CHAPTER 10

RESEARCH SUMMARY
VASCULAR REGENERATIVE MEDICINE

Regenerative medicine strives to regenerate viable and long-lasting tissues and tissue replacements after acute or chronic tissue injury [356]. Vascular regenerative medicine is a multidisciplinary research field comprising developmental biology, material science and biomedical engineering, amongst others. As such it aims to yield clinical benefit for patients suffering from vascular diseases by the generation of novel therapies, aimed to maintain, restore or replace vascular perfusion. One important approach is the generation of ‘designer’ blood vessels, i.e. vascular tissue engineering, which is the in vitro creation of replacement blood vessel conduits by combing vascular cells, i.e. endothelial cells (EC) and smooth muscle cells (SMC), and degradable materials. Another important approach, yet conceptually distinct, is (stem) cell therapy which aims to improve tissue perfusion by the delivery of cells capable of inducing neovascularization in vivo (chapter I).

EC are of prime interest to vascular regenerative medicine because of their inherent antithrombogenic potential and vessel-forming ability. However, the limited accessibility and longevity of mature EC [44] hampers development of such applications. Endothelial progenitor cells (EPC) exert the capacity to differentiate into cells of the endothelial lineage, i.e. endothelial outgrowth cells (EOC), and possess longevity. Therefore, EPC may add to the repair capacity of the vasculature by regenerating damaged endothelium [357].

EPC originate from the hematopoietic stem cell lineage in the bone marrow [296]. There are two known populations of circulating EPC, which can be distinguished based on the expression of marker proteins CD34 and CD14 [64], time of appearance of their progeny EOC in culture [62], and clonogenic behavior [63]. Typically, EPC adhere to gelatin or fibronectin, take up acetylated LDL, bind lectins from Ulex europaeus, and express marker proteins of the endothelial cell lineage, e.g. CD31, VE-Cadherin (CD144), von Willebrand Factor and endothelial cell Nitric Oxide Synthase, after cell culture [315;316].

The current thesis investigates the use of human EPC for both in vitro tissue engineering of ‘designer blood vessels’ (part I) and for in vivo cell therapy for therapeutic neovascularization of ischemic tissues (part II). The research described in this thesis particular focuses on the biological mechanisms behind vascular progenitor cell plasticity, since knowledge on these cellular processes will aid regenerative medicine approaches in the near future.

PART I: ENGINEERING ‘DESIGNER BLOOD VESSELS’

GENERATING ENDOTHELIAL MONOLAYERS ON (DEGRADABLE) BIOMATERIALS

Vascular tissue engineering aims at the creation of small-diameter replacement blood vessel conduits by differentiating autologous EPC on degradable biomaterials. Besides suturability and mechanical strength, the biomaterial ideally supports cell
adhesion, cell differentiation and growth. Degradation of the biomaterial, together with the generation of a new basement membrane by (seeded) EC and SMC, converts the implantable vessel into a fully functional native vessel in vivo.

Since the CD14+ EPC is more abundant in the peripheral blood than the CD34+ EPC (10-20% versus 0.01-0.1% of the mononuclear cell fraction, respectively); the CD14+ EPC seems more appropriate as EOC-source to engineer large tissues such as replacement vessels. We have explored the ability to isolate large quantities of CD14+ EPC from peripheral blood of healthy volunteers and investigated their differentiation behavior on different biodegradable scaffolds (chapter 2).

In vitro, the majority (>70%) of CD14+ EPC acquired an endothelial phenotype during the 21-day culture period, as was indicated by expression of EC specific proteins CD31, VE-Cadherin, von Willebrand Factor and endothelial cell Nitric Oxide Synthase on these cells. A minor fraction of CD14+ EPC (~15%) differentiated into macropages in time (chapter 2).

In order to create a small-diameter vascular conduit that is viable, remodelable in time and self-reparative after damage, cells need to hold the potential of longevity or self-renewal. The transient proliferation of CD14+ EPC (chapter 2) therefore may pose a major threat to the integrity of a small-diameter bioartificial vascular conduit created using these cells. However, in chapter 7 we have found that after co-culture of human CD34+ EPC and human CD14+ EPC, the proliferation of CD14+ EPC was no longer transient, but constitutive. In fact, around 12% of all cells displayed expression of cell proliferation marker Ki67 after 21 days in culture.

Since vascular tissue engineering aims to generate autologous small-diameter replacement vessels, we investigated the availability and functionality of EPC in patients affected by vascular disease. In chapter 3, we describe the numerical impairment of EPC in patients that suffer from chronic kidney disease and are at increased risk for cardiovascular complications. Furthermore, EPC from patients with increased cardiovascular risk did not only show numerical, but also functional impairment as was indicated by their relative inability to prevent thrombus formation in vitro (chapter 3). These impairments pose a new challenge for vascular tissue engineering and strategies to overcome such impairments have to be addressed in future research.

GENERATING SMOOTH MUSCLE CELLS IN (DEGRADABLE) BIOMATERIALS

Next to the luminal EC lining, a ‘designer blood vessel’ needs to incorporate an outer layer of vascular SMC that provides stability and contractility. These SMC can be isolated from blood vessel biopsies and may even be differentiated from circulating smooth muscle progenitors [139;181;358]. Sata and colleagues discovered that hematopoietic stem cells, the precursors of human EPC and their progeny EOC, can differentiate into SMC [359] through endothelial-to-mesenchymal transdifferentiation (EnMT). EnMT is a process wherein EC lose their EC phenotype and adopt the cellular phenotypes of cells from the mesenchymal lineage. This concept encouraged us to investigate the possibility of EnMT by EOC and its progeny EC (chapters 4 and 5).

As proof-of-principle, in chapter 4 we have shown that neonatal human umbilical vein endothelial cells (HUVECs) can transdifferentiate into vascular SMC via TGF-β1-dependent mechanisms. EnMT resulted in the emergence of a proliferating population of vascular SMC which were functionally indistinguishable from genuine vascular SMC [165]. Furthermore, to establish proof-of-concept for vascular tissue engineering, we analyzed if EnMT could be performed in the context of 3D (degradable) collagen scaffolds. Indeed, EnMT occurred in the 3D collagen scaffolds where transdifferentiated cells bound the collagen bundles with hemidesmosomes, adhered to other cells through tight junctions and formed an extensive actin cytoskeleton, resembling the cytoskeleton of true vascular SMC (chapter 4).

We next investigated the ability of human EOC, i.e. human CD14+ EPC and CD34+ EPC-derived progeny, to form SMC through EnMT (chapter 5). The addition of TGF-β1 and PDGF-BB to EOC cultures resulted in diminishment of marker proteins of the EC lineage, i.e. CD31, CD144, eNOS, vWF, VEGF-R2 and Tie-2, and increased expression of marker proteins of the SMC lineage, i.e. SM22α, αSMA, calponin, and SM-MHC2. EOC-derived SMC exhibited functional contractile capacity similar to genuine vascular SMC. Longevity, indicated by telomerase activity and a hallmark for stem- and progenitor cells like EOC, however was reduced during EnMT (chapter 5).

TOWARDS ‘DESIGNER BLOOD VESSELS’

Tissue engineering of small-diameter vascular conduits is more than differentiating the proper cell types (chapter 6). A natural blood vessel contains an intima of vascular cells and basement membrane and an outer adventitia of extracellular matrix which provides strength to the conduit. In chapter 6, we have summarized developmental principles that underlie EPC differentiation into EC and SMC and reviewed the current progress in material sciences that aid the in vitro creation of a small-diameter tissue engineered blood vessel. The biomaterial has to comply with several biological principles; (1) the biomaterial has to resemble tissue architecture (e.g. porosity, directionality), (2) the biomaterial must mimic tissue strength and dynamics, (3) the biomaterial must allow for cell adhesion, (4) the biomaterial must be able to instruct cell differentiation or maintenance, and (5) the biomaterial needs to degrade properly in order to become remodeled in time.

For every biological modality distinct biomaterials have been developed (chapter 6), however, the current biomaterial challenge in vascular tissue engineering is to combine these modalities into a new tissue engineering paradigm. From a developmental biology viewpoint, the molecular modalities that govern progenitor cell differentiation and plasticity have been identified (chapters 2, 4, 5 and 7), while in parallel advances in biomaterial research have enabled us to incorporate these factors, i.e. VEGFa, bFGF, TGF-β1 and PDGF-BB, within ‘smart’ materials (chapter 6), thus facilitating the creation of biologically-active small-diameter vascular conduits that contain the aptitude to regulate their own tissue development.

PART II: THERAPEUTIC NEOVASCULARIZATION

Therapeutic neovascularization uses the inherent angiogenic capacity of human EPC for the treatment of tissue ischemia. In theory, implantation of human EPC
improves the body’s capacity to repair the injured microvessels and may even aid in the induction of new microvessel formation.

We and others have attempted to induce the formation of a microvascular network in vivo by injection of human CD34+ EPC into nude mice [100;268;360;361]. Although microvascular density is increased following human CD34+ EPC implantation, proof of active engraftment of these human EPC into the murine neovessels is scarcely observed [100;268]. However, recruitment of murine CD14+ EPC is commonly observed after implantation of human CD34+ EPC. We therefore wondered if human CD34+ EPC could augment the angiogenic capacity of CD14+ EPC in an in vitro assay.

IN VITRO ENDOTHELIAL CELL DIFFERENTIATION

In chapter 7, we discovered apparent cell-cell contacts between human CD34+ EPC and human CD14+ EPC following angiogenic stimulation in vitro. We therefore contemplated that these cell-cell contacts may influence EC differentiation and proliferation of these cells. Using various in vitro assays, we discovered that indeed the human CD34+ EPC and the human CD14+ EPC interact during EC differentiation. However, contradictory to our hypothesis, the interaction does not rely on cell-cell contacts, but rather on paracrine signaling by the human CD34+ EPC (chapter 7).

The human CD34+ EPC is able to produce a large variety of growth factors and cytokines which can be pro-angiogenic [276]. We identified that human CD34+ EPC produce high amounts of the pro-angiogenic growth factor HGF, which innervates its receptor cMET on the CD14+ EPC. Following such innervation, EC differentiation by the CD14+ EPC increased from 65% to 95% of all adhered CD14+ EPC. Moreover, proliferation of CD14+ EPC increased approximately 10-fold (chapter 7). Thus, co-cultivation of human CD34+ EPC and CD14+ EPC leads to superior EC differentiation in vitro than either cell type alone. Hence, co-administration of human CD34+ EPC and human CD14+ EPC may be a promising approach to induce in vivo neovascularization.

IN VIVO NEOVASCULARIZATION

We next investigated if neovascularization could be amplified by simultaneous transplantation of human CD34+ EPC and human CD14+ EPC subcutaneously into nude mice using Matrigel as a carrier. Indeed, mice receiving both human CD34+ EPC and human CD14+ EPC formed more neovessels than mice receiving either cell type alone. Furthermore, human CD14+ EPC aligned with the neovascularature, albeit in low numbers. Hence, cellular incorporation of CD14+ EPC into the neovessels could not account for the increased neovascularization (chapter 8).

We therefore analyzed if human CD34+ EPC and human CD14+ EPC secrete pro-angiogenic factors that induce microvessel formation. In our in vitro co-culture system, both the human CD34+ EPC and human CD14+ EPC contributed to the formation of an angiogenic niche by producing angiogenic factors like VEGF, IL-8 and HGF. Furthermore, when co-cultured these EPC showed amplified production and secretion of MCP-1 and bFGF (chapter 8).

Since neovascularization by human CD34+ EPC and human CD14+ EPC seemed to be influenced solely through paracrine signaling events, we hypothesized that we could induce microvessel formation by the sole application of these paracrine factors. Therefore we implanted Matrigel carriers, loaded with a pentet of pro-angiogenic factors, i.e. IL-8, MCP-1, HGF, VEGF and bFGF, subcutaneously into nude mice. Confirming our hypothesis, microvessel formation was induced by this pentet of factors (chapter 8).

Taken together, therapeutic neovascularization is a multifactorial process which is orchestrated by human EPC through paracrine signaling events. Microvessel formation is initiated through the production of angiogenic factors, i.e. IL-8, MCP-1 bFGF, HGF and VEGFa, by both the human CD34+ EPC and human CD14+ EPC that induce sprouting angiogenesis by the surrounding endothelium.

CONCLUSIONS

The concept of vascular regenerative medicine has been around for three decades, however, the discovery of the EPC a mere decade ago, has provided new insights into the developmental processes that orchestrate EC differentiation, neovascularization and vascular repair. Understanding the significance of phenotypic variations in progenitor cell types and their plasticity enables us to use them to our advantage for vascular regenerative medicine.

For one, the concept of vascular tissue engineering has evolved from a technical era, in which we aimed to replicate the blood vessels gross anatomy, into an era in which we can apply nature’s developmental processes for the creation of genuine natural vessel. In this thesis we describe that human EPC contain the intrinsic capacity to differentiate into both mature and functional EC and SMC on degradable biomaterials. These EC and SMC can be employed to tissue engineer ‘designer blood vessels’ that contain the ability to respond to its environment, have self-renewal capacity and thus the ability to regenerate when damaged. Also, in studying the role of human EPC in neovascularization, we have elucidated the natural processes that govern microvascular formation and are now able to mimic these processes using slow-release depots that release pro-angiogenic factors like VEGFa, bFGF, HGF, MCP-1 and IL-8 (chapter 9). The induction of neovascularization in a clinical setting, may therefore find its future not in (stem) cell therapy, but in the use of ‘smart-release’ depots, containing multiple pro-angiogenic factors that are released in temporal distinct patterns and mimic the paracrine signaling events of EPC during neovascularization. Such materials can be made according to the ‘off the shelf’ principle, which is highly desirable for clinical application.
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