Bachelor Thesis

Pluripotent stem Cell-based therapy for patients suffering from Peripheral Arterial Disease

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I. Abstract:

Peripheral arterial disease (PAD) is characterized by limited blood flow to the limbs due to large artery obstruction, excluding coronary, carotid and aortic arteries. Many patients cannot be successfully treated with current surgical or endovascular procedures, often resulting in amputation of the affected limb. However, new cell therapies being developed in laboratories may provide a superior mechanism for treating the underlying cause of PAD. Relatively new progenitor cell and adult stem cell therapies have been shown to modestly improved patient conditions, while the use of human embryonic stem cells (hESC), and recently discovered induced pluripotent stem cells (iPSCs), are potentially superior platforms for vascular regeneration. When obstacles like the current viral production of iPSCs are overcome, vascular regeneration using iPSCs derived Endothelial Cells (ECs) transplantation may be possible as the standard of care for PAD patients.

II. Introduction:

PAD is generally secondary to atherosclerosis, which is a process that involves accumulation of lipid, vascular inflammation, cellular proliferation, and vascular calcification. PAD may affect the ileofemoral infrainguinal and/or infrapopliteal arteries, to reduce pulsatile blood flow.[1]

The main risk factors for PAD are smoking, diabetes, hypertension, hyperhomocysteinemia and dyslipidemia. Genetic factors may also play a role in the onset and progression of PAD. A number of genetic variants have been reported to be associated with peripheral arterial disease, but few have been confirmed in large genome wide association studies. [2, 3] Over 50% of PAD patients suffer cardiac disease as well, which is the principal mortality risk factor.[4] Surgical bypass or endovascular interventions may reduce symptoms or salvage tissue in a minority of patients. Medical therapy is limited, with only modest benefit from pentoxiffyline, cilostazol, or nicorandil, each of which are inferior to supervised exercise therapy.

Cell therapy is a promising new therapeutic approach that delivers adult-stem cells to the site of occlusion. In arteriosclerosis and PAD, the inner layer of cells that lines the luminal surface of blood vessels is dysfunctional or disrupted [5], leading many laboratories to investigate methods to regenerate this layer using cells with endothelial potential. Those cells might migrate into the endothelium and restore its function. Alternatively or in addition, these bone-marrow derived adult stem cells may secrete angiogenic cytokines that enhance the survival and proliferation of endothelial cells. It has already been demonstrated in several animal models that cell therapy improves perfusion of the ischemic limb. Cell therapies like human endothelial progenitor cells (hEPCs), bone marrow derived mononuclear cells (BN MNC), stem cell derived endothelial cells, and others have already been tested

in small clinical trials, and appear to modestly improve symptoms and function in patients suffering PAD.

The focus of this thesis is on embryonic stem cells because they hold a greater potential for differentiating into the desired cell types, like endothelial cells, have greater replicative capacity than adult stem cells, and they have been shown to improve blood flow in animal hindlimb models.[6] I will also describe the potential use of iPSCs and refer to unpublished data generated by my laboratory.

III. Cell therapy

1. Endothelial progenitor cells and Bone marrow derived cells.

Elevated atherosclerotic risk factors are associated with decreased number and function of circulating EPCs, leading research to believe that the key to effective cell replacement therapy lies in efficient replacement of these cells.[7] Studies indicate that EPCs potentially contribute to neoangiogenesis and vasculogenesis in mice and rabbit models after differentiation into functional endothelial cells.[8]

Angiogenesis is herby defined by the formation of new blood vessels from existing vascular beds, whereas vasculogenesis is characterized by a new formation of blood vessels from circulating endothelial progenitor cells or angioblasts.[9-11]

EPCs are mainly derived from the bone marrow, and thus it is unsurprising that bone marrow derived cells have been shown to have a regenerative capacity and improve blood flow and capillary density in mice.[12, 13]

Bone marrow derived cells have been shown to improve limb ischemia in human as well. Huang et al. injected BM-MNC as well as peripheral blood mononuclear cells (M-PBMNCs) into 105 patients. An improvement of Ankle Brachial Pressure Index (ABI) and Distal transcutaneous oxygen pressure measurement ($TcPO_2$) was shown within 12 weeks of treatment.[14] ABI is defined as the ratio of blood pressure in the lower legs to the blood pressure in the arms. Low blood pressure in the leg compared to the arm indicates PAD. $TcPO_2$ detects tissue hypoxemia in PAD.[15] The overall improvement observed in this study is consistent with previous clinical trials.[16 17 18]

However BM-MNC and EPCs of patients suffering from PAD were shown to have a compromised capacity to regenerate blood flow. Those patients had dysfunctional and a deficient number of angiogenesis promoting BM-MNCs. In addition BM-MNCs are not fully characterized yet and their ability to improve peripheral function is not fully understood.[19 20 21] Even though initial studies reported beneficial results, complications in the treatment with BM-MNC have been demonstrated by other -studies, including sudden death.[22 23]

To mitigate immunological complications, and to ensure the delivery of functional vascular-related endothelium, new cell based approaches are being developed with endothelial cells differentiated from pluripotent stem cells.

2. Stem Cells

Stem cells are defined by their capacity for self-renewal and capacity to differentiate into multiple cell types. Different stem cells include adult, embryonic and induced pluripotent stem cells. Adult stem cells are multipotent cells, which are partially lineage- committed and have the capacity to give rise to a limited set of cells within a specific germ layer. Embryonic stem cells (ESs) are pluripotent cells that are able to differentiate into all three primary germ layers. Induced pluripotent stem cells are reprogrammed somatic cells, which are able to differentiate into all three germ layers.

2.1 Adult stem cells

Qi Liu and colleagues demonstrated the regeneration of new blood vessels and skeletal muscle fibers after injection of adult bone marrow derived stem cells (BMCs) in the ischemic limb of mice. BMCs might have paracrine effects on myovascular progenitor cells due to an increase in proangiogenic cytokines like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2). However, mainly arterial vasculogenesis and almost no angiogenesis has been seen with this approach and therefore may not play an essential role in the myovascular regeneration in severe ischemic limbs because relatively few marked BMCs were seen to fuse to already resident endothelial cells or incorporate into existing blood vessels.[24]

BMCs have been studied in humans as well: Prohazka treated 37 patients with severe disease (Fontaine IV, ie. critical limb ischemia and ulceration) for whom all other therapeutic strategies failed. The patients showed improved ABI and TcO2 after intramuscular injection of autologous BMSC but their pain levels remained unaffected.[25] Several other research teams have confirmed an improvement in PAD after local injections of BMSCs.[14 26 17]

Other groups, however, have found less success with BMSC treatment. De Vriese´s trial on 16 PAD patients has shown modest improvement of the condition in eight patients, which were also the least affected ones. Four patients died during the trial, two of unrelated causes and two because of progressive gangrene. Three other patients required an amputation within 3 months. The small size of this study, and the lack of a control group, limit conclusions that can be drawn[16]

2.2 Embryonic stem cells

Adult stem cells represent a cell source that commonly has limited proliferative capacity, which makes it difficult to expand colonies to a cell number adequate for transplantation.[27] This is not a limitation of hESC-derived cell therapy. Many laboratories currently focus on the use of embryonic stem cells due to their nearly unlimited proliferative capability, and their capacity for differentiation into all somatic cell types.[28 29] hESCs therefore have the potential to renew and regenerate all cell types in the body and could be used for many therapeutic implications, including the repair of ischemic tissues. Here, generating a homogenous endothelial population from hESCs and determining their vascular potential is crucial.

2.2.1 Embryonic stem cell derived endothelial cells

2.2.1.1 Characteristics of normal human endothelial cells

It is essential to understand the characteristics of normal human endothelial cells before developing strategies to derive human ESC-derived endothelial cells. Human endothelial cells can be identified and characterized by assaying the expression of platelet endothelial cell adhesion molecule-1 (PECAM1), vascular endothelial cadherin (VE-cad), CD31, and human counter- part KDR, vascular endothelial growth factor receptor 2 (Flk1), von Willebrand factor (vWF), and Tyrosine kinase receptor 2 (tie2).[8, 30] To determine functionality, the ability for endothelial cells to uptake Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) can be assayed.[31, 32] Fully differentiated mature ECs can be identified by selective staining for CD144, endothelial nitric oxide synthase (eNOS), vWF, GATA2, Ac-LDL uptake, E-selectin proteins and capillary network formation. [8, 30, 32]

2.2.1.2 characteristics in mice:

Early endothelial markers in mice include PECAM, Flk1, VE-Cad, tie1, tie2, Plasmalemma vesicle associated protein (MECA-32), vWF, Spinocerebellar ataxia 1 (Sca1) and the uptake of Ac-LDL. [33,34] Mature endothelial cells can be identified by surface and cystol proteins PECAM, Flk1, VE-Cad, vWF, CD34, endoglin (CD105), VCAM1 (CD106), VE-cad**,** tie1, intracellular adhesion molecule 2 (ICAM 2), and functional responses such as Ac-LDL uptake, NO generation, and capillary-like network formation in 3D gels.[35,34]

However, it is important to consider, that hematopoietic and endothelial cells share CD34 as their marker.[36] Furthermore, endothelial cells share CD31 in mammals with other vascular cells like monocytes, and neutrophils.[37] In addition, hematopoietic progenitor cells and hematopoietic stem cells also express PECAM, vWF, Flk1 markers and are able to take up Dil-Ac-LDL. More mature EPC markers like VE-cad and Tie1 provide a more clear identification of ECs.[38] The current gold standard

for endothelial phenotype is the ability of a putative endothelial cell to integrate into a pre-existing network of endothelial cells (inosculation).[39]

2.3 differentiation of ESCs into ECs

Embryonic stem cells could be differentiated into functional endothelial cells, which may restore the vasculature. To differentiate hESCs into endothelial cells, three methods were established: 3D embryoid body (EB) differentiation, 2D growth factor-supplemented differentiation and feeder / and serum free monolayer directed differentiation.

2.3.1 3D differentiation system

In the EB differentiation system, hESCs are spontaneously differentiated into ECs. For EB formation the ES cells are removed from contact with the feeder cells, (or the presence of leukemia inhibitory factor), and are cultured in liquid or methylcellulose containing media in low attachment dishes. These plates inhibit ES cell adhesion to the plate surface, generating a clone of differentiated cells, which is called embryoid body.[40]

EB formation had been observed in 1988 by Risau et al. in mice ES systems.[41] Cells spontaneously started to differentiate, resulting in blood islands, which contain hematopoietic and endothelial progenitor cells. Those cells expressed endothelial markers, like CD31, and could take up DiI-Ac-LDL.[42] Frequently, vascular structures form in the ES derived EBs.[43] This whole development can be directly compared with the initial and steps of in vivo vasculogenesis.[43]

Levenberg et al. first identified and isolated hESC derived endothelial cells and characterized expression of endothelial markers during EB differentiation. Cells were not cultured in liquid or methylcellulose containing media, but in their normal culture media without lymphocyte inhibitory factor (LIF) and basic fibroblast growth factor (bFGF), a method used in other studies as well.[44-46] In undifferentiated hESC cells VEGFR2, Tie2 and CD133 were expressed but not PECAM1, CD34 and VE-cad. The expression of these markers was then highly ameliorated during spontaneous EB formation at day 13 to 15. They isolated hESC-ECs from the 13-days-old EB using PECAM1 antibodies for fluorescence-activated cell sorting (FACS) and expanded them in endothelial medium. The cells were able to form tube-like structures on matrigel and could differentiate. Expression of endothelial protein vWF, VE-cad, Flk1, CD34, and PECAM1 was shown and the cells were capable of taking up Dil-Ac-LDL. The function of these embryonic stem cell derived endothelial cells was determined by transplantation into immunodeficient (SCID), which showed microvessels expressing human CD34 and PECAM1.[30] This EB-based approach to generating ESC-derived EC is inefficient at generating ECs (1%-3% yield) as the EB simultaneously differentiates into many other cells from all 3 germ layers.[30, 47, 48]

Zongjin Li et al later transferred ESCs to collagen IV-coated plates and cultured them for 4 days. FACS was used to sort for the early EC marker Flk-1. After addition of VEGF to the media, Flk-1 positive cells were stained for the late EC marker VE-cad and were also positive for other endothelial markers and functional assays. The cells were maintained on EGM-2 medium for 1 to 2 passages on fibronectin-coated plates.[49]

An improvement of differentiation efficiency has been introduced by Maxim et al. using a coculture of OP9 stromal cells. Purity (95%) and efficiency was improved with yields of up to 20% CD34 positive cells after FACS. However this marker is shared with hematopoietic stem cells and the presence of an endothelial precursor population was not definitely proven.[50]

2.3.2 2D growth factor-supplemented differentiation

S. Gerecht-Nir et al. first showed the differentiation of hESCs into ECs without the use of EBs. In this approach hESCs were cultured as single cells to prevent EB formation. Furthermore the cells were seeded at a specific concentration using differentiation media. After culturing the cells on a Type-IV Collagen based matrix, cells were filtered trough a 40um mesh strainer and were exposed to angiogenic growth factors VEGF and platelet-derived growth factor-BB. This resulted in an upregulation of EC and smooth muscle cell markers.

In comparison to the 3 dimensional differentiation method, this approach benefits by not using feeder cells, exogenous agents or cell sorting procedures like FACS.[51]

In 2007 Wang et al showed a different 2D differentiation protocol. After plating hESCs on MEFs, supplemented with Fetal Growth Serum (FBS) CD34 positive cells were isolated by magnetic bead sorting. Wang tried to replace FBS by using BIT9500 with VEGF, bFGF and bone morphogenetic protein (BMP) and was able to isolate a similar amount of cells than with the use of FBS. Most of the derived cells were adherent and expressed endothelial markers like PECAM1 and VE-Cad. Adherent cells were shown to take up DiI-Ac-LDL and rapidly formed vascular like networks on matrigel. Additionally they expressed vWF, PECAM1, VE-Cad, tie 2, Ephrin type-B receptor 2 and 4 (eph b2 and eph b4) and CD105. [52]

2.3.3 feeder- and serum-free monolayer hESC-EC–directed differentiation protocol

Obstacles remain in the use of the 3D EB and 2D growth factor-supplemented differentiation method. Several issues need to be solved before applying these differentiated cells to patients. Animal-derived feeder cells and serum containing media represent animal product contamination. It is also important to develop efficient strategies to enable robust production and isolation of hESC-EC. N.M. Kane may have set the first step in a feeder- and serum-free monolayer hESC-EC directed differentiation

protocol. A special endothelial differentiation media was used, containing large-vessel endothelial growth media plus additional hydrocortisone, human epidermal growth factor, bFGF and heparin, to induce hESC-EC differentiation. Using this protocol a rapid down regulation of pluripotency markers was shown, concomitant with induction of vascular endothelial markers at the protein, miRNA and mRNA levels consistent with endothelial phenotype. hESC-ECs responded to NO stimulator, migrated, and spontaneously produced tube-like structures on Matrigel or in monolayer cultures. The transplantation of these cells into the ischemic limb of immunodeficient mice promoted neovascularization and blood flow recovery due to engraftment into the vasculature.[53]

2.4. Transplantation Method of ESC-ECs

2.4.1. ESC-ECs maintain their EC lineage

In 2007 Wang et al. implanted hES – ECs into cranial windows of SCID mice via a fibronectin-collagen gel. After one week the cells quickly declined in vivo and could not anastomose into the vasculature of the host. However, using a combination of hES-ECs and mouse mesenchymal precursors cells, ECs were shown to form cord like networks at day 2 and formed luminal structures after day 11. The functionality of these engineered vessels was proven by intravenous injection of labeled dextran to perfuse the cells. The hES-ECs were shown to form blood-perfused conduits some of which remained stable for more than 150 days. No undifferentiated hESCs were observed, demonstrated by lack of Oct4 and Nanog expression in the endothelial culture.[52]

2.4.2 homing of ESC-ECs into the ischemic sites

Huang at al. showed the survival of murine ESC derived -ECs in the ischemic limb of a murine model of hindlimb ischemia. ESC-ECs were injected intraarterial (IA), intramuscular (IM) or through through intrafemoral vein injections and were tracked for 2 weeks by bioluminescence imaging (BLI) while their functional improvement was determined by laser Doppler perfusion. Independent of the delivery modality, ESC-ECs localized to the ischemic limb. Intriguingly, ESC-ECs injected intravenously initially localized to the lung, but over a period of days, appeared to home to the ischemic limb. ESC-EC maintained their EC lineage and incorporated into the microvasculature of the ischemic limb. Neovascularization and limb perfusion was remarkably ameliorated by ESC-ECs compared to a vehicle control group or to parental ESCs.[6]

However transplantation of undifferentiated embryonic stem cells exhibit the risk of undirected growth like teratoma and teratocarcinoma formation. The transplantation of a predifferentiated cells obviate this obstacle.[54,49]

iPSCs in contrast could be derived from the patients directly and these cells would then maintain their genetic material after injection and no immune responses would be caused. Ethical concerns are also solved, since iPSCs can be derived from every adult somatic cell.

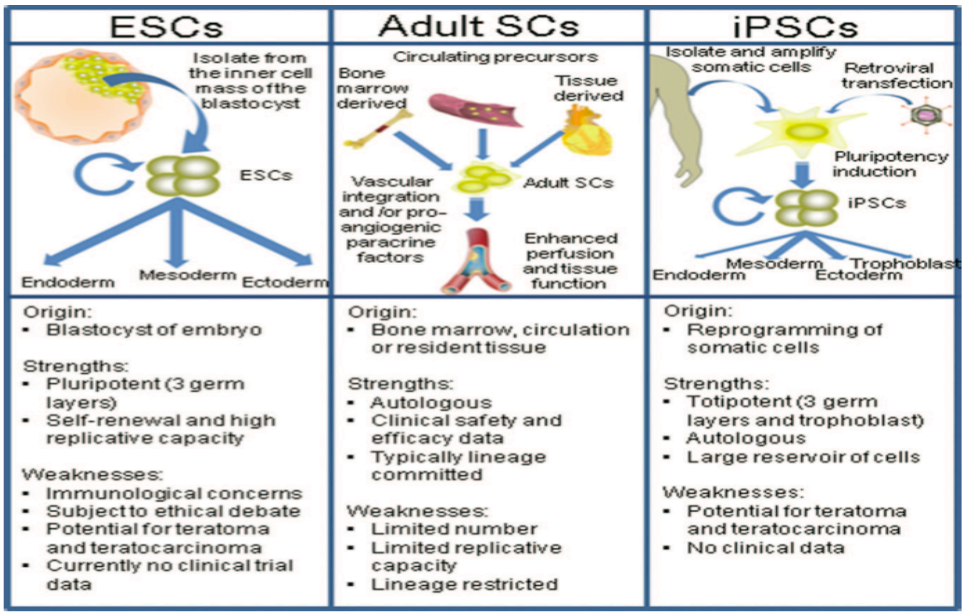

Figure 1: Summary of the promise and peril of ESCs, adult stem cells (SCs), and iPSCs for vascular regenerative therapy. Derived from reference [55]

3.Induced pluripotent stem cells

In 2006 Yamanaka et al reported that mouse fibroblasts and, a year later, human fibroblasts could be reprogrammed into induced pluripotent stem cells (iPSC) by viral transduction of the four transcription factors octamer 4 (Oct3/4), sex determining region Y)-box 2 (Sox2), krueppel like factor 4 (Klf4) and myc (c-Myc). Oct 3/4, Nanog and Sox2 were shown to be core transcription factors, which are required to maintain pluripotency.[56]

3.1 Molecular mechanism of reprogramming cells

The molecular mechanism of reprogramming is not completely understood.[55, 57] The proto-oncogene c-Myc is thought to promote histone acetylation and chromatin remodeling to recruit Oct3/4 and Sox2 to their binding sites, which results in accelerated cellular proliferation and reprogramming. Klf4 inhibits apoptosis by its p53 suppression and by the upregulation of Nanog, which functions as a gene inducing cell renewal. To induce reprogramming, Oct3/4 and Sox2 promote other crucial factors maintaining pluripotency and chromatin remodeling complexes. Lin28 for instance is a microRNA-related protein, which binds RNA and is important in the regulation of developmental timing.[55]

IPSCs represent a promising new cell source to be used in regenerative medicine as they create autologous stem cells that are patient specific and therefore do not face any immunological barrier. Furthermore they can be experimentally derived from easily accessible tissue sources, like skin or hair, and could help fill the deficit of donor tissue.

3.2 Reprogramming of fibroblasts into iPSCs

Reprogramming may be performed using pMX retroviruses encoding Oct-3/4, Sox-2, Klf-4 and c-Myc. Derived fibroblasts are infected with all four viruses at an MOI of 10 on day 1 and 3. On day 5 cells are seeded onto inactivated mouse embryonic fibroblasts (MEFs) and allowed to form colonies. Colonies are manually dissected based on ESC-like appearance and are further passaged and characterized.

3.3. Characterisation of iPSCs.

Characterization involves immunofluorescent staining for pluripotency markers and positive staining for alkaline phosphatase activity, spectral karyotyping for normal karyotype and reverse transcriptase PCR analysis to ensure silencing of exogenous genes. Bisulfite genomic sequencing analysis is also used to determine the methylation status at CpG dinucleotides. The sequences are analyzed and transformed into a meaningful data usually focusing on the promoter regions of genes related to pluripotentiality. An increase in methylation of a promoter region is an indication of the repression of the gene under its control. Those clones with normal karyotypes, silenced exogenous genes and proper expression of pluripotency markers are further characterized by their ability to form all three germ lines as demonstrated by their ability to form teratomas in severe combined immunodeficiency (SCID) mice.

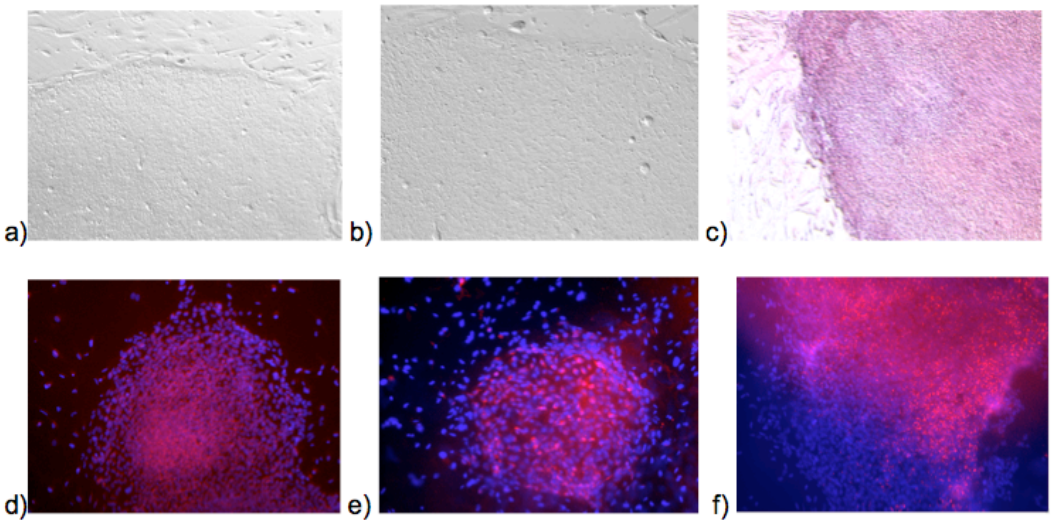

Figure 2: Characterization of iPSCs. iPSCs derived from human fibroblast reprogrammed with retroviral vectors expressing Oct-3/4, Sox2 and Klf4 maintain colony morphology characteristic of ESC (a: 10X, b: 20X), demonstrate alkaline phosphatase activity c) and express pluripotency markers as detected by immunocytochemistry including Nanog (e), TRA-1-60 (e) and SSEA-3 (f). For panels D-F pluripotency markers were detected using antibodies tagged by Alexa-594 (red) and nuclei are marked by DAPI staining (blue). Notably, control plates of differentiated fibroblasts that were mock transfected failed to give rise to any hESC-like colonies. (Unpublished data, Katharina Volz)

Unpublished data of our lab suggest these iPSCs can be induced to differentiate into arterial, -venous, and –lymphatic endothelial cells.

3.3 differentiation of induced pluripotent stem cells into endothelial cells

This procedure is similar to the manner in which EBs are formed from hESCs. hiPSCs are first cultured in suspension for four days in differentiation media, supplemented by BMP-4 and VEGF-A. Subsequently, four days old EBs are seeded on gelatin coated dishes and are cultured in differentiation media for 10 days. High VEGF-A and cyclic AMP concentrations support the specific differentiation into arterial endothelial cells by activating the Notch signaling pathway. A low VEGF concentration promotes differentiation into venous endothelial cells and exposure of VEGF-C, VEGF-A and Angiopoietin1 into lymphatic endothelial cells. (Jalil R. et al. unpublished).

VEGF contributes to angiogenesis by activating signaling pathways involved in EC proliferation, migration, and an increased number of capillaries in vivo. In vivo, VEGF promotes endothelial tube formation, representing the three dimensional structure of a vessel.[58] Plated endothelial cells proliferate and migrate in the presence of VEGF and form tube structures resembling capillaries. In the VEGF signaling pathway, VEGF binds to the VEGF receptor 2 (VEGFR-2), which triggers the tyrosine kinase-signaling cascade. Hereby eNOS is activated, producing NO, which increases endothelial survival, proliferation and permeability. The VEGF also stimulates the release of bFGF, which is important for EC proliferation and survival. EC migration is promoted by the factors ICAM, VCAM and matrix metalloproteinase (MMP). These factors contribute to a final differentiation into mature blood vessels. VEGF is also upregulated by increased blood flow, as are VEGF receptor 1 and 2. The increase in receptor production is coupled with the signaling cascade relating to angiogenesis. In the angiogenic signaling cascade, NO is a major contributor to the angiogenic response because NO inhibition reduces the effects of angiogenic growth factors.[59]

Other laboratories however, successfully differentiated iPSCs into hECs using no additional cytokines in growth media, but using a coculture of OP9 feeders. These feeders have been previously demonstrated[60] to induce formation of hESCs into CD34 positive cells, including CD34, PECAM1 and CD43 positive endothelial cells. [61]

hiPSC-ECs were successfully characterized by looking at vascular tube formation on matrigel, acetylated LDL uptake and upregulation of ICAM1 upon TNF alpha exposure. Furthermore all iPSCs-ECs were positive for the endothelial markers PECAM1 and CD144.

Unpublished data from my laboratory suggest that iPSC-derived ECs, when injected into the ischemic murine hindlimb, can induce an improvement in limb blood flow measured by laser doppler perfusion. A SCID mouse model of limb ischemia was used, and the animals were treated with intramuscular injections of iPSC-ECs or saline ($n \geq 8$). The localization and survival of iPSC-EC was tracked 2 weeks

by BLI. IPSC-ECs engrafted into the ischemic microvasculature and improved limb perfusion. (Huang et al., unpublished)

However, methods that rely on genetic modification of the cells to be reprogrammed suffer from limitations including low reprogramming frequencies, variability in number and location of insertions, variable iPSC phenotypes, concerns regarding safety due to insertional mutagenesis and in some cases, tumorigenicity. The induction of iPSCs, and their directed differentiation, remain inefficient.

IV. Future perspectives

Many laboratories intend to develop a chemically defined and rigorously tested approach to efficiently produce safe and functional hiPSCs. Approaches include reprogramming via protein delivery, small molecules and micro RNA.

For protein delivery, fusion peptides holding the individual reprogramming factor together with a 7-15mer polyarginine chain are used to promote protein transduction and are expressed as a fusion peptide with a short linker to the reprogramming factor. The target cells can thereby uptake the fusion peptides without cell cytotoxicity and potentially reprogram the somatic cell.[55]

These viral-free methods of reprogramming mitigate tumorigenic risk by eliminating the addition of exogenous genetic sequences to the host cell's DNA, an element that persists in the current method of iPSC reprogramming. Protein based reprogramming may generate safer iPSCs through a simpler and more rapid procedure than current non-integrative genetic methods, which require tedious and sequential selections of potentially integration-free iPSCs. Large-scale production of recombinant proteins and small molecules are well established; thus, chemically defined approaches will increase accessibility and utility with far greater consistency and convenience than genomic methods, while simultaneously allowing for simpler manufacturing procedures and reduced regulatory complications.

The use of effective differentiation protocols is critical to cell therapy, requiring optimized growth factors, media and substrates to generate the desired cell type(s). The ability to generate homogenous cell populations through differentiation and cell selection remains the most significant bottleneck for iPSC/hESC translation to clinical therapies. However, once these obstacles are overcome, vascular regeneration through iPSCs derived ECs transplantation may be the standard of care for PAD patients.

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