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RESEARCH HIGHLIGHT

The dawn of angiogenesis modeling: regenerating vasculature from human pluripotent stem cells

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Efficient generation of functional human vascular endothelial cells and smooth muscle cells from pluripotent stem cells is an extensively studied topic and of great interest in the stem cell field. Though thought to be technically complex and difficult, substantial progress has been made towards this direction. Here we aim to summarize and discuss the most recent advances in this topic and their future perspective in research and clinic.

Vasculature and blood cells together compose the blood circulatory system, which maintains extensive capacity for self-regeneration. Stem cells or lineage specified progenitor cells play an important role in this regeneration process. Unlike the well-accepted concept that hematopoietic stem cells (HSCs) are the stem cell populations of all blood cell lineages, no bipotent "vascular stem cell" population that contributes to the development of both vascular endothelial cells (VECs) and vascular smooth muscle cells (VSMCs) has been isolated. Even so, researchers have continued to seek new methods to generate functional VECs and VSMCs from stem cells by different routes. Conventionally, VECs are thought to be differentiated from VECs progenitors, which are postulated to arise from "hemangioblast" (common precursor of HSCs). In adults, VECs progenitors reside in the stem cell niche in bone marrow. Upon activation, the mobilized VECs progenitors enter the circulation and move to the site of ischemia, where they incorporate into the vasculature and cooperate with VSMCs to form new blood vessels (angiogenesis). VSMCs are a specific type of smooth muscle cell, which composes the majority of the wall of blood vessels. Unlike the relatively simple origin of VECs, VSMCs may arise from multiple independent sources during embryogenesis. Both VECs and VSMCs are important for the normal function and regeneration of human vasculature. In this regard, defects in either cell type may lead to severe manifestation. Many studies have demonstrated that VECs and VSMCs can be generated from human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) through stepwise directed cell differentiation. However, in spite of their great value in both pathological study of vascular disease and cell-based replacement therapy, effective methods for differentiating VECs and VSMCs from hPSCs are still challenging and elusive.

The generation of human VECs from hPSCs is thought to be technically difficult due to inefficient lineage induction during differentiation and lack of isolation methods to identify and purify target cells from a heterogeneous population. At present, there is no definitive surface marker that clearly distinguishes VEC progenitors. Therefore, novel selection markers are in urgent need. In a recent publication in *Nature Biotechnology*, Drukker *et al*. [1] reported the identification of novel markers of vascular endothelial progenitors after screening over 400 antibodies of cell surface markers on BMP4 or retinoic acid-induced putative lineage progenitors from hESCs. By combining and analyzing a massive set of information obtained from 1) profiling of gene expression signature; 2) marker expression comparison to mouse embryo development; and 3) subsequent functional assays, the authors identified that CD87 and CD60 selectively mark endothelial progenitors from BMP4-induced hPSCs differentiation. CD87+ cells transcriptionally resemble the signature of vasculogenesis and can extensively produce microvascular networks with further differentiation.

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Unlike the previously pan-endothelial somatic markers used for inefficient isolation of VECs from hESC differentiation, Drukker *et al*. demonstrated the first proof in principle that CD87 can be employed as a lineage marker for early-stage endothelial fate specification and can be applied to enrich the endothelial progenitor population. This finding creates new opportunities for better investigation of endothelial specification and isolation of therapeutic vascular endothelial progenitors.

As a specialized type of VECs, the brain microvascular endothelial cells (BMECs) compose the blood-brain barrier (BBB). BMECs are crucial for the physiological function of BBB as the complex intercellular tight junctions limit the massive diffusion of molecules into the circulation of the brain. This attribute also prevents the uptake of most neurotherapeutics and is commonly compromised in neurological diseases, thereby driving great interest from pharmacology as well as disease modeling studies. However, because of the limited availability and fidelity of human BMECs, most BBB models demonstrated to date are either borrowing brain microvessels from primary animal sources or utilizing immortalized BMECs. Inevitably, they commonly fail to faithfully recapitulate the barrier properties of human BBB. Very recently, a positive breakthrough has been made. Lippmann and colleagues reported the derivation of human BMECs with BBB properties from hPSCs while co-differentiating with neural cells [2]. Applying a differentiation strategy mimicking the microenvironment of the embryonic brain, Lippmann *et al*. [2] showed that co-differentiation of neural and endothelial cells followed by endothelial-lineage specification can generate substantial yields of BMECs from various lines of hESCs and hiPSCs. The authors indicated that Wnt-β-catenin signaling is significantly involved in the BMEC specification but is not exclusively responsible for this process. Furthermore, an effective and facile purification of monolayer BMECs can be subsequently achieved by extracellular matrix-based selective passaging. These enriched BMECs possess requisite BBB properties including well-organized tight junctions, elevated barrier formation, polarized efflux transporter activity and response to astrocytic cues, which all attest to their identity and maturation. Due to the high efficiency achieved (> 60%), this methodology may be scaled up for robust BBB modeling and pharmacological studies.

While the studies discussed above demonstrate progress related to the differentiation of PSCs to VECs, the differentiation of PSCs to functional human VSMCs represents a different goal and challenge. Like other higher vertebrates, human VSMCs are highly mosaic tissues that arise from multiple origins during embryogenesis. This origin-specific nascence adds an additional level of complexity into the studies of both VSMC-related diseases and cell replacement-based therapy. Indeed, the role of VSMC diversity has been increasingly appreciated in vascular pathogenesis [3]. Therefore, a better understanding of the intrinsic differences among different VSMC subtypes would be an obligatory task. Along this line, differentiation of hESCs into developmental origin-specific VSMC subtypes will provide an ideal platform to investigate and dissect the molecular basis of this lineage specification process. Recently, Cheung *et al*. [4] reported the generation of VSMC subtypes from both hESCs and hiPSCs in chemically defined conditions *in vitro*. Intermediate populations of neuroectoderm, lateral plate mesoderm and paraxial mesoderm were generated by treatments mimicking different signaling gradients and subsequently differentiated toward VSMCs in a unified inductive condition. These different embryological lineage-originated VSMC subtypes shared common VSMC hallmarks while recapitulating unique properties, which resembled their different *in vivo* counterparts in responding to different cytokine stimuli. Therefore, although phenotypically indistinguishable, the authors convincingly proved that VSMC subtypes generated *in vitro* faithfully represent their *in vivo* origin specificities. Importantly, they demonstrated that origin-specific VSMC subtypes can be used to predict properties of *in vivo* counterparts, and thus be quite valuable in modeling origin-specific VSMC disease susceptibility in the future. Moreover, this study provided a novel platform for readily accessible origin-specific human VSMCs of the same genetic background. Combining with hiPSCs generated from patients with pathological backgrounds, this method will promptly facilitate the study of many VSMC-related diseases.

Differentiation of hPSCs into many cell types, including VECs and VSMCs, has been heavily investigated due to their high potential for use in regenerative medicine and drug development. hiPSCs, which harbor specific genetic backgrounds, are an even more desirable source for personalized cell replacement therapy and modeling of inherited disease. Indeed, previous studies, including ours, have demonstrated that hPSC-derived VECs and VSMCs can be used as efficient tools for vascular-related disease modeling, *e.g.*, vascular aging-associated atherosclerosis [5, 6]. The successful establishment of disease-affected VECs and VSMCs may not only help to reveal cell type-specific manifestation, but also provide ideal platforms for cell type-specific high throughput drug screening in the future.

Parallel to direct differentiation from hPSCs, transdifferentiation has provided a new avenue for generating various cell types, including vascular cells. In fact, a recent work from Margariti *et al*. [7] reported the generation of human VECs by transdifferentiation. Adapting a partial reprogramming

strategy using four Yamanaka factors, Margariti and colleagues successfully transdifferentiated human fibroblasts into VECs that exhibited functions *in vitro* and more strikingly, demonstrated physiological potential *in vivo* [7]. A better understanding of different aspects of human vascular cells may sometimes render unexpected advantages in other related studies. For example, recent evidence suggests that endothelial cells and perivascular cells may have an underappreciated role in supporting HSCs. In mammals, HSCs reside and are maintained by the bone marrow stem cell niche. In a recent report, Morrison and colleagues demonstrate that the specific deletion of the Stem Cell Factor (SCF) gene in endothelial cells and perivascular stromal cells results in loss of HSCs in bone marrow, demonstrating an essential role for endothelial cells in the vascular stem cell niche [8].

On the other hand, as shown in many studies, directed differentiation from hPSCs requires extensive knowledge of the transcriptional network and epigenetic modification that is involved in the differentiation process. In this regard, genome-wide gene expression and epigenetic analysis, such as RNA sequencing, DNA methylation and histone modification-associated sequencing, and proteomics analysis may be needed to map and identify new VEC/VSMC-specific markers and signaling nodes in hPSC-differentiated vascular derivatives. Moreover, as repeatedly demonstrated in many cases, the complexity of the extracellular matrix as well as the microenvironment may play a pivotal role in the commitment of cell lineage. For instance, a cell co-culture or parallel-differentiation system may be generally required to achieve certain functionality or terminal maturation, which raises additional challenges for cell isolation and purification. In this regard, cell purity and functional maturation may represent interests on two opposing ends, which would not only require new thoughts but also perhaps new experimental approaches.

In summary, the recent advances in the generation of human VECs and VSMCs provides a renewed hope for the field. However, for either generating healthy therapeutic cells or establishing defective cells for disease modeling and drug screening, the methodology of directed cell differentiation still needs further investigation in order to reach desired efficiency and purity. One ultimate goal of generating human VECs and VSMCs is the potential application of cell transplantation-based therapy. For this to take place, several other important questions will need to be answered along the way: 1) how similar the hallmarks of these hPSC derivatives are to their physiological counterparts; 2) whether pure populations of VECs and VSMCs are achievable by improved methodology; 3) whether differentiated VECs and VSMCs are expandable *in vitro*; 4) whether differentiated VECs and VSMCs can re-construct vessels with specific matrices amenable to whole vessel transplantation; 5) whether differentiated VECs and VSMCs are epigenetically stable and resistant to carcinogenesis after transplantation. At the current pace in which the field is moving, we may not have to wait too long to have many of these questions answered.

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