LEIDEN UNIVERSITY MEDICAL CENTER

RECENT ADVANCES IN VASCULAR TISSUE ENGINEERING

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ABSTRACT

Correct functioning of the vascular system is crucial to avoid the development of cardiovascular pathologies. Despite the increasing economic burden of cardiovascular and peripheral vascular disease in developed societies, current pharmacological and surgical interventions still do not offer a definitive cure for many existing patients. However, vascular tissue engineering is emerging as a promising and powerful treatment option. The selection of an appropriate vascular cell source in addition to the creation of a proper microenvironment that would kept the cells alive and able to maintain or develop a specific differentiated state in the host body, is essential for the implementation of vascular tissue engineering as a feasible therapy. In this thesis, the main cell sources used at present for vascular regeneration are reviewed as well as the hydrogel-based biomaterials that can support and recreate a suitable cell microenvironment, with a special focus on recent advances in customized smart scaffolds. Finally, some relevant perspectives for the future of the vascular tissue engineering field are presented.

Chapter 1. INTRODUCTION

Blood vessels are the major component of the circulatory system, forming a closed branched structure of arteries, capillaries and veins that go all over the body. Arteries carry blood from the heart to the tissues and organs delivering nutrients and oxygen, capillaries distribute blood within the tissues and organs, and veins take away tissue waste matter transporting blood from the tissues back to the heart. Additionally, the vascular system plays a key role mediating immune defence and maintaining the body temperature and pH [2].

In response to specific local cues, endothelial precursor cells or angioblasts migrate and differentiate to create *de novo* blood vessels (vasculogenesis). An angiogenic process of sprouting from pre-existing vessels follows to create the mature vascular network (Figure 1). After such remodelling, complex structures of large and medium sized arteries and veins develop by the assembling of three main layers: the tunica intima, the tunica media and the tunica adventitia. Together they maintain, remodel and repair the blood vessels after injury. Essentially, a vessel is comprised of a lining of endothelial cells (ECs, the endothelium) located in the inner layer (tunica intima) surrounded by a thick and tough wall of connective tissue (tunica intima) and several layers of vascular smooth muscle cells (tunica media) with an outer layer (tunica adventitia) of a collagenous extracellular matrix, fibroblasts and nerves. Each of these strata is separated by elastic basal laminas. ECs are crucial for many physiological functions, but especially to promote structural integrity of the blood vessel and provide a thromboresistant wall. In addition, vascular smooth muscle cells and pericytes are required to stabilize the vessel and prevent further angiogenesis. In contrast to this thick and multi-layer structure required to deal with strong mechanical forces of the blood flow, vessels with a small diameter (microvessels) branch from these arteries and veins and organize themselves in vascular beds. They are mainly formed by an endothelial cell layer surrounded by a basement membrane together with a few scattered pericytes wrapped around the vessel [3].

The vascular system is important for the normal physiological functioning of our body, since when its functionality is compromised, major health

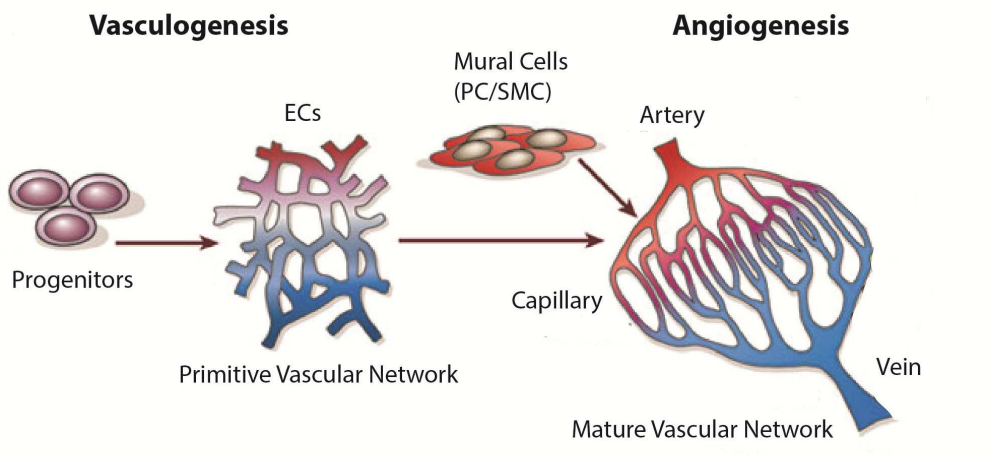

Figure 1. Schematic illustration of the formation of the vascular network. It proceeds first with the *de novo* formation of blood vessels (vasculogenesis) and secondly, with the creation of the mature vascular network from pre-existing vessels (angiogenesis). Mural cells, including both smooth muscle cells and pericytes, contribute to the stabilization of the newly synthesized blood vessels. ECs: Endothelial cells; PC: Pericytes; SMC: Smooth muscle cells. Adapted from *Carmeliet, P. (2005) [1]*

problems result. Actually, vascular and cardiac diseases are the main cause of mortality in the western society and account for up to 30% of all deaths worldwide, representing a major burden for the health system [4]. These diseases include, on the one hand, different venous diseases, such as venous insufficiency, deep venous thrombosis and pulmonary embolism. In most of the cases, excluding deep venous thrombosis, venous diseases are not life threatening. However, they can cause pain and discomfort. On the other hand, diseases that affect arteries (such as atherosclerosis, atherothrombosis and hypertension) contribute to and increase the risk of myocardial infarction, congestive heart failure, renal failure, stroke, valvular heart diseases and blood vessel obstruction or ischemia (myocardial, hindlimb, renal, cerebral). Despite the advanced pharmaceutical approaches and the medico-surgical efforts invested in their treatment, new therapies need to be developed for those patients for whom no adequate therapy is available. Specifically, occlusive atherosclerosis in coronary arterial circulation requires therapies that stimulate neovascularization into the ischemic area or stenting in vascular segments. Pulmonary hypertension involving chronic thromboembolism or hypoxia requires chronic intravenous treatment. Finally, obstructive peripheral vascular disease or microvascular dysfunction and ischemia that often occur in diabetic patients and can cause claudication, gangrene and amputation, are in need of new strategies to restore the vascular wall integrity and function [5]. Taken altogether, new translational therapies, including re-endothelialization, tissue engineering of vascular conduits, graft functionalization, plaque stabilization, organ vasculogenesis and adaptive remodeling are the prospective clinical applications in vascular regeneration.

A powerful solution to restore the damaged vasculature in the above-mentioned pathologies relies on the generation of blood vessels that recreates the original functional tissue. Actually, the discipline of tissue engineering goes back to the mid-1960 with the development of artificial biomaterials for skin-burn treatment [6]. However, the term tissue engineering was defined in the 80s as an “interdisciplinary field that applies the principles and methods of engineering, materials sciences and the life sciences towards the development of biological substitutes to restore, maintain, or improve tissue functions or organs” [7].

Following the historical perspective, pioneer studies in the vascular tissue engineering field include the first tissue engineered blood vessel (TEBV), which was created in 1980 by Weinberg and Bell by mixing bovine endothelial cells, smooth muscle cells and fibroblasts [8]. The mechanical strength of these first vessels was improved by L’Hereux in 1998 by culturing human umbilical vein, smooth muscle cells (SMC) and skin fibroblasts together with sodium ascorbate (vitamin C) which allowed the formation of a 3D extracellular matrix [9]. They showed a vessel wall organization comparable to that in native arteries. However, intramural blood infiltration was observed between vessel layers and long-term patency could not be verified. In 1999, Niklason and colleagues developed a TEBV based on bovine SMC and ECs seeded on polymeric tubes in a bioreactor under flow conditions. The grafts were shown to be thicker, with more smooth muscle cell and collagen density and remained for a longer time [10]. Kaushal *et al* used a different approach based on a decellularized vascular vessel from porcine iliac arteries which served as scaffold. On it, endothelial progenitor cells were seeded and the grafts were subjected to shear stress. The resulting vessels were implanted *in vivo* and remained stable up to 130 days [11]. Although the main restriction of these large-diameter vascular grafts is their poor long-term durability, nowadays they are commercially available for clinical use in a variety of chemistries (such as polyester, polyurethane and polyacrylate, among others).

The focus of current research is to develop polymers and scaffolds that maximally mimic the microenvironment of a specific tissue employing biopolymer chemistry. Thus, natural or synthetic modified polymers would allow the remodeling of

the new vascular tissue by releasing specific growth factors and providing specific mechanical forces and a certain topography, which will dictate a diversity of cell responses via chemical and physical cues [12]. The recreation of this optimized and provisional microenvironment aims to activate endogenous mechanisms of regeneration *in vivo* that will promote the neovascularization not only of damaged tissues but also of artificially created ones.

In essence, the three key basic components for tissue engineering are: the cell source; the signals, including bioreactive agents or growth factors that promote correct cell function; and the scaffolds, that host the cells providing a proper environment [13, 14]. In the following chapters, the cell sources currently available to derive ECs, smooth muscle cells and pericytes are discussed. These are namely adult stem cells (mesenchymal stem cells and endothelial progenitor cells), pluripotent stem cells (embryonic stem cells and human induced pluripotent stem cells) and direct reprogramming. Additionally, a section on biomaterials used to support the cells in the host tissue is also included. The chapter is particularly centered on hydrogel-based scaffolds and the application of customized smart matrices for the regeneration of the vascular tissue.

Chapter 2. CELL SOURCES

A reliable cell source to generate new vascular tissue with a long-lasting repair is a key aspect for success in vascular tissue engineering. Ideally, such a source should be (i) easy to expand in culture or to be produced in sufficient numbers, (ii) able to differentiate into a specific cell type, (iii) produce ECM and adopt an adequate 3D structure, (iv) functional and with the ability to integrate within native cells to avoid immunogenicity and (v) have minimal associated biological risks [15].

Depending on the species from which these cell sources are coming from, they can be classified into autologous (if they come from the patient itself), allogenic (if they come from another human different from the patient) and xenogenic (if the origin is other species than human) [16]. As long as the activity remains high, an autologous source of EC and SMCs is the best option to avoid immunogenicity. However, cells in adult blood vessels are terminally differentiated, meaning that they have a limited proliferative potential. Thus, an important handicap is the harvest of the sufficient amount of this type of cells. Although it would require time and a sterile protocol, a possible way to overcome this situation would be to expand them in cell culture. Taking any of the other non-autologous cell sources would imply the use of immunosuppressive therapy.

Recently, stem cells became a promising cell source in regenerative medicine. They are characterized by their capacity to self-renew thus preserving its undifferentiated state, and differentiate into lineage-specific cells under defined conditions [15]. Based on their differentiation capacity or potentiality, stem cells can be classified into (i) totipotent: cells that can differentiate into embryonic and extraembryonic cell types needed to form a viable organism (ii) pluripotent: cells that can give rise to any cell of the mesoderm, endoderm or ectoderm germ layers but not the extraembryonic tissues (iii) multipotent: cells that differentiate into a specific family of cells (iv) unipotent: cells that produce one cell type but have increased replicative capacity and self-renew. Besides this classification, they have been historically categorized into embryonic stem cells (ESCs) and adult stem cells. The former are pluripotent cells isolated from the inner cell mass of a blastocyst; whereas the latter have a multipotent capacity and are found in several tissue types with ability to proliferate actively in situations of tissue repair. Recently it became possible to generate so-called induced pluripotent stem cells (iPSCs) via reprogramming of somatic cells into cells with the same pluripotent features as embryonic stem cells [17, 18]. Important strengths of iPSCs include the simplicity of its technology in addition to its reproducibility and potential to differentiate into any adult cell of interest.

In this chapter, the focus is on adult stem cells and pluripotent stem cells, both human ESCs (hESC) and human iPSC (hiPSCs) as among the most attractive sources to derive functional ECs, smooth muscle cells (SMC) and pericytes for vascular tissue regeneration purposes. Additionally, direct reprogramming of somatic cells towards ECs is emerging as a very promising vascular cell source which may become of value in the near future (Figure 2).

2.1. ADULT STEM CELLS

Adult stem cells have a more restricted differentiation potential since they are multipotent, meaning that they can only give rise to cell types of the tissue in which they are found. However, adult stem cells have been shown to have important tissue restoration capacity. In vascular regeneration, the use of adult stem cells has primarily centered on mesenchymal stem (or more correctly, stromal) cells (MSCs) and endothelial progenitor cells (EPCs).

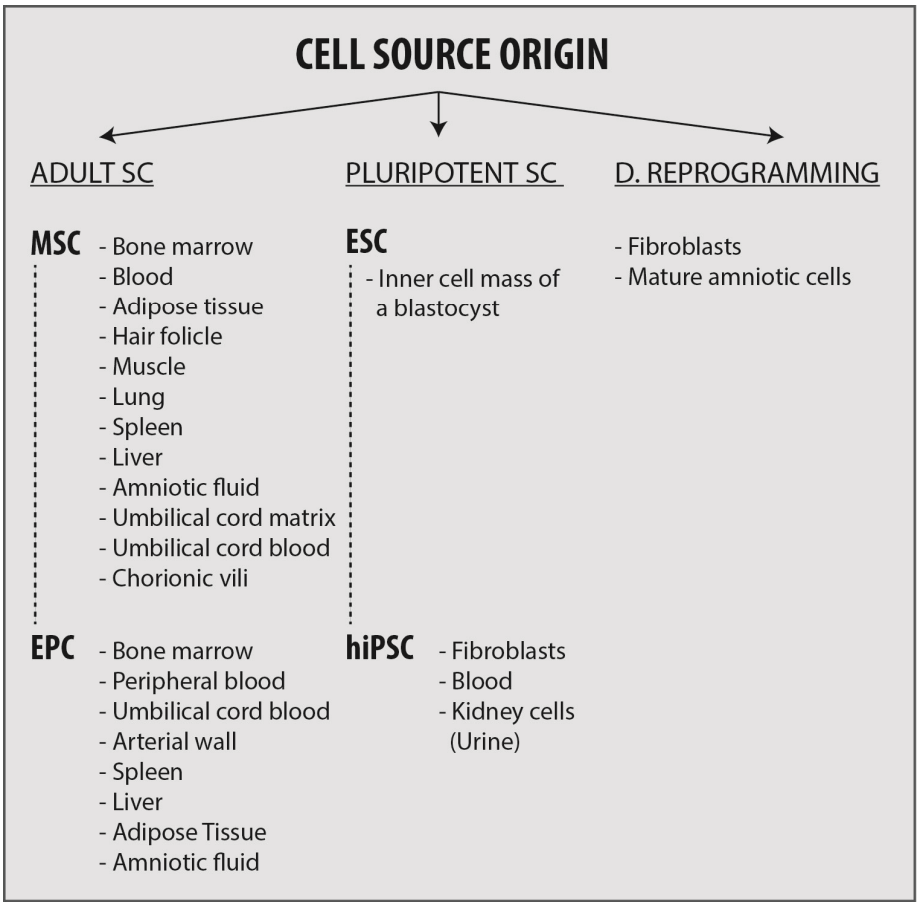

Figure 2. Diagram showing the tissue origin of the cell sources used for vascular tissue regeneration.
SC: Stem cell; MSC: Mesenchymal stem cell; EPC: Endothelial progenitor cell; ESC: Embryonic stem cell; hiPSC: human induced pluripotent stem cell; D. Reprogramming: Direct Reprogramming.

2.1.1. MESENCHYMAL STEM CELLS

Vascular cells can be derived from multipotent mesenchymal stromal (MSCs) cells present in the bone marrow (0.1-0.5%) supporting the HSC niche. Bone marrow-mesenchymal cells (BM-MSCs) adhere to the surface when plated on plastic culture flasks and they can differentiate to osteogenic, chondrogenic and adipogenic lineages. In addition to the bone marrow, other tissues also serve as reservoirs of MSCs, namely blood, adipose tissue, hair follicle, muscle, lung, spleen, liver, amniotic fluid, umbilical cord matrix, umbilical cord blood and chorionic villi [19, 20]. MSCs reside in the perivascular space of all tissues where they contribute to homeostasis and tissue repair [21]. BM-MSCs can be differentiated towards the SMC lineage and stabilize blood vessels *in vivo*. In particular, adipose (AD), hair follicle (HF), umbilical cord (UC) and umbilical cord blood (UCB)-derived stem cells have been shown to be a robust autologous source of vascular cells, including both EC and SMCs, while muscle-derived stem cells (MD-SCs) has been reported to better be a SMC source [19].

2.1.2. ENDOTHELIAL PROGENITOR CELLS

EPCs can be found in the bone marrow, peripheral blood or the umbilical cord blood [22-25]. Recently, tissues including the arterial wall, the spleen, liver, adipose tissue and amniotic fluid have been reported as other sources of autologous EPCs [26]. These cells have been classified using endothelial and progenitor markers for human EPCs, endothelial morphology,

lectin binding or formation of a tubular network in a substrate containing laminin and collagen IV, such as Matrigel™ [27].

The first method used to collect human EPCs was based on the isolation and growth of mononuclear cells from peripheral adult human blood seeded on fibronectin-coated plates in media containing specific endothelial growth factors [23]. As a result, the small population of adherent mononuclear cells (CD34+) showing endothelial features in culture was designated as EPCs. Some studies reported that these cells originate from the bone marrow and can be recruited into the circulation upon physiological signals from the periphery (chemokines, VEGF, erythropoietin, statins [28-30]) or pathological conditions, such as ischemia [22]. After their incorporation into the vasculature, they can acquire some endothelial and perivascular cell features. Although the ability of these cells to differentiate towards ECs, SMCs or pericytes remains controversial, they might contribute to tissue regeneration [31, 32]. In fact, it has been shown that monocytic EPCs could boost the angiogenic process through the release of paracrine signals [33, 34]. However, these primary EPCs have been later demonstrated to be heterogeneous in nature, meaning that they are a mixture of cells from different lineages, including both hematopoietic and endothelial.

A second approach using *in vitro* colony forming cell assays was used to isolate EPCs from peripheral adult human blood, bone marrow and umbilical cord blood. Notably, this technique resulted in the appearance of endothelial-like colonies after 2 to 3 weeks in culture. This last subset of cells was highly proliferative, expressed EC-specific markers, incorporated better into vessel-like structures and lacked expression of hematopoietic markers such as CD14. Therefore, they were truly of endothelial origin. They were termed as late out-growth endothelial cells (OECs) or endothelial cells with colony-forming cells (ECFC) ability [35-38]. At the present moment ECFCs are so-called “gold-standard” of EPCs. In the adult, OECs/ECFCs reside in the bone marrow, but they are also found in circulation. However, umbilical cord blood seem to be the best source of ECFCs since they form functioning blood vessels that last longer and have higher proliferation capacity than ECFCs derived from adult peripheral blood [39]. Despite relatively low numbers (2 ECFC/10^8 leukocytes) in peripheral blood, they have a tremendous regenerative capacity [40].

Finally, a third method that relies on the use of flow cytometry with antigen antibodies for specific EPC-cell surface molecules. However, due to the lack of a EPC specific profile for either the endothelial lineage or early endothelial progenitors, is difficult to separate EPCs from other populations of progenitor cells of different lineages via this procedure [41]. Nowadays, the only reliable approach to identify the endothelial lineage is using the CD133 surface marker or through morphological features [27].

Autologous EPCs could be collected, expanded *ex vivo* and administered to increase tissue reperfusion or to improve re-endothelialization of denuded arteries in hind limb and coronary-ischemia animal models [42-44]. So far, the EPCs used for vascular regeneration in humans are autologous bone marrow mononuclear cells (BMNCs) or G-CSF-expanded peripheral blood mononuclear cells (PBMNCs). Several clinical studies suggest modest but reliable improvements in contractility and vascular repair after some months post-implantation of BMNCs [45, 46]. Despite, the mechanism of how this repair occurs is still not completely understood.

More research needs to be carried out, in the first instance, to develop reproducible and efficient methods for EPC purification, expansion and specific delivery of angiogenic cells and, secondly, to better understand the mechanisms of interaction among EPCs so that the optimal therapeutic combination of progenitor cells can be defined.

The use of adult stem cells in vascular tissue repair presents the following advantages: (i) the cell source is autologous thus overcoming any type of immunogenic issue, (ii) some MSC sources are abundant and easy to access (iii) no ethical concerns apply to adult stem cells since no embryos or immortalization steps are involved and (iv) they are the type of stem cell that has undergone the least manipulation in culture so that most translational and human studies have had safe outcomes. Nevertheless, this approach also has some drawbacks: (i) delay in treatment due to the time required to expand progenitors *ex vivo* prior to implantation, (ii) adverse effects as a result of the delivery method (iii) requirement for tight control of the differentiation potential (iv) insufficient set of markers to accurately identify vasculogenic EPCs (v) their limited availability and proliferative capacity, and dysfunctionality in aged or diabetic patients. In order to overcome this last issue and increase the numbers of EPCs in the target area, several strategies have been used, among them: immobilizing CD34 antibodies to guide circulating stem cells to the injury ("EPC-capture stent"), fixing the cell transplant with injectable biomatrices, co-injecting angiogenic chemokines (SDF-1,VEGF) locally or genetically modify EPCs to make them overexpress factors that would increase their incorporation or their activity and viability (such as human telomerase reverse transcriptase -hTERT, cGMP-dependent protein kinase PKG or enhancing the Akt signaling pathway) [47-52].

2.2. PLURIPOTENT STEM CELLS

2.2.1. EMBRYONIC STEM CELLS

A second stem cell approach is based on ESCs, which are derived from the inner cell mass of a blastocyst. They are pluripotent and able to differentiate into any somatic cell type of the body plus germ cells. Three different strategies have been used to differentiate ECs from ESC: (i) 3D cell aggregates called embryoid bodies, (ii) stromal cells co-culture and (iii) ECM-guided differentiation [19, 53]. After this process, mixed cell types usually arise from which the EC populations have to be isolated and subsequently expanded in culture.

Both ECs and SMCs have been differentiated from ESCs and several studies determined the growth factors and the timing schedule need for vascular specification *in vitro*. In brief, within 2-4 days, the mesoderm fate is induced by cooperative interactions of the canonical Wnt, Activin/Nodal and BMP4 signaling pathways [54-56]. Furthermore, VEGF signaling activators (VEGFA, FGF2) and TGFβ signaling inhibitors (SB431542) that are essential for EC specification and proliferation need to be added [57].

ESC-derived ECs showed specific endothelial markers (such as CD31/PECAM1, VE-cadherin, von Willebrand factor), endothelial functions and they were able to form durable, stable and functional blood vessels *in vivo* [58, 59]. Moreover, several studies showed that ESC-derived ECs incorporate into ischemic murine vasculature [60, 61]. SMCs and pericytes could also be derived from embryonic stem cells. On the one hand, ESC-SMCs supported the formation of longer and thicker cord-like structures *in vitro,* and they also contributed to the development of vascular networks *in vivo* [62, 63]. On the other, ESC-derived pericytes expressed specific pericyte markers (NG2, PDGFb) and promoted the recovery of murine ischemic hind limb [64].

The strengths of such a cell source are its pluripotenciality, allowing ECs, SMCs and pericytes to be derived from one genetic source, in addition the high proliferative capacity. However, ESCs present several disadvantages for therapy, such as (i) the use of embryos is for some still an ethically controversial topic, (ii) cells derived from ESCs are allogenic, implying the administration of immunosuppressive agents if they are used and (iii) risk of teratoma formation after transplantation of ESCs. Therefore, more research

must be carried out to shed new light on the regulation of human cell differentiation and function, which will lead to the development of more robust differentiation and purification protocols in addition to an improvement in safety. For all these reasons, pre-clinical studies in vascular regeneration using ESC are still at a very early stage.

2.2.2. HUMAN INDUCED PLURIPOTENT STEM CELLS

An important breakthrough in the field of regenerative medicine was the development of human induced pluripotent stem cells from somatic tissue cells. In an independent manner, both Yamanaka and Thomson identified different sets of defined factors which when overexpressed in human fibroblasts resulted in the de-differentiation of the adult fibroblast phenotype into an undifferentiated pluripotent state, capable of giving rise to cells of all germ layers [17, 18]. This process was termed as reprogramming. The reprogramming factors used were the transcription factors Oct3/4, Sox2, Klf4 (Kruppel-like factor 4) and the proto-oncogene c-Myc (Yamanaka), or the transcription factors Oct3/4, Sox2, Nanog and the micro-RNA binding protein Lin28 (Thomson). Although these factors are required for the induction but not the maintenance of pluripotency, the exact mechanism by which de-differentiation is induced is not completely understood [65]. Apart from fibroblasts, other cell types can be targeted for reprogramming, such as blood cells or (kidney) cells in urine. In fact, the cell type of origin has a great impact in the efficiency of the process; this has been associated to differences in the epigenetic state and transcriptional activation of cells. After the reprogramming step and upon another specific cocktail of factors, the induced pluripotent cells have been differentiated into many cell types of interest, including ECs, SMC or pericytes [66-69].

Recently, ECs have been derived from hiPSCs that display a variety of genuine EC functionalities upon the application of several proinflammatory stimuli [66]. Orlova *et al* reported the simultaneous differentiation of ECs and pericytes from fibroblast or blood-derived hiPSCs. In this study, hiPSC-derived ECs were able to properly incorporate to the developing vasculature of a zebrafish embryo [67]. In other recent work from Samuel *et al*, ECs and mesenchymal precursor cells were also generated in parallel from hiPSCs. They demonstrated that both cell types together are able to form competent and durable vessels *in vivo* [68]. Kusuma and co-workers derived ECs and pericytes from both hiPSCs and ESCs and showed their functional integration into the vasculature of a 6-8-week-old mice [69]. Other studies showed the capability of hiPSC-derived ECs to integrate into ischemic vasculature improving its function [70, 71], and to augment perfusion and capillary density in a mouse model of peripheral arterial disease [72]. Moreover, hiPSC-derived ECs also demonstrated their ability to repair damaged vessels in a bleomycin-induced scleroderma murine model [73]. Interestingly, Lippmann *et al* showed that ECs derived from both hiPSCs and ESCs and co-differentiated with neural cells, expressed specific blood-brain barrier EC attributes [74].

hiPSCs have been considered to be of great therapeutic potential since they can be patient-specific (autologous), their derivation can be done from easily accessible cell sources, they are abundant, virtually indistinguishable from ESCs and with pluripotent properties but with less ethical problems, and they represent a good basis to investigate heritable vascular disorders and screen for novel therapeutics [75]. By contrast, clinical development was initially difficult due to the use of genome integrating retroviral or lentiviral systems for reprogramming, which make safety concerns arise. However, such concerns can be overcome using adenoviruses, episomal vectors, non-integrating Sendai virus, strategies to

silence or excise the viral elements or using non-viral approaches, namely microRNA or small molecules [76-80]. Moreover, the use of non-human products to produce hiPSCs (media, matrices) can also give rise to undesired immunological reactions. Other weaknesses are the low efficiency of the reprogramming process (0.01 to 0.1%) in addition to the accumulation of chromosomal abnormalities and the variation in the differentiation potential depending on the donor cell type [65, 81, 82]. It has to be pointed that in order to avoid teratoma formation, hiPSC have to be carefully guided and pluripotent cells must be eventually sorted out. As a final remark, the use of autologous hiPSC-derived cells will carry the genetic abnormalities already present in the patient from whom the original cells derive. This would be a potential hurdle if a hiPSCs-therapy would be applied to these patients with genetic disorders unless the gene mutations were corrected.

2.3. DIRECT REPROGRAMMING

Recent advances point towards the direct reprogramming of fibroblasts or mature amniotic cells into ECs using a defined subset of factors that convert one somatic cell type into the other [83-85]. The ECs derived expressed specific endothelial markers and formed vascular structures both *in vitro* and *in vivo*. Margariti and co-workers used the transcription factors Oct4, Sox2, Klf4 and the oncogene c-Myc to generate partial-iPS from fibroblasts. In a defined media and culture conditions they derived ECs with angiogenic properties able to improve neovascularization of impaired tissue in a hind limb ischemic model [83]. Moreover, Ginsberg and colleagues derived ECs from amniotic cells by co-expressing the ETS (E26 transformation-specific) transcription factors ETV2 (transiently) and FLI1/ERG1 (constitutively) together with the addition of the SB431542 compound to inhibit TGFβ signaling. The derived ECs showed to be durable and able to form stable vessels in Matrigel™ plugs and incorporate long-term into the vasculature of an injured liver after transplantation in mice [84]. Finally, Li *et al* derived ECs from fibroblasts making use of the transcription factors Oct4 and Klf4 together with the growth factors BMP4, VEGF, bFGF and the cAMP analog 8-Bromo-cAMP. They showed increased capillary density and enhanced tissue perfusion when the derived ECs were engrafted into a murine ischemic limb [85].

The advantage of such approach is the avoidance of the pluripotent step required in the iPS technology, then reducing the risk of tumor development in the host organism. More research still needs to be carried out to directly reprogram somatic cells into either smooth muscle cells or pericytes.

Chapter 3. BIOMATERIALS

The fate of transplanted cells, including their survival, proliferation and differentiation highly depends on the local tissue microenvironment [86, 87]. At present, one of the major limitations for a successful cell therapy, apart from obtaining the sufficient number of cells, is to maintain them alive during and after the transplantation procedure [88-90]. In addition, abnormal mechanical properties of the diseased tissues, such as the case in fibrosis, might also result in a poor transplantation efficiency due to impaired differentiation [91]. Furthermore, pre-transplantation treatment *in vitro*, such as trypsinization, might also affect the expression of certain adhesion proteins or surface receptors [92]. Finally, the non-existence of a pre-established vasculature or cavity to nourish the cells is an extra hurdle for the transplanted cells to survive and well-engraft [93].

Nowadays a great effort is being invested in trying to generate supports or scaffolds as a niche in which to place the cells to be transplanted, thus providing (i) both mechanical and chemical cell support to increase cell viability (ii) a spacious substrate for the cells to remodel (iii) a template to guide structure formation (iv) a carrier to deliver cells in the specific damaged location in the patient able to stimulate the function of endogenous stem cells (v) a carrier to locally deliver diffusible bioactive niche components, such as diffusible cytokines or regulatory proteins to enhance cell mobilization, survival and tissue-specific differentiation [94]. Besides this, a scaffold for vascular tissue engineering should be biocompatible, biodegradable, permeable, shapeable, with the ability to support several cell lineages, with continuous porosity and sufficient mechanical strength [12].

In this chapter recent advances in polymer chemistry and biomaterials required for successful cell transplantation will be discussed, with particular focus on therapeutic vascularization and engineering of small vascular grafts. Highlights will include: (i) the use of hydrogels as artificial biodegradable scaffolds, (ii) natural versus synthetic hydrogels and (iii) tailor-made smart scaffolds.

Hydrogels made from naturally occurring biopolymers or synthetic biomaterials offer many attractive properties for the vascular regenerative medicine field [12, 95] (Figure 3). Firstly, their high water content, which creates a 3D-crosslinked hydrophilic polymeric structure allows the diffusion of small molecules, gases and proteins. This presents them as a highly biocompatible and degradable matrix. Secondly, chemically-available side groups can be functionalized to release different growth factors to trigger specific functions of the embedded cells. Thirdly, they can be injected in a liquid form therefore minimizing the invasiveness of the implantation process in the patient [96-98].

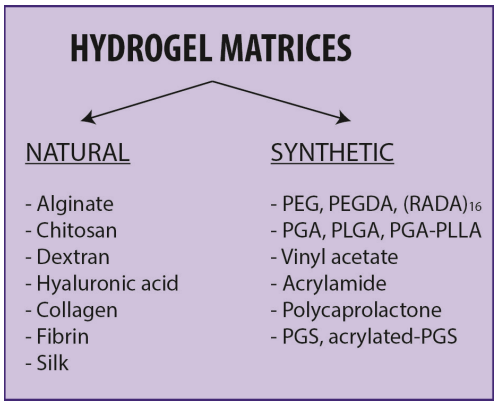

Figure 3. Diagram showing the different types of natural and synthetic hydrogel matrices.
PEG: Polyethylene glycol; PEGDA: diacrylate polyethylene glycol; $(RADA)_{16}$: arginine-alanine-aspartate-alanine tetrapetpide; PGA: polyglycolic acid; PLGA: poly(lactide-co-glycolide); PGA-PLLA: polyglycolic acid-poly-L-lactic acid; PGS: poly(glycerolcosebacate).

Natural hydrogels include alginate, chitosan, dextran, hyaluronic acid, collagen, fibrin and silk. The most well-known example of this group is Matrigel™. These natural matrices are well-tolerated by the body since they mimic the structure and backbone of the native extracellular matrix (ECM) with biologically recognizable groups. However, they have lot-to-lot variability (meaning variable batch composition), high degradation rates and poor ability to be custom-designed [99].

Synthetic matrices comprise polyethylene glycol (PEG) and its derivatives diacrylate (PEGDA) and arginine-alanine-aspartate-alanine tetrapeptide $(RADA)_{16}$, polyglycolic acid (PGA) and its derivates poly(lactide-co-glycolide) (PLGA) and polyglycolic acid-poly-L-lactic acid (PGA-PLLA) in addition to vinyl acetate, acrylamide, polycaprolactone and poly(glycerolcosebacate) (PGS and acrylated-PGS). These matrices are reproducible, able to be custom-designed and easily addressed accordance with regulatory protocols due to its defined composition. However, they require the engineering for bioactive properties. Although in this case one should be concerned about the ability of these synthetic polymers not to be rejected and to be permeable and properly degraded, PLLA, PLGA, polycaprolactone and PGS or PGSA have been of special interest due to their biocompatible, biodegradable and mechanical strength properties [99].

Both natural and synthetic hydrogels can be additionally modified by crosslinking the hydrogel structure to active groups that in turn will be chemically conjugated or will physically encapsulate bioactive ligands (including growth factors, cytokines, ECM components, adhesion peptides, surface proteins or drugs) to the surface of the hydrogel matrix [100]. For instance, the natural hydrogel based on hyaluronic acid has been conjugated to thiol or tyraminated groups [101], and PEG synthetic biopolymers have been crosslinked with binding sites for growth factors, integrin motifs to facilitate surface attachment, or metalloproteinase(MMP)-sensitive domains to add degradation signals to the matrix [102].

Such modifications make possible the creation of tailor-made biomimetic scaffolds with more controlled delivery of the niche signals, thus increasing stem cell survival and function in the host tissue [103]. In that respect, hydrogels can also be designed to shrink or expand in response to environmental stimuli. Such stimuli include physical changes in temperature, electrical signal, light, magnetic fields, ultrasonic irradiation, ionic strength or dynamic mechanical forces; chemical changes in pH or chemical agents; or biochemical changes caused by proteases, polysaccharides (glucose) or antigens [104]. For illustration, some polymers can release a specific growth factor or drug with the transition from gel to sol controlled by a decrease in temperature or external pH. These approaches have been shown to be useful in the treatment of ischemia, which is characterized by low tissue temperature and pH [105, 106]. Some other hydrogels are able to convert from a liquid to a gel state when reaching body temperature after its injection *in vivo* [107]. Interestingly, Zhao and colleagues developed a hydrogel coupled to iron oxide particles which under magnetic fields deformed and released bioactive molecules including drugs, chemokines or plasmid DNA [108]. All these systems triggered by external stimuli appear as a very exciting way to liberate specific factors in a fast, precise, reversible and localized mode.

Recent advances in biomimetic scaffolds include the incorporation of: (i) integrin binding sites and protease-sensitive substrates in a PEG-RGDS(Arg-Gly-Asp-Ser) hydrogel mixed with a MMP-sensitive motif or VEGF, resulting in a stable vascular structure both *in vitro* and *in vivo* [109] (ii) the heparin-binding domain II of fibronectin incorporated into a multifunctional PEG matrix mimicking the functionality of fibrin (crosslinked with the fibirin stabilizing factor XIIIa, a lysine donor peptide with MMP and plasmin-sensitive sequences, and several growth factor binding

sites), which improved wound healing in a diabetic mouse model [110] (iii) an engineered stromal cell-derived factor 1-α chemokine analog to be released along with the degradation of a sodium hyaluronate gel modified with hydroxyethyl methacrylate (HEMA-HA). They showed that it maintained the ventricular function in a rat model of myocardial infarction [111] (iv) hyaluronan and insulin-like growth factor (IGF-1) cues purified and dissolved together in serum-free culture medium regenerated elastin matrix structures and inhibit cell proliferation in smooth muscle cells [112] (v) a small bioactive peptide thymosin β4 (Tβ4) encapsulated in a PEG-vinylsulfone hydrogel crosslinked with integrin ligand and MMP peptides, which assisted in the attachment of human umbilical vein endothelial cells (HUVEC) adhesion and formation of a vascular network *in vitro* [113].

Interestingly, Chien and co-workers delivered a modified RNA for VEGF-A intramyocardially mixed with a synthetic hydrogel made of polyacrylic acid. In a murine model, it locally mobilized endogenous epicardial progenitors leading to cardiovascular differentiation and regeneration of the infarcted tissue [114].

Furthermore, photopatterning or the use of light-response building blocks embedded to hydrogel networks able to couple or cleavage specific bioactive molecules are also emerging as an important breakthrough in the field [115-117]. This way, one can tightly control both in space and time the behavior of the cells (namely adhesion or migration) attached to the material in either reversible or irreversible manners [118].

Some other works focused on the micropatterning of these synthetic hydrogels with either a cell adhesive ligand Arg-Gly-Asp-Ser (RGDS), thus providing a geometrical cue, or by assembling cell-laden polyelctrolyte hydrogel fibers to manipulate endothelial cell morphogenesis and enhance the prevascularization process in several tissues [119, 120]. Moreover, the use of hydrogel constructs able to create aligned cords of ECs improved the survival of the implanted tissue [121].

Other interesting approaches make use of other novel technologies, such as nanotechnology to deliver micro or nanoparticles as carriers of bioactive components of the stem cell niche. By selecting the optimal material composition and the particle size, one could have a more fine control on the component or drug release, but also on the cell-specific targeting and the immunological response [122, 123].

The recreation of the cellular microenvironment is not only essential for an effective vascular replacement, but also to make possible the study of the cell niche *in vitro*. Such an understanding will give new insights into specific pathways or interactions that regulate the formation of new vessels, providing essential knowledge for vascular tissue engineering and vascular disease modelling [124].

Chapter 4. FUTURE DIRECTIONS

Vascular regenerative medicine has developed enormously in the recent years thanks to both fundamental research on stem cells and technological advances in tissue engineering. In fact, it has already emerged as a promising solution for cardiovascular disease patients without any other pharmacological or surgical treatment options.

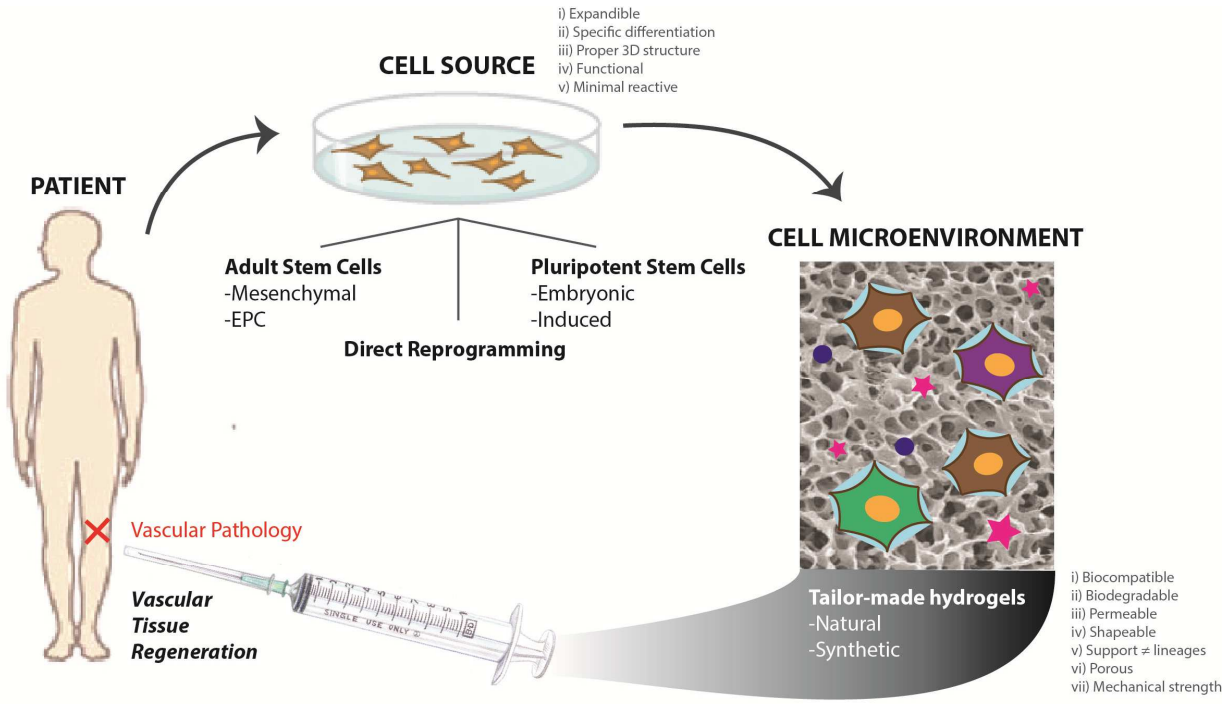

Figure 4. Schematic representation of *in situ* regeneration of the vascular tissue. EPC: Endothelial progenitor cell.

Nowadays, although the great efforts have already been made, the translation of laboratory research into relevant *in vivo* applications still remains challenging; mainly because of the need to recreate the precise 3D microenvironment required to promote vascular assembly. Actually, the way to solve this out may be as simple as finding or designing the right biomaterial for the right cells so that they can remodel and turn on endogenous mechanisms of vascular regeneration *in situ*. Furthermore, if such a material could have the properties of a hydrogel, it would be biocompatible, biodegradable and in a liquid format, which will mean easy to inject and less invasive for the patient (Figure 4). Recent advances in soft mosaic hydrogels offer a controlled and organized heterogeneous composition of polymers (using the microfluidic technology) to precisely create a specific microenvironment according to the requirements of each cell type [125].

The idea of using scaffold-free techniques, in which the cells by self-assembly and self-organization produce their own ECM, seems to be the approach with better perspectives at present since it avoids the harsh processing requirements and the immunological response involved in scaffold-based techniques [126]. In that regard, cells can be printed into a template pattern or cell sheets can be seeded in monolayers, mechanically stacked and rolled to fuse them together, and with a subsequent tissue organization carried out by the cells themselves [127-129]. Novel methods in scaffold-free approaches involve

microprinting using two immiscible aqueous solutions that allow a more defined cell positioning or the cell-sheet technology together with a perfusion bioreactor and collagen-based microchannels to vascularize thick tissue *in vitro* [130, 131].

Following that line, vascularization is a vital component for the reconstruction of a complex organ and to avoid its failure after the transplantation procedure. Apart from being crucial for tissue regeneration, vascularization is also important for a successful wound healing and the avoidance of fibrotic tissue in several diseased or transplanted organs. Among the latest breakthroughs, the generation of organoids or mini-organs in a dish stand out. They are a very interesting advance not only because they are a representative 3D model to study organ development, but also because they are a step forward in the transplantation into humans of complex organs generated *in vitro* [132, 133].

What is clear is that for the bench advances in vascular tissue engineering to succeed as clinical therapies, a multidisciplinary team of scientists, engineers and medical doctors will be required to work closely hand in hand. Hopefully then we will finally be able to belie Voltaire in his famous quote in which he said that *"Doctors are men who prescribe medicines of which they know little, to cure diseases of which they know less, in human beings of whom they know nothing".*

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