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Review

Building human artery and vein endothelial cells from pluripotent stem cells, and enduring mysteries surrounding arteriovenous development

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ABSTRACT

Owing to their manifold roles in health and disease, there have been intense efforts to synthetically generate blood vessels *in vitro* from human pluripotent stem cells (hPSCs). However, there are multiple types of blood vessel, including arteries and veins, which are molecularly and functionally different. How can we specifically generate either arterial or venous endothelial cells (ECs) from hPSCs *in vitro*? Here, we summarize how arterial or venous ECs arise during embryonic development. VEGF and NOTCH arbitrate the bifurcation of arterial *vs.* venous ECs *in vivo*. While manipulating these two signaling pathways biases hPSC differentiation towards arterial and venous identities, efficiently generating these two subtypes of ECs has remained challenging until recently. Numerous questions remain to be fully addressed. What is the complete identity, timing and combination of extracellular signals that specify arterial *vs.* venous identities? How do these extracellular signals intersect with fluid flow to modulate arteriovenous fate? What is a unified definition for endothelial progenitors or angioblasts, and when do arterial *vs.* venous potentials segregate? How can we regulate hPSC-derived arterial and venous ECs *in vitro*, and generate organ-specific ECs? In turn, answers to these questions could avail the production of arterial and venous ECs from hPSCs, accelerating vascular research, tissue engineering, and regenerative medicine.

1. Introduction

"Throughout the body of the animal, arteries are mingled with veins and veins with arteries … And of course the usefulness of such a complete interweaving is very evident, if, that is to say, it is a useful thing for all parts of the animal to be nourished."

—Galen, *On the Usefulness of the Parts of the Body*, 2nd century A.D.

As metaphorical highways throughout the human body, blood vessels span and interconnect all organs, delivering much-needed oxygen, sustenance, hormones, and immune cells to their client organs. Owing to the importance of blood vessels, there is considerable interest in generating synthetic human blood vessels for tissue engineering, regenerative medicine, and studies of vascular biology and disease [72,95]. For instance, vascularization has been termed "the key challenge in tissue engineering": all cells within the body are within ~200 μm of a blood vessel, and vascularization of engineered tissues is of paramount importance to suffuse them with oxygen and nutrients, thereby ensuring their survival [95]. Synthetic blood vessels are also proving to be powerful *in vitro* platforms to interrogate the underpinnings of vascular diseases. Vascular diseases are diverse in nature—including atherosclerosis, pulmonary arterial hypertension, diabetic vasculopathy, single ventricle disease, and various viral diseases, to name a few—and are estimated to cause one-third or one-half of deaths globally [7,14,17,48,84,142]. Additionally, blood vessels dictate whether circulating immune cells can enter inflamed tissues [2]. Strategically manipulating this process could be crucial to enhance the efficacy of cancer immunotherapies, especially to convey immune cells into "immunologically cold" tumors that poorly recruit immune cells [2]. These vignettes represent why generating synthetic human blood vessels is of broad importance.

Blood vessels are multi-layered structures, comprising an inner layer of endothelial cells (ECs) surrounded by overlying mesenchymal cells, variously known as pericytes or smooth muscle cells [102]. Here we specifically focus on ECs, which in turn comprise multiple subtypes—including arterial, venous, capillary, and lymphatic ECs—that are molecularly and functionally different from one another, and are often targeted by different diseases [27,39,80,102,129].

Our discussion will center on arterial and venous ECs. First, we

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briefly describe how arterial and venous ECs arise *in vivo*. Second, we examine efforts to generate arterial and venous ECs *in vitro* from human pluripotent stem cells (hPSCs, including embryonic and induced pluripotent stem cells). While there have been longstanding successes in differentiating hPSCs into ECs, until recently, they have often harbored an ambiguous arteriovenous identity [72,93]. However, it is now possible to generate arterial or venous ECs from hPSCs, creating new possibilities for studying vascular development, biology, and disease. Yet, challenges in generating arterial or venous ECs *in vitro* hint at mysteries that continue to surround arteriovenous development *in vivo*. Hence, we will dedicate a final "unanswered questions" section to these issues.

2. Arterial and venous development *in vivo*

First, we will briefly overview how arterial and venous ECs develop, focusing on the mouse embryo. For a fuller discussion, we refer the reader to excellent reviews on this subject [27,39,71,102,109,129].

2.1. Sequence of steps leading from pluripotency to arterial vs. venous endothelial cells

Pluripotent cells develop into ECs through a sequence of steps *in vivo*. First, pluripotent cells within the post-implantation epiblast (at embryonic day 5.0–5.5 [~E5.5]) differentiate into the primitive streak (~E6.0–6.5), which expresses transcription factors (TFs) including *Brachyury* [63,110] and *Mixl1* [100,111] (Fig. 1**a**). The primitive streak is induced by BMP, FGF, TGFβ and WNT (Fig. 1**a**) [21,23,31,76,85]. Subsequently, the primitive streak differentiates into multiple types of mesoderm, including the axial, paraxial (presomitic), intermediate, lateral, and extraembryonic mesoderm, which arise at ~E7.0-E7.5 [126,143] (Fig. 1**a**).

Consensus holds that lateral mesoderm generates ECs within the embryo, whereas extraembryonic mesoderm gives rise to ECs outside the embryo [36,105]. Intraembryonic ECs build the circulatory system that conveys blood between organs. In contrast, extraembryonic ECs constitute the lifesaving bridges between the embryo's circulation and the interface with the mother's blood, and deliver oxygen and nutrients before the baby takes its first breath upon birth [36].

What signals specify lateral mesoderm? *In vivo* and *in vitro*, BMP induces lateral mesoderm [3,128], while inhibition of WNT (which instead specifies paraxial mesoderm) and inhibition of TGFβ (which instead generates endoderm) consolidates lateral mesoderm identity [77] (Fig. 1**a**). The lateral mesoderm expresses multiple archetypic TFs, including *Hand1* [9,26,37] and *Isl1* [12,87,103]. Genetic lineage-tracing with *Hand1-Cre* [9] and *Isl1-Cre* [12] corroborates that lateral mesoderm generates intraembryonic ECs, including those found within the aorta. However, lateral mesoderm can generate a wide range of lineages, including heart, blood, ECs, limbs, and the visceral mesenchyme surrounding most internal organs [105]. Defining whether there truly exists a common lateral mesoderm progenitor to these lineages, and how lateral mesoderm becomes diversified into all these lineages, remain

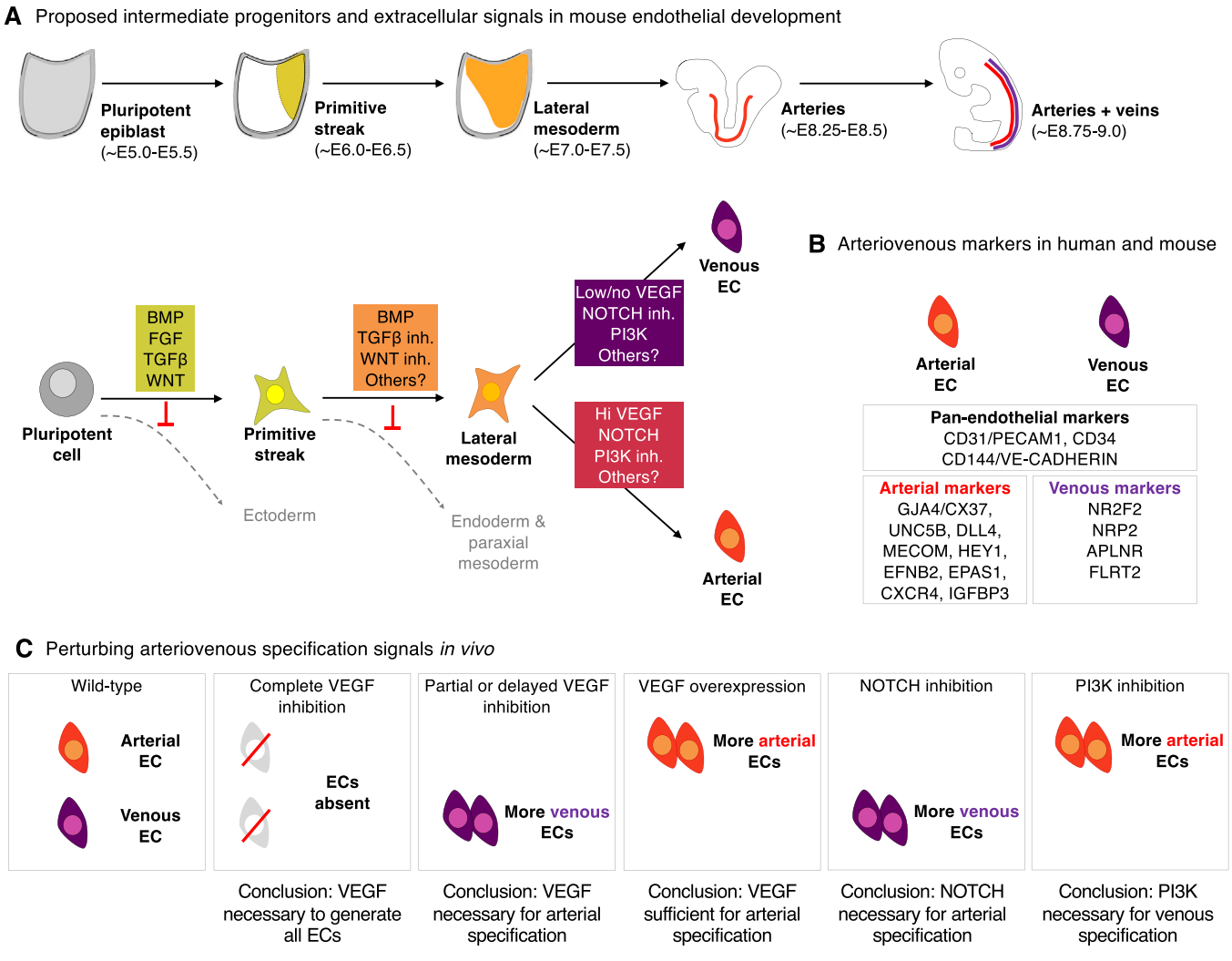

Fig. 1. Arterial and venous development *in vivo*.

important questions.

How does the lateral mesoderm generate ECs? A subset of lateral mesoderm expresses the endothelial-specifying TFs *Scl/Tal1*, *Lmo2*, *Fli1* and *Etv2* [20]. Attesting to the endothelial-specifying powers of these four TFs, their overexpression suffices to generate ectopic endothelial progenitors and/or ECs within zebrafish embryos, whereas their genetic inhibition compromises EC development [45,75,98,99,101,125]. Expression of these endothelial-specifying TFs precedes the emergence of ECs [20,30].

Subsequently, the first ECs—defined by expression of certain cell-surface markers such as CD31/PECAM1—arise at ~E8.0 or shortly thereafter [30,136]. Around E8.25-E8.5, a subset of ECs gain arterial identity, marking the first emergence of arterial ECs [18] (Fig. 1**a**). There are multiple interesting aspects of early arteriovenous development. First, are EC and arterial markers gained simultaneously, or in succession? Second, not all arterial markers are induced simultaneously, with *Dll4* expression preceding that of other arterial markers [18]. Third, venous ECs do not emerge contemporaneously with arterial ECs; unexpectedly, venous ECs instead emerge later during development [18]. Fourth, the earliest venous ECs appear to transiently "mis-express" certain arterial markers (*e.g.*, *Cx40* and *Nrp1*), and therefore the precise timing of when venous ECs first arise remains to be fully clarified, although it is estimated to be around ~E8.75 [18]. Why venous ECs arise later than arterial ECs is quite interesting and mysterious, as it implies that perhaps more time and/or additional signals are needed to specify venous identity.

2.2. Arterial and venous endothelial cells express mutually-exclusive markers

Although arterial and venous endothelial cells share the expression of "pan-endothelial" markers—including CD31/PECAM1, CD34, and CD144/VE-CADHERIN—they are defined by expression of other mutually-exclusive markers (Fig. 1**b**). For years, whether arterial and venous ECs were molecularly different from one another was unclear, but a pathbreaking study demonstrated that arterial ECs express *Efnb2*, whereas venous ECs express *Ephb4* [137]. This introduced the first molecular markers to distinguish arteriovenous identity, although more recent studies have suggested that *EPHB4* levels modestly differ between arterial and venous ECs [123]. Beyond these two markers, the advent of single-cell RNA-sequencing (scRNAseq) has led to an expansion in the number of arteriovenous signature markers, which may differ depending on animal species, developmental time, and organ, as reviewed elsewhere [58,123,129]. Additionally, scRNAseq comparisons of arterial and venous ECs isolated from both human and mouse embryos have identified a list of arterial and venous markers evolutionarily conserved across both species [53] (Fig. 1**b**). These *in vivo* markers could serve as benchmarks to determine the arteriovenous identity of hPSC-derived ECs generated *in vitro*.

Two prominent TFs, Sox17 and Nr2f2, are master regulators of arterial and venous identities, respectively. In mice, *Sox17* is expressed within early arteries [114], and its loss dramatically converts arteries into veins [24]. It should also be noted that *Sox17* is a member of the SoxF TF family, which includes *Sox7* and *Sox18*, and there is some degree of redundancy between these SoxF family members in specifying arterial fate [49]. Conversely, Nr2f2 is expressed in the earliest veins [74], and its loss reprograms veins into arteries [150]. The reciprocal phenotypes of *Sox17* or *Nr2f2* deletion—whereby arteries are converted into veins or *vice versa*—attest that arteries and veins are mutually-exclusive lineages specified by opposing TFs.

While these arteriovenous markers are often used to determine the arteriovenous identity of hPSC-derived cell-types *in vitro*, it is important to mention that these markers are not EC-specific. For instance, *Sox17* is also expressed by endoderm [34,59], whereas *Nr2f2* and *Efnb2* are additionally expressed by mesenchymal cells surrounding ECs [42,150]. Defining arterial or venous ECs produced *in vitro* thus relies on assaying whether cells co-express pan-EC and arteriovenous markers.

2.3. Extracellular signals that specify arterial vs. venous identities

VEGF and NOTCH signaling control the bifurcation of arterial *vs.* venous ECs *in vivo*: both induce arterial, at the expense of venous, identity [25,39,51,71,80,129]. Beyond these two signaling pathways, additional signals likely modulate arteriovenous fates *in vivo*, and remain to be discovered.

VEGF has multiple roles: it is required for the early specification of ECs, but also bestows them with arterial identity. Deletion of the VEGF receptor *Vegfr2* (otherwise known as *Flk1/Kdr*) eliminates all ECs within the mouse embryo [117] (Fig. 1**c**). Accordingly, VEGF is known as the master endothelial-specifying signal, and is an indispensable component of essentially every method to differentiate hPSCs into ECs (discussed below). Mechanistically, VEGF upregulates *Etv2* and *Scl* [15,46,108], and may therefore act directly on lateral mesoderm to generate the earliest endothelial progenitors and/or ECs. However, VEGF also controls the bifurcation of arterial *vs.* venous identities. While wholesale VEGF inhibition blocks EC formation altogether in zebrafish embryos, either delayed or partial VEGF inhibition instead promotes venous specification, while reducing arterial markers [15,67] (Fig. 1**c**). Therefore, VEGF induces arterial fate, while restricting venous identity. As discussed below, the multifaceted effects of VEGF have contributed to difficulties in generating venous ECs from hPSCs. VEGF can be a double-edged sword: it is clearly required to generate ECs *in vitro*, yet sustained or excessive VEGF arterializes ECs, and blocks venous formation.

What signaling cascades does VEGF trigger to specify arterial or venous identities? The prevailing view is that MAPK/ERK and PI3K signaling oppose one another to induce arterial *vs.* venous ECs [39,71]. VEGF rapidly and transiently induces phospho-ERK in ECs [38], and the earliest arterial ECs *in vivo* harbor phospho-ERK [52]. Excessive MAPK/ERK activation, through *Vegf* overexpression [67] or expression of constitutively-active RAF [28], ectopically induces arterial markers (Fig. 1**c**). By contrast, MAPK/ERK inhibition blocks arterial specification, and instead induces supernumerary venous ECs *in vivo* [52] (Fig. 1**c**). Altogether, this suggests that MAPK/ERK is necessary and sufficient to induce arterial fate, although other studies have challenged this view [118]. By contrast, PI3K instructs venous fate: PI3K inhibition abolishes venous identity, and expands arterial ECs *in vivo* [52] (Fig. 1**c**). Yet, paradoxes remain. For instance, while VEGF activates both MAPK/ERK and PI3K cascades, these cascades play opposing roles, respectively specifying arterial *vs.* venous identities [39,71]. Can these two cascades be simultaneously active, or does one assert primacy over the other?

NOTCH also instructs arterial fate: this has been extensively reviewed elsewhere [39,51] and is therefore only briefly mentioned here. Notch ligand *Dll4* is itself an arterial marker [79,119,133], and is the first arterial marker known to be expressed *in vivo*, inaugurating the emergence of the earliest arterial ECs in the ~E8.25 mouse embryo [18]. Accordingly, DLL4 is often used as a marker of hPSC-derived arterial ECs, as discussed below. Attesting to the importance of NOTCH in specifying arterial identity, NOTCH blockade eliminates arterial ECs, while expanding venous ECs, in both zebrafish and mouse embryos [39,51,66] (Fig. 1**c**). However, an unexpected molecular mechanism has been proposed for how NOTCH induces arterial identity. NOTCH signaling arrests cell division to specify arterial fate; remarkably, while NOTCH inhibition typically eliminates arteries, simultaneous inactivation of *Myc* reduces cell division and therefore re-enables arterial specification [35,78]. In short, NOTCH specifies arterial fate at least in part by blocking cell division.

Taken together, these *in vivo* studies provided a foundation for using PI3K inhibitors to bias differentiating hPSCs towards arterial identity, by blocking venous differentiation. Conversely, MAPK/ERK and NOTCH inhibitors have been used to differentiate hPSCs towards venous ECs, by

repressing arterialization, as discussed further below.

3. Arterial and venous differentiation *in vitro* from hPSCs

Although *in vivo* studies have provided an informative roadmap to generate arterial and venous ECs *in vitro*, until recently it has remained challenging to generate pure populations of these cell-types from hPSCs. At each step of differentiation—from pluripotency to primitive streak, lateral mesoderm, and subsequently the bifurcation into arterial *vs.* venous ECs—different timings and combinations of extracellular signals must be turned on or off. Consequently, a dizzying combinatorial matrix of signals could be screened to induce differentiation *in vitro*. Additionally, at each step of differentiation, intermediate progenitors can differentiate into two (or potentially more) cell-types, and cells that stray from the intended lineage can "mis-differentiate" into unwanted non-ECs that could contaminate the final cell product. These non-ECs could produce additional signals to aid or antagonize arteriovenous specification. At each differentiation step, stringent combinations of signals are thus required to exclusively differentiate progenitors into the desired lineage, while suppressing the formation of unwanted cell-types [40]. Suffice it to say, *in vitro* differentiation of hPSCs into arterial and venous ECs—or, for that matter, any cell-type—presents formidable challenges.

For concision, here we focus on the differentiation of hPSCs into arterial and venous ECs, cognizant that these were guided by preceding efforts to produce these cell-types from mouse PSCs.

3.1. To be 2D, or not to be 2D? (that is, 3D?)

Methods to generate arterial or venous ECs from hPSCs can be categorized into 2-dimensional (2D) or 3-dimensional (3D) differentiation approaches (Figs. 2–3). Historically, empirical or logistical considerations were used to decide between 2D or 3D differentiation approaches, yet recent years have provided mechanistic reasons to employ one or the other. In particular, cells in 2D *vs.* 3D differ in their access to gradients of developmental signals, nutrients and oxygens, and mechanical properties that may affect mechanotransduction pathways.

There has been a rich history of differentiating hPSCs in 3D aggregates (sometimes alternatively referred to as embryoid bodies, spheroids or organoids [65,115]), and such differentiating aggregates often produce multiple cell-types comprising both ECs and non-ECs, as discussed below.

Why do 3D differentiation approaches often generate heterogeneous cell populations? Certain extracellular signals, such as BMP, preferentially act on the periphery of hPSC clusters even when uniformly added to culture media [60,140], owing to the basolateral localization of BMP receptors within a given cell [154]. Therefore even when BMP is added to hPSC clusters, peripheral cells are affected, with deeper cells remaining largely untouched, leading to the creation of multiple cell-types along the width of the cell cluster [60,140]. Additionally,

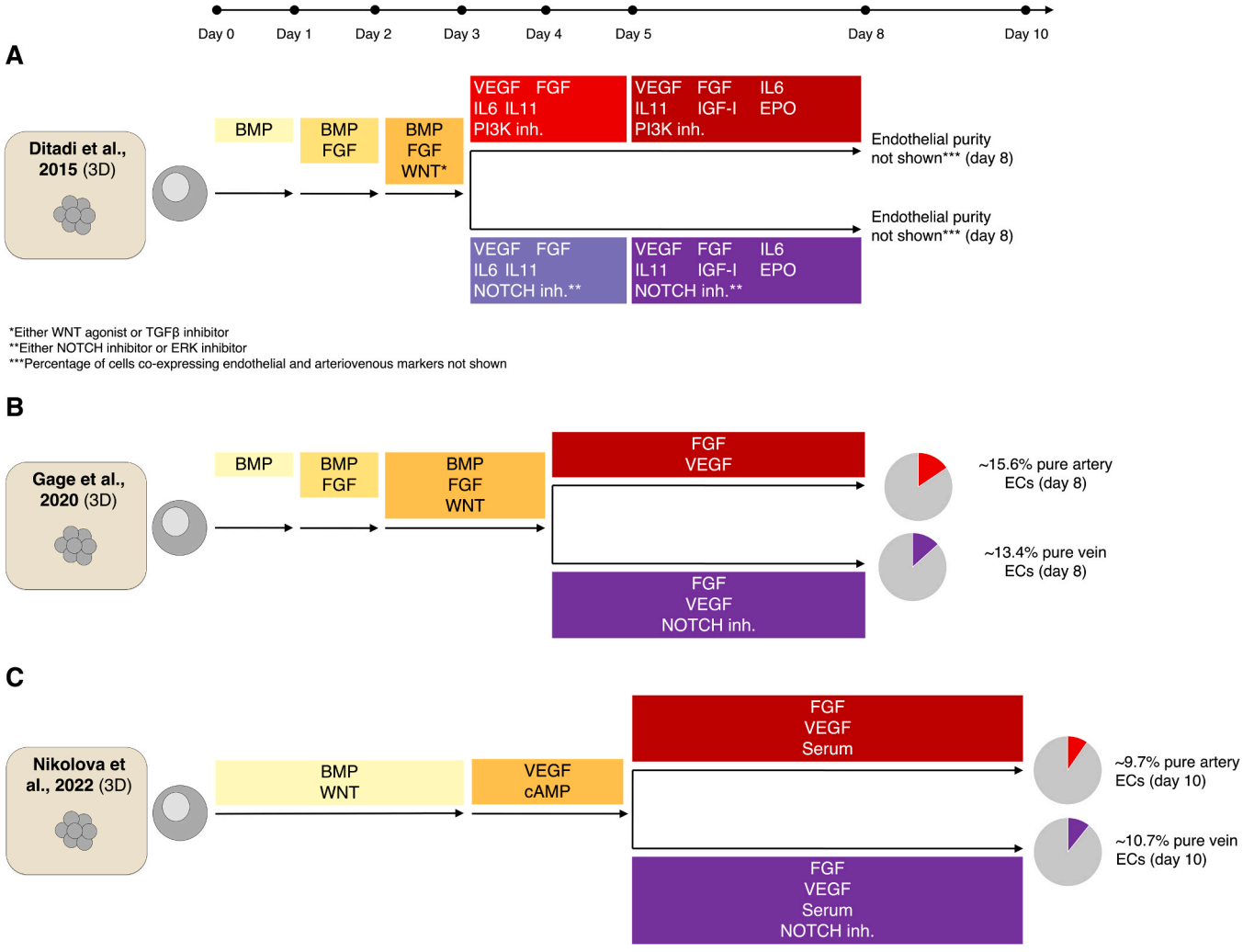

Fig. 2. Differentiation of hPSCs into arterial or venous ECs *in vitro*, in 3-dimensional aggregates Summary of current 3-dimensional hPSC differentiation protocols, with extracellular signals, days of differentiation, and purity of resultant arterial or venous ECs shown [29,41,94].

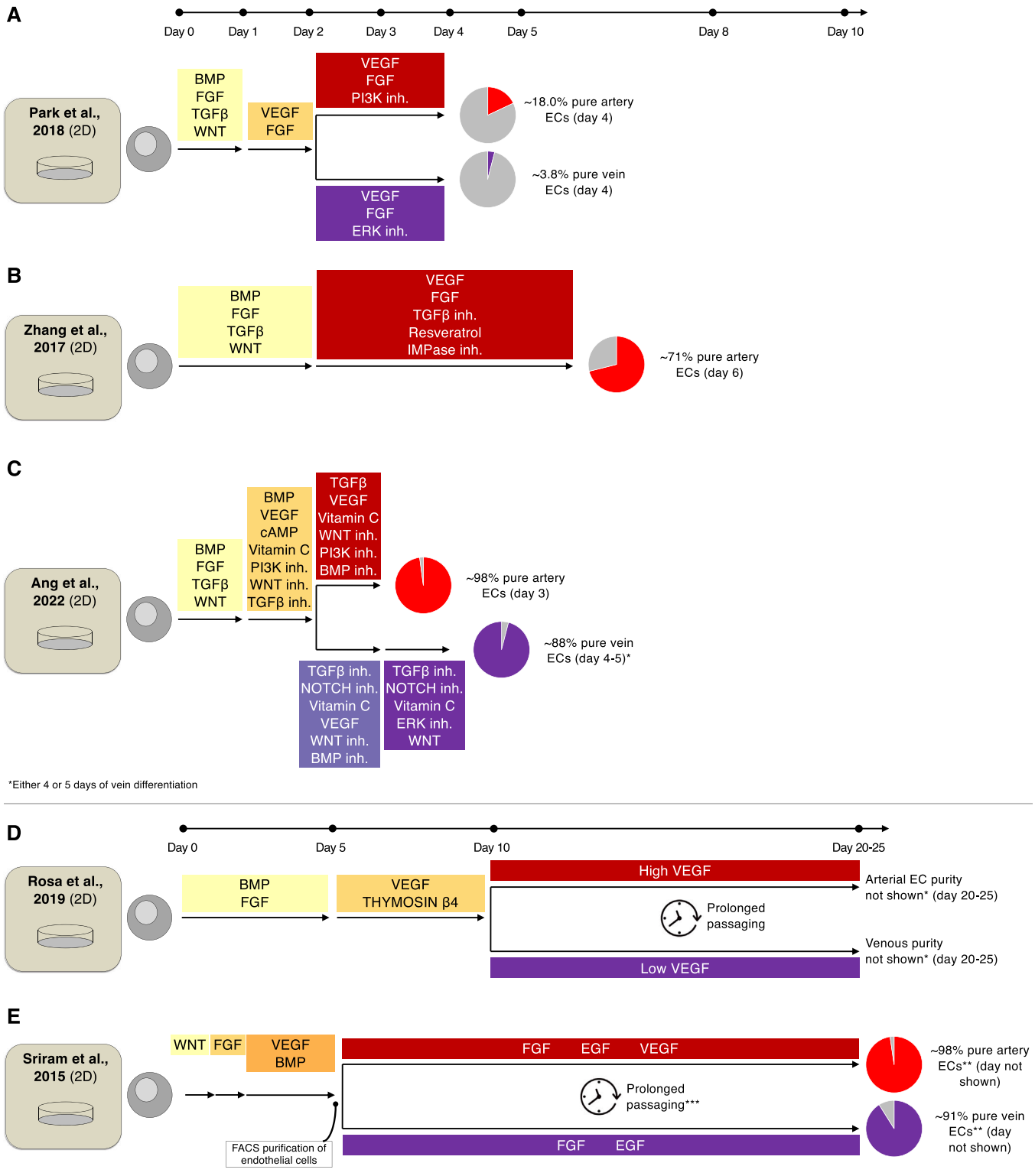

Fig. 3. Differentiation of hPSCs into arterial or venous ECs *in vitro*, in 2-dimensional monolayers Summary of current 2-dimensional hPSC differentiation protocols, with extracellular signals, days of differentiation, and purity of resultant arterial or venous ECs shown [4,96,112,121,152]. Note that different timescale lengths are used for differentiation methods entailing prolonged passaging.

VEGF—the quintessential endothelial-inducing factor—seemingly forms gradients, partly owing to its ability to bind extracellular matrix proteins [132], implying that it may not be able to deeply penetrate into hPSC clusters. Taken together, 3D spheroids likely present barriers to the penetration of media-borne extracellular signals, such that different signaling environments arise at different depths of an aggregate, culminating in heterogeneous differentiation. In line with this expectation, aggregate size appears to be a critical parameter: overly-large aggregates differentiate towards vascular lineages less efficiently [141], perhaps because inner cells might be "shielded" from differentiation-inducing signals in the media. Additionally, cells could differentiate asynchronously if cells in different cluster layers receive a given signal, but at different times.

Alternatively, arterial and venous ECs have also been generated from hPSCs in 2D monolayers. Monolayer differentiation can theoretically be more efficient than in 3D aggregates, as cells within a monolayer culture should have more equitable access to extracellular signals supplied by the media (in contrast with cells within 3D aggregates). Nevertheless, as detailed below, most 2D differentiation approaches continue to generate heterogeneous cell populations, and multiple challenges had to be overcome to achieve efficient arterial and venous EC generation within monolayers.

Multiple other parameters distinguish 2D *vs.* 3D differentiation approaches. First, oxygen and nutrients likely also form gradients within 3D cellular aggregates, with notable oxygen gradients arising within hPSC spheroids exceeding 200 μm in width [144]. Different levels of oxygen and nutrients could, in turn, affect the differentiation of superficial *vs.* deeper cells within aggregates. Second, cellular monolayers grown on plastic surfaces often experience extraordinary stiffnesses over a thousand times greater than most tissues, which can inactivate Hippo and other mechanotransduction pathways [134]. Cells grown in 3D aggregates are surrounded by liquid media and likely experience mechanically "softer" environments that activate Hippo signaling [148]. Going forth, it will be important to be cognizant of gradients (whether of developmental signals, oxygen or nutrients) and mechanical stiffness when differentiating hPSCs towards arterial or venous ECs—or for that matter, any cell-type—in 2D *vs.* 3D..

3.2. Generation of arterial or venous ECs from hPSCs in 3-dimensional cultures

Based on the roles of VEGF and NOTCH in specifying arterial *vs.* venous identities *in vivo*, manipulation of these two signaling pathways is at the crux of essentially all methods to generate arterial or venous ECs from hPSCs *in vitro*.

The Keller group differentiated hPSCs in 3D spheroids, initially with a sequence of BMP, FGF and WNT in the first 3–4 days of differentiation to generate mesoderm cells, followed by a second stage of differentiation with VEGF, FGF and other signals for several days to specify arterial or venous ECs [29,41] (Fig. 2**a,b**). In this second phase of differentiation, treatment with endothelial-inducing signals (*e.g.*, VEGF and FGF) together with PI3K inhibitors biased differentiation towards arterial ECs, whereas endothelial-inducing signals in concert with MAPK/ERK or NOTCH inhibitors preferentially generated venous ECs [29] (Fig. 2**a**). In a subsequent study, the second differentiation stage was further optimized such that high VEGF in concert with FGF generated ~16% pure arterial ECs, whereas low VEGF together with NOTCH inhibitor and FGF produced ~14% pure venous ECs [41] (Fig. 2**b**). Although the majority of cells (≥80%) within the differentiated population were non-endothelial, the ECs largely adopted either arterial or venous identities within the respective differentiation conditions: for instance, few vein ECs arose in "arterial" conditions and *vice versa* [29,41]. This demonstrated that manipulation of these select signaling pathways enabled guidance towards arterial or venous identities. That notwithstanding, an improved understanding of the signals that specify endothelial *vs.* non-endothelial fates could potentially enable more efficient

Table 1
Extant protocols to differentiate hPSCs towards arterial or venous endothelial cells.

Protocol	Purity	Duration	Format
Ditadi et al., ($year$)[29]: arterial differentiation	Purity of $CD34^+$ $CXCR4^+$ $CD73^{lo}$ arterial ECs not shown (% of $CD34^+$ ECs not quantified)	8 days	3-dimensional differentiation
Ditadi et al., ($year$)[29]: venous differentiation	Purity of $CD34^+$ $CXCR4^-$ $CD73^{hi}$ venous ECs not shown (% of $CD34^+$ ECs not quantified)	8 days	3-dimensional differentiation
Gage et al., ($year$)[41]: arterial differentiation	~15.6% $CD31^+$ $CD34^+$ $CXCR4^+$ $CD73^{lo}$ arterial ECs	8 days	3-dimensional differentiation
Gage et al., ($year$)[41]: venous differentiation	~13.4% $CD31^+$ $CD34^+$ $CXCR4^-$ $CD73^{hi}$ venous ECs	8 days	3-dimensional differentiation
Nikolova et al., ($year$)[94]: arterial differentiation	~9.7% arterial ECs (transcriptionally defined by scRNAseq)	10 days	3-dimensional differentiation
Nikolova et al., ($year$)[94]: venous differentiation	~10.7% venous ECs (transcriptionally defined by scRNAseq)	10 days	3-dimensional differentiation
Park et al., ($year$)[96]: arterial differentiation	~18.0% $CD144^+$ $CD73^-$ $CD43^-$ $CD235a^-$ $DLL4^+$ arterial ECs	4 days	2-dimensional differentiation
Park et al., ($year$)[96]: venous differentiation	~3.8% $CD144^+$ $CD73^+$ $CD43^-$ $CD235^-$ venous ECs	4 days	2-dimensional differentiation
Jung et al., ($year$)[57]: arterial differentiation	~5.8% $CD144^+$ $CD73^-$ $CD43^-$ $DLL4^+$ arterial ECs	4 days	2-dimensional differentiation and hypoxia (5% O_2)
Zhang et al., ($year$) [152]: arterial differentiation	71.1 ± 12.9% $CD144^+$ $DLL4^+$ and 56.03 ± 11.3% $CD144^+$ $CXCR4^+$ arterial ECs (average of 6 hPSC lines)	6 days	2-dimensional differentiation
Ang et al., ($year$)[4]: arterial differentiation	97.9 ± 2.4% $CD144^+$ $DLL4^+$ arterial ECs	3 days	2-dimensional differentiation
Ang et al., ($year$)[4]: venous differentiation	88.3% $CD144^+$ $NR2F2\text{-}GFP^+$ venous ECs	4–5 days	2-dimensional differentiation
Rosa et al., ($year$) [112]: arterial differentiation	~92.3% $CD144^+$ ECs and ~100% $EFNB2^+$ cells* (although % of arterial ECs not quantified by EC and arterial marker overlap)	20–25 days	2-dimensional differentiation, with prolonged passaging
Rosa et al., ($year$) [112]: venous differentiation	~89.9% $CD144^+$ ECs and ~55% $NR2F2^+$ cells*	20–25 days	2-dimensional differentiation, with prolonged passaging
Sriram et al., ($year$) [121]: arterial differentiation	99.2 ± 0.8% $CD144^+$ ECs and 97.7 ± 2.3% $DLL4^+$ cells* (average of 2 hPSC lines)	5 days + multiple passages (total duration not shown)	2-dimensional differentiation
Sriram et al., ($year$) [121]: venous differentiation	99.4 ± 0.1% $CD144^+$ ECs and 91.1 ± 8.9% $NRP2^+$ cells* (average of 2 hPSC lines)	5 days + multiple passages (total duration not shown)	2-dimensional differentiation

* In these studies, cells were not pre-gated on endothelial cells to quantify the percentage of endothelial cells that expressed either arterial or venous markers. As discussed in the main text, certain arteriovenous markers such as SOX17, EFNB2, and NR2F2 are expressed on non-ECs as well.

generation of ECs, by specifically blocking signals that generate non-ECs. However, at the end of differentiation, ECs could be enriched by fluorescence-activated cell sorting (FACS) or magnetically-activated cell sorting (MACS) to deplete non-ECs for subsequent experiments [29,41].

In a different approach, 3D hPSC spheroids were skewed to differentiate towards arterial or venous ECs by tuning NOTCH signaling [94] (Fig. 2**c**). Building on previous work [142], the Treutlein, Camp and Penninger laboratories developed a three-step differentiation protocol: initial treatment with BMP and WNT for 3 days, followed by VEGF and a cAMP-elevating agent (Forskolin) for 2 days, and finally, 5 days of treatment with endothelial-inducing signals (serum, VEGF and FGF) in the absence or presence of NOTCH inhibitor to respectively bias differentiation towards ~10% arterial or ~11% venous ECs, with mesenchymal cells emerging alongside ECs [94] (Fig. 2**c**).

Fascinatingly, when ECs and mesenchymal cells contemporaneously emerge within spheroids, they can spontaneously assemble into blood vessel-like structures [91,94,142]. Within these 3-dimensional blood vessel-like structures, ECs are encircled by mesenchymal cells, which in turn are surrounded by extracellular matrix proteins (*e.g.*, collagen IV) that may help stabilize the vascular structures [142]. There is skyrocketing interest in the ability of multiple cell-types to spontaneously assemble into more complex structures [65,115], and enabling such vascular self-assembly to occur is an innate advantage of 3D differentiation approaches. Nevertheless, these 3D aggregates must be dissociated to isolate ECs for certain types of downstream experiments, including functional assays [29,41,142]. Taken together, present approaches to generate arterial or venous ECs in 3D aggregates generate admixtures of ECs and non-ECs, but these disparate cell-types can self-assemble into 3D blood vessel-like structures in certain conditions. Therefore, cellular heterogeneity originating within 3D aggregates can provide powerful advantages, including the possibility of cellular self-organization into spatially-complex structures.

3.3. Generation of arterial or venous ECs from hPSCs in 2-dimensional cultures

Alternatively, hPSCs have been differentiated into arterial or venous ECs in monolayers. However, multiple challenges had to be surmounted to efficiently generate arterial and venous ECs.

The Slukvin group differentiated hPSCs towards arterial or venous ECs in 2D monolayers, using a 3-step protocol [96] (Fig. 3**a**). This entailed initial treatment with BMP, FGF, TGFβ, and WNT pathway agonists for 1 day to generate primitive streak, followed by endothelial-inducing signals (VEGF and FGF) for 1 day [96]. In the third phase, VEGF and FGF treatment was continued together with a PI3K inhibitor (to specify ~18% pure arterial ECs), or alternatively, an ERK inhibitor (to generate ~4% pure venous ECs) for 2 days [96]. Withholding the PI3K inhibitor generated arterial ECs less efficiently, reaffirming the importance of PI3K inhibition to generate arterial ECs [57,96]. Within 4 days of differentiation, over half of cells (54–62%) were endothelial, speaking to the efficiency and rapidity of endothelial differentiation [57,96]. However, only a subset of these ECs acquired arterial- or venous-specific identities [57,96], suggesting that additional arteriovenous specification signals could be incorporated in the future.

Multiple hPSC lines could be reproducibly differentiated into enriched populations of arterial ECs, using a 2-dimensional differentiation method developed by the Thomson group [152]. In this two-step method, BMP, FGF, TGFβ and WNT were activated for 2 days to differentiate hPSCs into mesodermal intermediates, followed by treatment with VEGF, FGF, TGFβ inhibitor, resveratrol and an inositol monophosphatase (IMPase) inhibitor (L690) for 4 days [152]. Impressively, this yielded on average 71.1 ± 12.9% pure $CD144^+$ $DLL4^+$ and 56.03 ± 11.3% $CD144^+$ $CXCR4^+$ pure arterial ECs within 6 days of hPSC differentiation, across 6 different hPSC lines [152]. This work also identified additional non-classical developmental signals, including resveratrol and IMPase inhibition, that potentially control arteriovenous specification. Intriguingly, reservatrol and IMPase inhibition were required for the upregulation of certain arterial markers (*e.g.*, EFNB2) but not others (*e.g.*, DLL4 and CXCR4) [152], hinting at complex roles of these two factors in arterial specification.

Finally, we developed 2D methods to differentiate hPSCs into > 95% pure arterial ECs or > 85% pure venous ECs, within 3 or 4 days of differentiation, respectively [4]. First, BMP, FGF, TGFβ, and WNT were activated for 24 h to differentiate hPSCs into > 97% pure $MIXL1^+$ primitive streak cells [4,77]. Second, day-1 primitive streak cells were further differentiated into day-2 lateral mesoderm cells by activating BMP, FGF and PKA signaling, while simultaneously inhibiting PI3K, TGFβ and WNT signaling, for 24 h [4]. At this stage of differentiation, inhibition of TGFβ blocked endoderm formation, whereas WNT blockade prevented paraxial mesoderm formation, thereby enhancing the purity of lateral mesoderm cells produced [4,77].

Subsequently, day-2 lateral mesoderm was further differentiated into day-3 arterial ECs or day-4 vein ECs through the application of mutually-exclusive signals. Activation of TGFβ and VEGF signaling, together with inhibition of BMP, WNT and PI3K signaling for 24 h generated arterial ECs with 97.9 ± 2.4% purity [4]. To summarize, arterial EC specification was achieved by simultaneously applying artery-specifying signals (TGFβ and VEGF) while simultaneously repressing the vein-specifying PI3K pathway. This study also nominated TGFβ as a new candidate artery-specifying signal, whose *in vivo* effects remain to be further assessed.

Conversely, inhibition of TGFβ and NOTCH signaling for 48 h generated ~88% pure venous ECs, when coupled with VEGF activation and WNT inhibition for the first 24-hour period, followed by VEGF/ERK inhibition and WNT activation for the second 24-hour period [4]. Indeed, VEGF is initially required to generate ECs *in vivo*, but subsequently it must be repressed at later stages to specify venous identity, as prolonged VEGF signaling instead specifies arterial fate [15,67,117]. In brief, an initial pulse of VEGF signaling, followed by VEGF/ERK pathway inhibition, in concert with continued blockade of artery-inducing signals (TGFβ and NOTCH), efficiently generated venous ECs *in vitro*.

3.4. Generation of arterial or venous ECs in 2D cultures, through endothelial progenitor intermediates

Alternative 2D approaches have instead differentiated hPSCs into early “endothelial progenitor cells” lacking arteriovenous identity, which were subsequently biased towards arterial or venous identities by toggling VEGF levels during prolonged culture [112,121].

The Ferreira group differentiated hPSCs for 5 days with BMP and FGF, followed by 5 days of VEGF and thymosin β4 to generate endothelial progenitors that expressed CD31, but low levels of VE-CADHERIN [112] (Fig. 3**d**). Subsequently, these endothelial progenitors were skewed towards arterial or venous ECs solely by culturing them with either high or low VEGF [112]. This yielded 90–92% pure ECs, which expressed elevated levels of arterial or venous markers in the respective differentiation conditions; however, the percentage of ECs that expressed arterial- or venous-specific markers was not shown [112]. Additionally, venous marker NR2F2 was expressed in a subpopulation of cells, suggesting that additional signals beyond low levels of VEGF may be important to efficiently steer ECs towards a venous identity [112].

The Cao group instead differentiated hPSCs into primitive streak by activating WNT for 1 day, followed by FGF for 1 day, and then BMP and VEGF for 3 days to generate 94% pure $CD31^+$ $CD34^+$ endothelial progenitors [121]. These ECs were purified by FACS, and then cultured for

three passages with endothelial signals (FGF and EGF) either in the presence or absence of VEGF to bias cells towards arterial ECs (~98% purity) or venous ECs (~91% purity), respectively [121].

An advantage of both differentiation protocols is that prolonged culture in endothelial media may enrich for ECs, by conferring them a proliferative and/or survival advantage in comparison to non-ECs within the culture. Both protocols revolve around the *in vitro* generation of an early "endothelial progenitor cell" lacking arteriovenous identity. However, the precise *in vivo* counterpart to such a cell remains to be clarified, as discussed below.

4. Outstanding questions

4.1. What is the in vivo significance of signals that specify arteriovenous fate in vitro?

The ability to rapidly screen large numbers of signaling perturbations on differentiating hPSCs has revealed

new signals that may instate arteriovenous fate *in vitro*; however, the *in vivo* significance of some of these signals remains to be rigorously investigated. Alternatively, certain signals used to drive EC differentiation *in vitro* may be artificial and may not have a clear *in vivo* counterpart, which could warrant concern.

Forskolin—an adenylate cyclase agonist that elevates cAMP levels—enhances EC differentiation from hPSCs [4,94,97,142]. Ligand binding to certain G-protein coupled receptors (GPCRs) triggers adenylate cyclase, thereby elevating cAMP levels, which in turn activates protein kinase A (PKA) [55]. cAMP-PKA signaling seemingly increases cellular sensitivity to VEGF *in vitro* [146], perhaps explaining why it boosts EC differentiation. Additionally, cAMP-PKA signaling may induce endothelial progenitor marker *Etv2*: in cultured mouse embryos, the PKA inhibitor H-89 decreased *Etv2* [147]. However, H-89 also inhibits multiple kinases in addition to PKA [8], thus calling for more precise perturbations to delineate the effects of cAMP-PKA signaling. It will therefore be important to establish whether cAMP promotes endothelial and/or arteriovenous development *in vivo*. If so, what are the upstream ligand-GPCR interactions that elevate cAMP *in vivo* within differentiating ECs?

FGF is another signal prevalently used, alongside VEGF, to specify ECs at later steps of hPSC differentiation [29,41,96,152]. In fact, one study suggested that FGF-containing media sufficed to generate ECs in the absence of exogenous VEGF [152]. This is quite remarkable, given the paramount requirement for VEGF in EC specification *in vivo*. Under artificial circumstances, can FGF partly replace VEGF in EC development? This warrants further investigation, as FGFR and VEGFR are closely-related receptor tyrosine kinases that activate similar signaling pathways (*e.g.*, MAPK/ERK) [69]. Intriguingly, despite the prevalent use of FGF to generate ECs *in vitro*, at present, there is no genetic evidence that ablation of FGF ligands or receptors impairs EC development *in vivo* [149]. Of note, the purported later role of FGF in inducing ECs is likely distinct from the earlier, well-described role of FGF in incipiently generating primitive streak or mesoderm cells at early steps of differentiation [4,29,41,96,112,121,152].

Vitamin C (ascorbic acid) promotes EC differentiation from hPSCs [4,70] and at first glance, appears to be far removed from a plausible developmental signal that may figure in EC development *in vivo*. However, Vitamin C enhances the enzymatic activity of multiple α-ketoglutarate-dependent chromatin regulators, including TET family DNA demethylases and JMJD family histone demethylases [11]. Vitamin C globally decreases DNA methylation in mouse PSCs and other cell-types, profoundly altering chromatin state [10,11]. The roles of chromatin regulators, including TET and JMJD family enzymes, in EC development *in vivo* are understudied and could be fruitfully explored in the future. Notably, common cell-culture media used for EC differentiation, including KnockOut Serum Replacement [43] and Essential 6 (E6) [16], contain Vitamin C, suggesting that Vitamin C may be an overlooked, yet implicit and important component of many EC differentiation protocols.

TGFβ signaling has been attributed to contradictory roles in either promoting [4] or inhibiting [152] arterial specification from hPSCs. This discrepancy may be partly attributable to temporally-dependent effects. It was initially reported that continuous TGFβ inhibition for four days specified arterial ECs [152]. However, another study showed that TGFβ inhibition initially differentiated primitive streak into lateral mesoderm (by blocking endoderm formation), but 24 h later, TGFβ activation further differentiated lateral mesoderm into arterial ECs, while blocking venous fate [4]. The hypothesis that TGFβ signaling specifies arterial fate at the expense of venous identity remains to be investigated *in vivo*. On the other hand, BMP signaling—which is related to, but often competes against TGFβ signaling—has been reported to specify venous ECs *in vivo* [89]. One interesting model would be that TGFβ and BMP compete against one another to respectively induce arterial and venous fates in mutually-exclusive fashion.

Finally, one study suggested that temporally-dynamic repression, followed by activation, of WNT signaling specified vein ECs from hPSCs [4]. Whether WNT signaling is active within, or specifies, arterial or venous ECs, remains controversial *in vivo* [39]. However, scRNAseq revealed that *LEF1*, a WNT pathway target gene and transcriptional effector of WNT signaling, is preferentially expressed in human embryonic venous (rather than arterial) ECs *in vivo* [151]. Therefore, the role of WNT signaling in arteriovenous specification *in vivo* warrants further attention.

In summary, *in vitro* differentiation approaches have nominated a host of signals, some classical developmental signals (*e.g.*, FGF, TGFβ and WNT) and others more unexpected (*e.g.*, cAMP and Vitamin C), with potential roles in endothelial and/or arteriovenous specification.

4.2. Mysteries of vein development

To date, most hPSC differentiation approaches tend to generate arterial ECs more efficiently than venous ECs (Figs. 2–3), suggesting that mysteries continue to surround vein development.

Indeed, arterial ECs arise before venous ECs *in vivo* [18], suggesting a protracted program for venous development *in vivo*, which may be more challenging to emulate *in vitro*. Paralleling this, one study found that hPSCs differentiated into arterial ECs one day earlier than venous ECs *in vitro* [4]. However, most other studies report that arterial or venous ECs arise synchronously *in vitro* [29,41,94,96,112,121]. That being said, why does it take longer for venous identity to be specified *in vivo*?

The extracellular signals that specify venous fate also remain to be fully explored: is it simply all about modulating the timing and levels of known extracellular signals (*e.g.*, VEGF and NOTCH), or are there "missing" vein-specifying signals that remain to be discovered? Part of the challenge in generating venous ECs undoubtedly lies with VEGF. As discussed above, VEGF is required to generate all ECs (including venous ECs), as *Vegfr2-/-* mice completely lack ECs [117]. However, higher VEGF levels or prolonged VEGF signaling specifies arterial identity, while blocking venous fate *in vivo* [15,67]. In summary, VEGF is paradoxically required for, yet also represses, the formation of venous ECs. The concentration or timing of VEGF, or other developmental signals provided in parallel, likely prove decisive in deciding between arterial *vs.* venous identities.

In vitro, there exists a fundamental tension between the prerequisite to add VEGF to induce ECs and yet also the need at some point to attenuate VEGF to generate venous ECs. Excessive VEGF activation generates arterial ECs, yet the solution to generate venous ECs cannot simply be to block VEGF, as inhibiting VEGF abrogates EC formation *in vitro* altogether [4,138]. In some ways, acquisition of pan-EC identity and arterial fate are inextricably linked at a mechanistic level. VEGF signaling induces ETS family TFs (*e.g.*, ERG and FLI1) that bind to, and activate, the enhancer of arterial marker *Dll4* [145], underpinning why VEGF seemingly induces ECs and arterializes them.

Navigating the EC- *vs.* artery-specifying effects of VEGF *in vitro* to

produce venous ECs efficiently has proven challenging. The first generation of hPSC differentiation approaches provided the same duration and concentration of VEGF to induce arterial or venous ECs. However, VEGF was combined with NOTCH blockade to generate venous ECs [29]. Other differentiation approaches used lower concentrations of VEGF, or even withheld exogenous VEGF altogether at later stages of differentiation, to generate venous ECs [41,112,121]. To take it one step further, VEGF signaling can be inhibited to install venous identity. We found that VEGF activation for 24 h, followed by explicit inhibition of MAPK/ERK signaling for 24 h, generated venous ECs more efficiently [4]. However, the optimal means to inhibit the arterializing effect of VEGF remains to be determined, as some studies suggest that VEGF activates other signaling cascades beyond MAPK/ERK to induce arterial fate [118].

Finally, it should be noted that most of our knowledge of venous development concerns the *inhibition* of arterializing signals: for instance, blocking NOTCH is integral to most venous EC differentiation protocols [4,29,41,94]. However, PI3K signaling has emerged as a "positively-acting" signal required to specify venous ECs *in vivo* [52], although the upstream signal activating PI3K signaling in venous ECs *in vivo* has not been definitively identified. (VEGF is a candidate, perhaps at low levels.) Recent studies suggest that BMP may also be a vein-specifying signal [89].

4.3. May the Force be with you—what about the effects of shear stress?

The prevailing model is that shear stress is dispensable for initial arteriovenous specification, but is subsequently required to maintain distinct arteriovenous identities [39]. While it was initially assumed that shear stress was required for arteriovenous specification, arterial and venous ECs arise *in vivo* in ~E8.25-E8.75 mouse embryos [18,137], before the onset of vigorous heartbeats—and thus strong shear stress—at ~E10.0 [83]. In chicken embryos, arterial and venous ECs likewise emerge before blood circulation [50]. In summary, arterial and venous ECs likely arise without strong shear stress *in vivo*, likely explaining why these cell-types can be generated *in vitro* in static media conditions.

However, could the identity of hPSC-derived arterial and venous ECs be modulated by shear stress, particularly different levels of shear stress? Shear stress can bias human or mouse PSC-derived ECs towards arterial fate [6,81,120]. This is consistent with a body of work suggesting influential roles for shear stress in arteriovenous specification. In particular, altering shear stress within avian embryos [68], or in explanted human and mouse ECs [18,86], can alter arteriovenous identities to some degree. However, shear stress may selectively promote certain aspects of arterial identity, but not arterial identity as a whole. Shear stress upregulates certain markers (*e.g.*, *Cx37* and *Cx40*), yet other arterial markers (*e.g.*, *Dll4*) can nevertheless be expressed in arterial ECs cultured in static conditions [18,35]. Going forward, it will be fascinating to understand how classical arteriovenous developmental signals intersect with mechanical forces (shear stress) to initially specify, and subsequently regulate arteriovenous identities. Indeed, shear stress and NOTCH may cooperate to induce arterial marker *Cx37* [35].

4.4. Cutting to the chase: overexpressing transcription factors to directly specify arterial or venous identities?

Thus far, this review has largely focused on extracellular signals that instill arteriovenous identity, but TF overexpression represents another avenue for hPSC differentiation. In one approach, hPSCs were differentiated into BRACHYURY^{+} primitive streak/mesoderm cells by activating WNT for 2 days, which were further differentiated into 89–95% pure ECs within 2 additional days by overexpressing the master endothelial-specifying TF *ETV2* [138]. Remarkably, this method proved effective across 13 different hPSC lines and was unfazed by VEGF blockade, suggesting that *ETV2* overexpression overrides the need for VEGF to induce ECs *in vitro* [138]. However, the hPSC-derived ECs obtained by *ETV2* overexpression did not reportedly possess a clear arteriovenous identity.

Do these *ETV2*-induced ECs correspond to a *tabula rasa* that can be further biased into arterial or venous ECs by overexpressing other TFs in parallel? One example of an artery-specifying TF is *ETS1*, whose overexpression skews differentiating hPSCs towards an arterial identity [96]. Going forward, it would be interesting to combine pan-EC TFs (*e.g.*, *ETV2*) alongside either artery-specifying TFs (e.g., *SOX17* or *ETS1*), or vein-specifying TFs (*e.g.*, *NR2F2*), thereby artificially "building" EC arteriovenous identity in modular fashion. As a further measure, TF overexpression could also be combined with the aforementioned extracellular signals that specify arteriovenous fates to "sharpen" the identity of the induced ECs. Indeed, TF overexpression alongside with provision of lineage-specifying extracellular signals may prove to be a powerful strategy to efficiently generate desired cell-types from hPSCs [40].

However, in considering such TF-driven differentiation approaches, the timing of TF overexpression is of primary importance [40]. While *ETV2* overexpression in undifferentiated hPSCs converts them into ECs [32,90,124], it is worth noting that *ETV2* is normally expressed in lateral mesoderm or endothelial progenitors, and *ETV2* overexpression at intermediate steps of hPSC differentiation has proven more effective [73,138]. By analogy, it may be similarly critical to overexpress arteriovenous-specifying TFs at later stages of differentiation; indeed, *SOX17* overexpression directly in hPSCs generates endoderm, not ECs [116].

4.5. Do starting points matter: do different mesoderm subtypes generate different types of ECs?

Although this review has emphasized lateral mesoderm as a source of ECs, multiple mesoderm subtypes may form ECs. Other potential sources of ECs include endoderm, paraxial mesoderm and extraembryonic mesoderm. The endoderm has been claimed to generate ECs in zebrafish [88], although this is not supported by *Foxa2-Cre* lineage-tracing in mouse embryos [131]. Classical embryological studies suggest that paraxial mesoderm—which forms the somites—also generates ECs [1,19,143]. Indeed, recent zebrafish studies also report that paraxial mesoderm can generate lymphatic ECs [122], which will be interesting to test in mouse embryos using genetic lineage-tracing. Finally, recent live-imaging studies have also blurred the lines between extraembryonic and intraembryonic ECs, by suggesting that certain extraembryonic ECs can invade the embryo proper [22,127]. It will be fascinating to deconvolute which mesoderm subtype(s) contribute to each blood vessel within the body. If multiple parallel progenitors converge to form ECs, what are the relative proportions of ECs do they each supply?

If multiple subtypes of mesoderm can form ECs *in vivo*, this piques the question of whether each mesoderm subtype gives rise to different EC subtype(s). That is, do ancestral differences in early mesodermal precursors translate into lasting differences in the resultant ECs? Multiple protocols to differentiate hPSCs into ECs proceed through HAND1+ mesoderm intermediates [4,94,106,130,153]. HAND1 is a marker of lateral and extraembryonic mesoderm [26], and it has not been determined whether the hPSC-derived HAND1+ mesoderm corresponds to lateral and/or extraembryonic mesoderm, owing to the need for additional markers to distinguish the two. Recent methods have purported to generate paraxial mesoderm-derived ECs from hPSCs [13], setting the stage to compare ECs derived from various mesoderm subtypes *in vitro*.

4.6. What is the exact order and sequence of endothelial lineage decisions?

Defining the exact order and sequence of endothelial lineage decisions, particularly when bipotent progenitors bifurcate into arterial *vs.*

venous ECs, remains an outstanding task. Most *in vitro* differentiation approaches implicitly assume the existence of bipotent arterial-venous progenitors. Studies of zebrafish embryos have challenged this notion and instead suggest separate arterial *vs.* venous precursors [64]. At some level, this is a question of what exact time arterial *vs.* venous potentials become separated, as ultimately pluripotent cells (*e.g.*, hPSCs) can give rise to both cell-types. Do arterial *vs.* venous potentials become segregated during early differentiation, at the primitive streak, lateral mesoderm or endothelial precursor stages? Alternatively, other models postulate the existence of "primordial" ECs that are imbued with the potential to generate both arterial and venous ECs [57,80,112,121,130]. In the "primordial EC" model, single ECs retain bipotentiality, that is, arterial *vs.* venous potentials have not been segregated at earlier developmental steps. Investigating the precise stage of arterial *vs.* venous lineage separation will be important.

Another related question concerns the definition of "endothelial progenitor" or "angioblast". Multiple definitions have been proposed for these cells. VEGFR2 (FLK1/KDR), SCL, LMO2 and/or ETV2 have been used to identify endothelial progenitors or angioblasts [18,30,39,64]. However, the earliest VEGFR2$^+$ cells are primitive streak cells with the broad potential to generate heart and skeletal muscle, in addition to ECs [33,54], although at later stages, VEGFR2 expression is probably confined to endothelial progenitors. VEGFR2 is turned on before SCL, LMO2 and ETV2 [20]. At what stage along this continuum can cells be truly said to be "endothelial progenitors", then? Genetic lineage-tracing suggests that SCL$^+$, LMO2$^+$ and ETV2$^+$ progenitors are largely committed to ECs (although they also generate blood cells, which are thought to arise from ECs) [47,107,139]. That notwithstanding, these genetic lineage-tracing studies were performed with transgenic promoter and/or enhancer fragments for *SCL*, *LMO2* and *ETV2* [47,107,139]. Inserting Cre or another recombinase into the endogenous respective genes could be helpful to assess whether SCL, LMO2 and ETV2 mark EC-restricted progenitors.

What is the precise relationship between lateral mesoderm and angioblasts/endothelial progenitors: are they interchangeable or does one precede the other? SCL$^+$ LMO2$^+$ cells are contained within lateral mesoderm [20], but are they still "lateral mesoderm" or should they be defined as another cell-type (*e.g.*, "angioblasts")? Adding another wrinkle, even after they emerge from mesoderm, others continue to refer to early arterial or venous ECs as angioblasts [25].

The field will need to arrive at unified terminology to categorize these various cellular states, recognizing that differentiation is a continuum that may not be easy to parcellate using rigid labels. One proposal is that once cells upregulate endothelial cell-surface markers (*e.g.*, CD31/PECAM1, CD34 and CD144/VE-CADHERIN) they can be considered ECs. In this definition, "endothelial progenitors" would be CD31$^-$ CD34$^-$ CD144$^-$ cells that precede the emergence of ECs. However, it will be important to pinpoint when and where endothelial-restricted progenitors precisely exist along the FLK1-SCL-LMO2-ETV2 continuum.

Adding further complexity, the earliest cellular products of the arterial-venous bifurcation also deserve further attention. Intriguingly, the earliest venous ECs "mis-express" certain arterial markers before exclusively adopting venous identity [18]. This calls for further investigation. For instance, are artery ECs specified first and then "trans-differentiate" into venous ECs? Or do the earliest venous ECs have a mixed arteriovenous identity, which resolves with time?

4.7. Can we expand hPSC-derived arterial and venous ECs in vitro?

Despite successes in generating hPSC-derived arterial or venous ECs, methods must be devised to maintain and massively expand these cells. From a pragmatic perspective, expanding arterial or venous ECs could supply massive numbers of these cells for basic research, tissue engineering, and regenerative therapies. hPSC-derived ECs can be massively expanded [56,104], but their arteriovenous identity remains to be assessed.

A landmark study demonstrated that primary human arterial and venous ECs can expand in the commercially-available Endothelial Growth Medium 2 (EGM2) [5]. Nevertheless, these ECs swiftly relinquish their respective arteriovenous identities *in vitro* [5]. Therefore, although cultured arterial and venous ECs are widely used for vascular research, a proviso is that their arteriovenous specificity may gradually degrade in conventional culture conditions.

Indeed, hPSC-derived venous ECs cultured for several days in conventional media "drifted" and upregulated arterial markers [4,41]. This is perhaps not surprising, given the evidence that VEGF—a ubiquitous component of all EC media—is arterializing *in vivo*. Inhibition of artery-specifying signals (NOTCH and TGFβ) was required to maintain hPSC-derived venous ECs *in vitro*, suggesting that repression of arterializing signals is required to establish and maintain venous identity [4]. While it is possible to maintain hPSC-derived arterial and venous ECs for several days *in vitro* [4], methods to massively expand them while preserving their arteriovenous identity are sorely needed.

4.8. How can we produce organ-specific ECs in vitro from hPSCs?

Generation of organ-specific ECs is a major goal for the field [92], and we will only briefly touch on this topic. Arterial and venous ECs within different organs (*e.g.*, brain, lung, liver, and heart) are transcriptionally different from one another [58,113]. However, current-generation arterial and venous ECs derived from hPSCs typically lack organ-specific markers [4]. This may be because the earliest arterial and venous ECs originate from the mesoderm germ layer, and arise before most major organs have formed [18]. One hypothesis suggests that these early hPSC-derived ECs, having recently arisen from mesoderm, might be uniquely plastic and could diversify into organ-specific ECs upon encountering organ-specific cues. In one notable study, hPSC-derived venous ECs transplanted into the mouse liver expressed liver-specific EC markers, such as the coagulation factor F8 [41]. Alternatively, naïve hPSC-derived ECs treated with WNT *in vitro* acquired certain blood-brain barrier properties, while more mature ECs appeared resistant to WNT [44].

4.9. When do mesenchymal cells split off from the endothelial lineage?

Multiple methods to differentiate hPSCs into ECs generate contaminating mesenchyme-like cells, identified by various markers such as PDGFRβ or SMAα/ACTA2 [4,82,94,142]. Which mesenchyme subtype (s) do these hPSC-derived cells correspond to? Are they pericytes, smooth muscle cells, fibroblasts, or another as-of-yet-unidentified mesenchymal cell?

In any case, the repeated co-emergence of mesenchyme and ECs across multiple hPSC differentiation protocols is intriguing, and could reflect a shared mesenchymal-EC precursor [135], perhaps residing in the primitive streak [54] or lateral mesoderm [61,62]. Our understanding of how lateral mesoderm progenitors bifurcate into mesenchyme *vs.* EC identities is incomplete. Discovering the signals that control this lineage decision may allow us to suppress unwanted mesenchymal differentiation during hPSC-to-EC differentiation *in vitro*.

5. Conclusion

Vertebrate embryology has provided a roadmap to differentiate hPSCs into arterial or venous ECs. *In vivo* studies emphasized the importance of VEGF and NOTCH in the bifurcation of these two cell-types. Manipulation of these two signals biases hPSC differentiation towards arterial or venous identities. However, modulation of additional signals, in various combinations and timings, is required to efficiently generate arterial or venous ECs *in vitro*.

It is an exciting time for vascular development, with the field burgeoning with questions that warrant further investigation. What is the precise sequence of branching lineage decisions that lead from

pluripotency to arterial *vs.* venous identities? What are the combinations and timings extracellular signals that must be turned on or off at each step along the way? To efficiently generate venous ECs, how can we wield VEGF in a precise way to generate incipient ECs but without arterializing them? Can we arrive at unified definitions of endothelial progenitors and angioblasts and pinpoint when they arise along the continuous process of pluripotent cell differentiation into ECs? At what stage of development do arterial *vs.* venous potentials separate, and when does this happen relative to the segregation of mesenchymal potential in early progenitors? After they are specified, how do the earliest arterial and venous ECs expand in number and adopt organ-specific identities *in vivo*, and can we recreate these processes *in vitro*?

Declaration of Competing Interest

None.

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