelialisation	Speed of epithelial coverage	speed of epithelial coverage
	epidermal thickness	
Dermal matrix	Organisation	Production

Table 11. Complementarity of BOEC and DFS in wound healing.

Many commercially available dermal substitutes are seeded with allogeneic dermal fibroblasts. However, allogeneic fibroblasts induce an inflammatory reaction, display higher numbers of myofibroblasts and have reduced collagen organisation ²¹⁸. Moreover, seeding dermal fibroblasts in a dermal equivalent might result in phenotypic changes that might negatively influence dermal regeneration after transplantation ¹⁵¹. We therefore tried to mimic the composition of natural dermis by superimposing several human dermal fibroblast sheets, which can easily be derived from a donor, and stimulating these sheets to produce their own interstitial matrix over time. We applied these DFS to our mouse wound healing model and noticed decreased contraction. Importantly, BOEC alone did not affect the contraction rate of the wounds, thus the presence of DFS was absolutely required to limit contraction.

The underlying mechanisms for counteracting contraction may be multifactorial. For instance, in accordance to studies where fibroblasts were seeded in dermal substitutes ²¹⁹, DFS showed in vivo increased collagen deposition. As total collagen deposition correlates with remaining wound surface, matrix deposition by DFS might provide an explanation for reduced wound contraction. On the other hand, a causal link between myofibroblasts

and wound contraction has been previously suggested ²¹⁷. In our model, the presence of DFS correlated with a reduction in myofibroblast content at the wound edges, thus providing an alternative explanation for reduced wound contraction.

While wound contraction was significantly reduced when using DFS in our wound healing model, we did not look for skin elasticity and aesthetic appearance, as this can only be appreciated later on. Furthermore, we believe the mouse model is not the right animal model to score the latter items, as mice have a naturally elastic skin and the appearance is completely different from human skin.

8.2. Re-vascularisation

There is a body of data suggesting that fibroblasts produce several factors that induce angiogenesis (for review, see: ref. ¹²⁸). This is in agreement with our findings that DFS produce angiogenic growth factors, such as VEGF and bFGF (Table 7). These findings correlated with enhanced revascularisation in DFS treated conditions in vivo (Table 9).

It should however be noted that the increased vascularisation in the DFS-treated condition was limited to the initial healing phases (day 5), whereas this effect had disappeared by day 10. Therefore, notwithstanding the angiogenic effect of DFS, it makes sense to combine DFS and BOEC, the latter which may guarantee a sustained vascularisation of DFS. In order for vessels to be sustained in the wound bed, it is important that the endothelial layer is stabilised by a smooth muscle or pericyte coat. In agreement with such a stabilising effect, BOEC, unlike DFS, secreted chemoattractant factors for peri-endothelial cells, such as PDGF-BB and PIGF^{128, 134, 136, 173}.

8.3. Re-epithelialisation

BOEC alone were sufficient to accelerate the epithelial coverage of the wound. This accelerated movement of the epithelial tongues over the wound bed correlated inversely with the number of connexin43 expressing keratinocytes. Connexin43 is an anchoring molecule that is expressed on basal keratinocytes and down-regulated during keratinocyte migration ²²⁰. Consistent with such an effect, BOEC abundantly expressed/secreted some factors that have been associated with keratinocyte behaviour, such as KGF and GM-CSF. Here we identified another factor, PIGF, which was previously not associated with keratinocyte migration and proliferation.

Nevertheless, the presence of DFS was also sufficient for an improved re-epithelialisation and was to some extent more effective than BOEC. This more dramatic effect may be related to the secretion of higher amounts of keratinocyte-responsive factors (e.g.

KGF and HGF) and/or by providing a scaffold on top of which keratinocytes could efficiently move forward to the wound centre. On the other hand, the added value of BOEC was to increase the thickness of the epithelial cover, perhaps related to the stimulation of keratinocyte proliferation.

8.4. Dermal matrix deposition and organisation

Ultra-structural analysis and histology revealed that DFS in vitro produced their own collagen matrix. In accordance with studies where fibroblasts were seeded in dermal substitutes ²¹⁹, DFS showed in vivo increased collagen deposition, whereas collagen organisation was not altered. Matrix organisation by DFS could be improved by increasing oxygen levels and adding BOEC-conditioned media to the growth media. While the BOEC transplantation had a stimulating effect on collagen deposition, this effect was not additive to that of DFS. Thus, while DFS are important for collagen deposition, the BOEC are determinant for organisation of the deposited collagen. In fact combining both in a dermal construct resulted in the formation of a matrix, the abundance and organisation of which started to resemble the dense basket-weave pattern of normal skin (Fig. 38). Exactly which factors in BOEC-conditioned media are responsible for this improved organisation, remains to be determined. However, we suggested a potential mechanism for this by considering a number of growth factors and MMP (see 7.).

9. Wound repair or wound regeneration?

In contrast to foetal wound repair, postnatal wound healing always results in scar tissue that is distinct from uninjured skin. The final aim of skin tissue engineering, however, is to create a skin substitute for reconstruction of large skin defects that, after healing, resembles normal skin as much as possible. We wish to underscore that our initial goal in this doctoral project was to test the effects of improved vascularisation on the wound healing process in the earlier stages and that our latest observation end-point (i.e. 10 days after wounding) therefore is well before reaching that final aim of obtaining a normal skin texture. Indeed, when comparing normal skin to wounds treated with the combination of BOEC and DFS, we acknowledge that certain aspects are still quite different, such as the incomplete remodelling of the dermal matrix (see above; Fig. 38), the intense hyper-vascularity of the wound bed, and the presence of an epidermal layer that is quite thicker than the one covering normal skin.

While the high metabolic demands of the initial stages of wound healing require intense vascularisation, the continuous abundance of blood vessels seems undesirable. Therefore, during the later stages, the wound is further extensively remodelled and EC, macrophages and (myo)fibroblasts undergo apoptosis or exit

from the wound, leaving a scar consisting mostly of collagen and only few cells and vessels ²²¹.

In our wound model, we did not examine scar tissue at later time points to look for scar maturity or persisting hypervascularisation, but we believe this item should be addressed before clinical application of BOEC in wound healing. In our model, BOEC were only sporadically found in the wound bed at 10 days after wounding. Nevertheless, this temporary presence was however sufficient to sustain the effect on re-vascularisation, perhaps due to a prolonged effect of the trophic factors they released.

On the other hand, the fact that BOEC were no longer abundantly present at day 10 may be compatible with vascular regression in later stages of the wound healing process beyond day 10. The reason why BOEC persisted longer (e.g. 2 to 3 weeks) in wound healing studies reported by others ^{78,67} may be related to the use of another genetic background of the recipient mice, the use of another matrix or the continuous replenishment with bone marrow-derived cells from the donor marrow following marrow reconstitution.

Along the same lines, restoration of the epidermal barrier in wound healing should mimic the natural situation. Epithelial tongues in wound healing however display firm keratinocyte proliferation, with several layers of nucleated keratinocytes superimposed, unlike the thin epidermis in non-injured skin. The primary goal of wound healing is the restoration of the epidermal barrier and therefore keratinocyte proliferation and subsequent migration is needed, leading to increased thickness of the epithelial tongues in wound healing. After wound closure, i.e. total re-epithelialisation, the keratinocytes further differentiate gradually towards corneocytes ¹²⁸, thus leading to the different epidermal layers (stratum basale, stratum granulosum, stratum spinosum, stratum corneum). Our results clearly demonstrate that BOEC-induced wound oxygenation and growth factor production is likely to stimulate this epidermal healing response. The BOEC-driven increased keratinocyte proliferation and migration we observed *in vitro* was translated in thicker epithelial tongues *in vivo* and increased keratinocyte migration (as evidenced by connexin43 down-regulation). However, at our latest observation time point, epithelial coverage of the wound was just completed and the process of keratinocyte differentiation was only just started. Therefore, to fully score, keratinocyte differentiation, it might be interesting to examine the epidermis at a later time point in future studies.

10. Is there a backside of the medal?

We successfully applied BOEC to induce vascularisation in secondary wound healing and in combination with a dermal substitute. In addition, we demonstrated the beneficial effects of BOEC on dermal and epidermal healing. Are BOEC and DFS therefore the ideal candidates for clinical use in chronic wound healing and for vascularisation of all kind of dermal substitutes? Although both cell components appear to be very promising, some considerations must be made.

10.1. BOEC need time to expand

Isolating and expanding BOEC is a time-consuming process. It easily takes 6 weeks before BOEC are expanded to a clinical scale, which may impede autologous use of these cells. In case of burn wounds, surgical debridement is classically postponed until clinical evidence has arisen on the depth of the burn – and therefore the likeliness to properly heal without surgical intervention – which is normally after 3 weeks. Moreover, large burns often require staged reconstruction with multiple surgical interventions, and the total treatment often takes months. Thus, in case of burn patients, the long-term procedure for BOEC upscaling is not an impediment. Furthermore, if BOEC are intended to treat chronic wounds, time lag for BOEC expansion neither is a major drawback.

Whereas we almost always succeeded in isolating BOEC (success ratio: 26/28 isolations, in 7/8 individuals), other authors report lower isolation success rates^{222, 223}. This may be related to the fact that we worked with young donors from whom it may be easier to retrieve BOEC than from older individuals. An unsuccessful isolation can easily delay treatment with 4 weeks, which is regrettable not only for the patient, but also for the hospital and society, in terms of prolonged hospital stay and increased costs related to the new isolation. To optimise BOEC isolation, Kolbe *et al.*²²³ recently proposed to fit in an additional passaging step at day 7 of BOEC isolation, thereby increasing outgrowth efficiency.

10.2. The need for animal-related ingredients for cell-based tissue engineering?

For both the isolation and expansion of BOEC and DFS, FBS is used, which inherently raises questions on disease transmission. In a recent report however, Reinisch *et al.*²²⁴ describe a large scale, animal protein-free humanised expansion strategy for BOEC that preserves their proliferation potential. They sidestep the histopaque density centrifugation step by plating whole blood and replace FBS by human pooled platelet lysate from more than 40 donors. This humanised protocol is an important headway towards clinical application of BOEC. Serum-free isolation and culture of

fibroblasts has lately been described as well ²²⁵. Whether or not the thus obtained fibroblasts are able to be grown in multiple layers, produce matrix in vitro and be transformed in an applicable DFS, needs to be determined. It should however be noted that animal-free protocols are not indispensable for allowance of clinical use. Keratinocyte sheets, for example, are used clinically, even though their expansion and differentiation depends on (irradiated) FBS and cholera toxin.

10.3. Lower numbers and dysfunctionality of BOEC?

The number and function of EPC derived from patients with chronic wounds and burns might be altered due to co-morbidity. Patients with chronic wounds often have cardiovascular co-morbidity. Except for an acute ischemic event, where the number of early EPC is increased in the circulating blood ^{226, 227}, chronic cardiovascular disease is known to be related to lower numbers of early EPC and therefore several isolation methods of early EPC are used to predict clinical outcome in cardiovascular disease ^{182, 183}. Heiss *et al.* ²²⁸ demonstrated that – although the numbers of early EPC are not altered with age – their function, survival, proliferation and migration was significantly reduced in healthy elderly (average 61 year old) people, compared to healthy young (average 25 year old) people. Likewise, dysfunctional early EPC were found in patients with ischemic cardiomyopathy ^{229, 230}. Sugihara *et al.* ²³¹ noted decreased incorporation of bone marrow cells derived from elder mice in a mouse hind limb ischemia model.

These findings suggest that the vasculogenic potential of undifferentiated mononuclear cells derived from aged bone marrow is decreased. Equivalent studies examining the in vivo vasculogenic potential of BOEC in pathological conditions have not been carried out yet. Nevertheless, like early EPC, BOEC number/outgrowth appear to be increased in patients with acute myocardial infarction ²³². Moreover, in a pig model, Huang *et al.* describe that besides the number of colonies, the expansion capacity of BOEC is dramatically increased following acute myocardial ischemia ²³³. However, unlike with early EPC, in the absence of acute ischemic events, the number of BOEC is also *increased*, not decreased. There even appears to be a positive correlation between the severity of coronary stenosis and the number of BOEC ²³⁴. Besides, there are no differences in functional behaviour in vitro between BOEC isolated from patients with coronary artery disease and BOEC isolated from young healthy donors ²²². Thus, from these initial observations it seems that, in contrast to early EPC, ‘true EPC’ function and release into circulation is stimulated in both acute *and* chronic pathologic conditions, yielding increased BOEC outgrowth. On the other hand, it appears that even though the number of BOEC is increased in chronic diabetic disease with retinopathy, their function was impaired ²³⁵. Additional studies will be needed to confirm these initial observations.

The occurrence of burns (which can be considered as an acute event) is associated with increased mobilisation of bone marrow-derived cells. Increased levels of CD34⁺CD133⁺VEGFR2⁺ cells (corresponding to early EPC) were detected upon burn injury²³⁶. In a porcine burn injury model²³⁷ and in human burn patients²³⁸ increased levels of CD34⁺ mobilised cells were detected. As BOEC originate from CD34⁺ precursors (Fig. 37), BOEC outgrowth might be increased upon burn injury. However, no reports have been made on BOEC outgrowth or characteristics following burn injuries. We isolated BOEC from a single burn patient and noted increased outgrowth (data not shown), but further research is indispensable to verify this finding.

In summary, as BOEC levels appear to be increased in acute and chronic pathologic conditions that are linked to extensive or chronic wounds, BOEC therapy – combined or not with DFS – seems an appealing approach. We are currently investigating the use of BOEC in pathologic wound healing, such as in diabetes.

10.4. Off-target vascularisation or tumour formation by BOEC?

BOEC may theoretically assist in undesired vessel growth in vivo. Depending on the animal model used, type of tumour and time point of analysis, the contribution of bone marrow derived cells to tumour angiogenesis is either limited^{239, 240} or substantial²⁴¹. However, when BOEC are considered for clinical use in wound healing, they are ex vivo expanded to large quantities and subsequently applied *locally* to the wound. It seems therefore unlikely that they will exert considerable systemic effects on any possible malignant process at a distant site. Yet, it appears that in patients with age-related macular degeneration, BOEC number is significantly increased, in comparison with age-matched non-affected controls²⁴². It remains to be determined whether the raised BOEC levels directly give rise to the pathologic vessel formation in the retina, or BOEC mobilisation occurs secondarily to the pathologic circumstances in macular degeneration.

On the other hand, regarding their vast expansion capacity in vitro, BOEC might theoretically give rise to endothelial cell tumours in vivo. Cord blood-derived BOEC are known to have a greater expansion potential compared to peripheral blood-derived BOEC, but also have a high incidence of cytogenetic aberrations whereas no karyotype alterations have been observed in peripheral blood-derived BOEC³⁰. Therefore, it is unlikely that peripheral blood-derived BOEC would form tumours upon in vivo application. Furthermore, while BOEC display a large proliferative capacity in vitro, it is questionable whether BOEC maintain this large proliferation capacity to the same extent upon transplantation in vivo. Indeed, we noticed firmly decreased numbers of BOEC 10 days after transplantation to the wound in mice, suggesting that their proliferative features can only be maintained under angiogenic conditions in vitro.

L'heure de la fin des découvertes ne sonne jamais

(Collette)

Conclusions & Perspectives

1. A vascularised three-dimensional skin substitute for clinical use: are we there yet?

Restoring the epidermis is hardly ever a problem in non-infected, well-debrided wounds, using very thin split thickness grafts or even cultured keratinocyte grafts. Even patients with very extensive full thickness burns can successfully be grafted, re-using donorsites and massively expanding split thickness skin grafts. Nevertheless, cultured keratinocyte grafts often lead to very unstable epidermal healing ²⁴³. However, restoring the deeper layers of skin, i.e. the dermis and the subcutis, is more complicated and therefore we focussed on vascularization of a dermal substitute.

The work in this thesis demonstrates a body of evidence on the beneficial effects of both BOEC and DFS for dermal skin tissue engineering. However, some important steps lay ahead before clinical translation of this concept is feasible.

1.1. The stop-over in the pig model

As mentioned above, wound healing in mice is different from human wound healing in terms of composition of skin and degree of contraction. Before making the switch from this mouse model to clinical trials in humans, exploration of the effect of BOEC in a large animal model should be done. As porcine skin much resembles human skin, we focused on isolating porcine BOEC for use in a porcine wound healing model. We successfully isolated porcine BOEC and demonstrated the phenotypic and functional resemblance with human BOEC. We already described application of porcine BOEC to full thickness wounds in pigs, in combination with an autologous platelet-rich plasma gel, and noted firmly increased vascularisation in BOEC treated conditions ²⁴⁴. Although these data corroborate the findings in our mouse wound healing models further research in pigs will give a decisive answer about the effect of BOEC +/- DFS on dermal and epidermal wound healing. Also, we included no epidermal layer in our skin construct, as the wounds were limited in size and easily re-epithelialized spontaneously. This limited the number of clinical groups, which allowed us to clearly dissect out the mechanisms by which BOEC interact with fibroblasts and keratinocytes. It is beyond doubt that an epidermal layer should be included when aiming at restoration of big defects and we therefore included a multilayered keratinocyte sheet with pDFS and pBOEC in our experiments in a porcine model with larger wounds (data not shown).

1.2. The temptation of pre-vascularisation

Considering the in vitro vasculogenic effect of BOEC, another strategy might be to combine BOEC and DFS and culture them for some time in vitro, in order to make a pre-vascularised dermal substitute. This procedure complicates the construction of the dermal template, but has the theoretic advantage of allowing direct inosculation of the pre-formed vessels in the dermal substitute to the host vasculature, whereas in the current setting BOEC have to form vessels upon transplantation in vivo⁵, leading to initial hypoxia of the dermal fibroblasts. However, as hypoxia initially triggers the healing response^{143, 147, 245} and DFS appear to be quickly vascularised by BOEC, the need for in vitro pre-vascularisation is a matter of dispute. To our knowledge, no BOEC-mediated in vitro pre-vascularisation experiments prior to in vivo transplantation of dermal substitutes have been described. However, the idea of a turnkey vascularised dermal substitute is very appealing and needs further exploration.

1.3. Digging deeper into engineered skin

When aiming at restoring full thickness skin, attention should be paid to the subcutaneous fat layer as well, since it is important for thermoregulation, skin elasticity and aesthetics (Fig. 41). MSC of different origins can differentiate into adipocytes¹⁰⁴, and can thus be used as a source of adipocytes in skin substitutes²⁴⁶. However, fat itself is highly vascular and a rich vascular network is essential to support the demands of an adipose construct¹⁰¹ and a rather thick subcutaneous layer tremendously impedes oxygen supply to the more superficial layers. Adipose tissue-derived MSC could also be differentiated towards angiogenic cells and provide therefore interesting candidates for subcutis tissue engineering. Growth factor therapy has been used for vascularisation of subcutaneous fat grafts, however leading to unstable vessels¹⁰¹. Several pre-lamination or pre-fabrication steps will be mandatory to induce quick neo-vascularisation in the fat tissue in first stage, to allow for survival of the superficial dermal and epidermal layers. Alternatively, subcutaneous fat can be restored after wound repair with a dermal substitute. This can be achieved either by classical fat grafting²⁴⁷ or by in vitro expansion of pre-adipocytes and subsequent application under the reconstructed skin²⁴⁶.

Figure 41. Future challenges in STE. While vascularization is an important cornerstone, future challenges in skin tissue engineering (STE) include the implementation of additional anatomical features into the STE construct, such as lymphatic vessels (in green) and a subcutaneous fat layer (in yellow).

1.4. Lymphatic vessels: the often neglected counterparts of blood vessels

In addition to blood vessels, the skin also contains another vascular network, the lymphatic vessels which drain fluid ('lymph') and inflammatory cells from the tissues (Fig. 41). From studies in diabetic mice that feature impaired wound healing, it was shown that the reduced healing process is in part due to lymphatic defects²⁴⁸. Impaired lymph drainage may lead to fluid accumulation and lymphedema. Therefore, ideal cell-based approaches for skin tissue engineering may have to consider including a lymphatic network in addition to blood vessels to avoid edema formation. Recently, it was shown that after implantation of AlloDerm, an acellular dermal matrix, host-derived lymphatic EC infiltrated into this matrix after two weeks²⁴⁹. So far, this paradigm has however not been tested in an in vivo wound setting using exogenous cells.

2. General conclusion

In this project, a dermal tissue engineering strategy that allows a completely autologous approach is described. BOEC appear to be extremely potent in wound vascularisation. Besides, they exert multiple advantageous effects on dermal and epidermal healing. The dermal substitute consisting of multilayered dermal fibroblast sheets meets the needs of dermal matrix in terms of inhibition of contraction. Integration of BOEC in this dermal substitute combined the positive effects of both components and is therefore very attractive for clinical use. Future studies will have to focus on applying this strategy in a diabetic wound healing model as well as on expanding this vascularised dermal layer with a subcutis and a lymphatic vessel network.

*Croire à la médecine serait la suprême folie
si n'y pas croire n'en était pas une plus grande,
car de cet amoncellement d'erreurs se sont dégagées,
à la longue, quelques vérités.*

(Marcel Proust, A l'ombre des jeunes filles en fleurs, 1920)

Summary

Vascularisation is the cornerstone of wound healing. We therefore aimed to test a re-vascularisation strategy for wound healing based on the use of human blood outgrowth endothelial cells (hBOEC), a subset of endothelial progenitor cells (EPC), combined or not with a self-assembled human dermal fibroblast sheet (hDFS), intended as a tissue-engineered dermal substitute with inherent vascular potential.

We first thoroughly characterised hBOEC by comparing them to early EPC and human umbilical vein endothelial cells (HUVEC) and firmly established that hBOEC are molecularly and functionally quite distinct from both other cell types. We next tested hBOEC in a preclinical mouse model of wound healing. Unlike HUVEC, hBOEC alone efficiently re-vascularised and re-oxygenated the wound bed, both by active incorporation into new vessels and by trophic stimulation of host angiogenesis. Furthermore, hBOEC alone accelerated epithelial coverage and matrix organisation of the wound bed. In addition, integration of hBOEC in hDFS not only further improved vascularisation, epithelial coverage and matrix organisation, but also prevented excessive wound contraction.

In vitro analyses with hBOEC, fibroblasts and keratinocytes revealed that these effects were both due to growth factor cross-talk and to short cutting hypoxia. These in vitro studies also revealed that hBOEC-derived Placenta Growth Factor (PIGF) was – at least in part – responsible for stimulating keratinocyte migration and proliferation. Furthermore, we found that the growth factor expression profile of hBOEC and hDFS and their effects on re-vascularisation, re-epithelialisation and dermal matrix remodelling was remarkably complementary, which likely explained the success of using them in combination.

Overall, this combined tissue engineering approach paves the way for clinical development of a fully autologous vascularised dermal substitute for patients with large full thickness skin defects.

Science is but an image of the truth.

(Francis Bacon)

Dutch summary

Vascularisatie is de hoeksteen van wondgenezing. We beoogden daarom een manier te ontwikkelen om de vorming van bloedvaten in een wonde te verbeteren met behulp van een bepaald type Endotheliale Progenitor Cel (EPC), met name human Blood Outgrowth Endothelial Cells (hBOEC). Deze cellen werden, al dan niet in combinatie met dermale fibroblasten enten (hDFS), gebruikt om via tissue engineering een dermaal substituuat te genereren dat de capaciteit heeft om bloedvaten te vormen.

Vooreerst hebben we hBOEC zeer grondig gekarakteriseerd door ze te vergelijken met een ander type EPC, met name 'vroeg EPC', en met endotheliale cellen uit de navelstrengvene (HUVEC). We hebben aangetoond dat hBOEC uitermate verschillend zijn van deze celtypes, zowel op moleculair als op functioneel niveau. Vervolgens hebben we hBOEC getest in een pre-klinisch wondgenezingsmodel in muizen. In tegenstelling tot HUVEC, waren hBOEC op zich in staat de wonde efficiënt te voorzien van bloedvaten en zuurstof, deels door zelf actief bloedvaten te vormen (vasculogenese), deels door de vorming van bloedvaten door de gastheer te stimuleren (angiogenese). hBOEC zorgden verder voor een snellere genezing van de opperhuid en betere organisatie van matrix proteïnen in de lederhuid. De integratie van hBOEC in hDFS leidde bovendien niet alleen tot een verdere verbetering van zowel de vascularisatie van het wondbed als de genezing van de opper- en lederhuid, maar verminderde daarnaast de wondcontractie ook aanzienlijk.

Met behulp van in vitro experimenten toonden we aan dat deze effecten enerzijds te wijten waren aan een intensieve groeifactor-communicatie tussen hBOEC, fibroblasten (uit de lederhuid) en keratinocyten (uit de opperhuid), en anderzijds aan het verbeteren van de zuurstofvoorziening van het wondbed. We konden bovendien aantonen dat de door hBOEC aangemaakte groeifactor Placenta Growth Factor (PIGF) – op zijn minst ten dele – verantwoordelijk was voor het gunstige effect op keratinocyten. Daarenboven stelden we vast dat de aanmaak van groeifactoren door hBOEC en hDFS en hun effecten op vascularisatie en genezing van de opper- en lederhuid opmerkelijk complementair was, hetgeen hoogstwaarschijnlijk het succes van de combinatie van beide in wondgenezing verklaart.

Deze gecombineerde aanpak om huid te herstellen effent het pad voor de ontwikkeling van een volledig autoloog, gevasculariseerd, dermaal substituuat om patiënten met grote huiddefecten te behandelen.

*Il faut n'appeler science que l'ensemble
des recettes qui réussissent toujours.
Tout le reste est littérature.*

(Paul Valéry)

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*But in science the credit goes to the man who convinces the world,
not to the man to whom the idea first occurs.*

(Sir Francis Darwin; Eugenics Review 6: I (1914))

The child is the father of the man.

(William Wordsworth)

Curriculum vitae

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*Le vent se lève! . . . il faut tenter de vivre!
L'air immense ouvre et referme mon livre,
La vague en poudre ose jaillir des rocs!
Envolez-vous, pages tout éblouies!*

(Paul Valéry)

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