Research Article

Evaluating the Impact of Human Amnion Epithelial Cells on Angiogenesis

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The effects of human amnion epithelial cells (hAECs) on angiogenesis remain controversial. It is yet unknown if the presence of inflammation and/or gestational age of hAEC donors have an impact on angiogenesis. In this study, we examined the differences between term and preterm hAECs on angiogenesis in vitro and in vivo. Conditioned media from term hAECs induced the formation of longer huVEC tubules on Matrigel. Both term and preterm hAECs expressed VEGFA, PDGFB, ANGPT1, and FOXC1, which significantly increased after TNFα and IFNγ stimulation. In the presence of TNFα and IFNγ, coculture with term hAECs reduced gene transcription of Tie-2 and Foxc1 in huVECs, while coculture with preterm hAECs increased gene transcription of PDGFRα and PDGFRβ and reduced gene transcription of FOXC1 in huVECs. In vivo assessment of angiogenesis using vWF immunostaining revealed that hAEC treatment decreased angiogenesis in a bleomycin model of lung fibrosis but increased angiogenesis in a neonatal model of hyperoxia-induced lung injury. In summary, our findings suggested that the impact of hAECs on angiogenesis may be influenced by the presence of inflammation and underlying pathology.

1. Background

Human amnion epithelial cells (hAECs) isolated from the amniotic membrane are an attractive source of cell therapy. In addition to their anti-inflammatory and antifibrotic effects, they are nontumorigenic and they exhibit low antigenicity and multipotent differentiation potential [1–3]. Furthermore, they can be isolated in large numbers adequate for clinical applications without requiring serial expansion [2]. We previously applied hAECs isolated from term pregnancies to a mouse model of bleomycin-induced lung fibrosis. We showed that hAEC administration prevented lung inflammation and fibrosis and prevented decline in lung function [4]. In studies, we have also shown that term hAECs can reduce markers of lung inflammation and mitigate structural damage when administered following lung injury induced by either hyperoxia [5] or ventilation [6]. Furthermore, studies on the therapeutic effects of hAECs showed that the engraftment of amnion cells is very rare [5, 7], and hAEC-conditioned media have been shown to contain soluble factors that exert profound biological effects [8]. These studies indicate that hAECs may exert their function in a paracrine fashion.

Given that angiogenesis plays a critical role in wound healing and resolution of inflammation [9, 10], it is important to assess the contribution of angiogenesis to hAEC-augmented repair. There have been contradictory reports on the angiogenic effects of hAECs to date. Specifically, hAECs have been reported to secrete several angiogenic factors in vitro. These include tissue inhibitors of metalloproteinases (TIMP)-1 and 2, epidermal growth factor (EGF), angiogenin, vascular endothelial growth factor (VEGF), platelet derived growth factor B (PDGFB), and angiogenin [11]. However, when functionally assessed in a rodent dorsal skinfold chamber model hAECs did not increase vessel lengths or vessel sprouts number [12]. It is possible that the effect of hAECs on angiogenesis is altered in an inflammatory environment.
Indeed, when exposed to a bleomycin-induced lung injury model a week following bleomycin administration when lung inflammation was at its peak, hAECs were unable to mitigate injury [3].

We have also shown that preterm hAECs exert significantly less protective effects *in vivo* compared to term hAECs [13]. This suggests that there may be functional differences between term and preterm hAECs. As such, we sought to assess potential differences in the angiogenic effects of hAECs isolated from different gestational ages. Furthermore, angiogenesis can be either beneficial or detrimental to wound healing, depending on the disease context. Accordingly, we sought to assess the impact of hAECs on angiogenesis using two models of lung injury. The first is a bleomycin-induced lung fibrosis model where angiogenesis is detrimental to the outcome, and the second is a hyperoxia-induced lung injury model where angiogenesis is beneficial.

## 2. Materials and Methods

### 2.1. huVEC and hAEC Isolation

Human umbilical vein endothelial cells (huVECs) were isolated from healthy term human umbilical cords and hAECs were isolated from amnion collected from women undergoing a caesarean section preterm (<37 weeks gestation) or at term as previously described [2,14]. Preterm donors included women delivering prematurely due to preeclampsia, gestational hypertension, fetal growth restriction, placenta praevia, and discordant twin growth. Donors with pregnancies complicated by chorioamnionitis and preexisting maternal diseases were excluded. The term donors were women with a healthy pregnancy undergoing an elective repeat caesarean section. The mean gestational age for preterm birth was 230 ± 7 days and for term birth 268 ± 1 days (Table 1). All collection and isolation procedures were undertaken with the approval from Monash Health Human Ethics Committee and with written informed consent.

### 2.2. Collection of hAEC-Conditioned Media

The collection of hAEC-conditioned media followed previous protocol [15]. Briefly, hAECs were plated at a density of 10 million cells per T175 flask and conditioned media were collected following 96 hours under standard tissue culture conditions.

### 2.3. In Vitro Angiogenesis Assay

On a Matrigel (50 μL/well; Corning Life Sciences) coated 96-well plate, 1 × 10⁷ huVECs at passage 3 were seeded in each well. Cells were cultured in either M199 media (Invitrogen) [16] or hAEC-conditioned media. Phase contrast images were taken every 30 minutes for a total of 40 hours. The capillary-like structures were detected and the average length of tubes between branches was calculated using Image J (National Institutes of Health, Bethesda, MA) and Metamorph software (Molecular Devices, Sunnyvale, CA) programs.

### 2.4. Stimulation of hAECs

Preterm (*n* = 6) and term (*n* = 6) hAECs were cultured in the presence or absence of tumor necrosis factor α (TNFα) (20 ng/mL, PHC3015, Life Technologies) and interferon γ (IFNγ) (20 ng/mL, PHC4031, Life technologies) for 24 hrs and 48 hrs. Concentrations of TNFα and IFNγ were based on a previous study by Liu and colleagues who reported an increase in angiogenic potential of MSCs following exposure to inflammatory cytokines [17].

### 2.5. Coculture of huVECs and hAECs

hAECs were seeded at a density of 5 × 10⁵ cells in each well of a 6-well plate, while 1 × 10⁵ huVECs were seeded into each 0.4 μm pore transwell insert (BD Bioscience, San Jose, CA) in M199 media in the presence or absence of TNFα and IFNγ.

### 2.6. Gene Expression Assays

RNA was isolated using the RNeasy Mini Kit (Qiagen, Limburg, Netherlands) and 1 μg total RNA was converted to cDNA using the Thermoscript Reverse Transcription System (Invitrogen). qRT-PCR was performed using SensiMix SYBR and Rotor-Gene (Qiagen). Primers for hAECs were directed against *VEGFA*, *PDGFB*, angiogenin-1 (*ANGPT1*), and Forkhead box (FOX) transcription factor *FOXC1*. Primers for huVECs were directed against vascular endothelial growth factor receptors 1 and 2 (*VEGFR1* and *VEGFR2*), platelet derived growth factor receptors alpha and beta (*PDGFRα* and *PDGFRβ*), *Tie-2*, and *FOXC1*. Gene expression was normalised to 18S and expressed relative to either unstimulated hAECs or huVECs. Detailed information on primer sequences is listed in Table 2.

### 2.7. Animals and Experimental Groups

All animal experiments were approved by the Monash Medical Centre Animal Ethics Committee and were conducted in accordance with the Australia Code of Practice for Care and Use of Animals for Scientific Purpose (2006). In the bleomycin-induced mouse lung injury model, 6–8-week-old female C57/BL6 mice weighing 16–20 g were housed in a specific pathogen-free animal facility during this study. Experimental groups included saline + saline, saline + term hAECs, bleomycin + saline, bleomycin + term hAECs, and bleomycin + preterm hAECs. The mice were given either saline or 0.3 IU bleomycin (Blenoxane, Hospira, Lake Forest, IL, USA) intranasally, followed by intraperitoneal administration of 4 million term hAECs or preterm hAECs, or 200 μL saline 24 hours later, as previously described [13]. Mice were culled 14 days following intranasal instillation of bleomycin by

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### Table 1: Gestational age of both term and preterm birth.

| Gestational age (d) | Minimum | Maximum | Mean ± SEM | Complications |
|---------------------|---------|---------|------------|---------------|
| Preterm birth (*n* = 9) | 196     | 257     | 230.4 ± 7.37 | IUGR (*n* = 5); PE (*n* = 4) |
| Term birth (*n* = 16)  | 257     | 277     | 268.3 ± 1.03 | Nil |

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Table 2: Primer sequences and annealing temperatures.

| Gene     | Primer sequence     | Annealing temperature |
|----------|---------------------|-----------------------|
| VEGFA    | Fwd: CTCCTCCACCATGCAAGTG  |
|          | Rev: TGGTCTGCCCCCTCCTCTCCT  | 60°C                  |
| PDGFB    | Fwd: CCTGCCAGCCAGTTTGG  |
|          | Rev: CTGGCATGCAAGTGTGAGAC  | 60°C                  |
| ANGPT1   | Fwd: CTCGATCTACCGTGCTGATT  |
|          | Rev: GTCCCGCAGTATAGAACATTCCA  | 60°C                  |
| VEGFR1   | Fwd: CCGGGGATTTACCTGTACATCT  |
|          | Rev: AAGCAAAAACACTGGGTCCTTC  | 60°C                  |
| VEGFR2   | Fwd: CCTGCCGTGTTGAAGAGTT  |
|          | Rev: GGACAGGGGGAAGAACAAAA  | 60°C                  |
| PDGFRα   | Fwd: AGCTGCGGTGTTGAGAGTT  |
|          | Rev: CTCCATGTGCTTGGACATTCA  | 60°C                  |
| PDGFRβ   | Fwd: CAGGAGAGACAGCAACAGCA  |
|          | Rev: TGTCCAGAGCCTGGAACTGT  | 60°C                  |
| Tie-2    | Fwd: AGTCTATGTGTCTGCTTGCTGACC  |
|          | Rev: TCATCCCTCGGTATGGCCCTCTCCTC  | 60°C                  |
| FOXC1    | Fwd: ACCTTGAGGAAGCACTGTT  |
|          | Rev: CGGATCTACCAGTTCCATCA  | 60°C                  |

carbon dioxide asphyxiation. The right lungs were inflated and fixed with 4% (w/v) paraformaldehyde processed for immunofluorescence.

In the neonatal mouse model of hyperoxia-induced lung injury, newborn C57/BL6 mouse pups were randomised to either normoxia (inspired O2 content (FiO2) = 0.21) or hyperoxia (FiO2 = 0.85). Experimental groups included normoxia + saline, normoxia + term hAECs, hyperoxia + saline, and hyperoxia + term hAECs. On postnatal days (PND) 4, 5, and 6, a total of 4.5 million term hAECs or 50 μL sterile saline (control) was administered intraperitoneally as previously described [5]. On postnatal day 14, mice were culled and lungs were collected in 4% PFA prior to processing and embedding in paraffin.

2.8. Immunohistochemistry Staining for vWF. Pulmonary vessels were assessed by von Willebrand factor (vWF) staining of paraffin embedded lung tissue sections. The slides were subjected to proteinase K retrieval, followed by incubation with primary antibody polyclonal rabbit anti-human vWF (1:400, Dako, A0082, Germany) overnight at 4°C. After washing with PBS, LSAB+/HRP kit (Dako, K0690, Germany) was applied to the sections for 1 hour at room temperature, followed by labelling with streptavidin-horseradish peroxidase and diaminobenzidine (DAB, Dako, K3408, Germany). Nuclei were counterstained with haematoxylin. The percentage of vWF positive area was determined in bleomycin mice by Image J (NIH). In the hyperoxia study, the number of vWF positive vessels with diameter less than 50 μm was counted in 15 random images at 200x magnification using Image J (NIH).

2.9. Data Analysis. Data were expressed as mean ± standard error of mean (SEM). Statistical significance was determined using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) with one-way ANOVA accompanied by the Bonferroni post hoc test for multiple groups or the Mann-Whitney test when comparing between two groups. Statistical significance was accorded when p < 0.05.

3. Results

3.1. Vascular Tube Formation in huVECs on Matrigel. The maximum length of vascular tubes formed by huVECs appeared at 6 hours following culture in term hAEC-conditioned media and M199 media and appeared at 4 hours following culture in preterm hAEC-conditioned media. Changes in average vascular tube lengths over a 40-hour period are depicted in Figure 1(a). The average maximum length of vascular tubes formed in term hAEC-conditioned media was significantly greater compared with M199 control media (Figure 1(b), 88.03 ± 5.77 μm versus 59.76 ± 2.19 μm, p < 0.05) but not the preterm hAEC-conditioned media (73.52 ± 2.86 μm), which was not significantly different to either control conditions or term hAEC-conditioned media. On average, the tubules of huVECs cultured in term hAEC-conditioned media were significantly greater compared with M199 control media (Figure 1(b), 88.03 ± 5.77 μm versus 59.76 ± 2.19 μm, p < 0.05) but not the preterm hAEC-conditioned media (73.52 ± 2.86 μm), which was not significantly different to either control conditions or term hAEC-conditioned media. On average, the tubules of huVECs cultured in term hAEC-conditioned media and control media were stable for up to 40 hours. In contrast, the average length of huVEC tubules cultured in preterm hAEC-conditioned media decreased after 16 hours, such that by 20 hours they were significantly shorter than tubules cultured in either term hAEC-conditioned media or control media (p < 0.05). This suggests that preterm hAEC-conditioned media contain soluble factors that reduce tubule stability. Representative images of huVEC tubules formed following culture in term hAEC-conditioned media and control media are shown in Figure 1(c).

3.2. Gene Expression of Angiogenic Ligands by hAECs. We assessed gene transcription of VEGFA, PDGFB, ANGPT1, and FOXC1 in term and preterm hAECs under basal conditions
Figure 1: The vascular tube formation in huVECs on Matrigel in vitro. ((a) and (b)) huVECs formed longer vascular tubules following culture in term hAEC-conditioned media compared to control media \((p < 0.05)\), and tubule lengths were stable for 40 hours. huVECs cultured in preterm hAEC-conditioned media did not support tubule formation where tubule length was significantly reduced at 20 hours \((p < 0.05)\). (c) Representative pictures of vascular tubules of huVECs following culture in term hAEC-conditioned media and control media \(\ast p < 0.05\).

3.3. Gene Expression of Angiogenic Receptors by huVECs. We next assessed the impact of term hAECs on huVECs stimulated by TNF\(\alpha\) and IFN\(\gamma\). When cocultured with stimulated term hAECs, huVECs significantly increased gene transcription of VEGFA, PDGFB, and FOXC1 (Figure 2(a), 2.82 \(\pm\) 0.66, \(p = 0.0411\); 5.27 \(\pm\) 2.68, \(p = 0.0087\), and 7.72 \(\pm\) 3.69, \(p = 0.0411\), resp.). Gene transcription of ANGPT1 remained unchanged. In preterm hAECs, stimulation with TNF\(\alpha\) and IFN\(\gamma\) increased gene transcription of PDGFB and ANGPT1 (Figure 2(b), 6.14 \(\pm\) 2.73, \(p = 0.0462\) and 90.92 \(\pm\) 55.21, \(p = 0.0079\), resp.). Gene transcription of VEGFA and FOXC1 remained unchanged.

We next assessed the impact of term and preterm hAECs on huVECs stimulated by TNF\(\alpha\) and IFN\(\gamma\). When cocultured with stimulated term hAECs, huVECs significantly reduced gene transcription of Tie-2 (Figure 4(e), 0.40 \(\pm\) 0.07 versus 0.67 \(\pm\) 0.08, \(p = 0.0476\)) and Foxc1 (Figure 4(f), 0.59 \(\pm\) 0.04 versus 0.88 \(\pm\) 0.09, \(p = 0.0238\)). When cocultured with stimulated preterm hAECs, huVECs significantly increased gene transcription of PDGFR\(\alpha\) (Figure 5(c), 0.64 \(\pm\) 0.12 versus 0.18 \(\pm\) 0.06, \(p = 0.0159\)) and \(\beta\) (Figure 5(d), 27.92 \(\pm\) 1.96 versus 11.6 \(\pm\) 5.33, \(p = 0.0119\)). Gene expression of Foxc1 was also significantly reduced (Figure 5(f), 0.42 \(\pm\) 0.03 versus 0.78 \(\pm\) 0.12, \(p = 0.0286\)).

3.4. Assessment of Angiogenesis In Vivo. Term but not preterm hAEC administration reduced pulmonary fibrosis following bleomycin challenge (Figure 6(a)). The percentage of vWF positive staining increased significantly in the lungs of bleomycin-alone animals \((9.31 \pm 0.69\% \text{ versus } 3.12 \pm 0.47\%, p < 0.0001)\). This was mitigated by the administration of term \((5.34 \pm 0.25\%, p < 0.001)\) but not preterm hAECs (Figure 6(b)). Representative images of immunohistochemical staining are shown in Figure 6(c).

Given that only term but not preterm hAECs result in an effect in the adult mice, we proceeded to assess the impact
Figure 2: Gene expression of angiogenic ligands by hAECs following stimulation with TNFα and IFNγ. (a) Gene expressions of VEGFA \((p = 0.0411)\), PDGFB \((p = 0.0087)\), and Foxc1 \((p = 0.0411)\) increased in term hAECs. (b) Gene expression of PDGFB \((p = 0.0462)\) and ANGPT1 \((p = 0.0079)\) increased in preterm hAECs.

of term hAECs on angiogenesis in a hyperoxia-induced lung injury model in neonatal mice. Here we observed that mice in hyperoxia group had simplified lung structure and enlarged alveoli, and term hAEC treatment improved lung structure (Figure 7(a)). The number of small pulmonary vessels (diameter < 50 μm) decreased in hyperoxia-injured neonatal mice compared with normoxia mice \((5.84 \pm 0.58\% \text{ versus } 8.80 \pm 0.21\%, \ p < 0.001)\). Term hAEC administration restored the number of small pulmonary vessels in hyperoxia-induced lung injury animals \((7.88 \pm 0.33\%, \ p < 0.05)\) (Figure 7(b)). Representative images of immunohistochemical staining are shown in Figure 7(c).

4. Discussion

The ability of hAECs to support angiogenesis is poorly understood. Additionally, little is known about the importance of gestational age of hAEC donors to the contribution of angiogenesis during repair. In this study, we showed that in vitro tubule formation by huVECs was best supported by term hAECs compared to preterm hAECs. Both term and preterm hAECs transcribed genes of proangiogenic ligands VEGFA, PDGFB, and ANGPT1 and transcription factor, FOXC1. These were upregulated by inflammatory cytokines, IFNγ and TNFα. However, coculture with both term and preterm hAECs did not consistently increase gene transcription of proangiogenic receptor ligands in huVECs. When we assess the effects of hAEC treatment on angiogenesis in vivo using a bleomycin model of lung fibrosis, we found that while term hAECs reduced vWF staining in the lungs, consistent with resolution of lung fibrosis, treatment with preterm hAECs had no effect. This observation coincided with our previous study showing that preterm hAECs had diminished reparative effects. When we assess the effects of term hAEC treatment on angiogenesis in a model of hyperoxia-induced neonatal lung injury, we found instead that hAEC treatment was associated with improvement in pathological lung remodelling.

The Matrigel tubule formation assay is an established method for evaluating the angiogenic effects of soluble factors in endothelial cells in vitro [18]. Using this assay, we determined that term hAECs release more proangiogenic factors compared to their preterm counterparts, supporting endothelial cell tubule formation as previously reported with bone marrow-derived MSCs [19]. In order to elucidate the nature of these proangiogenic factors, we compared the gene expressions of proangiogenic ligands. Angiogenic factors, such as ANGPT1, PDGFB, and VEGFA, have been previously detected in the secretome of human MSC from different tissue sources [20]. Stimulation by TNFα and LPS increased the production of VEGFA by adipose-derived MSCs [21] while transforming growth factor α (TGF-α) induced the secretion of VEGFA and PDGFB in bone marrow-derived MSCs [22]. Similarly, amnion derived mesenchymal stromal cells (MSCs) secrete angiogenic factors including EGF, VEGF, TIMP-1, and TIMP-2 [11].

In our current study we found that both preterm and term hAECs expressed VEGFA, PDGFB, ANGPT1, and FOXC1 under basal conditions. We then looked to see if the hAECs altered transcription of these genes in response to
Figure 3: Gene expression of angiogenic factors by huVECs cocultured with term hAECs under basal conditions. ((a)–(f)) huVECs expressed VEGFR1, VEGFR2, PDGFRα, PDGFRβ, Tie-2, and FOXC1. Following coculture with term hAECs, ((c) and (e)) gene expression of PDGFRα increased \((p = 0.0286)\), but gene expression of Tie-2 decreased \((p = 0.0333)\), ((a), (b), (d), and (f)) while there was no change in gene expression of VEGFR1, VEGFR2, PDGFRβ, and FOXC1.
Figure 4: Gene expression of angiogenic factors by huVECs cocultured with term hAECs in the presence of TNFα and IFNγ. (a)–(d) There was no change in gene expression of VEGFR1, VEGFR2, PDGFRα, and PDGFRβ, ((e) and (f)) while gene expression of Tie-2 and FOXC1 was significantly reduced (p = 0.0476 and p = 0.0238, resp.).
Figure 5: Gene expression of angiogenic factors in huVECs cocultured with preterm hAECs in the presence of TNFα and IFNγ. ((a), (b), and (e)) There was no change of gene expression of VEGFR1, VEGFR2, and Tie-2. ((c), (d), and (f)) While gene expression of FOXC1 was also significantly reduced ($p = 0.0286$), gene expression of PDGFRα and β was significantly increased ($p = 0.0159$ and $p = 0.0119$, resp.).
Saline + saline

Bleomycin + saline

Bleomycin + preterm hAECs

Bleomycin + term hAECs

200 µm

200 µm

200 µm

200 µm

(a)

Saline + saline

Bleomycin + saline

Bleomycin + saline

Bleomycin + preterm hAECs

Bleomycin + term hAECs

200 µm

200 µm

(b)

Figure 6: Collagen staining and vWF immunohistochemistry in bleomycin challenged mouse lung tissue. (a) Bleomycin challenged mice had more fibrotic tissues in the lung compared to control group; term hAEC treatment, but not preterm hAEC treatment, reduced the fibrotic tissues. (b) The percentage of vWF positive staining increased in bleomycin-injured animals compared to control group ($p < 0.0001$) and decreased after term ($p < 0.001$) but not preterm hAEC administration. (c) The representative images for vWF immunohistochemistry in mouse lung tissues ($^* p < 0.05$, $^{**} p < 0.001$, and $^{***} p < 0.0001$).

proinflammatory stimuli by exposing them to a combination of TNFα and IFNγ. VEGFA stimulates the generation of new, immature, and leaky blood vessels by disrupting the basement membrane of the preexisting vessels, inducing endothelial cell migration and proliferation [23, 24], while PDGF and ANGPT1 are essential for the stabilisation of new vessels. These angiogenic ligands promote angiogenesis, induce vascular maturation, and decrease vascular permeability by mediating migration, adhesion, and survival of endothelial cells [25]. Interestingly, transcription of VEGFA, PDGFB, and FOXC1 was elevated in term hAECs, while PDGFB and ANGPT1 were increased in preterm hAECs. This suggests that the gestational age of the hAEC donor can influence differential response of hAECs towards an inflammatory stimulus. Given our current understanding of how stem cells and stem-like cells can respond to environmental priming [26, 27], the findings from this study may have implications on the application of hAECs collected from donors across different gestational ages.

While hAECs have been reported to secrete angiogenic factors in vitro [11], this is the first time that FOXC1 expression by hAECs has been reported. FOXC1 is a transcription factor involved in regulating vascular development. It is critical for pericyte regulation of vascular development in the mouse fetal brain [28] and is essential for maintaining the integrity of basement membrane and decreasing vascular permeability in zebrafish [29]. FOXC1 also reportedly regulates proangiogenic factors such as matrix metalloproteinases (MMPs) as well as VEGF receptor-ligand signalling [30, 31]. Although VEGFRI has higher affinity for VEGF, it has...
Figure 7: H&E staining and vWF immunohistochemistry in neonatal mouse lung tissue. (a) Hyperoxia-induced lung injury mice had simplified lung structure and enlarged alveoli, and term hAEC treatment improved lung structure. (b) The number of vessels (diameter < 50 μm) decreased in hyperoxia-injured animals compared with normoxia animals (p < 0.001). Term hAEC administration restored the number of small pulmonary vessels in hyperoxia-induced lung injury animals (p < 0.05). (c) The representative images for vWF immunohistochemistry in mouse lung tissues (*p < 0.05, ***p < 0.001).

Given the transcriptional changes to proangiogenic ligands in hAECs, we next assessed changes to their receptors in huVECs in a coculture system. When huVECs were cocultured with term hAECs under basal conditions, we saw an increase in PDGFRα transcription but a reduction in Tie-2. In the presence of IFNγ and TNFα, however, we observed a reduction in Tie-2 as well as FOXC1. When huVECs were cocultured with preterm hAECs in the presence of IFNγ and TNFα, we observed an increase in PDGFRα and PDGFRβ but a reduction in FOXC1 gene transcription. These findings indicate that the relationship between hAECs and endothelial...
cells is complex, and multiple receptor-ligand signalling pathways are likely to be activated during hAEC-mediated angiogenesis. For example, MSCs exert proangiogenic effects through VEGF during wound healing [26] and yet they suppress neovascularisation in chemically injured rat corneas [27].

Next we employed two animal models of lung injury to determine how hAECs affect angiogenesis in vivo and if these effects are dependent on the underlying pathology of the injury. Bleomycin-induced pulmonary fibrosis is associated with neovascularisation, where the imbalance of pro- and antiangiogenic mediators is a perpetuator of lung fibrosis [39]. Indeed inhibitors of angiogenesis such as intedanib have been investigated as treatments for lung fibrosis [40]. In our current study we observed that excessive angiogenesis was inhibited in bleomycin challenged mice after the administration of term but not preterm hAECs. This concurs with our previous findings where we showed that term but not preterm hAECs mitigated bleomycin-induced lung injury [13]. In keeping with our in vitro findings where we showed that the expression levels of Tie-2 and FoxC1 in huVECs were reduced following coculture with term hAECs in the presence of IFNγ and TNFα, hAECs may reduce excessive angiogenesis by downregulating angiogenic factor receptors on the endothelial cells.

Since there were only observable differences in the vWF staining following term and hAEC treatment in the bleomycin model, we next applied only term hAECs to the hyperoxia neonatal lung injury model. Alveolarisation and angiogenesis are essential for normal lung development and the blood vessels in the lung promote normal alveolar development and contribute to maintenance of alveolar structure [41]. We observed that the number of smaller blood vessels was reduced following hyperoxia and this was restored by hAEC treatment, indicating that hAEC treatment may have affected angiogenesis in conjunction with the reversal of alveolar simplification as previously reported [5]. Our findings from the two lung injury models suggest that angiogenesis may be the driving force for hAEC-mediated lung repair; however, it is equally important to appreciate that angiogenic factors such as VEGF and PDGF can also act as proinflammatory cytokines rather than solely as angiogenic factors during lung injury [4, 6]. In particular, the process of angiogenesis can perpetuate inflammation depending on concurrent events such as enhanced adhesion and increased endothelial permeability [42].

In conclusion, we showed that angiogenesis may be one of the mechanisms through which hAECs augment lung repair. We also report on differential angiogenic potentials between term and preterm hAECs, which may have profound implications on donor sourcing and clinical applications of these cells. Further, we show that inflammatory cytokines such as IFNγ and TNFα can impact the angiogenic properties of hAECs and these findings may extend to other stem cells and stem-like cells as well as other mechanisms of action. Given that stem cell priming has become a topical discussion point of late, the impact of microenvironmental cues on stem cell functionality should be considered when identifying optimal times of cell administration.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| huVECs | Human umbilical vein endothelial cells |
| hAECs | Human amnion epithelial cells |
| TNFα | Tumor necrosis factor α |
| IFNγ | Interferon γ |
| VEGF | Vascular endothelial growth factor |
| PDGF | Platelet derived growth factor |
| ANGPT | Angiogenin |
| FoxC1 | Forkhead box (Fox) transcription factor |
| VEGFR1 | Vascular endothelial growth factor receptors 1 and 2 |
| VEGFR2 | Platelet derived growth factor receptors alpha and beta |
| PDGFRA | Platelet derived growth factor receptors alpha |
| PDGFRA | Platelet derived growth factor receptors alpha |
| vWF | von Willebrand factor |

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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