

1 **Inhalational delivery of induced pluripotent stem cell secretome improves**
2 **postpneumonectomy lung structure and function**

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28 **Abstract**

29 Cell-free secretory products (secretome) of human induced pluripotent stem cells (iPSCs) have
30 been shown to attenuate tissue injury and facilitate repair and recovery. To examine whether
31 iPSC secretome facilitates mechanically-induced compensatory responses following unilateral
32 pneumonectomy (PNX), litter-matched young adult female hounds underwent right PNX
33 (removing 55-58% of lung units) followed by inhalational delivery of either the nebulized
34 conditioned media containing iPSCs secretome (iPSC CM) or control cell-free media (CFM);
35 inhalation was repeated every 5 days for 10 treatments. Lung function was measured under
36 anesthesia pre-PNX and 10 d after the last treatment (8 weeks post-PNX); detailed quantitative
37 analysis of lung ultrastructure was performed postmortem. Pre-PNX lung function was similar
38 between groups. Compared to CFM control, treatment with iPSC CM attenuated the post-PNX
39 decline in DL_{CO} and DM_{CO} , accompanied by a 24% larger postmortem lobar volume and distal
40 air space enlargement. Alveolar double-capillary profiles were 39% more prevalent consistent
41 with enhanced intussusceptive angiogenesis. Frequency distribution of the harmonic mean
42 thickness of alveolar blood-gas barrier shifted towards the lowest values while alveolar septal
43 tissue volume and arithmetic septal thickness were similar, indicating septal remodeling and
44 reduced diffusive resistance of the blood-gas barrier. Thus, repetitive inhalational delivery of
45 iPSC secretome enhanced post-PNX alveolar angiogenesis and septal remodeling that are
46 associated with improved gas exchange compensation. Results highlight the plasticity of the
47 remaining lung units following major loss of lung mass that are responsive to broad-based
48 modulation provided by the iPSC secretome.

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50 **Key words:** induced pluripotent stem cells, secretome, compensatory lung growth, alveolar
51 remodeling, lung diffusing capacity.

52 **Noteworthy:**

53 To examine whether the secreted products of human induced pluripotent stem cells (iPSC)
54 facilitate innate adaptive responses following loss of lung tissue, adult dogs underwent surgical
55 removal of one lung, then received repeated administration of iPSC secretory products via
56 inhalational delivery compared to control treatment. Inhalation of iPSC secretory products
57 enhanced capillary formation and beneficial structural remodeling in the remaining lung, leading
58 to improved lung function.

59

60 Introduction

61 Major lung resection by pneumonectomy (PNX) mimics the consequences of destructive lung
62 disease regardless of specific etiology and is a useful model for studying the mechanisms and
63 adaptive potential of the remaining functioning units. Following right PNX, the markedly
64 increased supra-threshold mechanical stresses on the remaining lung units result in vigorous
65 expansion and compensatory alveolar tissue-capillary growth (5, 7, 37, 38), leading to balanced
66 generation of new acinar structural components, progressive remodeling of existing structure,
67 and eventually augmentation of lung function. Robust post-PNX responses have been
68 documented in multiple species and in both young and adult animals (18); compensation is
69 more complete in young than adult animals, suggesting a need for exploring interventions to
70 amplify the innate response and fully harness the potential plasticity in the adult lung. In the
71 presence of sufficient mechanical signals, active compensatory responses may be
72 pharmacologically augmented; supplementation with individual growth promoters in adult
73 canines enhanced selected aspects of post-PNX structural growth, angiogenesis and acinar and
74 alveolar septal remodeling but not global lung function (4, 6, 34, 51). This “*structure-function*
75 *gap*” in response may be the result of a) supra-physiologic and/or skewed pharmacological
76 stimulation, b) inadequate tissue protection from mechanically-induced oxidative stress damage
77 and c) inadequate architectural remodeling. These considerations suggest a need for a broad
78 panel of factors such as those produced by stem cells capable of modulating the myriad
79 homeostatic pathways involved in a balanced interactive response, to maximize the innate
80 potential for compensation and improve lung function.

81 The role of stem cells during post-PNX compensation remains poorly understood (26, 30). In
82 addition to alveolar type-2 epithelial cells classically considered to be progenitors, putative distal
83 airway progenitor cells also increase in number post-PNX (9) and exhibit age-related
84 proliferative and reparative potential (31). Delivery of exogenous stem cells including induced

85 pluripotent stem cells (iPSCs) has been reported to alleviate acute lung injury and facilitate
86 repair and regrowth (3, 11, 15, 29, 42, 47, 53). However, in vivo delivery of intact stem cells has
87 been limited by a low rate of engraftment and retention, and risks of immunogenicity and
88 tumorigenicity (1, 16, 45). Instead, the modest beneficial effects of stem cell delivery are thought
89 to be mediated mainly through the production of growth factors, cytokines, exosomes and
90 microvesicles, that constitute the secretome (2); therefore, an alternative approach is targeted
91 local delivery of cell-free conditioned media (CM) containing the stem cell secretome. We have
92 shown that tracheal delivery of iPSC CM containing the secretome alleviated bleomycin- and
93 hyperoxia-induced acute lung injury (10, 11); treatment activated endogenous antioxidant
94 proteins, enhanced antioxidant capacity and ameliorated oxidative damage to DNA, lipid, and
95 protein (11).

96 Based on the above observations, we hypothesized that pulmonary delivery of cell-free iPSC
97 secretome protects lung tissue from damage arising from post-PNX mechanical stress and
98 facilitates compensatory growth and remodeling, leading to enhanced function of the remaining
99 lung. To test this hypothesis and establish the feasibility of pulmonary delivery of stem cell
100 products in a large animal model, we nebulized the CM containing well-characterized iPSC
101 secretome (10, 11) for repeated inhalational delivery into the lungs of adult canines over ~8
102 weeks following right PNx. Control animals received cell-free media (CFM) in a similar fashion.
103 Blood oxidative damage markers and lung function were measured before and after PNx, and
104 detailed postmortem lung morphometry was performed. Results show that inhalation of iPSC
105 secretome enhanced post-PNX alveolar angiogenesis and septal remodeling, leading to
106 reduced alveolar-capillary diffusion resistance and improved diffusing capacity in the remaining
107 lung.

108 **Methods**

109 *Animals and experiments* The Institutional Animal Care and Use Committee of the University
110 of Texas Southwestern Medical Center approved all procedures. Litter-matched young adult
111 mixed-breed female hounds (total n=13, 10 months old, body weight 18.3 ± 1.1 kg, mean \pm SD),
112 were obtained from approved vendor (Marshall Farms, Rose, NY). A flowchart of experimental
113 design is shown in **Figure 1**.

114 *Right pneumonectomy* Following completion of baseline (pre-PNX) measurements, the
115 animal underwent right PNx following established procedures (5, 7). Briefly, the animal was
116 premedicated, anesthetized, intubated, and mechanically ventilated. Rectal temperature, heart
117 rate, blood pressure, and transcutaneous O₂ saturation were continuously monitored. Through a
118 small right lateral fifth intercostal space thoracotomy, individual hilar vessel ligation and stapling
119 of the bronchus was performed, covering the bronchus by oversewing adjacent healthy regional
120 tissue. The bronchial stump was checked for leaks and then oversewn with loose hilar tissue for
121 added protection. Lidocaine (1%) was applied to the intercostal nerves and the chest wall
122 closed in layers. Residual thoracic air was evacuated to underwater seal. Supplemental O₂ was
123 administered as needed. Intraoperative fluid administration was minimized (<50 mL). Analgesia
124 (Buprenorphine) was administered postoperatively for 48 h and then as needed. The animal
125 was monitored daily; skin stitches were removed in 7–10 days. The remaining left lobes were
126 estimated to comprise on average 42% of the pre-PNX total lung volume (38).

127 *iPSC conditioned media* Production of iPSC CM followed established procedures (10, 11).
128 Human foreskin dermal fibroblasts were reprogrammed into iPSCs using established protocols
129 (10). For producing the cell-free iPSC conditioned media (CM), iPSCs (1×10^6) were grown in
130 Corning ultra-low attachment flask (75cm²); once the cells formed spheres the culture media
131 was changed to a serum free media without additional supplements and the cells were grown
132 for 24 hours. Cell-free CM containing the iPSC secretome was harvested and characterized,

133 then kept deeply frozen (-80°C) until use. An aliquot (10 mL) was defrosted (4°C) overnight and
134 gently vortexed before use. Control cell-free media (CFM) was similarly processed.

135 *Inhalational delivery* Following PNX and chest wall closure, animals received the nebulized
136 compound (iPSC CM or CFM) before recovery from anesthesia; subsequent treatments were
137 given every 5 days for a total of 10 treatments. For each treatment (6), the animal was fasted
138 overnight and premedicated with acepromazine (0.05 mg/kg IM) and atropine (0.04 mg/kg IM).
139 Anesthesia was induced with propofol (4 mg/kg IV) and maintained with propofol as needed
140 (approximately 0.5-1.0 ml/min). Each animal was intubated and mechanically ventilated in the
141 supine position (16-18 breaths/min, 50/50 inspiration/expiration ratio, tidal volume 10-12 ml/kg).
142 Mouth pressure, O₂ saturation and heart rate were monitored. The animal pre-breathed 100%
143 oxygen in an open circuit for 1-2 min and then were switched to a closed circuit connected to a
144 reservoir bag and a nebulizer (Aerogen Aeroneb Pro, Tri Anim, Sylmar CA). The desired media
145 (10 mL) was nebulized (4 µm droplets) into the inspiratory limb of the breathing circuit and
146 delivered via the tracheal tube (average flow rate 0.4 mL/min, minimum flow rate 0.2 mL/min),
147 followed by nebulization of two 1 mL saline rinses to ensure complete delivery. Oxygen was
148 added to the circuit as needed to keep O₂ saturation above 90%. The entire procedure was
149 complete in 20-30 min.

150 *Biochemical assays* Peripheral venous blood (2 mL each) was collected before, during, and
151 after the first two treatments and then at every other treatment. Plasma was used for
152 measurement of oxidative stress markers 8-hydroxy-2'-deoxyguanosine (8-OHdG, Cell Biolabs,
153 San Diego, CA) and 8-isoprostane (Cayman Chemical, Ann Arbor, MI). Serum was used for
154 measurement of total antioxidant capacity (OxiSelect STA-360, Cell Biolabs). Complete blood
155 counts and biochemical panels (Superchem + CBC) were measured pre-PNX and at 1 h, 5 d,
156 30 d and 55 d post-PNX.

157 *Physiological studies* Lung function was measured pre-PNX and 55 d post-PNX (10 d
158 after completion of inhalation treatments) (6, 7). The animal was fasted overnight, pre-
159 medicated with acepromazine (0.05 mg/kg IM) and atropine (0.04 mg/kg IM). Anesthesia was
160 induced with propofol (4 mg/kg IV) and maintained with intravenous ketamine and diazepam
161 infusion at a dose titrated to effect. Animal was intubated with a cuffed endotracheal tube and
162 mechanically ventilated supine (tidal volume 10-12 ml/kg, 16-18 breaths/min) to eliminate
163 spontaneous breathing effort. Rectal temperature, heart rate, transcutaneous O₂ saturation, and
164 mouth and esophageal pressures, were monitored. Static transpulmonary pressure-lung volume
165 (PV) curves were measured using a calibrated syringe inflating the lungs to 15, 30, 45, and 60
166 ml/kg above end-expiratory lung volume (EELV), or up to a transpulmonary pressure of 30 cm
167 H₂O, in increasing and then decreasing order. End-inspiratory and end-expiratory lung volume
168 (EILV), pulmonary blood flow, lung diffusing capacity for carbon monoxide (DL_{CO}) and nitric
169 oxide (DL_{NO}), and septal tissue (including microvascular blood) volume were measured
170 simultaneously using an established rebreathing technique (4, 23) at two inspired O₂
171 concentrations (21% and 99%) and a lung volume of 45 ml/kg above EELV. The components of
172 DL_{CO}: membrane diffusing capacity (DM_{CO}) and pulmonary capillary blood volume (V_c), were
173 calculated from DL_{CO} measurements obtained at the two alveolar O₂ levels using established
174 methods (19, 39). Duplicate measures under each condition were averaged. PV curves were
175 analyzed using established methods (32, 41). Specific lung compliance was calculated from the
176 changes in lung volume and transpulmonary pressure between 10 and 30 cm H₂O and
177 normalized by the lung volume at 10 cm H₂O.

178 *Lung fixation* Under deep anesthesia, a tracheostomy was performed; a cuffed endotracheal
179 tube was inserted and tied securely. The chest was opened via a left lateral thoracotomy. An
180 overdose of pentobarbital (120 mg/kg IV) was administered and the remaining lung re-inflated
181 within the thorax by tracheal instillation of 2.5% buffered glutaraldehyde at a hydrostatic

182 pressure of 25 cmH₂O above the sternum. After the flow of fixative ceased, the tracheal tube
183 was closed to maintain airway pressure. The lungs were removed intact, immersed in buffered
184 2.5% glutaraldehyde, floated on a water bath, and stored at 4°C for at least 4 weeks before
185 processing.

186 *Lung morphometry* Volume of the left caudal lobe was measured by saline immersion; then
187 the lobe was serially sectioned (2 cm thickness). Volume of the sectioned stress-free lobe was
188 measured using the Cavalieri Principle, an established method of measuring the volume of
189 irregularly shaped objects. The total area of serial slices is estimated by point counting, then
190 multiplied by slice thickness to yield lobe volume (52). An unbiased systematic random sampling
191 scheme was used to select 8 blocks per lobe (21). Sectioned slices were arranged on a tray in
192 the same orientation with a grid overlay. From a random start, tissue samples (1.5 cm in each
193 dimension) were systematically selected at a fixed interval along grid points (4 each at
194 subpleural and interior locations). Tissue blocks were post-fixed with 1% osmium tetroxide in
195 0.1M cacodylate buffer, treated with 2% uranyl acetate, dehydrated through graded alcohol, and
196 embedded in Spurr resin (Electron Microscopy Sciences, Hartfield, PA). The other remaining
197 lobes were processed separately for other studies.

198 An established stratified analytical scheme (21) was used under low and high power light
199 microscopy (LM; 275x and 550x) and transmission electron microscopy (TEM; ~16,000x). For
200 LM, each block was sectioned (1µm) and stained (toluidine blue). One section per block was
201 overlaid with a test grid. At 275x, at least 20 non-overlapping fields were systematically sampled
202 from a random start. Excluding the structures between 20 µm and 1 mm in diameter; the volume
203 densities of fine parenchyma, alveolar sac and alveolar duct were estimated using point
204 counting. At 550x, at least 20 non-overlapping fields were systematically sampled to estimate
205 the volume density of alveolar septa. For TEM, each block was sectioned (70 nm) and mounted
206 on copper grids. Each grid was examined at ~16,000x (JEOL EXII). At least 30 non-overlapping

207 fields per grid were systematically sampled. Volume densities of epithelium type I and II,
208 interstitium, endothelium and capillaries were estimated using point counting with alveolar
209 septum as the reference space. Surface densities of alveolar epithelial and capillary were
210 estimated using intersection counting (21). At least 300 points or intersections were counted per
211 grid. Harmonic mean barrier thickness of the blood-gas barrier (τ_{hb}) was measured from the
212 lengths of intercept lines between alveolar surface and erythrocyte membrane.

213 Absolute volumes and surface areas of individual structures were calculated from the products
214 of fractional quantities estimated at each level. Prevalence of double capillary profiles, an index
215 of intussusceptive capillary formation (8, 22, 38), was calculated by completely sampling 2 grids
216 under TEM (~2,500x) and expressed as a ratio of (double capillaries)/(total number of
217 capillaries).

218 *Statistical analysis* Results (mean±SD) were normalized by body weight where appropriate.
219 Pressure-volume curves and temporal changes (pre- to post-PNX) between treatment groups
220 were compared by factorial and/or repeated measures ANOVA with post hoc test by Fisher's
221 protected least significant difference (STATVIEW v.5.0). Morphometric parameters were
222 compared between groups by unpaired t test. A p value ≤ 0.05 was considered significant.

223 **Results**

224 One animal in in iPSC CM group died from acute post-operative pulmonary bleeding; one
225 animal in CFM group was terminated due to post-operative vomiting and weight loss. The
226 remaining 11 animals (6 iPSC CM, 5 CFM) completed the study without complications.
227 Physiological data are summarized in **Table 1 and Figures 2-3**. Body weight, systemic
228 hematocrit and pre-PNX lung function were similar between groups (**Table 1**). In both groups,
229 post-PNX static lung volume at a given transpulmonary pressure was lower (**Figure 2**) while
230 specific lung compliance was similarly unchanged compared to pre-PNX (**Table 1**). During

231 rebreathing, mean alveolar O₂ tension and septal tissue volume were unchanged pre- to post-
232 PNx. Mean lung volumes were similarly maintained post-PNx in both groups (**Figure 3A-B**).
233 Pulmonary blood flow decreased post-PNx in CFM group and changed variably in iPSC CM
234 group (**Figure 3C**). Post-PNx DL_{CO} declined in both groups; the magnitude of decline from pre-
235 PNx in iPSC CM group (29%) was attenuated compared to that in the CFM group (48%,
236 p=0.026) by paired analysis (**Figure 3D**). In a similar pattern, DM_{CO} declined pre- to post-PNx in
237 CFM group (42%, p=0.04) whereas the post-PNx decline in iPSC CM group was less and not
238 significantly different from pre-PNx (14%, p=0.19) (**Figure 3E**). Baseline V_c was highly variable
239 in CFM group and pre-to-post-PNx paired comparison did not reach significance (p=0.19)
240 (**Figure 3F**). In iPSC group, pre-PNx V_c was less variable and declined consistently post-PNx
241 (p=0.03). The average V_c magnitude did not differ significantly between groups pre- or post-
242 PNx (p=0.15 and 0.44, respectively) and the average decline (post/pre-PNx ratio) also did not
243 differ (0.68 vs. 0.60 in CFM and iPSC groups, respectively, p=0.69).

244 Plasma oxidative damage markers and total antioxidant capacity (**Figure 4A to 4C**) increased
245 post-operatively then declined at different rates. Plasma 8-OHdG, a marker of DNA oxidative
246 damage, steadily increased up to four-fold post-PNx, peaking around day 10 then slowly
247 declined but still remained elevated at 55 d (**Figure 4A**). Plasma 8-isoprostane, a marker of lipid
248 oxidation, increased only mildly post-PNx then declined below pre-PNx baseline (**Figure 4B**).
249 Total antioxidant capacity also increased modestly post-PNx then returned to baseline in ~10
250 days. At this arbitrary secretome dose these profiles did not differ significantly between
251 treatment groups.

252 Morphometric results are available in 5 animals per group (**Tables 2-4** and **Figures 5-7**). The
253 lung from one animal (iPSC CM group) was excluded due to inadequate inflation and fixation. In
254 the iPSC CM group, volume of the remaining left caudal lobe was 26% larger (p<0.05)
255 compared to the control group (p=0.04) due to larger alveolar ducts and sacs (**Table 2, Figure**

256 **6A-6B**) as shown by representative micrographs (**Figure 5**). Volume and surface densities
257 (**Table 3**) and absolute volumes and surface areas (**Table 4**) of septal tissue and capillary
258 components did not differ between groups. With iPSC the alveolar surface area was 21% higher
259 but did not reach statistical significance ($p=0.09$) (**Table 3, Figure 6C**). The prevalence of
260 double capillary profiles was significantly (39%) higher in the iPSC CM group ($p=0.006$) (**Table**
261 **4, Figure 6D**), consistent with enhanced intussusceptive capillary formation. The frequency
262 distribution of harmonic mean blood-gas barrier thickness (τ_{hb}) shifted significantly towards the
263 lowest value category (**Figure 7**), suggesting remodeling and re-arrangement of septal
264 constituents and reduced barrier resistance to diffusion while the arithmetic thickness of alveolar
265 septum was similar between groups (**Table 2**). The combined results indicate larger volume and
266 air spaces, enhanced intussusceptive alveolar capillary formation, and septal remodeling that
267 reduced barrier resistance to diffusion in secretome-treated lungs. These changes are
268 associated with a 25% higher estimated morphometric diffusing capacity of the tissue-plasma
269 barrier (DB_{O_2}) ($p=0.068$) (**Table 4**) and correspond to the better preservation of post-PNX whole
270 lung DL_{CO} measured by a physiological method (**Figure 3D**).

271 **Discussion**

272 *Summary of the main findings* This is the first report to establish the feasibility and
273 efficacy of inhaled iPSC-derived cell-free secretome for enhancing post-PNX compensation in a
274 large animal model. Pre-PNX lung function was similar between groups. Post-PNX plasma 8-
275 OHdG level remained elevated for 55 d post-PNX, indicating persistent DNA oxidative stress
276 while plasma 8-isoprostane and total antioxidant capacity levels increased mildly and
277 transiently; the temporal profiles did not differ between groups at this treatment dose. In
278 secretome-treated compared to control animals, physiologic whole lung volume measured at
279 near total lung capacity was similar. However, postmortem stress-free volume of the caudal lobe
280 was 26-30% larger in secretome-treated animals. Differences between antemortem and

281 postmortem volumes suggest dynamic extra-pulmonary factors, e.g., thoracic and/or
282 diaphragmatic restriction, that limited lung expansion in the living animal. Secretome-treated
283 animals exhibited increased intussusceptive alveolar angiogenesis (double capillaries) and
284 septal remodeling with enlarged terminal airspaces and reduced barrier resistance to diffusion;
285 absolute alveolar surface area was also higher without reaching statistical significance. These
286 structural changes resulted in a 25% higher conductance of the blood-gas barrier (borderline
287 significance), which is consistent with physiological findings of better preservation of DM_{CO} and
288 a 23% higher physiologic DL_{CO} in post-PNX animals treated with iPSC CM. We conclude that
289 repetitive inhalational delivery of iPSC secretome enhanced endogenous post-PNX
290 angiogenesis and acinar and alveolar septal remodeling leading to modest and significant
291 improvement in gas exchange compensation. These findings highlight the plasticity of adult lung
292 units that remain following destructive processes and the responsiveness of these units to
293 broad-based modulation provided by iPSC secretome.

294 *Critique of the methods* Both iPSCs (10, 11, 33, 44) and mesenchymal stem cells (MSCs)
295 (12, 27-29, 49) or their secretome possess injury-alleviating and tissue regenerative potential. A
296 Phase I trial of systemic infusion of allogenic MSCs on aging frailty showed promising results
297 compared to placebo (13); however, more studies are needed in this and other conditions. Here
298 we chose to test a cell-free iPSC secretome preparation for enhancing post-PNX compensation;
299 the preparation has been characterized and shown to alleviate experimental lung injury (10, 11,
300 44). The iPSCs may be derived from readily available sources, e.g., human dermal fibroblasts,
301 and can differentiate into a variety of cell types including organ-specific MSCs (43). Other cell
302 types, e.g., fibroblasts, may secrete some of the same ingredients but lack the overall injury-
303 alleviating capacity of iPSCs (10, 11). There are no direct comparisons between iPSCs and
304 MSCs for post-PNX compensation; this comparison is beyond the scope of our report but may
305 be pursued in the future. The inhalational approach (6) and physiologic and morphometric

306 assessment (21) are established. An earlier canine study of post-PNX inhalation treatment of
307 exogenous erythropoietin documented the delivery of exogenous protein to the caudal lobe (6)
308 and showed that alveolar septal changes and the magnitude of enhancement of angiogenesis
309 were comparable among all post-PNX remaining lobes; structural response in the caudal lobe
310 was representative of that of the remaining lung (6, 7). In the current study, the largest
311 remaining lobe (left caudal, ~55% of total left lung volume) was sampled for morphometry. Both
312 subpleural and central lobar regions were sampled.

313 The derived components of DL_{CO} (DM_{CO} and V_c) are interdependent quantities that typically
314 exhibit larger variability than DL_{CO} . In CFM group post-PNX DM_{CO} declined 42% ($p=0.04$ vs. pre-
315 PNX) whereas in iPSC CM group the decline was less (14%) and the pre-to-post-PNX paired
316 values were not significantly different ($p=0.19$), suggesting that iPSC CM minimized post-PNX
317 decline in DM_{CO} , i.e., a similar pattern as DL_{CO} . V_c is sensitive to hemodynamic fluctuations,
318 exhibiting a variable baseline in CFM group that hampered inter-group comparisons.
319 Nevertheless, post-PNX V_c was less variable and reached similar levels in both groups
320 consistent with morphometric results where the average postmortem alveolar capillary blood
321 volume also did not differ significantly between groups ($p=0.82$) (**Table 4**). Variability in baseline
322 V_c does not alter the conclusion that DL_{CO} , the primary measure of diffusive gas exchange, was
323 better preserved in post-PNX animals treated with iPSC CM.

324 Two plasma biomarkers, 8-isoprostane and 8-OHdG, that reflect oxidative stress damage to
325 lipid and DNA, respectively, and are known to increase post-PNX (6), were measured along with
326 total antioxidant capacity. Plasma 8-OHdG was persistently elevated at 55 d post-PNX (**Figure**
327 **4A**), indicating ongoing DNA oxidative stress most likely related to the still increased mechanical
328 stresses on the remaining lung. Pulmonary delivery of iPSC secretome attenuates oxidative
329 damage in acute hyperoxic lung injury (11); a higher dose or more frequent dosing may be
330 needed to attenuate persistent post-PNX mechanically induced oxidative stress. The optimal

331 dose-response relationships remain to be determined. Treatment duration (~8 weeks) spanned
332 the early post-PNX period marked by active cell proliferation and matrix deposition; progressive
333 architectural remodeling and further gains in lung function continue well beyond this period (20,
334 55, 56). Therefore, a longer period of therapy and monitoring will be needed to assess the
335 ultimate outcome. Physiological assessment was made under anesthesia; the responses while
336 awake or during exercise may differ. This study used female animals; sex differences in
337 response to PNx are largely explained by body size (Dane, Kernstine, Hsia, unpublished data).
338 Further studies will be required to determine any sex differences in response to iPSC secretome
339 treatment.

340 *Mechanically induced post-PNX lung growth and remodeling* Post-PNX increases in lung
341 volume, perfusion and blood volume of the remaining lobes transduce compensatory responses
342 (5, 7, 37, 59). We previously documented a threshold, optimal range and upper limit of post-
343 PNx compensatory response, summarized in (38). Following left PNx (42% of lung units
344 removed), most of the remaining lobes compensated via recruitment of existing alveolar-
345 capillary reserves without new tissue growth, except the right infracardiac lobe that underwent
346 the largest expansion across the midline anterior and caudal to the cardiac fossa with nearly
347 two-fold increases in tissue-capillary volumes. Following right PNx (58% resection), mechanical
348 stress on the remaining lobes exceeded a growth-stimulating threshold and all the remaining
349 lobes exhibited 2 to 2.5 fold increase in alveolar septal components. Following bilateral
350 resection removing up to 70% of lung units (~35% each side) the remaining lobes exhibited
351 significant though diminished alveolar-capillary growth than that after 58% resection, suggesting
352 that an optimal stimulus-response range was exceeded.

353 *Interventions to augment compensatory lung growth* Attempts to enhance innate post-
354 PNx responses using individual growth promoters in rodent models led to mixed cellular and
355 structural effects (14, 24, 25, 40, 57) and few studies assessed the functional outcome on gas

356 exchange. In our earlier canine studies (4, 6, 34, 51, 54), oral *all-trans* retinoic acid significantly
357 enhanced active alveolar re-growth after right PNX (34, 51) but had no effect after left PNX (50),
358 suggesting that pharmacological agents modify active mechanically-induced lung growth but
359 cannot re-initiate growth de novo in the absence of sufficient mechanical stimuli. Delivery of
360 retinoic acid or recombinant erythropoietin enhanced post-PNX alveolar double-capillary
361 formation, consistent with the notion of intussusceptive angiogenesis as an essential event in
362 compensatory lung growth (22). Retinoic acid augmented post-PNX volumes of alveolar type-1
363 epithelium, interstitial collagen and matrix, endothelium and pulmonary capillary blood, but the
364 volume increase in type-2 epithelium lagged behind; structural distortion developed with thicker
365 alveolar septa and basement membrane and smaller air spaces indicating inadequate
366 remodeling (4, 34, 51). Erythropoietin possesses potent cytoprotective and pro-angiogenic
367 properties; inhaled recombinant human erythropoietin improved post-PNX in vivo distribution of
368 pulmonary blood flow (54) and abrogated oxidative stress damage (6), but exerted only minor
369 effects on extravascular alveolar tissue compartments. In spite of enhancing certain aspects of
370 the post-PNX response, neither of the above agents augmented lung function above that in
371 vehicle-treated post-PNX control animals. This “*structure-function gap*” in response to
372 pharmacological intervention is not surprising as each agent can stimulate only a subset of
373 interacting growth-related pathways, and may elicit supra-physiologic responses causing
374 distortion at micro- and macro-scopic levels. Furthermore, the selective structural alterations
375 were not accompanied by appropriate acinar or alveolar septal remodeling to optimize gas
376 exchange efficiency. This structure-function gap provides rationale for a cocktail approach such
377 as that offered by the iPSC secretome consisting of a broad panel of counter-acting mediators
378 capable of supporting balanced modulation of post-PNX adaptation without distortion to
379 ultimately attain functional benefit.

380 *The iPSC secretome* Delivery of stem cell secretome has been called an empirical “*shotgun*”
381 approach; yet this approach directly and mechanistically addresses the pre-requisite for useful
382 lung growth and remodeling leading to functional compensation, i.e., the need for balanced
383 physiologic modulation of all relevant mediator pathways and gas exchange structures with
384 minimal distortion. Few studies focused on iPSC secretome; our preparation has been
385 characterized by proteomic analysis, and found to contain >1,200 proteins (10, 11) including a
386 markedly enriched α Klotho content (10-25 fold of that in normal serum). α Klotho is an essential
387 cell maintenance and cytoprotective protein with pleiotropic actions including anti-apoptosis and
388 potent antioxidation via activation of the nuclear factor (erythroid 2)-related factor 2 (Nrf2)
389 network of endogenous antioxidant proteins (11, 35, 36). The lung normally does not express
390 α Klotho (58) but depends on kidney-derived circulating α Klotho for cytoprotection (35, 36).
391 Immunodepletion of α Klotho from iPSC CM reduced in vitro cytoprotective effects of iPSC CM
392 by ~50% (11). Tracheal delivery of this secretome preparation alleviated bleomycin and
393 hyperoxia induced acute lung injury compared to fibroblast conditioned media (10, 11),
394 enhanced total antioxidant capacity, ameliorated oxidative damage to DNA, lipid, and protein,
395 and broadly activated endogenous antioxidant proteins (11). Others reported that iPSCs or their
396 secretory products reduced pro-inflammatory and pro-fibrotic cytokines and chemokines (17)
397 and iPSC-derived exosomes/microvesicles protected against cardiac ischemia/reperfusion
398 injury (46). Gene network analysis of lung interstitial macrophages from bleomycin-injured rats
399 treated with this secretome preparation demonstrated modulation of multiple pathways involved
400 in immunomodulation, branching morphogenesis and canonical Wnt signaling (44). Our data
401 support the benefit of *intact iPSC secretome* in modulating the manifold adaptive mechanisms
402 required for compensatory alveolar angiogenesis and remodeling to facilitate more efficient
403 diffusive gas exchange. iPSC-derived cytoprotective factors such as α Klotho may have
404 permitted fuller expression of the innate compensatory potential of the remaining lung.

405 Comparisons between the single-agent and our broad-based interventional approaches offer
406 several useful insights:

407 1) Pharmacological augmentation of the formation of new alveolar tissue-capillary elements
408 alone is insufficient for achieving functional benefit unless the increase in these elements is
409 accompanied by appropriate architectural remodeling to optimize gas exchange.

410 2) Since lung growth and regeneration involve multiple dynamic processes, a broad-based
411 cocktail approach is superior to single or a few agents for bridging the structure-function gap in
412 attempts to amplify innate adaptive responses. This general concept is analogous to the
413 “cocktail” therapy routinely used in cancer chemotherapy to broadly target the myriad factors
414 that promote tumor growth and spread.

415 3) Individual secretome-induced structural modulations may be modest or borderline
416 significant, but assume cumulative significance with respect to global lung function. For
417 example, the 5% lower ($p < 0.05$) average harmonic mean barrier thickness (τ_{hb}) in secretome-
418 treated lungs was due to a preferential shift in the frequency distribution of τ_{hb} to the thinnest
419 part of the barrier (a 9% increase, $p = 0.02$, **Figure 7**). This modest shift disproportionately
420 increases gas transfer efficiency, because alveolar gas conductance is proportional to the
421 reciprocal of τ_{hb} (48) and >90% of alveolar gas exchange takes place across the thinnest part of
422 the septa while the thicker parts mainly provide physical support. Combined with the 17-20%
423 higher ($p = 0.09$) alveolar-capillary surface areas, these changes could account for a 25% higher
424 morphometric estimate of barrier conductance ($p = 0.068$) and a 23% higher physiologic DL_{CO}
425 ($p = 0.04$) in animals treated with iPSC CM compared to CFM over ~8 weeks. These are very
426 reasonable rates of improvement considering the need to maintain balanced structure-function
427 responses and minimize distortion throughout all regions of a large stratified lung.

428 *Conclusions* Previous attempts to amplify post-PNX compensatory responses were partially
429 successful but identified an important challenge of *structure-function dissociation*, i.e., individual
430 exogenous growth promoters augmented selective aspects of tissue-capillary growth but not
431 architectural remodeling or functional outcome. We report here that inhalation of a well-
432 characterized cell-free iPSC secretome preparation overcame this dissociation by enhancing
433 post-PNX angiogenesis and alveolar remodeling leading to more efficient gas exchange. These
434 novel findings reinforce the concept that post-PNX compensation is a highly orchestrated multi-
435 phasic process involving myriad pathways and mediators many of which are not classically
436 considered to be growth promoters. In addition to mechanically stimulated cell proliferation and
437 tissue-capillary growth, progressive structural remodeling minimized resistance of the diffusion
438 barrier, a critical requirement for achieving functional compensation. Innate post-PNX
439 compensation may be augmented by supplementing with iPSC secretome composed of broad-
440 based mediators in a physiologically relevant cocktail. These results established the feasibility
441 and efficacy of inhalational delivery of iPSC secretome in the canine model.

442 Many aspects of this emergent approach require further investigation, including characterization
443 and optimization of secretome composition and bioactivity, elucidation of the mechanisms of
444 action and interaction among the components, and determination of the degrees to which the
445 major components contribute to the observed effects. Our premise is that the *entire secretome*
446 is responsible for coordinated enhancement of post-PNX angiogenesis and remodeling required
447 for translating post-PNX tissue growth into functional gain; future studies may compare intact
448 secretome with subfractions such as exosomes and microvesicles. Unlike the short-term use of
449 secretome in acute lung injury, the post-PNX remaining lung undergoes progressive remodeling
450 over many months with gradual functional improvement (56), and may be susceptible to
451 secretome action throughout this period as the existing acinar scaffold is slowly modified.
452 Therefore, sustained modest and balanced modulation is preferable to acute supra-physiologic

453 skewed stimulation. Prolonged secretome therapy may be needed to maximize long-term
454 structural and functional gain and facilitate realization of the full innate compensatory potential.
455 Measurement of lung function during exercise may accentuate treatment effects. Impact of the
456 secretome delivery approach extends beyond the PNX model to regenerative therapy for
457 parenchymal lung diseases irrespective of the specific etiology.

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464 **Conflict of interest**

465 The authors have no conflict of interest to declare.

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657

658 **Figure legend**

659 **Figure 1. Timeline of the studies.** CFM: cell-free media; iPSC CM: iPSC conditioned media.

660 **Figure 2. Lung volume-transpulmonary pressure relationship pre- and post-PNX.** Lung
661 volume at a given transpulmonary pressure was similarly lower post-PNX compared to pre-PNX
662 in animals treated with cell-free media (CFM) or iPSC conditioned media (iPSC CM). Mean \pm
663 SD. Repeated measures ANOVA. Number of animals: 5 CFM, 6 iPSC CM.

664 **Figure 3. Lung function pre- and post-PNX** in animals treated with cell-free media (CFM) or
665 iPSC conditioned media (iPSC CM). **A.** End-inspiratory lung volume (EILV). **B.** End-expiratory
666 lung volume (EELV). **C.** Pulmonary blood flow. **D.** DL_{CO} measured at an inflation volume of 45
667 $ml \cdot kg^{-1}$ and inspired O_2 concentration of 21% was expressed under standard conditions
668 (hematocrit = 0.45, alveolar $PO_2 = 120$ mmHg). **E.** Membrane diffusing capacity (DM_{CO}). **F.**
669 pulmonary capillary blood volume (V_c). Box: Mean \pm SD; whiskers extend to maximum and
670 minimum values. Factorial and repeated measures ANOVA. Number of animals: 5 CFM, 6 iPSC
671 CM.

672 **Figure 4. Changes (post/pre-PNX ratio) in plasma biomarkers.** **(A)** 8-hydroxy-2'-
673 deoxyguanosine (8-OHdG), **(B)** 8-isoprostane, and **(C)** total antioxidant capacity (copper
674 reducing equivalents) in animals treated with cell-free media (CFM) or iPSC conditioned media
675 (iPSC CM). Mean \pm SD. Symbols for $p < 0.05$ with respect to time post-PNX: † vs. Pre-PNX, ‡
676 vs. 1 h post-PNX (0 d), § vs. 5 d, # vs. 10 d, @ vs. 20 d, ¶ vs. 30 d, \$ vs. 40 d by factorial
677 ANOVA. Overall comparison between treatment groups by repeated measures ANOVA: **(A)**
678 $p = 0.69$, **(B)** $p = 0.39$, and **(C)** $p = 0.25$. Number of animals: 5 CFM, 6 iPSC CM.

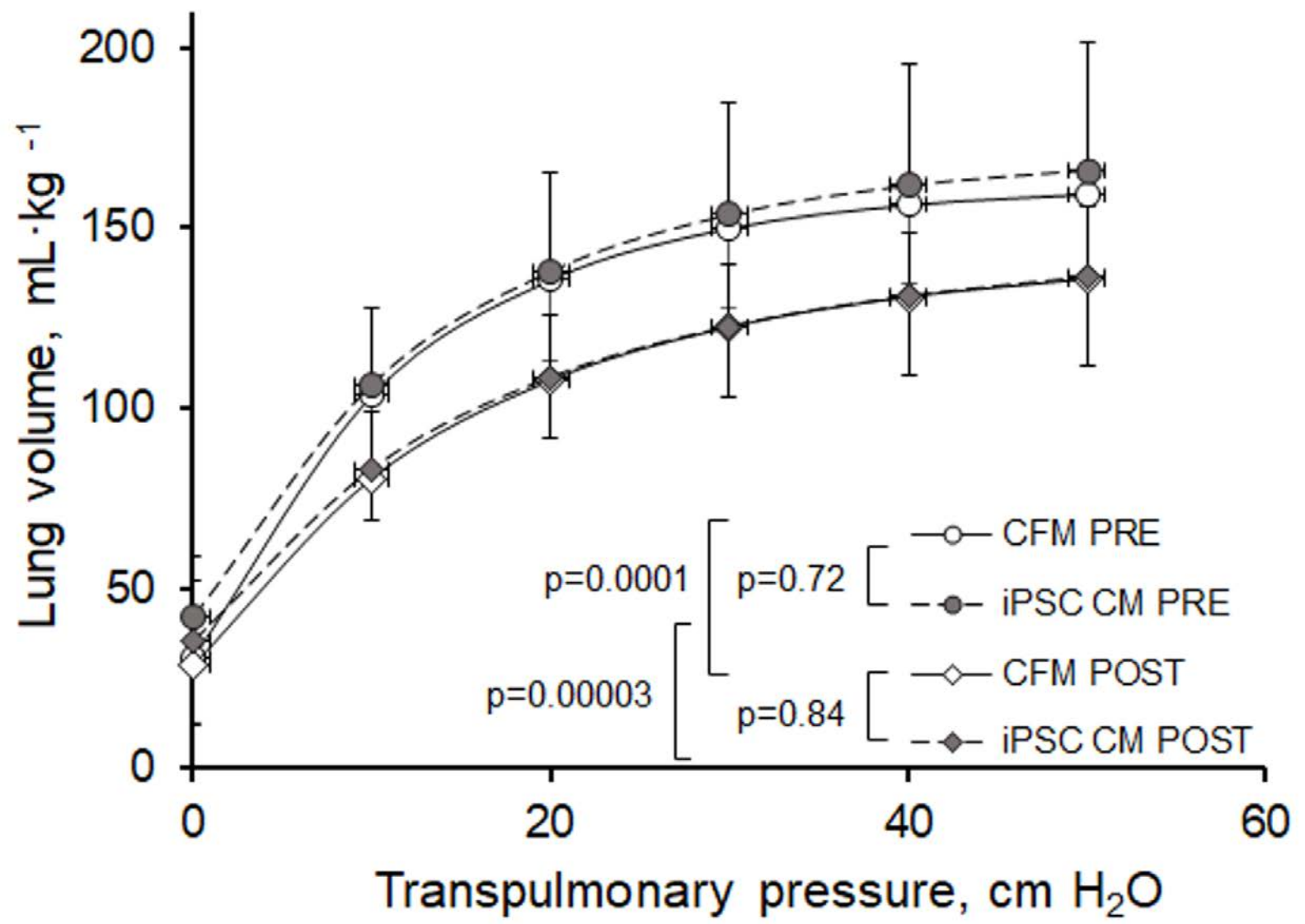
679 **Figure 5.** Representative distal lung morphology under light and electron microscopy in post-
680 PNX animals treated with cell-free media (CFM) or iPSC conditioned media (iPSC CM),

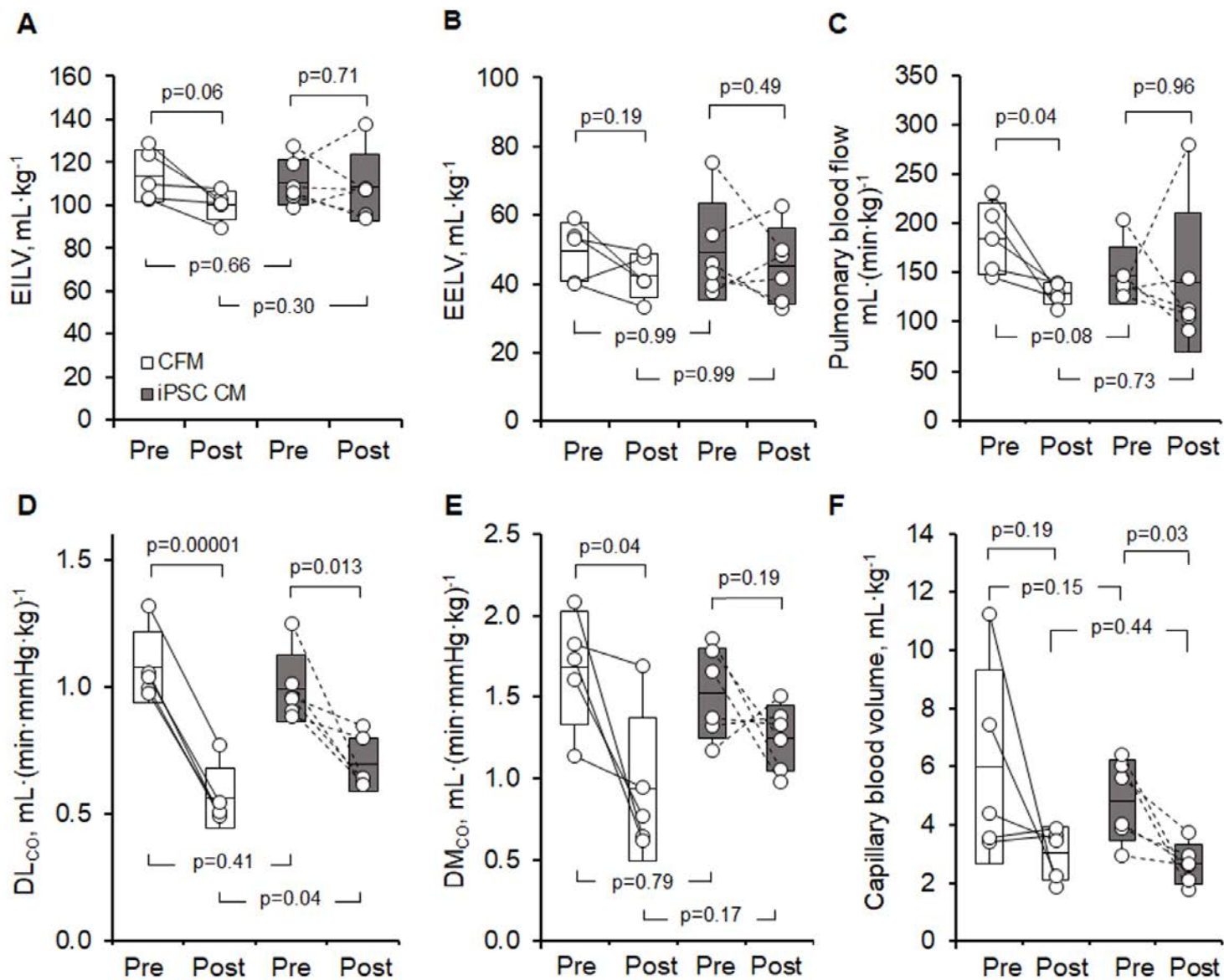
681 illustrating a thinner septal tissue layer in iPSC CM group on the “thin side” of the blood-gas
682 barrier where the bulk of alveolar gas exchange takes place. Top panels: bar = 50 μm . Lower
683 panels: bar = 2 μm .

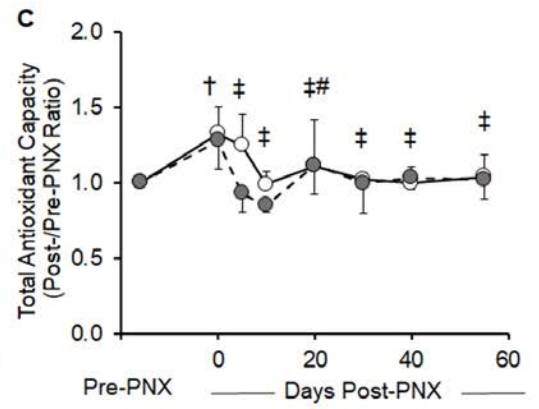
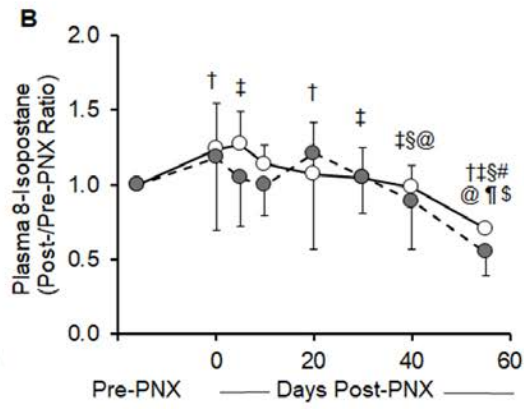
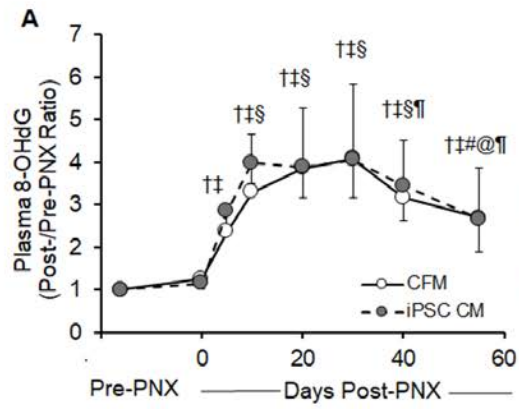
684 **Figure 6. Morphometric results** in the left caudal lobe in post-PNX animals treated with cell-
685 free media (CFM) or iPSC conditioned media (iPSC CM). **(A)** Total lobe volume (intact or serial
686 sectioned). **(B)** Volume of alveolar sacs and ducts. **(C)** Alveolar surface area. **(D)** Prevalence of
687 alveolar double capillary profiles. Box: Mean \pm SD; whiskers extend to maximum and minimum
688 values. P values indicate iPSC CM vs. CFM by unpaired t-test. Five animals per group.

689 **Figure 7. Frequency distribution of harmonic mean thickness of the tissue-plasma barrier**
690 in post-PNX animals treated with cell-free media (CFM) or iPSC conditioned media (iPSC CM).
691 Box: Mean \pm SD; whiskers extend to maximum and minimum values. P values shown indicate
692 iPSC CM vs. CFM in each barrier thickness category by unpaired t-test. Overall comparison
693 between treatment groups by repeated measures ANOVA ($p=0.16$). Five animals per group.
694

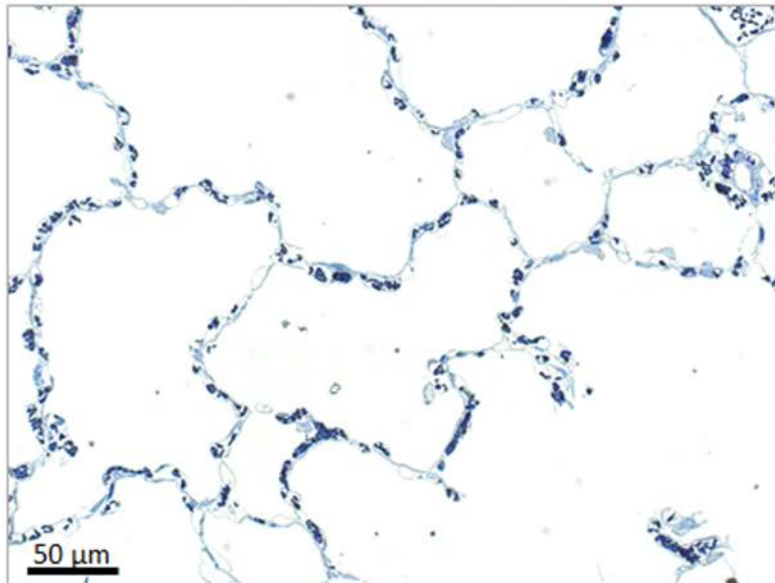




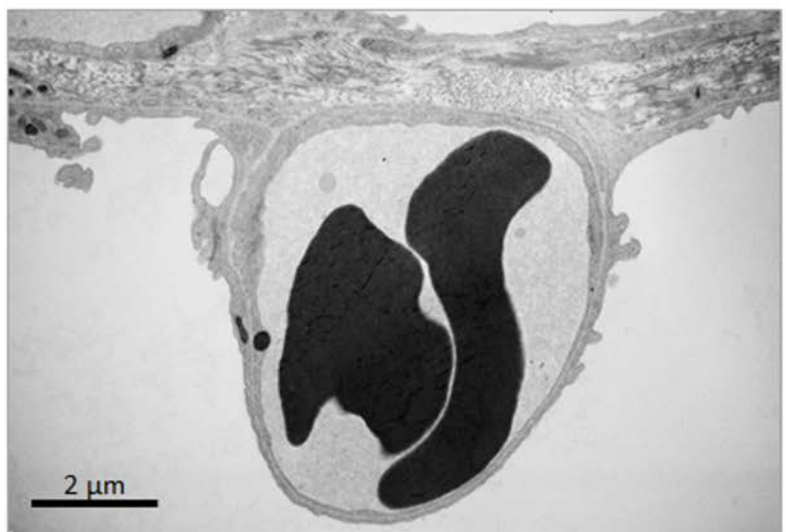
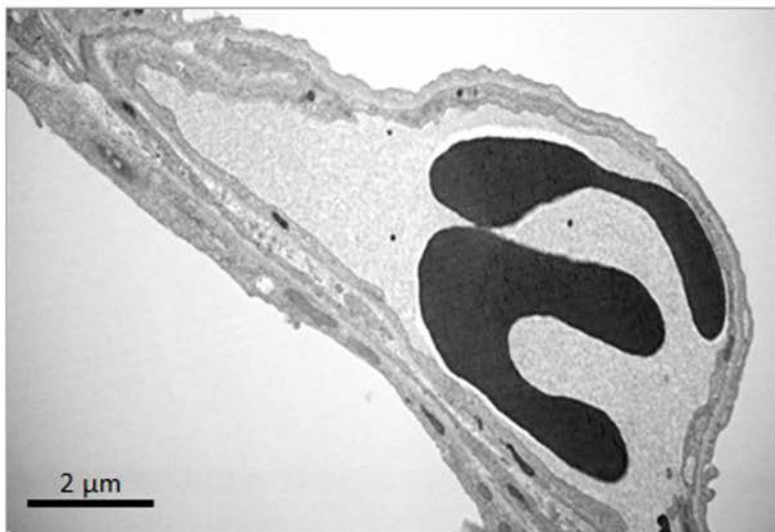
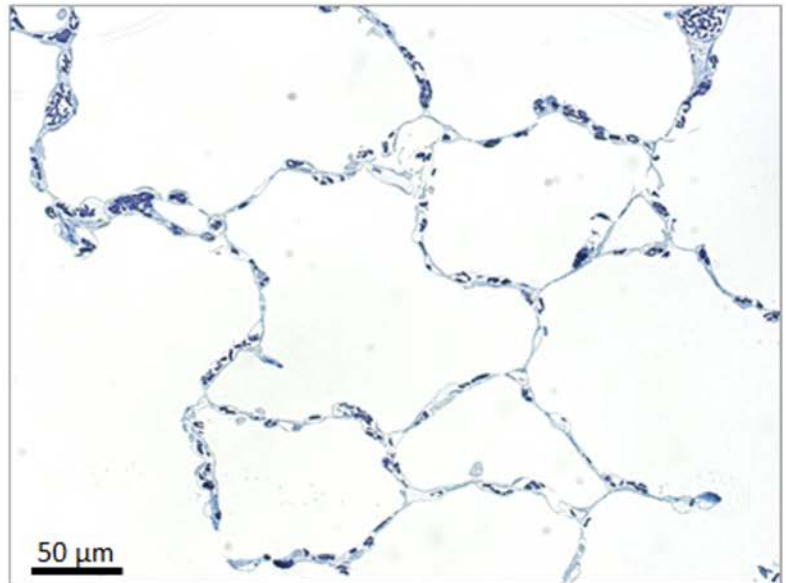


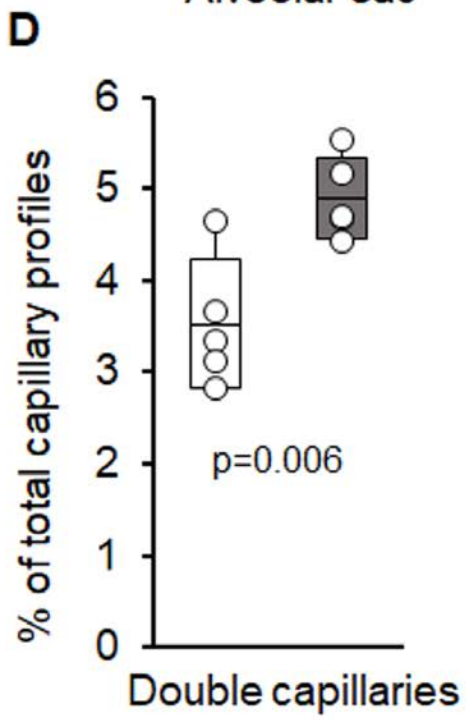
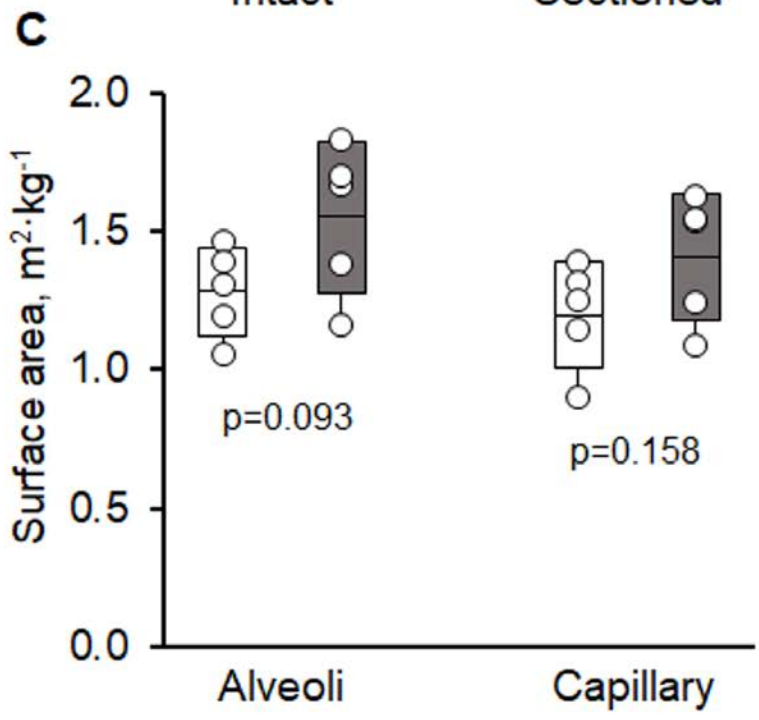
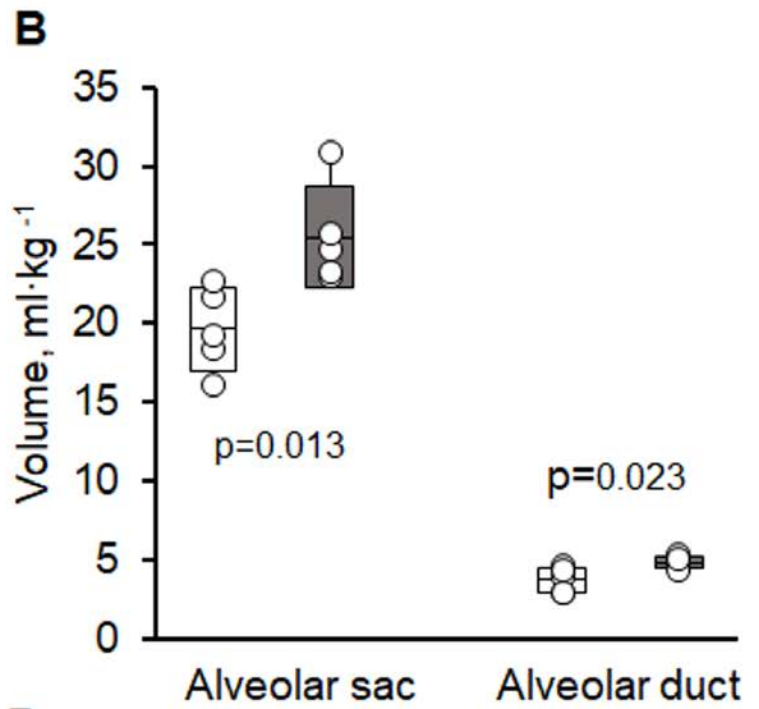
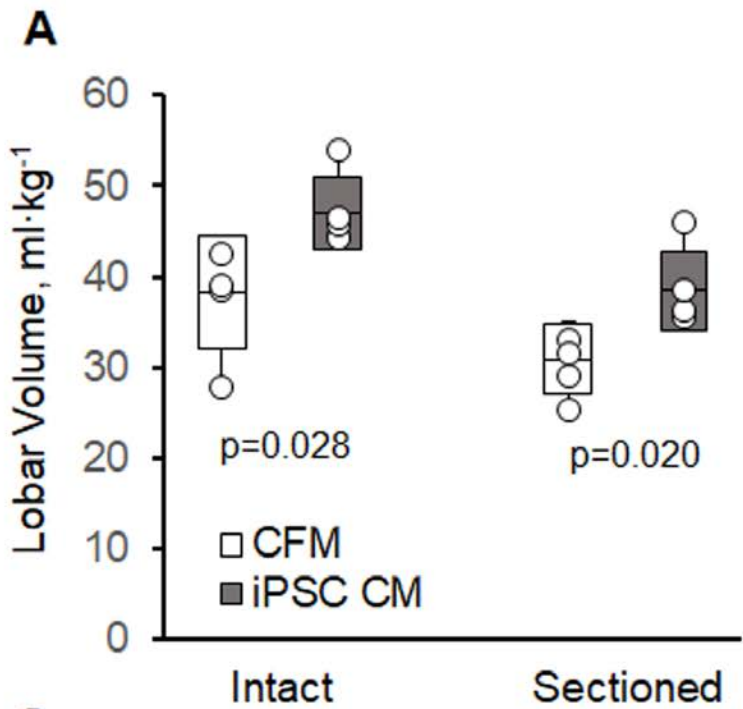


Cell-free media



iPSC CM





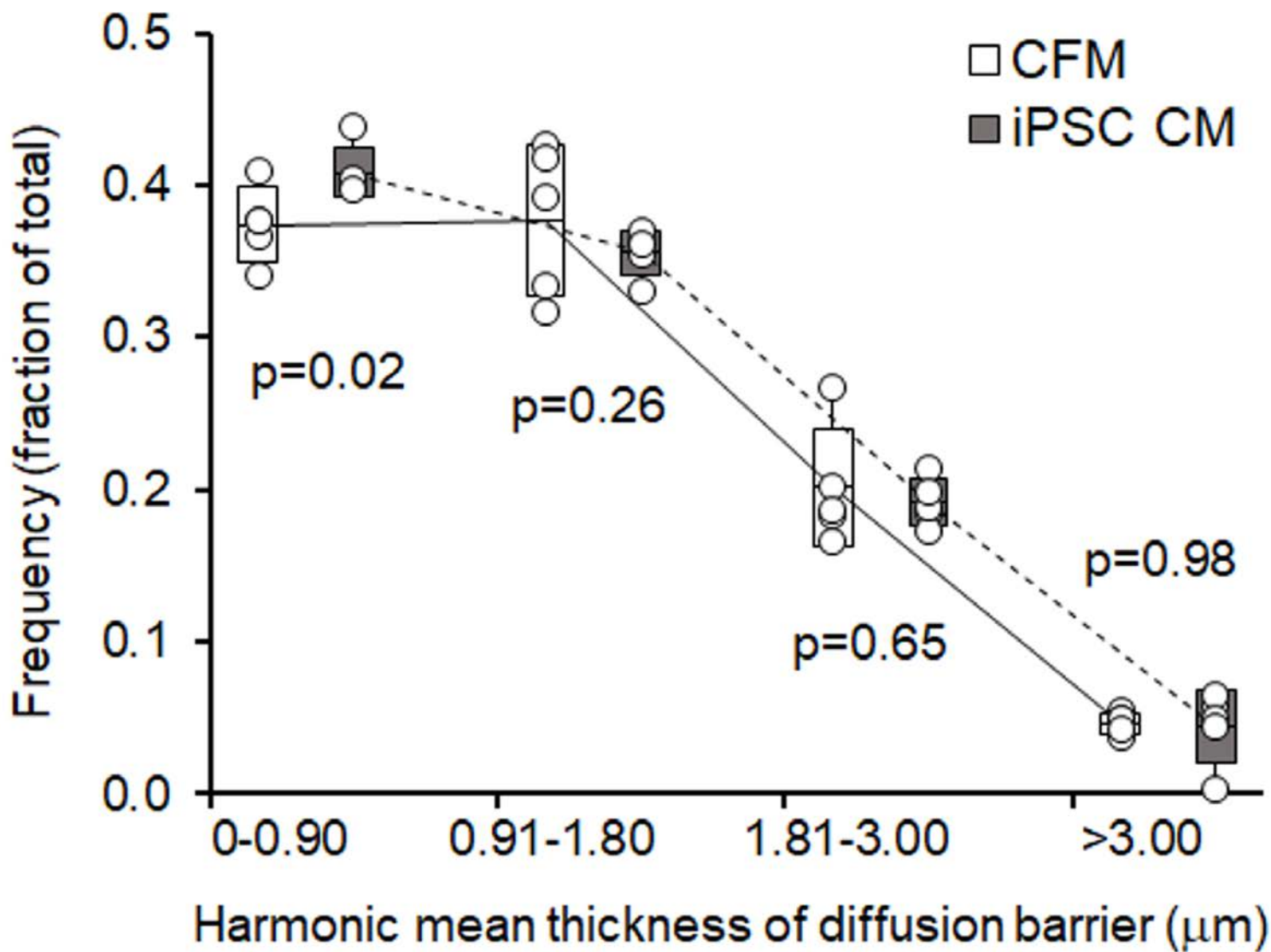


Table 1. Lung function

Group	CFM		iPSC CM		P value		
	PRE-PNX	POST-PNX	PRE-PNX	POST-PNX	vs. Group	vs. PNx	Group*PNx
N	5	5	6	6			
Body weight, kg	18.4 ± 1.6	18.5 ± 2.1	18.2 ± 0.3	18.5 ± 1.8	0.87	0.78	0.83
Hematocrit, %, sedated	42.8 ± 1.8	39.9 ± 3.2	42.0 ± 2.7	40.0 ± 2.8	0.78	0.048	0.68
Specific lung compliance, mL·(cmH ₂ O·L) ⁻¹	23.0 ± 5.8	27.7 ± 5.9	22.0 ± 2.4	24.5 ± 5.1	0.32	0.13	0.63
Alveolar PO ₂ breathing 21% O ₂ , mm Hg	125.3 ± 36.4	129.3 ± 19.5	128.8 ± 30.9	135.9 ± 18.5	0.70	0.60	0.88
Alveolar PO ₂ breathing 99% O ₂ , mm Hg	664.7 ± 14.3	669.9 ± 4.0	663.1 ± 4.8	670.1 ± 4.4	0.86	0.12	0.77
End-expiratory lung volume, mL·kg ⁻¹	55.7 ± 11.9	49.0 ± 9.1	52.5 ± 10.6	50.1 ± 16.4	0.85	0.38	0.67
End-inspiratory lung volume, mL·kg ⁻¹	113.8 ± 12.0	100.2 ± 6.8 #	110.7 ± 10.6	108.4 ± 15.6	0.68	0.08	0.19
Pulmonary blood flow, mL·(min·kg) ⁻¹	184.5 ± 35.8	129.2 ± 11.6 †	146.8 ± 28.8	140.4 ± 70.8	0.45	0.17	0.26
DL _{NO} , mL·(min·mm Hg·kg) ⁻¹	3.46 ± 1.25	2.61 ± 0.46	3.22 ± 0.54	2.58 ± 0.64	0.65	0.07	0.78
DL _{CO} measured, mL·(min·mm Hg·kg) ⁻¹	1.01 ± 0.16	0.50 ± 0.06 †	0.87 ± 0.04	0.58 ± 0.11 †	0.60	<.0001 †	0.004 ‡
DL _{CO-std} , mL·(min·mm Hg·kg) ⁻¹	1.08 ± 0.14	0.56 ± 0.12 †	0.99 ± 0.13	0.69 ± 0.10 *†	0.71	<.0001 †	0.04 ‡
DM _{CO} , mL·(min·mm Hg·kg) ⁻¹	1.68 ± 0.35	0.93 ± 0.44 †	1.53 ± 0.28	1.25 ± 0.20	0.51	0.01 †	0.17
Capillary blood volume, mL·kg ⁻¹	6.00 ± 3.34	3.03 ± 0.91	4.82 ± 1.38	2.65 ± 0.69 †	0.22	0.02 †	0.68
Septal tissue volume, mL·kg ⁻¹	6.62 ± 1.70	6.60 ± 2.65	5.96 ± 3.01	6.14 ± 3.85	0.66	0.95	0.94

Mean±SD. Repeated measures ANOVA: * p<0.05 vs. CFM group post-PNX. † p<0.05, # p=0.06 vs. corresponding Pre-PNX group. ‡ p<0.05 Group*PNx interaction. Specific lung compliance was measured at transpulmonary pressure between 10 and 30 cm H₂O, normalized by lung volume at 10 cm H₂O. DL_{CO} was measured at 45 mL/kg inflation volume. DL_{CO-std}: results were expressed at standardized conditions (hematocrit = 0.45 and alveolar PO₂ =120 mmHg).

Table 2. Morphometric Data

	CFM	iPSC CM	P value
Number of animals	5	5	
Terminal body weight (kg)	18.5 ± 2.1	18.6 ± 1.9	0.902
Total lobar volume, mL·kg ⁻¹			
Intact (Immersion method)	38.2 ± 6.2	47.0 ± 4.0	* 0.028
Sectioned (Cavalieri method)	30.9 ± 3.9	38.5 ± 4.3	* 0.020
Morphometric hematocrit, %	43.0 ± 2.4	44.4 ± 2.4	0.357
Arithmetic mean septal thickness, μm	4.73 ± 0.56	4.41 ± 0.49	0.356
Harmonic mean barrier thickness, (τ_{hb}), μm	0.97 ± 0.03	0.92 ± 0.03	* 0.046
Double capillary profiles, %	3.52 ± 0.70	4.90 ± 0.44	* 0.006

Mean±SD. * p<0.05 iPSC CM vs. CFM by unpaired t test.

Table 3. Volume-to-Volume and surface-to-volume ratios of alveolar structures

	CFM	iPSC CM	p value
<i>Volume per unit lung volume</i>			
Coarse parenchyma	0.8870 ± 0.0234	0.9039 ± 0.0154	0.213
Fine parenchyma	0.8723 ± 0.0225	0.8914 ± 0.0133	0.141
Respiratory bronchioles	0.0286 ± 0.0096	0.0292 ± 0.0049	0.904
Alveolar sac	0.6370 ± 0.0165	0.6620 ± 0.0148	* 0.035
Alveolar duct	0.1211 ± 0.0175	0.1267 ± 0.0079	0.536
Septum (tissue+blood)	0.0982 ± 0.0095	0.0886 ± 0.0141	0.246
Total epithelium	0.0166 ± 0.0011	0.0156 ± 0.0027	0.467
Type I epithelium	0.0101 ± 0.0012	0.0094 ± 0.0022	0.555
Type II epithelium	0.0065 ± 0.0005	0.0062 ± 0.0015	0.677
Interstitialium	0.0232 ± 0.0048	0.0210 ± 0.0037	0.438
Collagen fibers	0.0184 ± 0.0031	0.0163 ± 0.0032	0.320
Cells and matrix	0.0049 ± 0.0022	0.0048 ± 0.0011	0.924
Endothelium	0.0118 ± 0.0022	0.0122 ± 0.0031	0.828
Septal Extravascular tissue	0.0517 ± 0.0068	0.0489 ± 0.0087	0.583
Capillaries	0.0465 ± 0.0136	0.0398 ± 0.0093	0.391
<i>Surface area per unit lung volume, cm⁻¹</i>			
Alveolar Surface	416 ± 18	403 ± 57	0.643
Capillary Surface	387 ± 24	366 ± 51	0.428

Mean±SD. * p<0.05 IPS vs. CFM by unpaired t test.

Table 4. Absolute volumes, surface areas, and conductance for oxygen

	CFM	iPSC CM	P value
	<i>Volume, ml·kg⁻¹</i>		
Coarse parenchyma	27.41 ± 3.87	34.80 ± 4.16	* 0.020
Fine parenchyma	26.96 ± 3.80	34.32 ± 4.09	* 0.018
Alveolar sac	19.67 ± 2.64	25.49 ± 3.16	* 0.013
Alveolar duct	3.75 ± 0.78	4.86 ± 0.41	* 0.023
Respiratory bronchioles	0.89 ± 0.31	1.13 ± 0.24	0.212
Septum	3.04 ± 0.58	3.40 ± 0.54	0.348
Total epithelium	0.51 ± 0.08	0.61 ± 0.15	0.253
Type I	0.31 ± 0.06	0.36 ± 0.10	0.369
Type II	0.20 ± 0.02	0.24 ± 0.08	0.260
Interstitialium	0.72 ± 0.18	0.81 ± 0.14	0.409
Collagen fibers	0.57 ± 0.13	0.62 ± 0.12	0.496
Cells and matrix	0.15 ± 0.07	0.18 ± 0.04	0.385
Endothelium	0.36 ± 0.06	0.47 ± 0.14	0.155
Extravascular tissue	1.59 ± 0.28	1.88 ± 0.41	0.224
Capillaries	1.45 ± 0.54	1.51 ± 0.29	0.825
	<i>Surface area, m²·kg⁻¹</i>		
Alveolar surface	1.28 ± 0.16	1.55 ± 0.27	0.093
Capillary surface	1.20 ± 0.19	1.41 ± 0.23	0.158
O ₂ conductance of tissue-plasma barrier (Db _{O₂}), mL·(min·mmHg·kg) ⁻¹	1.95 ± 0.29	2.46 ± 0.46	0.068

Mean±SD. * p<0.05 iPSC CM vs. CFM by unpaired t test.