Amnion Epithelial Cell-Derived Exosomes Restrict Lung Injury and Enhance Endogenous Lung Repair

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Key Words. Fetal stem cells • Lung • Progenitor cells • Tissue-specific stem cells

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is characterized by chronic inflammation, severe scarring, and stem senescence. Stem cell-based therapies modulate inflammatory and fibrogenic pathways by release of soluble factors. Stem cell-derived extracellular vesicles should be explored as a potential therapy for IPF. Human amnion epithelial cell-derived exosomes (hAEC Exo) were isolated and compared against human lung fibroblasts exosomes. hAEC Exo were assessed as a potential therapy for lung fibrosis. Exosomes were isolated and evaluated for their protein and miRNA cargo. Direct effects of hAEC Exo on immune cell function including macrophage polarization, phagocytosis, neutrophil myeloperoxidase activity and T cell proliferation and uptake were measured. The impact on immune response, histological outcomes, and bronchioalveolar stem cell (BASC) response were assessed in vivo following bleomycin challenge in young and aged mice. hAEC Exo carry protein cargo enriched for MAPK signaling pathways, apoptotic and developmental biology pathways and miRNA enriched for PI3K-Akt, Ras, Hippo, TGFβ, and focal adhesion pathways. hAEC Exo polarized and increased macrophage phagocytosis, reduced neutrophil myeloperoxidases, and suppressed T cell proliferation directly. Intranasal instillation of 10 μg hAEC Exo 1 day following bleomycin challenge reduced lung inflammation, while treatment at day 7 improved tissue-to-airspace ratio and reduced fibrosis. Administration of hAEC Exo coincided with the proliferation of BASC. These effects were reproducible in bleomycin-challenged aged mice. The paracrine effects of hAECS can be largely attributed to their exosomes and exploitation of hAEC Exo as a therapy for IPF should be explored further. Stem Cells Translational Medicine 2018;00:00-000

SIGNIFICANCE STATEMENT

Idiopathic pulmonary fibrosis (IPF), a condition characterized by chronic inflammation and progressive fibrosis, is thought to arise from an impaired endogenous stem/progenitor cell response. Current treatment using nintedanib and pirfenidone targets the fibrotic phase and have minimal effect in treating the disease. This prompts the need for novel therapy targeting all aspects of this disease. Soluble factors from human amnion epithelial cell affect inflammatory, anti-fibrotic, and pro-regenerative making them a potential novel therapy for IPF.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive fibrosing interstitial pneumonia of unknown cause that results in worsening lung function [1], with a prognosis worse than many cancers [2]. Mortality rates are estimated at about 2.54 to 11.08 per 100,000 depending on country with rates significantly higher with increasing age [1]. Lung transplantation is currently the most effective therapy, with a 50% chance survival rate 5 years post-transplantation. As a bridge to lung transplantation and for patients who are ineligible for transplantation, drugs such as nintedanib and pirfenidone have been used in an attempt to slow progression of disease [3]. Notably, these drugs target the progression of fibrosis but neither are curative. Given the contribution of telomerase mutations resulting in premature cellular senescence in patients with familial and sporadic pulmonary fibrosis [4], there is little wonder that drugs targeting the fibrotic process alone have limited therapeutic capability and as such a multimodal approach is warranted. To this end, a regenerative approach, has been suggested to address this degenerative disease [5].

Mesenchymal stem/stromal cells (MSCs) from the bone marrow [6–8], adipose tissue [9,10], placenta [11], and umbilical cord [12,13] have been shown to exert beneficial effects in experimental models of lung fibrosis. Clinical testing of allogeneic stem cells has thus far shown them to...
be safe [14, 15], however clinical translation of cell therapies has been fraught with technical challenges in terms of scalability and reproducibility. Alternatives such as stem cell-derived exosomes, or more broadly extracellular vesicles (EVs), are thus being concurrently pursued in applications such as myocardial infarction, pulmonary and renal fibrosis, and cutaneous wound healing [16–19].

The most well-characterized subclass of EVs to date are the exosomes, which range from 40 to 120 nm and have a flotation density of 1.13 to 1.19 g/ml [20–22]. Exosomes from MSCs were reported to have the same immunomodulatory properties as the MSCs themselves [23]. MSC-derived exosomes carrying miR-125b reportedly reduced liver fibrosis in a carbon tetrachloride model of liver fibrosis by targeting Hedgehog signaling [24]. Human amnion epithelial cells (hAEC), which have comparable properties to MSCs, can be isolated from healthy term placenta with far greater yields (hundreds of millions isolated from a single donor) [25]. This study characterized hAEC-derived exosomes and their cargo content. We also investigated the anti-inflammatory, anti-fibrotic and pro-regenerative properties of hAEC-derived exosomes in a mouse model of lung injury, specifically testing preclinical efficacy in aged mice to model the clinical setting of increasing mortality in older IPF patients.

**MATERIALS AND METHODS**

A complete description of the material and methods has been provided as online Supporting Information.

**Human Amnion Epithelial Cell Isolation**

After obtaining written informed consent, hAECs were isolated from placenta of women undergoing elective cesarean section as previously described [26] in accordance with guidelines and approval from Monash Health Human Research Ethics Committee (H01067B). Mean gestational age of collection was 38 ± 4 weeks. EVs were isolated from 10 amnions, pooled and used for all experiments.

**Human Lung Fibroblast**

Exosomes from healthy human lung fibroblasts (HLF) were used controls for hAEC Exo given their previous use as control cells for comparison against hAECs [27]. HLFs were cultured under the same condition as hAECs to obtain EVs.

**In Vitro Immune Potency Assays.** In vitro assays to assess neutrophil, macrophage, T-cell behavior, and phenotype were performed to determine the immune potency of hAEC Exo and HLF Exo.

**Mice**

Animal experiments were carried out with approval by Monash University Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. C57Bl/6 mice aged 6 to 8 weeks or 12 months were housed in pathogen free Monash Medical Centre Animal facility with ad libitum access to food and water.

**Bleomycin Challenge.** C57Bl/6 mice received 0.04 units of bleomycin intranasally (Willow Pharmaceuticals, NSW, Australia). Each mouse was randomly allocated to receive either 10 μg hAEC Exo or HLF Exo in 0.1 ml of saline or equal volumes of saline alone via intravenous injection. Young mice (6–8 weeks old) were treated either 24 hours (early intervention) or 7 days (late intervention) after bleomycin challenge. Aged mice (12 months old) were treated with intranasal administration of exosomes 7 days after
bleomycin challenge. Mice were culled 7 days later. Lung tissues were collected as previously described [28]. Each group had an $n$ of 6 to 8 per group.

**RESULTS**

**Characterization of Extracellular Vesicles Shed by Human Amnion Epithelial Cells**

There are a number of methods to isolate EV with varying purity, yield and levels of complexity. Here we show that EVs released by hAEC (hAEC-EV) can be isolated using serial ultracentrifugation with relative purity. The isolated hAEC-EV fell within the exosome size range (i.e., 80–120 nm) as determined by nanoparticle tracking analysis (Fig. 1A). Morphological assessment by transmission electron microscopy revealed a typical cup-shaped morphology (Fig. 1B). Ultrathin sections of embedded hAEC showed evidence of intracellular multivesicular bodies (Fig. 1C) and the budding of vesicles from the cell surface (Fig. 1D). Using a combination of Western blotting and bead-based flow cytometry, we observed the presence of Alix, CD81, and CD9 as well as HLA-G, a protein that is highly abundant in hAECs, with relatively low abundance of Grp94 and Cyt C (Fig. 1E–1G). Together, these findings indicated that hAEC-EV fulfilled the minimal experimental criteria of exosomes described in the position statement by the International Society for Extracellular Vesicles [29]. The EVs derived from hAECs are hereafter referred to as hAEC-derived exosomes (hAEC Exo).

**Characterization of hAEC Exo**

The protein composition of exosomes isolated from conditioned media from hAECs was compared with that of exosomes isolated from HLF. Protein content was analyzed by liquid chromatography followed by mass spectrometry. The data are summarized in Figure 2 and show proteins that are enriched in hAEC Exo compared with HLF Exo. There were 84 proteins associated with the Reactome pathway by hAEC Exo, which are significantly different from HLF Exo with significance shown in Supporting Information Table 1. Proteins in hAEC Exo cargo were enriched for pathways associated with apoptosis, developmental growth, MAP kinase, inflammation mediated pathway, EGF, PDGF, and FGF signaling compared with HLF Exo cargo where pathways were centered around pathways associated with fibrosis. A priori analysis of gene union enrichment showed overrepresentation of some miRNAs in the cargo of hAEC Exo (Supporting Information Table 2). When significantly overrepresented pathways were ranked according to the numbers of genes targeted, we noted enrichment in a number of signaling pathways associated with fibrosis. These included the PI3K-Akt, MAPK, Ras, Hippo, TGFβ, and focal adhesion signaling pathways. A posteriori analysis of pathways union identified a dozen pathways significantly targeted by the miRNA in hAEC Exo. We observed that 110 genes associated with the KEGG pathway ‘proteoglycans in cancer’ were predicted targets of the known miRNA cargo, followed by similarly large numbers of predicted gene targets associated with Hippo signaling and stem cell pluripotency (Supporting Information Table 3). Additionally, reportedly anti-fibrotic microRNAs including miR-23a, miR-203a, miR-150, and miR-194 were packaged in hAEC Exo cargo.

**Immunomodulatory Properties of hAEC Exo**

Given previous reports on the beneficial effects of hAEC-mediated immunomodulation, we assessed the potency of hAEC Exo on a number of immune cell subsets where HLF Exo were used for comparison. Neutrophils are the first line of defense at the onset of inflammation where they oxidize tissue debris and pathogens
through myeloperoxidases (MPO) then apoptose resulting in the release of inflammatory signals to propagate downstream inflammatory responses. Here, we showed that neutrophils treated with either 1 μg or 5 μg of hAEC Exo (1 μg; p = .022 and 5 μg; p = .042, respectively, Fig. 3A) and HLF Exo (1 μg; p = .003 and 5 μg; p = .012, Fig. 3A) showed significantly lower myeloperoxidase activity than controls. Neutrophil cell death induced by hAEC Exo (1 μg; p = .006, 5 μg; p = .003, Fig. 3B) and HLF Exo (1 μg; p = .002, 5 μg; p = .001, Fig. 3B) was also higher than controls. Correspondingly, inhibiting exosome production by incubating hAEcs with 20 μM of GW4869, reversed inhibition of neutrophil MPO production by hAEcs. Neutrophil cell death with GW4869 primed hAEcs was 6.660 ± 0.433 significantly higher than hAEcs alone (4.943 ± 0.315, p = .022, Fig. 3D). However, these effects were similar in comparison to HLF treatment. Next, we targeted the proceeding step in resolution where polarization of inflammatory (M1) macrophages to an anti-inflammatory (M2) state coupled with increased phagocytic clearance of apopotic

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neutrophils are required to rid the microenvironment of further inflammatory stresses. We analyzed the effects of hAEC Exo on macrophage function and phenotype and report that 5 μg hAEC Exo doubled the phagocytic activity of macrophages (p = .00003, Fig. 3E, 3F) which was not achieved by the same amount of HLF Exo. Blocking hAEC Exo release also ameliorated this effect (Fig. 3G), however with a significant increase in cell death with 20 μM of GW4869. At 10 μM of GW4869 hAEC-mediated increase in macrophage phagocytosis was reversed without affecting macrophage survival (Fig. 3H, 3I). Similarly, the percentage of CD206+ (M2) macrophages was significantly higher following stimulation with 5 μg hAEC Exo than HLF Exo (p = .002) and controls (p = .002, Fig. 3J). Conversely, the percentage of CD86+ (M1) macrophages was lower with 5μg of hAEC Exo compared with HLF Exo and controls (Fig. 3I, p < .0001 and p = .002, respectively). Finally, we evaluated the effect of hAEC Exo on T cell proliferation in response to CD3/CD28 stimulus and showed significant suppression of T cell proliferation index with 5 μg of hAEC Exo at 0.400 ± 0.245 in comparison to HLF Exo (1.087 ± 0.038, p = .002) and controls (1.090 ± 0.020, p = .002, Fig. 3K). This effect was dependent on hAEC Exo, since blocking exosomal release using GW4869 ameliorated hAEC Exo mediated T cell suppressive effects (Fig. 3L). Next, we assessed Treg induction in CD4+ T cells in the context of hAEC Exo uptake in vitro. We labeled exosomal RNA of endothelial cells (MECs). Specifically, migration of CD11b+ macrophages was lower with 5 μM of GW4869. At 10 μM of GW4869 hAEC-mediated increase in macrophage phagocytosis was reversed without affecting macrophage survival (Fig. 3H, 3I). Similarly, the percentage of CD4+ T cells (p = .0140, Table 1) and neutrophils (p = .0018, Table 1) in the spleen, concurrently reducing the percentage of CD8+ T cells (p = .0265, Table 1), and interstitial macrophages (p = .062, Table 1) in the lung. Next, we evaluated the role of hAEC Exo early intervention on transcriptional changes in BASCs and MECs. Early administration of hAEC Exo also increased expression of canonical Wnt targets; β-catenin (p = .0082, Fig. 4B), BMP4 (p = .018, Fig. 4B), BMPRIA (p = .024, Fig. 4B), and NFATC1 (p = .046, Fig. 4B) in comparison to bleomycin injured mice. Interestingly, c-MYC transcription was significantly reduced in BASCs from mice given hAEC Exo in comparison to bleomycin controls (p = .041, Fig. 4B), while Axin1 and Axin 2 (both p = .028, Fig. 4D) were significantly elevated in MECs following administration of hAEC Exo compared with bleomycin controls, thus pointing to an inhibitory effect for Wnt/β-catenin signaling in MECs. In addition to Wnt target genes, growth factor FGFR1 was significantly elevated following hAEC Exo administration in comparison to bleomycin controls (p = .002, Fig. 4D). No significant differences in CNND1, FOXM1, LEF1, PGK1, PTN, Sca1, TGFβ, and WLS transcription were seen.

**hAEC Exo Prevent Bleomycin Induced Lung Injury in Young Mice**

We assessed the impact of hAEC Exo in a bleomycin model of lung fibrosis when administered at Day 1 (early) and Day 7 (late) following bleomycin challenge. Given previous reports that the immune response impacts regeneration of lung tissue, we assessed hAEC Exo administration at Day 1 its impact on the immune response, bronchioalveolar stem cells (BASCs), and endothelial cells (MECs). Specifically, migration of CD11b+ myeloid cells during injury is shown to aid lung regeneration, and mice lacking the CD11b/CD18 heterodimer have impaired leukocyte trafficking following partial pneumonectomy and have poor lung growth [30]. In our study, we first assessed the impact of early hAEC Exo administration on inflammation as lavage fluid from IPF patients show an accumulation of neutrophils, monocytes, and T cells. Immunomodulatory effects were measured at day 7 where early intervention resulted in the reduction of CD4+ T cells (p = .0140, Table 1) and neutrophils (p = .0018, Table 1) in the spleen, concurrently reducing the percentage of CD4+ T cells (p = .0265, Table 1), and interstitial macrophages (p = .062, Table 1) in the lung. Next, we evaluated the role of hAEC Exo early intervention on transcriptional changes in BASCs and MECs. Early administration of hAEC Exo also increased expression of canonical Wnt targets; β-catenin (p = .0082, Fig. 4B), BMP4 (p = .018, Fig. 4B), BMPRIA (p = .024, Fig. 4B), and NFATC1 (p = .046, Fig. 4B) in comparison to bleomycin injured mice. Interestingly, c-MYC transcription was significantly reduced in BASCs from mice given hAEC Exo in comparison to bleomycin controls (p = .041, Fig. 4B), while Axin1 and Axin 2 (both p = .028, Fig. 4D) were significantly elevated in MECs following administration of hAEC Exo compared with bleomycin controls, thus pointing to an inhibitory effect for Wnt/β-catenin signaling in MECs. In addition to Wnt target genes, growth factor FGFR1 was significantly elevated following hAEC Exo administration in comparison to bleomycin controls (p = .002, Fig. 4D). No significant differences in CNND1, FOXM1, LEF1, PGK1, PTN, Sca1, TGFβ, and WLS transcription were seen.

**hAEC Exo Resolve Bleomycin Induced Lung Injury in Young Mice**

We then evaluated the effect of hAEC Exo administration on ongoing lung injury by administering the hAEC Exo 7 days after bleomycin challenge (late intervention). Histological outcomes of ongoing lung injury by administering the hAEC Exo 7 days after bleomycin challenge (late intervention). Histological outcomes of injury, fibrosis, and progenitor cell counts were measured at day 14. The percentage of tissue-to-airspace ratio was improved (p ≤ .001, Fig. 4E), lung collagen content and αSMA expression were significantly lower (p ≤ .01, Fig. 4F, p = .176, Fig. 4G). Additionally, the average number of BASCs per terminal bronchiole was significantly higher in hAEC Exo than bleomycin controls (p = .017, Fig. 4I), and hAEC Exo increased the percentage of ATII, another endogenous lung progenitor cell subtype (p = .018, Fig. 4J), while ATI remained unchanged (Fig. 4K). Next, we evaluated the gene expression of sorted BASCs and MECs and found that fewer genes were affected by late intervention compared with early intervention in both cell population. In the late intervention group, hAEC Exo did not alter gene expression of BASCs in...
comparison to bleomycin controls but significantly increased MEC expression of FZD6 (p = .0287, Fig. 4P) and GATA6 (p = .0288, Fig. 4P) compared with bleomycin controls. These findings show that hAEC Exo given at different phases of lung injury can attenuate inflammation and alter transcriptional changes in BASCs and MECs.

**hAEC Exo Prevent Bleomycin Induced Lung Injury in Aged Mice**

Pulmonary fibrosis is a disease affecting the aged population who are predisposed to mutations in telomerase resulting in telomere shortening and potential loss of regenerative capacity. Accordingly, we evaluated the effects of delayed hAEC Exo administration in aged mice with established pulmonary fibrosis. In our hands, hAEC Exo administration ameliorated lung fibrosis in aged mice compared with bleomycin and HLF Exo controls. Specifically, there was no evidence of bronchoalveolar hyperplasia in hAEC Exo treated aged mice and an approximate twofold reduction in pulmonary collagen (p < .01, Fig. 5A) and depletion of α-SMA+ cells compared with bleomycin controls (p < .05, Fig. 5B). The average number of BASCs per terminal bronchiole was higher in hAEC Exo groups than bleomycin controls (p = .036, Fig. 5E). The mean percentage of alveolar type II (SPC+ CC10+) and type I (Pdpn+) cells were also higher in the hAEC Exo group compared with bleomycin controls (ATII; p = .02, ATI; p = .02, Fig. 5F, 5G).

**Pro-Regenerative Properties of hAEC Exo**

Current evidence suggests that in IPF, an imbalance in cell signaling leads to alveolar epithelial senescence and over activation of myofibroblast. Hence, our next step was to assess the

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**Figure 3.** Immunomodulatory potency of amnion epithelial cell-derived exosomes. Neutrophil myeloperoxidase (MPO) production was significantly reduced in the presence of both hAEC [1 μg; p = .022 and 5 μg; p = .042 respectively, (A)] and HLF exosomes [1 μg; p = .003 and 5 μg; p = .012, (A)]. Exosomes from hAECs and HLF increases percentage of Annexin V/PI positive cells indicative of increased cell death [1 μg; p = .006, 5 μg; p = .003, (B)]. Inhibition of exosomal release by GW4869 compound reverse inhibition of neutrophil MPO activity (C) but not survival of neutrophils [GW4869 primed hAECs; 6.660 ± 0.433 vs. hAECs; 4.943 ± 0.315, p = .022, (D)]. Exosomes at 5 μg increased macrophage phagocytic activity for pHRodo labeled bacterial particles [p = .00003, (E)]. Representative flow cytometry gating strategy of pHRodo positive macrophages pre-gated for live-dead, F4–80 and CD11b [p = .00003, (F)]. GW4869 neutralized hAEC Exo mediated increase in macrophage phagocytosis (G). Percentage of live:dead macrophages was unchanged in the presence of hAEC and HLF exosomes (H). Addition of 20 μM of GW4869 and equal volumes of DMSO increased macrophage cell death (I). Macrophage polarization was skewed toward M2 CD206+ phenotype and away from M1 CD86+ phenotype with increasing dose of hAEC exosomes [p < .0001 and p = .002 respectively, (J)]. T cell proliferation was significantly suppressed with 5 μg of hAEC exosomes [5 μg; 0.400 ± 0.245 vs. control; 1.090 ± 0.020, p = .002, (K)]. Addition of GW4869 reversed this effect but was not significant (L). Representative images of control (Exo-Red label only) against Exo-Red labeled hAEC Exo uptake by GFP-Foxp3 CD4+ T cells with red arrows pointing uptake of RNA exosome labeled in red by transgenic GFP-Foxp3 CD4 T cells (M). Only red signals of high abundance are labeled with red arrows. Green arrows point to GFP-Foxp3 positive cells. Colocalization of green and red are represented by both arrows. A video of hAEC Exo uptake can be found on Supporting Information data. (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Abbreviations: hAEC Exo, human amnion epithelial cell-derived exosomes; HLF exo, HLF exosomes; DMSO, dimethyl sulfoxide.
regenerative and anti-fibrotic ability of hAEC Exo in culture. To address this, we assessed the direct effects of hAEC Exo on BASC growth and fibroblast activation. BASCs from healthy mice cultured with hAEC Exo formed differentiated lung cells including alveolar, bronchiolar, and bronchioalveolar colonies after 21 days in culture (Fig. 6A). The addition of hAEC Exo resulted in more bronchiolar colonies and colonies were larger compared with those cultured in media alone ($p < .0001$, Fig. 6B and $p < .0001$, Figure 3. (Continued)).
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<td></td>
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<tr>
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<td>9.918 ± 2.116</td>
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<td>CD11b DCs</td>
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**Abbreviations:** hAEC EV, human amnion epithelial cell extracellular vesicle; HLF EV, human lung fibroblasts extracellular vesicle.
Exposed to either hAEC Exo or pirfenidone. The impact of hAEC colonies treated with 5 mM expression of club cell marker (CC10) was higher in bronchiolar expression in MECs [5].

Representative heat map of genes expressed by MECs sorted from healthy, bleomycin, bleomycin + hAEC exo, and bleomycin + HLF exo mice (A). Quantitative PCR analysis on sorted BASCs showed increase in CTNNB1, BMP4, FOXM1, LEF1, NFATC1, PGK1, PTN, SCA1, and WLS with a reduction of cMYC gene expression (B). Representative heat map of genes expressed by mouse endothelial cells (MECs) sorted from healthy, bleomycin, bleomycin + hAEC exo, and bleomycin + HLF exo mice (C). Sorted MECs showed increased Ang2, Axin1, Axin2, BMPR2, EFNB2, FGFR1, LEF1, NFATC3, and THBS1 gene expression (D). Day 7 hAEC Exo intervention on fibrotic lung injury in young mice. Exosomes given 7 days after bleomycin challenge then culled at day 14 showed significant reduction in inflammation; H&E stain, collagen content; Sirius red and α-SMA staining [5]. Average bronchoalveolar stem cell (BASC) counts per terminal bronchiol increased with hAEC Exo intervention (p = .017) (I). Average ATII counts increased with hAEC intervention (p = .018) (J). No changes were seen in ATII cells (K). Representative image of BASCs stained with CC10 in green, ATII cells stained with SPC in red and CC10, ATI cells, stained with Pdpn in white over nuclear DAPI stain in blue [scale bar = 50 μm] (H). Average bronchoalveolar stem cell (BASC) counts per terminal bronchiol with Pdpn in white over nuclear DAPI stain in blue [scale bar = 50 μm] (L). Representative heat map of genes expressed by BASCs sorted from healthy, bleomycin, bleomycin + hAEC Exo, and bleomycin + HLF Exo mice (M). Exosomes given 7 days post bleomycin significantly increased BMP4 and CNDN1 gene expression in BASCs (N). Representative heat map of genes expressed by MECs sorted from healthy, bleomycin, bleomycin + hAEC exo and bleomycin + HLF exo mice (O). Exosomes given 7 days post bleomycin increased FZD6 and GATA6, gene expression in MECs [FZD6; p = .0287, GATA6; p = .0288 (P)]. (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Abbreviations: BASCs, bronchioalveolar stem cells; hAEC Exo, human amnion epithelial cell-derived exosomes; HLF exo, HLF exosomes.

DISCUSSION

Lung transplantation remains the only effective therapy for IPF. Current preclinical studies using cell-based therapies to treat lung fibrosis has yielded mixed results between cell types. In particular, studies using MSCs when given 7 to 14 days after bleomycin regardless of its source has shown varying outcomes in amelioration of fibrosis. Specifically, in the case of hAECs, we have shown that given 14 days post bleomycin resulted in significant amelioration of fibrosis in comparison to administration at day 7. We
believe that hAECs respond to their microenvironment by releasing exosomes and the contents in hAEC Exo released in an inflammatory environment would not contain potent pro-regenerative, anti-fibrotic, and pro-inflammatory properties. To our knowledge, this is the first study to report that hAEC Exo treatment targets the inflammatory, fibrotic, and regenerative cascades of pulmonary fibrosis. In line with our interests in clinical translation, we assessed the ability of hAEC Exo to prevent and reverse lung inflammation and fibrosis, and we chose a delivery method (intra-nasal) that would be clinically useful. Given the degenerative nature of IPF, we also sought to ascertain the impact of hAEC Exo on endogenous lung stem/progenitor cells, and in aged animals.

In response to damage, immune cells particularly neutrophils and CD4 T cells release cytokines, growth factors, and stimuli to

Figure 4. (Continued).
modulate fibroblast and macrophage behavior [31]. However, an aberrant immune response contributes to the pathogenesis of IPF, and thus we first show that hAEC Exo cargo were enriched for proteins involved in immunomodulation. Specifically, we found that proteins involved in Fc-gamma receptor dependent phagocytosis and regulation of actin dynamics for phagocytic cup...
formation pathways were enriched in hAEC Exo. This was in line with our observations of increased macrophage phagocytic activity mediated by hAEC Exo, and supports earlier work by others showing that hAEC-conditioned media exerts similar effects [32, 33]. hAEC Exo also contained protein cargo enriched for P38 and PI3K-Akt pathways. P38 is critical to tissue regeneration, where p38 deletion results in immature and hyperproliferative lung epithelium [34]. Similarly, the PI3K-Akt pathway is critical for cell survival, cell cycle, and macrophage polarization [35]. Next, we evaluated the immunomodulatory properties of hAEC Exo. Here, we showed that hAEC Exo at 5 μg increased their phagocytic activity and changed their polarization state, which was possibly mediated by the PI3K-Akt signaling pathway [36]. Neutrophil myeloperoxidase activity was also reduced with both hAEC and HLF Exo, in conjunction with increased neutrophil apoptosis. This was particularly interesting, since HLF Exo were unable to prevent lung fibrosis in vivo despite inhibiting neutrophil activation in vitro. It is suggestive that exosomes from healthy lung fibroblast have the ability to modulate neutrophil response. However, this effect alone is insufficient to abolish this disease and targeting more than one cascade of this disease may be fundamental [37]. Neutrophil and macrophage activity encompasses the innate inflammatory response and is accompanied by the T cell adaptive immune response. As such, we evaluated the behavior of T cells and showed that hAEC Exo can suppress CD3/CD28 activated T cell proliferation. Additionally, this suppression of T cell proliferation coincided with hAEC Exo uptake, which occurred within minutes and was sufficient for Treg activation. This is in line with previous observations that dendritic cell-derived exosomes suppressed collagen-reactive T cells through their packaging of Fas-ligands [38]. Using a mouse model of bleomycin induced lung injury, we found that early intervention with hAEC Exo significantly decreased the percentage of splenic CD4+ CD3+ T cells, and neutrophils, with concurrent reduction of CD4+ T cells and interstitial macrophages in the lungs. These findings suggest that hAEC Exo may directly modulate the behavior and phenotype of neutrophils, macrophages, and T cells both in vitro and that in vivo outcomes maybe be as a consequence of their uptake by specific target cells.

Next, evaluation of hAEC Exo anti-fibrotic protein cargo revealed an enrichment of proteins associated with the PDGF, FGF, and EGF signaling pathways, which have been implicated in vascular remodeling, regulation of fibroblast proliferation and development, migration, adhesion, and cell survival, respectively. We found an overrepresentation of proteins in hAEC Exo that were involved in negatively regulating FGFR1 and FGFR2, and PDGF-mediated regulation of RAS through GAPs target pathways common to those targeted by nintedanib. RNA-Seq analysis showed that hAEC Exo contain miRNAs that converge on key pathways associated with fibrosis as well as cancer and stem cell pluripotency. In keeping with the apparent impact of hAEC Exo administration on lung fibrosis, we noted the presence of a number of reportedly anti-fibrotic miRNAs. Among the 10 most abundant miRNAs were miR-27a, which reportedly targets myofibroblast differentiation, and lentiviral delivery of miR-27a-3p diminished bleomycin induced lung fibrosis [39]. hAEC Exo also packaged miRNAs that target various aspects of TGFβ signaling including miR-23a, which inhibits Smad 2 [40], miR-203a which
inhibits Smad 3 and thereby contributes to the repression of TGFβ mediated epithelial-mesenchymal transition as well [41], and miR-34a which induces lung fibroblast senescence and suppresses fibroblast proliferation [42]. Furthermore, miR-150 and miR-194, both of which are enriched in hAEC Exo, inhibit the activation of hepatic stellate cells and reduce collagen deposition in the setting of liver fibrosis by inhibiting c-MYB and RAC 1 expression [43].

Future studies validating the contribution of each miRNA in the anti-fibrotic effects of hAEC Exo must be undertaken in order to ascertain their relevance and enable the development of hAEC Exo as a therapeutic for IPF. Interestingly, hAEC Exo miRNA cargo was also found to converge on the Hippo signaling pathway, which has been implicated in regeneration and repair. Specifically, the Hippo pathway regulates lung

Figure 5. Late intervention in aged mice. hAEC Exo given 7 days after bleomycin challenge showed significant reduction in collagen content; Sirius red and α-SMA staining [p ≤ .01, (A), p ≤ .05, (B)]. Representative images of Sirius red and α-SMA staining in aged mice [scale bar = 50 μm, (C, D)]. Average bronchioalveolar stem cell (BASC) counts per terminal bronchiole increased with hAEC Exo [p = .0167, (E)]. Alveolar type I and type II epithelial cell counts were higher in hAEC Exo [ATII; p = .02, ATI; p = .02, (F, G)]. Representative images of BASCs, ATII, and ATI staining [scale bar = 50 μm, (H)]. (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Abbreviations: hAEC Exo, human amnion epithelial cell-derived exosomes; HLF exo, human lung fibroblasts exosomes.
Figure 5. (Continued).

Figure 6. Regenerative potency of exosomes in vitro. BASCs sorted from healthy mice formed alveolar, bronchiolar, and bronchioalveolar organoids in culture. [scale bar = 10 μm, (A)]. Culture with hAEC Exo significantly elevated the number and relative area of BASC colonies per well \( p < .0001 \), (B) and \( p < .0001 \), (C). hAEC Exo also increased the number and relative area of AT2 colonies per well \( p = .003 \), (D); \( p = .0001 \), (E). Quantitative PCR analysis showed hAEC Exo significantly increased expression of CC10 in bronchiolar and FOXJ1 in mixed colonies \( 5 \mu g; p = .001 \), (Fi) and \( 10 \mu g; p = .00028 \), (Fi), \( 5 \mu g; p = .033 \), (Fiii), and \( 10 \mu g; p = .030 \), (Fiii). No changes were seen in expression of SPC (Fii) and Pdpn (Fiv). Volcano plot showing collection of proteins enriched in hAEC Exo, in green and yellow over proteins enriched in HLF Exo, in red and blue (G). Human lung fibroblasts activation by TGFβ measured by collagen deposition was significantly reduced when cultured with hAEC Exo at a dose dependent manner with effects closely resembling pirfenidone \( 5 \mu g; p = .003, 1 \mu g; p = .040 \), respectively, (H). Inhibition of HLF differentiation into myofibroblasts were inhibited by 5 μg of hAEC Exo similar to effects by pirfenidone \( 5 \mu g; p = .887 \), (I)]. (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \)). Abbreviations: BASC, bronchioalveolar stem cell; hAEC Exo, human amnion epithelial cell-derived exosomes.

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progenitor cell response and differentiation [44]. The converse role of Hippo signaling in cancer and indeed the convergence of miRNAs on proteoglycans in cancer should not be ignored, and the impact of hAEC Exo on tumorigenesis should be investigated further.

The significant improvement observed with the late intervention cohort is in keeping with the direct anti-fibrotic effects of hAEC Exo. We noted that hAEC Exo were able to directly suppress myofibroblast differentiation and decrease collagen production in vitro, comparable to pirfenidone. Since there is growing interest in the role of aging and telomerase mutations in the development of IPF [45, 46], we sought to assess the impact of delayed hAEC Exo in aged mice and found them to be comparably effective as they were in young mice. It is encouraging to show that hAEC Exo can abolish fibrosis and may have regenerative capabilities which can overcome aged-related cellular senescence in aged mice which are predisposed to bronchiolar and alveolar senescence as shown by Calhoun and colleagues [47].

Finally, given the relevance of stem cell senescence in the context of IPF, we assessed the regenerative role of hAEC Exo. Early hAEC Exo intervention increased gene expression of β-catenin, BMP4, LEF, and NFATC1 in BASCs. Notably, human umbilical cord MSC-derived exosomes have also been shown to deliver Wnt4 to stimulate migration and proliferation of skin cells for wound repair [48]. However, aberrant Wnt/β-catenin has been implicated in IPF and should be further studied to understand the effect of hAEC Exo-mediated Wnt signaling.

Proliferation of BASCs has been shown to be dependent on c-MYC, which regulates G1 and S phase transitioning during cell cycle and an increase in β-catenin is known to initiate transcriptional activation of c-MYC and CNN1 [49]. A study by Dong and colleagues showed that depletion of c-MYC in BASCs isolated from mice resulted in inhibition of BASC self-renewal [49]. BASC differentiation into alveolar cells is dependent on NFATC1 binding to THBS1 triggered by BMP4 [50]. Here, we showed that hAEC Exo treatment increased BMP4 expression in BASCs and increased NFATC1 and THBS1 expression in MECS. This suggests that early intervention with hAEC Exo encouraged BASC differentiation by stimulating the recently described NFATC1/BMP4/THBS1 signaling axis [50].

Late hAEC Exo intervention also increased the number of BASCs, which also showed an increased expression of β-catenin and BMP4. However, unlike in the early intervention group, hAEC Exo treated animals had MECS that showed increased expression of FZD6; a Wnt ligand receptor and inhibitor of GSK3β, and GATA6, which is expressed in distal lung epithelium and regulates differentiation [51, 52]. This increase in BASC expression of β-catenin suggests that hAEC Exo intervention may have encouraged BASC proliferation through increased canonical Wnt signaling. In support of this, primary BASCs cultured in the presence of hAEC Exo gave rise to more bronchiolar, alveolar, and bronchioalveolar colonies. Quantitative PCR analysis showed that colonies cultured with hAEC Exo highly expressed CC10 and FoxJ1 indicating that hAEC Exo may help retain the BASCs in their undifferentiated state. This is promising given the current hypothesis that the propensity for developing IPF is ultimately an exhaustion of stem/progenitor cells.

Asides from BASCs, we saw a difference in the percentage between ATI and ATII when compared between young and aged. In the lung, ATI cells account for the majority cells in the alveolar epithelium in comparison to ATIIs. In response to injury, ATIIs regenerate the lung through clonal expansion and differentiation into ATI cells. This process is deregulated in diseases such as IPF. In saying so, ATI and ATII expansion and differentiation are also dependent on age, as the literature has shown that aged mice are susceptible to increased ATI and ATII cell loss and delayed ATII recovery. In our study, we showed that in young mice, at day 14 bleomycin challenged mice had percentage of ATII cells, which sat between 15% and 30% while ATI cells were approximately 60% and hAEC Exo treatment increased ATII percentage by twofold while ATI cells were not affected. However, in aged mice the percentage bleomycin challenged resulted in an ATII cell percentage between 4% and 8% while the ATI cells were approximately 60%, suggesting aged related alveolar loss. However, despite this age-related disadvantage, hAEC Exo treatment increased the percentage of ATI cells by twofold and slightly increased the percentage of ATII cells.

**CONCLUSION**

In summary, this study shows that hAEC Exo can exert potent anti-fibrotic, immunomodulatory, and regenerative properties in the setting of bleomycin induced lung injury. This study paves the way for pharmacological development of cell-derived products that may overcome the hurdles associated with cell-based therapies. Intranasal delivery and ease of long-term storage without the need for liquid nitrogen and the ability to produce exosomes in a cost-effective and scalable manner are some of the advantages of exosome-based therapeutics over cell-based therapies. Furthermore, this multi-modal approach may be more efficacious compared with current drugs such as pirfenidone and nintedanib by addressing the degenerative aspects of IPF and similar diseases. This is the first study describing the anti-fibrotic and pro-regenerative potential of hAEC Exo in the context of lung injury. Future studies should be directed at validating the pathways where miRNA and protein cargo are enriched and optimizing cell conditions to obtain the most potent exosomes for this application.

**AUTHOR CONTRIBUTIONS**

J.L.T.: designed, performed experiments, analyzed data, prepared and edited the manuscript. S.N.L. and S.T.C.: performed experiments and analyzed data. B.L., M.I.S., and D.D.Z.: were responsible for immunohistochemistry and immunofluorescence experiments. M.K., H.N., and L.S.: contributed to exosome-based therapeutics over cell-based therapies. Furthermore, this multi-modal approach may be more efficacious compared with current drugs such as pirfenidone and nintedanib by addressing the degenerative aspects of IPF and similar diseases. This is the first study describing the anti-fibrotic and pro-regenerative potential of hAEC Exo in the context of lung injury. Future studies should be directed at validating the pathways where miRNA and protein cargo are enriched and optimizing cell conditions to obtain the most potent exosomes for this application.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

Dr. Wallace is named inventor on patents for the application of amnion cells in lung diseases and amnion exosomes as a regenerative therapy. Dr. Lim has intellectual property rights with Regenosphere Pty Ltd, has a consultant/advisory role with Meluha Capital, and has research funding from United Therapeutics. The other authors indicated no potential conflicts of interest.


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