Reply: Transitioning endothelial cells contribute to pulmonary fibrosis

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Reply to S.S. Sohal:

We sincerely appreciate the correspondence from S.S. Sohal, whose recent discoveries in human pulmonary fibrosis [1, 2] are complementary to our report on an abnormal endothelial differentiation trajectory contributing myofibroblasts to pulmonary fibrosis in matrix Gla protein (MGP)-deficient mice [3]. S.S. Sohal and his collaborators have provided strong evidence for an essential role of endothelial to mesenchymal transitions (EndMTs) in human pulmonary fibrosis [1, 2]. He proposed that the endothelial cell (EC)-like myofibroblasts identified in our mouse model might also undergo EndMTs as they transition from ECs towards myofibroblasts in the fibrotic process.

We agree with his suggestion that the differentiation trajectory from ECs to EC-like myofibroblasts and myofibroblasts passes through a mesenchymal stage. In addition, we hypothesise that this differentiation trajectory exists in a quiescent state in healthy lungs and may be activated when pathological stimuli trigger pulmonary fibrosis. The results of our study showed that the mesenchymal markers Snail1 and 2, and Zeb1 and 2, were expressed in EC-like myofibroblasts in the normal lungs of wild-type mice [3]. Here, we also examine the expression of mesenchymal markers vimentin, N-cadherin and S100A4, which all were elevated in human pulmonary fibrosis, as reported by S.S. Sohal and co-workers [1, 2]. We find that Vimentin is expressed in EC-like myofibroblasts in lungs of wild-type mice (figure 1a). We also examine the same markers in human pulmonary fibrosis, and find an increase in vimentin, S100A4, Zeb2 and Snail1 in EC-like myofibroblasts (figure 1b). The increased marker expression suggests that EC-like myofibroblasts acquire mesenchymal characteristics. Moreover, we previously reported that the fraction of ECs decreased in human pulmonary fibrosis compared to healthy lungs [3], which occurred in combination with an increase in EC-like myofibroblasts [3], suggesting that the ECs feed cells to the differentiation trajectory leading to EC-like myofibroblasts. Together, the results are highly supportive of the differentiation trajectory between ECs, EC-like myofibroblasts and myofibroblasts being mediated by EndMTs. Although EndMTs are part of normal developmental processes, they may contribute to disease if poorly regulated [1, 4]. Indeed, our results showed low levels of EC-like myofibroblasts in the lungs of healthy humans and wild-type mice, although far less than in the fibrotic lungs [3]. This would be consistent with the existence of a well-controlled, quiescent differentiation trajectory that is activated by pathological stimuli, such as a decrease of functional MGP after warfarin treatment [5].

S.S. Sohal's hypothesis was also supported by another report from our laboratory [6], which showed that infection with coronavirus disease 2019 (COVID-19) or influenza A viruses might drive ECs towards myofibroblasts in pulmonary fibrosis. We found that the fraction of EC-like myofibroblasts that expressed both endothelial and myofibroblast markers was robustly enhanced in pulmonary fibrosis in patients with COVID-19 [6]. The differentiation trajectory projected a clear direction from ECs towards EC-like myofibroblasts and myofibroblasts [6]. We discovered a similar differentiation trajectory in mouse lungs after influenza A infection and showed that the fraction of EC-like myofibroblasts dramatically increased 1 day after infection and continued to increase as the infection progressed [6]. It is possible that cytokines from immune cells or injured pulmonary cells in the infected lungs trigger a transition of ECs towards myofibroblasts [6].

We previously discovered that MGP is critical to maintaining pulmonary integrity and restricting EndMTs. MGP is highly expressed in pulmonary epithelial cells where it is essential for the regulation of endothelial

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Differentiation trajectory from ECs to EC-like myofibroblasts and myofibroblasts may pass through a mesenchymal stage. This trajectory exists in a quiescent state in healthy lungs and may be activated when pathological stimuli trigger pulmonary fibrosis. https://bit.ly/3QWfgyS

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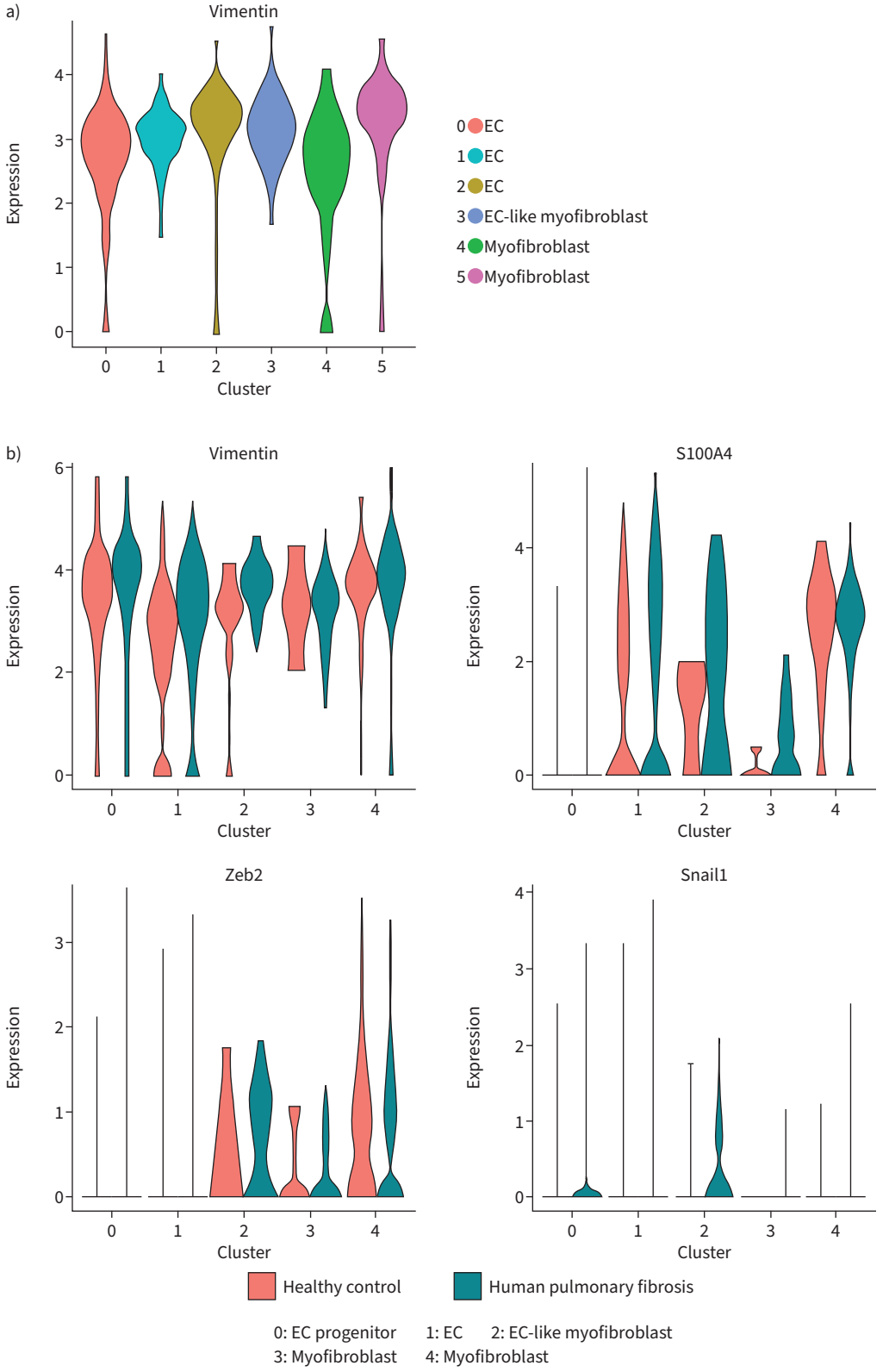

FIGURE 1 Endothelial cell (EC)-like myofibroblasts express mesenchymal markers in healthy pulmonary tissues and mesenchymal markers are elevated in human pulmonary fibrosis. a) Violin plots of vimentin expression in cell subclusters from $CD34^{+}CD45^{-}$ pulmonary cells of wild-type mice. b) Violin plots of gene expression of mesenchymal lineage markers in cell subclusters from $CD34^{+}CD45^{-}$ pulmonary cells of healthy human lungs and human pulmonary fibrosis.

differentiation [7]. Mutations in MGP may cause peripheral pulmonary stenoses in humans [8], and overexpression of MGP limits pulmonary arteriovenous malformations (AVMs) in a mouse model of hereditary haemorrhagic telangiectasia [9]. In our pulmonary fibrosis studies, we showed that MGP was induced in the EC-like myofibroblasts as compared to ECs, and loss of MGP in the EC-like myofibroblasts resulted in pulmonary fibrosis [3]. MGP expression was also elevated in EC-like myofibroblasts present in idiopathic pulmonary fibrosis and COVID-19-associated pulmonary fibrosis [3, 6], suggesting a feedback response that induced MGP to counteract unwanted EC transitions. MGP is an efficient inhibitor of bone morphogenetic proteins (BMPs) [10]. Besides its role in pulmonary fibrosis, the ability of MGP to restrain BMP signalling is essential in limiting EndMTs that lead to vascular calcification or AVMs depending on the vascular location [10]. Given the ubiquity of BMPs in various tissues, there may be wide-ranging effects on ECs when MGP levels or functionality are reduced. We expect future investigations to reveal the impact of tissue-specific BMP signalling on endothelial differentiation.

Xiuju Wu [1], Kristina I. Boström [1,2] and Yucheng Yao [1]

[1]Division of Cardiology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA. [2]The Molecular Biology Institute at UCLA, Los Angeles, CA, USA.

Corresponding author: Yucheng Yao (yyao@mednet.ucla.edu)

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