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Smooth muscle diversity from human pluripotent cells

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Abstract

A method for differentiating human pluripotent stem cells into several subtypes of smooth muscle cell should aid the study and treatment of vascular disease.

Diseases of the blood vessel wall occur at specific sites in the vascular system even though most identified risk factors, such as hyper-cholesterolemia, hypertension and diabetes, are systemic¹. A report in this issue promises to greatly facilitate research on the site-specific origins of vascular disease. Sinha and colleagues² describe an *in vitro* strategy for generating large numbers of lineage-specific vascular smooth muscle cells (VSMCs) from human pluripotent stem cells (hPSCs) using a chemically defined protocol. Their approach offers a solution to the difficulty of obtaining VSMC subtype progenitors from early embryos in sufficient quantities for study.

In the search for an explanation of the anatomic specificity of vascular diseases, some investigators have turned to the developmental pathways by which blood vessels are formed in embryos. One intriguing clue resulting from these experiments is the surprising finding that VSMCs arise from multiple independent origins in development (reviewed by one of us in ref. 3). VSMCs originating from different types of embryonic progenitor show lineage-specific differences in growth, gene expression and functional properties^{4–7}. Mouse studies using lineage-restricted *cre*-recombinase alleles, together with classic grafting and ablation experiments in avian embryos, have shown that vascular smooth muscle is a highly mosaic tissue arising from at least eight unique origins in higher vertebrates³. The boundaries between VSMCs of different origins within a given artery wall are often very abrupt, with little or no intermixing^{8–11}. Moreover, lineage-specific differences in growth and in transcriptional responses to transforming growth factor- β (TGF β) have been reported for VSMCs *in vitro*^{4,5} and *in vivo*⁷.

These observations from basic studies of vascular development support the notion that VSMC lineage diversity may contribute to site-specific patterns of vascular disease in adults. They also suggest that optimal cell replacement therapies—for example, using bioengineered vascular grafts—should attempt to match the appropriate origin-specific VSMC subtype to the particular vascular segment undergoing treatment. This principle may

also be extended to stem cell–based repair protocols for vascular smooth muscle and to cell therapies for other types of specialized smooth muscle tissue (e.g., airway, enteric, ciliary and urogenital). A better understanding of the intrinsic differences between different VSMC subtypes could even form the basis for novel approaches to disease management that involve replacing highly disease-susceptible VSMCs with disease-resistant VSMCs. For example, experiments in which atherosclerosis-susceptible segments of abdominal aorta were transplanted to atherosclerosis-resistant positions in the thoracic aorta, and vice versa, revealed that aortic disease maps to segments with an intrinsic predisposition to acquire or resist disease rather than to their anatomic position *per se*¹². Indeed, a role for VSMC lineage diversity has now been suggested in the pathogenesis of aortic aneurysms¹³, vascular calcification¹⁴ and medial dissection¹⁵.

The growing recognition of the importance of VSMC lineage diversity in the nonuniform distribution of artery-wall diseases raises a number of fundamental questions about its molecular-genetic basis. How are VSMC progenitors of such diverse embryonic origins and lineage histories specified to differentiate to a common cell type? What is the molecular basis for the reported VSMC origin-specific differences in response to TGF β signaling^{4,5,7}? Do VSMCs exhibit origin-specific differences in their responses to inflammatory cytokines, oxidized lipids, extracellular matrix proteases or other stimuli related to vessel wall disease? Until now, the ability to address these questions, particularly in human cells, has been limited by the unavailability of VSMC progenitor subtypes and of well-characterized origin-specific VSMC cell lines.

To generate lineage-specific VSMC subtypes in chemically defined media, Sinha and colleagues² exposed either human embryonic stem cells or induced pluripotent stem cells (collectively referred to as hPSCs) to a defined mixture of FGF2 (20 ng/ml), LY294002 (a PI3K inhibitor; 10 μ M) and BMP4 (10 ng/ml), according to a previously described protocol for mesoderm differentiation from human embryonic stem cells¹⁶ (Fig. 1). From there, guided by studies describing a natural gradient of BMP signaling along the anterior-posterior axis of the primitive streak¹⁷, the authors manipulated BMP4 and LY294002 concentrations and found that treatment for 5 days with FGF2 (20 ng/ml, F20) and BMP4 (50 ng/ml) optimally generated cells expressing lateral plate mesoderm markers, whereas F20 plus LY294002 (10 μ M) in the absence of BMP4 was optimal for producing cells that expressed paraxial mesoderm markers. Progenitor cells selectively expressing neuroectoderm markers were prepared according to a previously reported protocol employing FGF2 (12 ng/ml) and the activin-nodal inhibitor SB431542 (10 μ M)¹⁸.

Sinha and colleagues² went on to direct their neuroectoderm, paraxial mesoderm and lateral plate mesoderm progenitors to a smooth muscle fate using a combination of PDGF-BB (10 ng/ml) and TGF β 1 (2 ng/ml) for 12 days. The resultant differentiated cells were then carefully and extensively characterized for gene expression phenotypes and functional properties. Microarray studies provided evidence for differentiation to cells that expressed a broad panel of smooth muscle markers with little or no expression of neural, cardiac, endothelial, hematopoietic or skeletal myocyte markers—all of which are possible fates from neuroectoderm, paraxial mesoderm and lateral plate mesoderm progenitors.

The smooth muscle cells (SMCs) derived from the three progenitor types also exhibited sustained Ca²⁺ influx and prolonged tonic contractions in response to carbachol that are characteristic of physiological smooth muscle contraction. In addition, in Matrigel angiogenesis assays, the cells were recruited to a perivascular position closely associated with endothelial cells. Based on the relative expression of SMTN (smoothelin) isoforms (SMTN-A and SMTN-B), Sinha and colleagues² concluded that the SMCs most closely resembled vascular, rather than visceral, smooth muscle.

The evidence described thus far indicates that the protocols produce VSMCs, but it is not sufficient to establish the critical point that origin-specific VSMC subtypes have been made. The strength of the paper lies in the characterization studies done to establish that, in all likelihood, origin-specific VSMC subtypes were produced, and with surprisingly high fidelity. For example, it is well established that SMC differentiation requires a core complex of the DNA binding protein serum response factor (SRF) together with at least one member of the myocardin family of SRF-binding transcriptional coactivators¹⁹. Gene deletion studies in mice have shown that neural crest-derived VSMCs have a unique requirement for myocardin-related transcription factor-B (Mrtf-B, also called Mkl2) that is not shared by VSMCs originating from paraxial or lateral plate mesoderm^{20,21}. Using short interfering RNAs to silence MRTF-B/MKL2, Sinha and colleagues² found that SMC differentiation in neuroectoderm cultures was inhibited whereas SMC differentiation in paraxial or lateral plate mesoderm cultures proceeded normally.

A second line of evidence that the SMCs are origin specific is based on previous reports that TGFβ1 stimulates cell proliferation of cultured primary neuroectodermal SMCs but inhibits the growth of paraxial mesodermal SMCs isolated from the same vessels and maintained under identical conditions⁴. Likewise, angiotensin II (Ang II) promotes hyperplasia of neuroectodermal SMCs and causes hypertrophic growth of paraxial mesodermal SMCs in the same arteries⁶. Consistent with these findings, Sinha and colleagues² showed that neuroectoderm-derived VSMCs exposed to TGFβ1 or Ang II increased in cell numbers over 3 days, whereas VSMCs derived from paraxial mesoderm or lateral plate mesoderm cultures did not.

Finally, in certain cases, growth factors stimulate their own production, a feed-forward process called autoinduction. Neural crest-derived VSMCs exhibit strong autoinduction responses when stimulated with TGFβ1, whereas mesoderm-derived VSMCs isolated from the same vessels and maintained under identical conditions do not display any detectable autoinduction response⁴. When Sinha and colleagues² tested their hPSC-derived VSMC cultures, they found that only neuroectoderm-derived VSMCs exhibited autoinduction responses to TGFβ1—highly reminiscent of the previous report using native origin-specific SMC subtypes⁴.

Taken together, the authors' results make for a strong case that the *in vitro*-generated embryonic lineage-specific VSMCs closely resemble their *in vivo* counterparts. They also suggest exciting possibilities for scalable production of these cells for use in detailed studies of the biology of VSMC development and for the generation of novel bioengineered vascular grafts and other stem cell-based therapies. We highlight here several directions for future research.

First, smooth muscle tissue is present in many different organ systems other than blood vessels (e.g., airway, visceral, urogenital, lymphatic), where it carries out highly specialized functions specific to that organ system. Additional work is needed, beyond characterizing SMTN isoforms, to define the identity of the SMC types produced with the authors' protocols, particularly with regard to the possible formation of a hybrid SMC with mixed identity. Given the large number of environmental cues that normally guide development and differentiation of SMCs in embryos, it would be surprising if a gradient of BMP4 turns out to have sufficient information to replace the embryo.

Second, differentiation of VSMC subtypes in developing embryos occurs around midgestation and is followed by a prolonged maturation period during which the functional phenotype is 'fine tuned'. Given the short time frame for differentiation reported by Sinha and colleagues², it is possible that one or more maturation steps have yet to be modeled *in*

vitro. These maturation steps may become important as protocols to optimize the use of origin-specific VSMCs for clinical applications are developed.

Third, the question of whether pericytes are an intermediate step in VSMC differentiation from progenitor cells or are the products of a separate differentiation pathway has not yet been resolved. The authors' finding that CSPG4-positive cells did not coexpress Myh11 suggests that yet another selective pathway for progenitor cell specification to a pericyte fate remains to be characterized.

Fourth, the fact that paraxial mesoderm VSMCs did not proliferate in response to any of the growth-promoting stimuli tested raises the question of whether they were more fully differentiated than the neuroectoderm and lateral plate mesoderm VSMCs under the conditions used, or whether they were deficient in pathways required for growth factor-stimulated cell proliferation. Given the results of aortic homograft transplantation studies described above¹², which suggest that certain types of VSMC may be resistant to atherogenesis, this may be an important question to resolve.

In summary, expertise in hPSC biology combined here with exquisite knowledge of VSMC development has culminated in a method that will allow ready access to different types of human VSMCs from a single genetic origin. If applied to patient induced pluripotent stem cells or to human embryonic stem cells with disease mutations, this approach will undoubtedly open up unprecedented opportunities to study aspects of vascular disease. With the phenotypic subtleties of mesoderm derivatives from hPSCs beginning to match those described for the neural lineages²², the impact on understanding human vascular development and disease can now be eagerly awaited.

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Figure 1.

Derivation of origin-specific vascular smooth muscle cells from human pluripotent stem cells (hPSCs). Vascular smooth muscle cells (SMCs) have different developmental origins: the neuroectoderm-derived neural crest (gray) and two regions of the embryonic mesoderm, the paraxial mesoderm (orange) and the lateral plate mesoderm (yellow). **(a)** In the early postimplantation embryo, the fates of the neural crest, lateral plate mesoderm and paraxial mesoderm are controlled by growth factors such as bone morphogenetic protein (BMP). **(b, c)** By mimicking these growth factor gradients in the correct sequence, Sinha and colleagues² differentiated hPSCs toward the same fates *in vitro* **(b)** and, from there, to specific vascular SMC subtypes that cover different regions of the arteries **(c)**. LM, lateral plate mesoderm; PM, paraxial mesoderm; NE, neuroectoderm. Treatments in panel **b** are as follows: FB50: FGF2 (20 ng/ml), BMP4 (50 ng/ml); FLy: F+LY294002 (10 μ M); F12S: F+SB431542 (10 μ M).