ELSEVIER

Applications of nuclear reprogramming and directed differentiation in vascular regenerative medicine

Research Paper

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As vertebrates proceed through embryonic development the growing organism cannot survive on diffusion of oxygen and nutrients alone and establishment of vascular system is fundamental for embryonic development to proceed. Dysfunction of the vascular system in adults is at the heart of many disease states such as hypertension and atherosclerosis. In this review we will focus on attempts to generate the key cells of the vascular system, the endothelial and smooth muscle cells, using human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs). Regardless of their origin, be it embryonic or via somatic cell reprogramming, pluripotent stem cells provide limitlessly self-renewing populations of material suitable for the generation of multi-lineage isogenic vascular cells-types that can be used as tools to study normal cell and tissue biology, model disease states and also as tools for drug screening and future cell therapies.

Introduction

The vascular system permeates every organ and tissue of the human body. Acting as the conduit delivering oxygen and nutrients around the body, it is also necessary to allow the translocation of various factors, signals and by-products. Blood vessels consist of endothelial cell (EC) networks, which are often associated with mural cells including smooth muscle cells (SMCs) and pericytes.

The vascular system is divided into arterial and venous portions, in which the vessel architecture and function are distinct. The arterial system carries oxygenated blood to target tissues. It has higher blood pressures and has a higher component of smooth muscle cells underlying the endothelium. The venous system, whose goal is to deliver the deoxygenated blood back to the heart, has developed valves to deal with pressure changes and veins tend to have a larger luminal area in cross section compared to arteries. In addition, the vascular branch has macro- and micro-vascular components, from large vessels such as the aorta to capillary networks in the peripheral regions.

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Endothelial cells

Normal function and pathological aberrations of ECs

Endothelial cells line the innermost surface of blood and lymphatic vessels. Under normal conditions they form a mono-layered structure that provides a semi-selective yet dynamic barrier function between the lumen of the vessel and the surrounding tissues [1]. This permits the controlled passage of factors and cells, such as those of the immune system, from the blood or lymph into the tissue the vessel passes through. It is also responsible for regulating blood flow, vascular tone and vascular remodelling, including the proliferation of smooth muscle cells. In this review we shall focus our attention on vascular ECs.

Endothelial cell dysfunction, whether environmental, genetic or a combination of both is at the heart of a number of cardiovascular conditions including hypertension and atherosclerosis [2,3]. The normal state of endothelial cells lacks the expression of pro-inflammatory factors and maintains the expression of anti-thrombotic characteristics. However endothelial cells can become 'activated' in disease processes where pro-inflammatory signalling increases and expression of anti-thrombotic characteristics increases allowing interactions with white blood cells such as

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leucocytes. This is one of the key functions of endothelial cells and central to both normal and disease processes ([4] and references therein). Interestingly endothelial cells have also been found to enhance regeneration of a number of tissues such as the liver and pancreas [5,6]. Taken together there is a clear need for easily accessible and defined ECs populations for a number of regenerative medicine applications including disease modelling, drug discovery and future cell replacement therapies. One possibility is to use endothelial progenitors, the most accessible being blood-outgrowth endothelial progenitors [7]. These are highly proliferative vascular ECs but have a limited self-renewal capacity. In addition, it is not clear if progenitor cells could replace all types of vascular endothelial cells. Pluripotent stem cells provide a way to overcome these hurdles to produce potentially limitless and specific populations of ECs.

Development of endothelial cells

There is much heterogeneity between EC populations in the body, which is either genetically determined or acquired or maintained through environmental cues [8]. Derivation of ECs from pluripotent cells requires knowledge of the developmental biology of ECs and also the processes of vasculogenesis and angiogenesis. Given the heterogeneity that exists, it is clear that this knowledge is incomplete; however, there is an accepted general consensus for the origin of ECs and the basic program of their differentiation.

ECs, like SMCs described below, are derived from mesoderm. In human pluripotent cell differentiation studies, mesoderm, lateral mesoderm and pre-cardiac mesoderm have been induced by combinations of Activin, bFGF, BMP and PI3K and GSK3 inhibitors (LY294002 and Chiron) (for example [9–11]). Following mesoderm induction specific populations within the mesoderm start to express the ETS family related transcription factor ETV2/ER71 [12]. ETV2/ER71 lies up stream of Kinase insert domain receptor, KDR, also known as Vascular endothelial growth factor 2 (VEGFR2) and Fetal Liver Kinase 1 (FLK1), and KDR start to be expressed. These events mark the earliest known stage of EC differentiation. Exposure of KDR+ cells to VEGF secreted from surrounding mesoderm, in response to hedgehog signalling, helps aid these KDR+ cells towards an EC fate; however, it may not regulate their direct differentiation into ECs, more likely acting as a survival factor [13]. At this point the KDR+ cells also make a fate decision to adopt either arterial or venous or lymphatic identity. This decision is in part due to the concentration of VEGF signalling that the cells are exposed to. High VEGF induces Notch signalling and expression of arterial genes such as Hey1, Hey2, EphrinB2 and Nrp1 [14–17]. Additionally, the double knockout of forkhead transcription factors FoxC1 and FoxC2 has demonstrated their critical role in arterial fate specification [18,19]. In contrast low VEGF induces CoupTF-II expression, which inhibits Notch and Nrp1, and upregulates Eph4 and Nrp2 establishing a venous fate.

EC differentiation protocols

A variety of EC differentiation protocols have been published over the past 3 years [4,9–11,20–26]. Although some are reliant on embryoid bodies, more recent ones are not. In general, however, they follow the same principles of mesoderm induction followed by addition of VEGF and also cell sorting for either KDR+ or CD31+ or VE-cad+ expression. Interestingly, cell sorting seems to be a necessary step as no protocol currently produces very high yields without it. However, post-sorting cell purities are commonly around 95%. We shall now discuss four studies, which are ideal exemplars of the protocols most recently developed.

In the first by White et al. [9], the authors used an embryoid body approach to first generate KDR+ cells using bFGF (5 ng/ml), Activin (6 ng/ml) and BMP4 (12 ng/ml). On Days 4 and 5 of differentiation, the embryoid bodies were differentiated further to pre-cardiac mesoderm using bFGF (10 ng/ml) and VEGF (10 ng/ml) and on Day 5.4 the cells were attached to fibronectin. At Day 6 the cells were dissociated and sorted for KDR+ expression, and the medium to high expressers were split onto fibronectin in serum-containing EC medium. The percentage of KDR+ cells was very much variable between cell lines, but most lines produced >95% CD31+:CD144+ cells by Day 14. A major focus of this work concentrated on gene expression in differentiated ECs, and an interesting point argued by the authors of this work was that there were minimal gene expression differences in ECs differentiated from human embryonic stem cell and induced pluripotent stem cells, which is an important observation for the utility of iPSC-derived ECs in regenerative medicine applications.

The work by White et al. [9] was one of the number of helpful steps in EC differentiation, but as stated not all ECs are the same and it is important to be able to model different parts of the vascular branch. To this end Rufaihah et al. [26] attempted to differentiate specific EC types, namely arterial, venous and lymphatic. These authors also took an embryoid body approach and used either 50 ng/ml or 10 ng/ml of VEGF-A to induce arterial and venous fates respectively. The 14-day protocol started with the generation of embryoid bodies in a 20% serum-containing differentiation medium. This was supplemented with BMP4 and VEGF at 50 ng/ml each for 4 days. The embryoid bodies were then laid onto gelatin-coated plates in the same basal medium containing 20% serum and treated with either 50 ng/ml VEGF-A and 0.5 mmol/L 8-bromoadenosine-3′:5′-cyclic monophosphate or 10 ng/ml VEGF-A to induce arterial or venous fate respectively until Day 14, at which point he cells were FACS-sorted for CD31+ expression. Gene expression analysis revealed the expression of arterial and venous specific markers consistent with *in vivo* expression profiles such that the arterial differentiation produced cells expressing higher levels of *ephrinB2*, *Notch1*, *Notch4*, *Dll4*, *Jag1*, *Jag2*, *Hey2*, *FoxC1*, *FoxC2* and *Nrp1*, whereas the venous differentiation produced cells expressing higher levels of *EphB4* and *CoupTF-II*. This demonstrated that the authors had succeeded in producing arterial- and venous-like EC-like cells and has been a helpful step forward in this field.

However, gene expression alone is not a sufficient evidence to be satisfied that the cells produced via plutipotent differentiations are equivalent to their adult counterparts; thus we must also test how the cells function. Both White et al. [9] and Rufaihah et al. [26] performed a variety of standard EC functional analyses including assembly of vascular network-like structures, incorporation of 1,1′-dioctadecyl-3,3,3′,3′-tetramethlyindocarbocyanine perchlorate-acetylatedlow density lipoprotein (Dil-Ac-LDL), migration in response to VEGF, production of nitric oxide, response to tumor necrosis factor-α (TNF-α). Adams et al. [4] performed a series of further highly stringent functional analyses of pluripotent

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