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Cell-Based Vascularization Strategies for Skin Tissue Engineering

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Providing a blood–vascular network to promote survival and integration of cells in thick dermal substitutes for application in full-thickness wounds is essential for the successful outcome of skin tissue engineering. Nevertheless, promoting vascularization also represents a critical bottleneck in today's skin tissue engineering practice. Several cell types have been considered and tested, mostly in preclinical studies, to increase vascularization. When the clinical situation allows delayed reconstruction of the defect, an autologous approach is preferable, whereas in acute cases allogeneic therapy is needed. In both cases, the cells should be harvested with minimal donor-site morbidity and should be available in large amounts and safe in terms of tumor formation and transmission of animal diseases. Here, we outline the different mechanisms of cell-based vascularization and subsequently elaborate in more detail on the candidate cell types and their pros and cons in terms of clinical application and regulation of the wound healing process.

Introduction

The most widespread treatment for reconstruction of large superficial and full-thickness skin defects (e.g., after deep burns, trauma, or chronic wounds) still is the use of split-thickness skin grafts.[1] These very thin grafts survive initially by imbibition. After 6 days, connections are made between the host and graft vascular networks through inosculation.[2] As such grafts merely restore the epidermis and only a small part of the dermis, they often result in excessive contraction and ugly, brittle scars, with detrimental results at delicate, visible locations (e.g., the face) or in joint regions, where mobility threatens to be impaired.

Skin tissue engineering (STE) emerged in the 1980s as an alternative or adjuvant to skin grafting for the indications mentioned above where skin grafts are often not available in sufficient amounts. Initially, STE aimed at restoration of the epidermal barrier using confluent keratinocyte layers.[1,3] Subsequently, the development of a dermal substitute targeted the missing dermis in full-thickness wounds.[4,5] Nowadays, such dermal substitutes are commonly used for treatment of full-thickness burn wounds in the face and over exposed fragile structures, such as blood vessels and tendons with encouraging results in terms of healing, pain relief, and cosmetic outcome.[6] More recent skin substitutes combine an epidermal and dermal layer by introducing fibroblasts and keratinocytes into an acellular matrix.[6] However, the more layers are combined, the more likely the cell layers by further from the wound surface will die due to hypoxia. Although acute hypoxia initially triggers fibroblast[7] and keratinocyte[8] healing responses, sustained hypoxia leads to fibroblast dysfunction,[7] decreased keratinocyte migration and proliferation,[9] and tissue loss.[10] Hence, a major threat for clinical use of dermal substitutes is insufficient vascularization leading to loosening, infection or partial necrosis of the substitute.[11]

Many approaches have been tested to vascularize skin substitutes. Several growth factor (GF) delivery techniques have been suggested to increase vascularization.[12–14] Since these approaches often require repetitive administration, skin substitutes seeded with cells producing GF on a continuous basis provide an attractive alternative. For example, seeding fibroblasts or keratinocytes results in faster vascularization due to GF secretion[15] and we have shown vascular endothelial growth factor (VEGF) overexpression in keratinocytes to augment wound vascularization.[16,17] Aside from GF supplementation, incorporating cells into a skin substitute may also have other advantages. For instance, fibroblasts may serve as a living scaffold producing their own matrix that facilitates migration and proliferation of other cells required for restoration of full-thickness wounds.

Delivering cells of endothelial origin remains the most appealing option to enhance neovascularization, as these cells are destined to directly contribute to vessel formation. The skin substitute can either be seeded with endothelial cells (EC) to form a vascular network *in vitro*, before

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application to the wound—the so-called prevascularization technique—or be combined with the EC directly before transfer to the wound. *In vitro* prevascularization results in faster inosculation to the host vascular network,[18] but sometimes raises the problem of unequal cell distribution in the skin substitute, leading to incomplete vascular networks. Several rather complex methods such as low pressure centrifugation have been described to equally distribute cells over matrices.[19,20] A valuable alternative seems therefore to opt for *in vivo* (*in situ*) assembly of vascular networks within skin substitutes that have been preseeded *in vitro* with endothelial (precursor) cells that organize themselves in cooperation with endogenous EC into vascular tubes upon implantation. Furtherstill, stable and functional vascular networks depend on the recruitment of pericytes (small vessels) and vascular smooth muscle cells (SMC; large vessels) around the endothelial tubes, a process that is most likely easier to accomplish *in situ.*[21]

Formation of an endothelial network can either happen through angiogenesis, that is sprouting from existing endothelial tubes, or through vasculogenesis, that is *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/exogenously added to the skin substitute and subsequent connection to/incorporation into the host's vascular network (Fig. 1). 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, in a clinical setting, allogeneic cells may have a limited life-span due to immunologic rejection.[22,23] Autologous cells are not subject to immunologic rejection, but often they are available in only very small numbers, necessitating time-consuming *ex vivo* expansion of these cells to therapeutic amounts.

Many cell types may contribute to vascularization in STE through angiogenesis, vasculogenesis, or both. Preferably, in addition to enhanced formation of an endothelial network, the cells may also support vessel stabilization by pericytes/SMC and have a trophic effect on wound healing beyond vascularization, that is, by communication with other cell types present in the wound bed such as fibroblasts and keratinocytes that altogether orchestrate the sequential phases of the wound healing process (Fig. 1). Further, the ideal cell candidate for vascularization of engineered skin substitutes in a clinical setting should (1) be easy to harvest, with minimal discomfort/risk to the donor; (2) be easily and rapidly expandable to amounts sufficient for vascularization of large skin substitutes; (3) present no risk of malignant transformation; and (4) be nonimmunogenic. In this overview we discuss a number of candidate cell types for vascularization, discuss their use in STE *in vitro* and *in vivo* and their mechanism of action, and we score them according to the above-listed criteria related to clinical applicability (Table 1).

Cell Sources for Vascularization Strategies

Different (stem/progenitor) cells can be considered for vascularization of skin substitutes. Hereafter, we give an overview of these, thereby using the (endothelial) differentiation potential/status of the cells as a way to categorize them in a hierarchical system (Fig. 2). 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

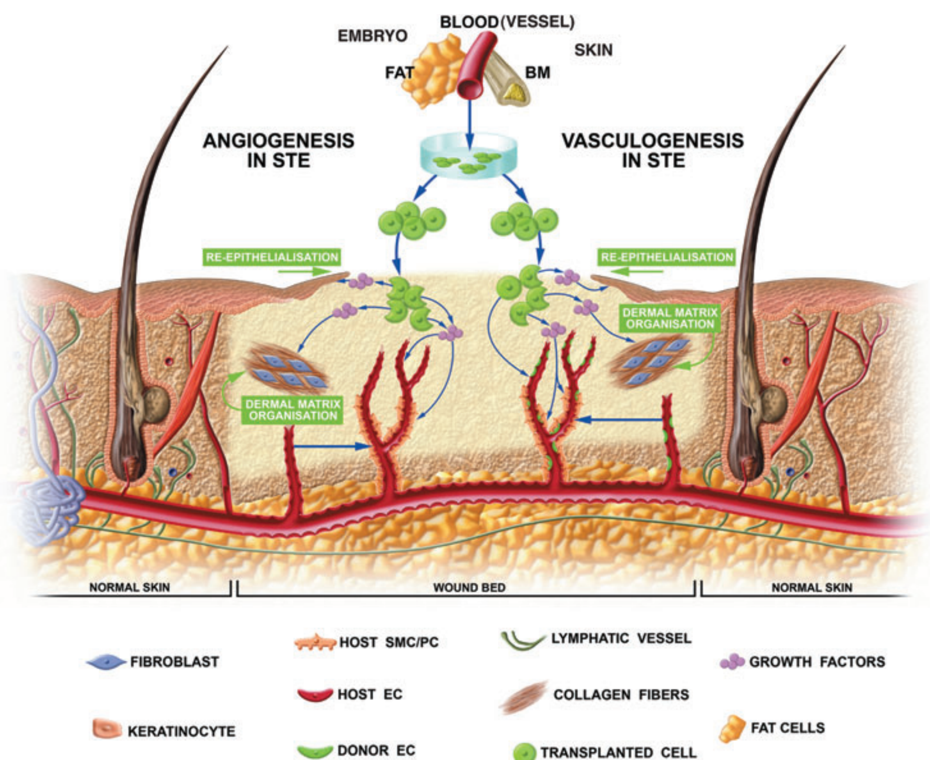

FIG. 1. Angiogenesis versus vasculogenesis in STE. *Ex vivo* expanded cells (in green) of different origins (fat, blood [vessels], and BM) are applied to the wound bed and stimulate vessel formation either by secretion of angiogenic factors (in purple) that communicate with endogenous vascular cells (angiogenesis) or by direct incorporation into nascent vessels (vasculogenesis). Moreover, these cells also secrete trophic factors (in purple) that can stimulate dermal and epidermal healing by communication with fibroblasts and keratinocytes, respectively. STE, skin tissue engineering; BM, bone marrow; EC, endothelial cells; SMC, smooth muscle cells; PC, pericytes. Color images available online at www.liebertonline.com/ten.

Table 1. Advantages and Disadvantages of Different Cell Types for Vascularization in Skin Tissue Engineering

		Clinical applicability				Mechanism of action			
Cell type	*Origin*	*Autologous approach possible?*	*Genetic stability*	*Number of cells after expansion*	*Harvest*	*Vasculogenic*	*Angiogenic*	*Effect on vessel stability*	*Trophic effect on wound healing*
Human dermal microvascular EC	Patient's skin	Y	+	+[11]	Skin sample needed	+[29,a]	?	+[29]	?
	Neonatal foreskin	N	+	+	Discarded tissue	+	?	?	?
Human umbilical vein EC	Umbilical cord	N	+	++	Discarded tissue	+[9,a]	−[9,52]	−[9]/+[36] (Bcl-2)	−[9]
Early endothelial progenitor cells	AB	Y	+	+	Peripheral blood	−[9,46]	+[45]	?	+[47]
OEC	CB	N	+/−[55]	++++	Discarded tissue	+	+[52]	+[52]	?
	AB	Y	+[55]	+++[9]	Peripheral blood	+[9]	+[9,52]	+[9,52]	+++[9]
Mesenchymal stem cells	BM	Y	+/−[69,b]	+++	BM punction	+[74]/−[76,126]	+[75]	+[74,76]	+[79,81]
	Fat	Y	+/−[70,b]	+++	Lipo-aspiration	+[85–87]/−[89,90]	+[94]	+[94,96]	+[94,98]
	CB	N	?	++	Discarded tissue	−[103]	−[103]	?	?
Multipotent adult progenitor cells	BM	N	+	+++++	BM punction	+[109]	+[109]	+[109]	+[c]
Embryonic stem cells	Embryo	N	−[115,d]	++++++[115]	Sacrifice of human embryos	+[127]	+[114]	+[114,127]	?
Induced pluripotent stem cells	e.g., skin	Y	−[d]	++++++	Skin sample needed	?	?	?	?

Increased proliferation/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.

[a]Functional connection to host vascularization not documented or apparent.

[b]Only unstable in later passages *in vitro*.

[c]Unpublished results.

[d]Giving rise to tumors (teratomas) *in vivo*.

EC, endothelial cells; AB, adult blood; CB, cord blood; Y, yes; N, no; BM, bone marrow; ?, no literature evidence; +/−, confounding data in literature or context dependent effect; (Bcl-2), after Bcl-2 transduction.

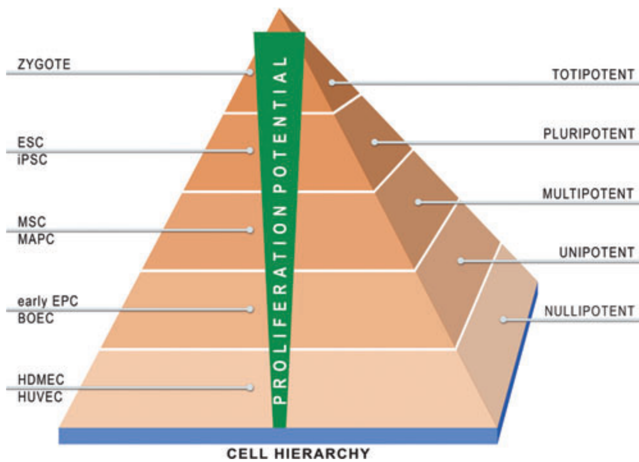

FIG. 2. Hierarchy of cell sources for vascularization in STE. Candidate cell populations for vascularization can be categorized according to their differentiation potential that inversely correlates with proliferation capacity. Cells with the broadest differentiation potential are on top of the pyramid, whereas 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. Color images available online at www.liebertonline.com/ten.

cells that 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.

Mature EC

Human dermal microvascular EC. Microvascular EC can be isolated from skin samples[24] and from neonatal foreskin.[25] Human dermal microvascular EC (HDMEC) are also commercially available as a standardized EC line that is suitable for preclinical testing, such as in *in vitro* wound assays,[26] as well as in *in vivo* blood vessel formation assays.[27] Further refinement of the culture protocol has led to the development of serum-free culture techniques, making these cells safer for potential clinical use in STE.[28] However, despite optimization of techniques to obtain HDMEC, the limited yield requires time-consuming culture expansion (up to 6 weeks) and contamination with fibroblasts remains a challenging problem,[11,25] which currently impedes their use in a clinical setting. Although Nör *et al.* succeeded in creating functional vessels in a subcutaneous pocket when combining HDMEC with matrigel, Supp *et al.* did not demonstrate functional connection to the host vasculature in a skin substitute.[27,29] Human fibroblast sheets have been combined with both human umbilical vein EC (HUVEC) and HDMEC, giving rise to endothelial-lined tube-like structures with apparent lumina *in vitro*. These endothelialized fibroblast sheets have, however, not been used *in vivo* so far.[30] To the best of our knowledge, no reports have been published about the ability of HDMEC to support trophic communication with wound cells (Table 1).

Human umbilical vein EC. When derived from umbilical cord veins, human EC can be expanded relatively well.[31] HUVEC are the most widely used standardized EC line and serve as a reference in many vascularization studies (e.g., refs.[9,32]). 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.*[33] 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.[34] In combination with fibroblasts and keratinocytes in a collagen matrix *in vitro*, HUVEC were able to form an organized vascular network. Notwithstanding the presence of collagen, fibroblasts were required to produce extracellular matrix proteins as a guidance grid for migration of HUVEC.[35] Seven years later, the same team was able to demonstrate quick inosculation of this *in vitro* prevascularized skin substitute, resulting in functional hybrid vessels, 4 days after transplantation.[18] We recently demonstrated that HUVEC seeded alone in a full-thickness wound participated in the formation of hybrid vessels; however, many of the HUVEC-containing vascular structures did not connect to the host vasculature.[9] To prolong survival, HUVEC have been transduced with a virus encoding the antiapoptotic protein Bcl-2, leading to a dense and mature (SMC-coated) vascular network when administered in subcutaneously implanted collagen plugs[36] and in a decellularized dermal matrix.[37] Even though Bcl-2 overexpression on itself did not cause malignant transformation of EC *in vitro*[38] or *in vivo*,[36] 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.[39] In our full-thickness wound model, HUVEC did not support vessel stabilization, nor did they have a trophic effect on host vascular cells, keratinocytes, or dermal fibroblasts[9] (Table 1).

Unipotent progenitor cells: Endothelial progenitor cells

It has long been accepted that vasculogenesis involving endothelial precursors only occurred *in utero* and that postnatal vessel formation was merely the result of angiogenesis. Since the initial description of circulating endothelial progenitors,[40] there is now clear evidence that circulating endothelial progenitor cells (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.[41,42] Currently, two major types of EPC are acknowledged when regarding their isolation kinetics and functional characteristics: early outgrowth EPC and late outgrowth EPC, of which only the latter have the capacity to incorporate into growing vessels.

Early outgrowth EPC. Early outgrowth EPC are obtained by adherence depletion on fibronectin of the mononuclear fraction of cord or adult blood (AB). After 4–9 days, colonies of cells, referred to as Colony Forming Unit-EC, appear with

limited proliferation capacity. Early outgrowth EPC are derived from $CD14^+$ (monocytic) cells[43] and regulate the angiogenic response through secretion of GF,[44,45] without integration into the endothelial intima[46] (Table 1). Application of early outgrowth EPC has proven to increase angiogenesis and epidermal wound healing.[47,48] Although their ease of harvest, defined angiogenic potential, and trophic effect on wound healing make them appealing candidates for STE, their limited proliferation capacities may diminish their clinical applicability. Compared to peripheral blood, cord blood (CB) gives a higher cell yield,[49] but as the latter are allogeneic, they are at risk for immunologic rejection[23] (Table 1).

Late outgrowth EPC. The groups of Hebbel and Yoder characterized late outgrowing endothelial-like colonies of collagen-adherent cells that had a much higher proliferation capacity than early outgrowth EPC. These cells have also been called endothelial colony forming cells or blood outgrowth EC (BOEC).[43,50,51] In contrast to early EPC, BOEC actively incorporate into intimal endothelium and can be expanded to high numbers *in vitro*. Besides the direct incorporation into vessels, BOEC indirectly stimulate host angiogenesis.[52] Unlike previously assumed,[53] we and others have shown that BOEC secrete several GFs that modulate angiogenesis and wound healing[9,52] (Table 1). Interestingly, early outgrowth EPC (or the factors they secrete) have a stimulatory effect on tube formation by BOEC,[53] which makes a combination therapy of both cell types attractive. Cotransplantation of early outgrowth EPC and BOEC had indeed a synergistic effect on revascularization in an ischemic limb model,[54] but there are no reports so far of this combined approach in STE. BOEC can either be derived from cord or AB, with the former showing higher proliferation potential.[55]

1. Cord blood: Many studies highlight the excellent outgrowth of BOEC from CB (CB-BOEC) and suggest them, due to their high expansion and vasculogenic potential, to be ideal candidates for (S)TE purposes.[43,52,56,57] 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 stability in comparison with AB-derived BOEC (AB-BOEC).[56] CB-BOEC formed stable vessels in a dermal PGA-PLLA matrix when cotransplanted with SMC.[58] They also efficiently formed a vascular network when seeded in a decellularized dermal matrix, to a comparable extent as Bcl-2-transduced HUVEC.[52] 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 application of CB-BOEC may not be free of risk for clinical application.[55] Moreover, in the absence of cell banking, autologous CB-BOEC are not available and as an allogeneic cell source are therefore at risk for immune rejection[23] (Table 1).
2. Adult blood: Although AB samples yield fewer BOEC, there is evidence that this discrepancy can be overcome by VEGF stimulation during culture expansion.[59] AB-BOEC have successfully been used in endothelialization of a decellularized aorta in sheep[60] or *in vitro* TE vessel constructs,[61] blood vessel growth in tissue-engineered bone scaffolds,[62] and for vascularization of a decellularized dermal skin substitute, in which they remained traceable for more than 4 weeks *in vivo*, without Bcl-2 transduction.[63] We have introduced AB-BOEC in a dermal fibroblast matrix, leading to enhanced wound vascularization, dermal matrix organization, and re-epithelialization[9] (Table 1). Interestingly, AB-BOEC also induced coverage of the vessels with SMC/pericytes.[9,52] As AB-BOEC and dermal fibroblasts can be isolated from an individual patient, expanded in culture, and engineered in a skin substitute, a fully autologous STE approach is possible. Recently, Reinisch *et al.* have described a large-scale, animal protein-free, humanized expansion strategy for AB-BOEC that preserves proliferation and functional characteristics, even after cryopreservation.[64]

Multipotent progenitor cells

Mesenchymal stem cells. Mesenchymal stem cells (MSC) represent a heterogeneous group of multipotent progenitor cells that can be harvested from several tissues, including bone marrow (BM), skeletal muscle, fat, (umbilical cord) blood, amniotic fluid, and different fetal tissues.[65] MSCs act on wound healing through transdifferentiation or cell fusion to wound healing cells,[66] production of cytokines and GF,[67] maintenance of the extracellular matrix, modulation of the immune system, and wound contraction.[65,68] There is evidence that adipose-derived MSC and BM-MSC kept in culture for long time undergo spontaneous transformation and form tumors *in vivo*, which might jeopardize clinical application[69,70] (Table 1). On the other hand, MSC display immunosuppressive characteristics through interactions with Natural Killer cells, B and T lymphocytes, and monocytes, making them compatible for allogeneic transplantation,[71] Indeed, Bartholomew *et al.* found that survival of allogeneic skin transplants was prolonged when combined with BM-MSC.[72] When considering clinical use in STE, only few sources are possible.

1. BM-MSC: Markowicz *et al.* noted increased vascularization when BM-derived MSC were seeded in a collagen sponge *in vivo*, as well as enhanced collagen production.[73] There are only few reports of endothelial differentiation of BM-MSC *in vivo*[74] and their contribution to neovascularization is most likely related to their trophic effect on host vessels.[75] BM-MSC also stimulate vessel maturation, through differentiation into perivascular cells,[74] and an increased angiopoietin (Ang)1/Ang2 secretion ratio.[76] Their ability to differentiate into α-SMC $actin^+$ cells, however, enhanced wound contraction, even in the presence of a dermal scaffold.[77] Combination of BM-MSC with BM-derived hematopoietic cells with EPC-like characteristics seems to have a synergistic effect on vascularization *in vivo*.[78] There is also evidence that BM-MSC can differentiate into epidermal cells, although in very small amounts.[74] BM-MSC were shown to augment vascularization in a dermal scaffold in a pig model[5] and to induce healing of burns and chronic wounds when combined with an artificial dermis in preliminary clinical trials[79–81] or acute wounds when sprayed in a fibrin glue.[82] None of these clinical trials, however, used a control population, making the results difficult to interpret. Standard MSC

culture protocols include the use of fetal calf serum, possibly leading to zoonoses or immune reactions in the host. Platelet lysate has been advocated as a surrogate for fetal calf serum in isolation of BM-MSC, but it is not yet clear whether this isolation procedure gives rise to cells with identical properties as BM-MSC obtained through the standard isolation protocol.[83,84]

2. Adipose-derived MSC: In contrast to BM-MSC, there is more evidence of differentiation of adipose-derived MSC into EC[85–88] even though some authors are unable to prove direct incorporation into host vessels.[89,90] Most authors use (a modification of) the isolation protocol as initially described by Zuk and coworkers,[91] which consists of enzymatic digestion of lipo-aspirate and centrifugation to obtain the stromal vascular fraction. Adherence depletion on plastic selects the cell fraction that is referred to as processed lipo-aspirate cells,[92] adipose-derived stromal cells,[93] adipose-derived stem cells,[94] or adipose-derived MSC,[89] and that consists of a heterogeneous population that contains vascular (pericytes and endothelial progenitor) cells, besides multipotent MSC.[95] Adipose-derived MSC can also differentiate toward perivascular cells, thereby increasing vessel stability.[93,96] They can relatively easily be harvested and applied in an autologous manner. Moreover, they appear to have a high expansion capacity *in vitro*[97] and are successfully used in STE, leading to increased vascularization and wound closure.[94] They stimulate fibroblast behavior by direct interaction, upregulation of matrix protein synthesis, and secretion of paracrine factors, besides enhanced re-epithelialization *in vivo*.[98] Lipoaspirate-derived MSC are currently used in a phase I/II clinical trial in the treatment of perianal fistula[99,100] and in phase IV trials for restoration of lumpectomy defects in the breast. These trials so far demonstrate the safety and the feasibility of the clinical use of these cells.[99,100]
3. CB-MSC: MSC can also be grown from CB and can be differentiated *in vitro* toward cells of all three germ layers,[101] including cells with endothelial characteristics.[102] However, application in a hind limb ischemia model did not result in increased angiogenesis, but rather in myogenesis.[103] They have therefore been used in a clinical setting where myogenesis might be beneficial, such as in dilated cardiomyopathy[104] and Buerger's disease,[105] but not yet for STE vascularization purposes.

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.[97] 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 vascularization.[87,92,97] Combining human CB-BOEC and BM- or CB-MSC in a subcutaneously implanted matrigel plug in mice resulted in a functional human vessel network that was stabilized by MSC-derived perivascular cells, suggesting for the benefit of a combined BOEC-MSC approach.[106]

Multipotent adult progenitor cells. Other investigators reported 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.[107] These multipotent adult progenitor cells (MAPC) are morphologically, molecularly, and functionally different from BM-MSC and can *in vitro* differentiate into EC that contribute to vasculogenesis *in vivo*[108–111] (Table 1). Although MAPC represent an almost inexhaustible source of cells that can be used in STE vascularization, the long-term procedure required to derive MAPC makes an autologous approach unfeasible. Nevertheless, similar to MSC, MAPC also seem to have immunesuppressive capacity, which may be favorable in an allogeneic setting.[112,113] Despite their large expansion capacity, no karyotypic instabilities have been reported for human MAPC. Our recent data reveal that MAPC transplantation improves wound vascularization as well as re-epithelialization in a full-thickness wound model in immunedeficient mice (unpublished results) (Table 1).

Pluripotent progenitor cells

Embryonic stem cells. Embryonic stem cells (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 toward EC that have been used for therapeutic revascularization of ischemic limbs, where they were shown to directly contribute to vessels.[114] ESC have an unlimited self-renewal capacity and therefore represent an inexhaustible source of EC. They are, however, due to this vast proliferative potential, prone to teratoma formation[115] (Table 1). Transplantation of selected endothelial-predifferentiated ESC can prevent teratoma formation; however, the presence of residual undifferentiated ESC remains a concern. Immortalized ESC-derived EC have been used for tissue-engineering of blood vessels,[116] but we have no record of ESC studies on STE so far.

Induced pluripotent stem cells. 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 four genes (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*).[117] In 2007, induced pluripotent stem cells (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 vitro*,[119–121] but has never been used for vasculogenic purposes *in vivo* so far. Although iPSC are not entirely similar to ESC, they also carry the risk of teratoma formation and genetic instability.[122,123] Moreover, the need for reprogramming by genetic manipulation through viral integration renders an additional risk for transformation.

From Bench to Bedside: How to Make a Choice?

Autologous or not autologous: That's the (first) question

As summarized in Table 1, the choice for the ideal cell candidate is dependent on criteria that are either related to the biological mechanism of action or to the feasibility of clinical application. Importantly, this choice is also largely

and perhaps predominantly determined by the type of patients that need skin reconstruction. Indeed, the urgency of intervention imposes a first choice between autologous or allogeneic cell therapy. For acute situations, such as trauma or extensive burns, the cells should be readily available in sufficient amounts for therapy, which precludes the use of autologous cell sources for vascularization. Studies with HDMEC have shown that it may take up to 6 weeks to prepare therapeutic amounts for STE.[11] Our own experience with AB-BOEC also indicates that it may take several weeks to expand them to a clinically useful number. An important advantage of the latter compared to HDMEC is that the total amount of cells that can be generated is larger, so that excess cells may be cryopreserved for later use in the same patient. Hence, when aiming to cultivate autologous cell-based skin substitutes, one must bear in mind that isolation and expansion of fibroblasts and keratinocytes also takes several weeks, so that all cell types must/can be grown simultaneously.[11] In some clinical cases, however, such as in chronic wounds, reconstruction can be delayed, which leaves sufficient time for isolation of autologous cells. Even in certain large burn cases, staged reconstruction is often required with multiple surgical interventions over a period of several months, such that these patients can still benefit from autologous cell approaches. When skin substitutes are needed in an immediate reconstruction, cells from a frozen stock have to be used, cells that are—in the absence of cell banking—per definition allogeneic, and thus prone to immunologic rejection. Cells with immunosuppressive characteristics, such as MSC and MAPC, offer an important advantage in this context.

Secondary criteria

Genetic instability remains a primary concern for clinical use; therefore, cell sources with documented tumorigenic potential *in vivo*, such as ESC and iPSC, need further preclinical optimization studies before transfer to patients. In addition, clinical use of iPSC awaits development of new nonviral reprogramming protocols to avoid tumorigenicity. Since for MSC the incidence of malignant transformation increases with passage number,[69,70] it seems safer to consider only cells from initial passages for clinical application. When an autologous approach is considered, attention should be paid to donor-site morbidity as well. If large skin samples are needed to obtain sufficient amounts of HDMEC for reconstruction of a tissue defect, cells with a more pronounced proliferation potential are preferable. The ease of harvesting also deserves consideration. Harvesting BM is a painful and not entirely risk-free procedure and the benefits of BM-derived cells should be weighed against these disadvantages. On the other hand, peripheral blood and, to a lesser degree, fat are more easily accessible and give rise to autologous cells that are very promising for vascularization purposes in STE with minimal donor-site morbidity. Finally, even though this has not been an impediment for clinical use of some cell sources, such as MSC,[79–82] the availability of serum-free and xenobiotic-free cell products should offer a safer therapy. Protocols for serum-free culture are under development for several cell sources, including BOEC and MSC.[64,83,84] Similar protocols have also been developed for keratinocyte and fibroblast cocultures.[124] Together, this paves the way for development of a fully autologous, serum-free, vascularized skin substitute.

Which mechanistic criteria prevail?

In addition to—or rather in parallel with—clinical feasibility criteria, another important issue is of course that the cells have the desired biological effect, which means in this context that they should improve vascular supply of thick STE constructs and thereby prevent infection and premature loss of the construct. Vascularization can be either through vasculogenic, angiogenic, or arteriogenic (support of SMC coating) mechanisms. Although it is currently not clear which of these mechanisms is most crucial, one may assume that cells, like BOEC and MAPC, that combine these three properties may have a more robust and functional effect on vascularization. To make choices based on vascular potential, preclinical comparative studies are mandatory. We found that the vascular potential of AB-BOEC was superior to that of HUVEC.[9] Information from comparative studies in other clinical areas where vascularization is important, such as ischemia (for review, see ref.[125]), may also provide useful information. Moreover, as illustrated above, to obtain an optimal and additive or complementary benefit, it may be a good practise to combine different cell types. In addition to improving vascularization, trophic effects on fibroblasts and keratinocytes may be an important additional factor in deciding between two cell sources with equal vascular potential. Unfortunately, this has not been evaluated for many of the cell types under study (Table 1). Finally, in addition to the degree and type of vascularization, an important issue is the duration of the effect. Indeed, while the metabolic demands of the cells within the construct may be high in the initial stages of grafting and therefore require intense vascularization, continuous hypervascularity is not desired and the vascular response needs to eventually subside to return to the baseline vascular profile of normal skin. To evaluate this, longer-term preclinical studies are needed. In our recent wound healing study, we noticed that the number of AB-BOEC in the wound bed was significantly reduced at the time of complete re-epithelialization.[9]

Summarizing Conclusion

Providing blood vessels is essential in STE to promote survival and integration of dermal substitutes into the wound. Several cell types have been considered and tested mostly in preclinical studies to promote vascularization. When the clinical situation allows delayed reconstruction of the defect, an autologous approach is preferable, whereas in acute cases allogeneic therapy is needed. In both cases, the cells should be harvested with minimal donor-site morbidity and should be available in large amounts and safe in terms of tumor formation and transmission of animal diseases. Mechanistically, besides an effect on vascularization, communication through trophic support with other wound cells could be an additional asset. Taking together these clinical and mechanistic criteria, we consider AB-BOEC a very promising cell type for autologous vascularization purposes in STE. In case of allogeneic treatments, fat-derived MSC currently seem promising given their immunesuppressive features. Nevertheless, additional direct comparative and long-term preclinical studies will further help to decide which cells or combination of cells are/is most optimal to use for which patients.

Disclosure Statement

No competing financial interests exist.

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