

1 **Attenuating endogenous Fgfr2b ligands during bleomycin-induced lung fibrosis does**
2 **not compromise murine lung repair**

3

4 BreAnne MacKenzie¹, Ingrid Henneke¹, Stefanie Hezel¹, Denise Al Alam², Elie El Agha¹,
5 Cho-Ming Chao¹, Jennifer Quantius¹, Jochen Wilhelm¹, Matthew Jones¹, Kerstin Goth¹,
6 Xiaokun Li³, Werner Seeger¹, Melanie Koenigshoff⁴, Susanne Herold¹, Albert A. Rizvanov⁷,
7 Andreas Günther^{1,5,6}, and Saverio Bellusci,^{1,2,7,#}

8

9 ¹German Center for Lung Research, Excellence Cluster Cardio-Pulmonary System,
10 Universities of Giessen and Marburg Lung Center, Giessen, Hessen, Germany.

11

12 ²Developmental Biology Program, Division of Surgery, Saban Research Institute of
13 Children's Hospital Los Angeles, University of Southern California Keck School of Medicine,
14 Los Angeles, California

15

16 ³School of Pharmacy, Wenzhou Medical College, China

17

18 ⁴Comprehensive Pneumology Center, Ludwig Maximilians University, University Hospital
19 Grosshadern, and Helmholtz Zentrum München, Munich, Bavaria, Germany.

20

21 ⁵AGAPLESION Lung Clinic Waldhof-Elgershausen, Greifenstein, Germany

22

23 ⁶Member of the German Center for Lung Research

24

25 ⁷Institute of Fundamental Medicine and Biology, Kazan
26 Federal University, 18 Kremlyovskaya Street, Kazan 420008, Russian
27 Federation

28

29 # Corresponding author

30 Saverio.Bellusci@innere.med.uni-giessen.de

31 Abstract

32 Fibroblast growth factors (Fgfs) mediate organ repair. Lung epithelial cell overexpression of
33 *Fgf10* post-bleomycin injury is both protective and therapeutic, characterized by increased
34 survival and attenuated fibrosis. Exogenous administration of FGF7 (Palifermin) also showed
35 prophylactic survival benefits in mice. The role of endogenous Fgfr2b ligands upon
36 bleomycin-induced lung fibrosis is still elusive. This study reports the expression of
37 endogenous Fgfr2b ligands, receptors and signaling targets in wild type mice following
38 bleomycin lung injury. In addition, the impact of attenuating endogenous Fgfr2b-ligands
39 following bleomycin-induced fibrosis was tested using a doxycycline (dox)-based inducible,
40 soluble, dominant-negative form of the Fgfr2b receptor. Double transgenic (DTG)
41 *Rosa26^{rtTA+};tet(O)solFgfr2b* mice were validated for the expression and activity of soluble
42 Fgfr2b (failure to regenerate maxillary incisors, attenuated recombinant FGF7 signal in the
43 lung). As previously reported, no defects in lung morphometry were detected in DTG (+dox)
44 mice exposed from post-natal (PN) 1 through PN105. Female single transgenic (STG) and
45 DTG mice were subjected to various levels of bleomycin injury (1.0, 2.0, and 3.0U/kg). Fgfr2b
46 ligands were attenuated either throughout injury (d0-d11; d0-28) or during later stages (d6-
47 d28 and d14-d28). No significant changes in survival, weight, lung function, confluent areas of
48 fibrosis, or hydroxyproline deposition were detected in DTG mice. These results indicate that
49 endogenous Fgfr2b ligands do not significantly protect against bleomycin injury, nor do they
50 expedite the resolution of bleomycin lung induced injury in mice.

51

52

53

54 **Introduction**

55 The interstitial lung disease, Idiopathic Pulmonary Fibrosis (IPF) occurs between the sixth
56 and seventh decades of life at a rate of 2-4/10000 (21). 5-year survival rates approximate just
57 10-15% (21). The pathomechanism of IPF is not yet fully understood, however it is thought to
58 occur as a result of chronic epithelial injury or stress, resulting in the accumulation of
59 myofibroblasts which express high levels of extracellular matrix (19). Genetic manipulation of
60 lung development pathways in the context of bleomycin-lung injury; including: Notch,
61 transforming growth factor beta (Tgf β), Bone morphogenetic protein (Bmp), Sonic hedgehog
62 (Shh), fibroblast growth factors (Fgfs), epidermal growth factor (Egf), and wingless-type
63 MMTV integration site family, (Wnt), combined with results based on studies using IPF patient
64 materials have led to the development of potential therapeutic treatments for IPF (31, 38).

65
66 Fgf7 and Fgf10 signal in a paracrine fashion via epithelially-expressed Fgfr2b receptor (43).
67 The signal results in a phosphorylation cascade, mediated by fibroblast growth factor receptor
68 substrate (Frs2), which activates PI3k- and Mapk-signaling pathways and/or activation of
69 phospholipase C gamma (Plc- γ). Depending on the cell type and context, Fgfr2b-signaling
70 culminates in survival, growth and differentiation of epithelial cells. Fgf10/Fgfr2b signaling is
71 critical for murine lung development while Fgf7 is dispensable (2, 16, 27). Fgfs have been
72 reported to act upstream of Wnt-signaling (23). Interestingly, bleomycin-injured mice with
73 epithelial specific deletion of β -catenin-signaling, a downstream target of both Fgf- (23) and
74 Wnt-signaling (26), suffered increased fibrosis (37). While Wnt-signaling was previously
75 thought to play an exclusively pro-fibrotic role by mediating epithelial-to-mesenchymal
76 transition (EMT), this report revealed the importance of β -catenin-mediated protection of
77 epithelial cells against bleomycin-injury.

78
79 In the bleomycin mouse model, past studies have focused primarily on the beneficial effect of
80 prophylactic treatment with Palifermin, a pharmacological agent composed of a truncated
81 form of keratinocyte growth factor (KGF), also known as, FGF7 (7, 36). While Palifermin
82 demonstrated a protective, prophylactic effect, genetic *Fgf10* expression post-bleomycin
83 injury (5) resulted in increased survival as well as prevention and accelerated resolution of
84 lung fibrosis in mice. While current therapies target tyrosine kinases for the treatment of IPF
85 (1), whether endogenous FGF-signaling plays a protective, pathogenic, or ambivalent role in
86 IPF, is still unknown. Given the beneficial effects of exogenous Fgfr2b ligands on lung repair,
87 the authors hypothesized that endogenous Fgfr2b ligands play a critical role in repair
88 following bleomycin-injury. However, endogenous Fgfr2b ligands as well as Fgfr2b receptor
89 expression, were decreased following bleomycin-injury in wild type mice. Thus unsurprisingly,
90 attenuating endogenous Fgfr2b-ligands during bleomycin-induced lung injury did not lead to
91 significantly increased fibrosis or decreased survival. In summary, although endogenous
92 Fgfr2b ligand signaling failed to play a critical role in limiting fibrosis, these results do not

93 negate the potential benefit of exogenously stimulating developmental pathways to protect
94 against lung fibrosis.

95

96

97

98

99

100

101

102

103

104

105

106

107

108

109

110

111

112

113

114

115

116

117

118

119

120

121

122

123

124

125

126

127

128

129

130

131 **Methods**

132 **Animal Care**

133 All experiments were performed in accordance with the National Institutes of Health
134 Guidelines for the Use of Laboratory Animals. Animal experiments were approved by the
135 Institutional Animal Care and Use Committee at Children's Hospital Los Angeles protocol
136 193-12 and the Federal Authorities for Animal Research of the Regierungspraesidium
137 Giessen, Hessen, Germany protocols 72/2012 and 73/2012.

138

139 **Generation of Mice**

140 *CMV-Cre* mice (33) were crossed with *rtTA^{flox}* mice (9) to generate mice expressing *rtTA*
141 under the ubiquitous *Rosa26* promoter. This constitutive *Rosa26^{rtTA/+}* mouse line was then
142 crossed with the *tet(O)solFgfr2b/+* responder line to generate *Rosa26^{rtTA/+};tet(O)sFgfr2b/+*
143 double heterozygous animals, allowing ubiquitous expression of dominant-negative soluble
144 *Fgfr2b* (28). All mice were generated on a CD1 mixed background. Attenuation of *Fgfr2b*
145 ligand activity was achieved by administration of doxycycline-containing food; normal rodent
146 diet with 0.0625% doxycycline (Harlan Teklad). Tet(O)Cre (B6.Cg-Tg(tetO-cre)1Jaw/J) (30)
147 and *Tomato^{flox/flox}* reporter mice (B6;129S6-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J) (24)
148 were purchased from Jackson lab. Mice were genotyped as described previously (3, 12, 34).

149

150 **Bleomycin administration**

151 10–14 week-old female mice were anaesthetized with a mixture of 0.6 µl/g Ketamine 10%
152 (100 mg/ml) and 0.3 µl/g Dormitor 10% (0.5 mg/ml) dissolved in 0.7% saline. A microsyringe
153 (PennCentury) was used to administer an intra-tracheal dose of either 0.7% saline or
154 bleomycin (1.0–3.0U/kg) (Hexal, Germany or Sigma-Aldrich, USA). Weight, activity,
155 respiration and temperature was monitored daily and mice were sacrificed if they showed a
156 significant decline in health parameters.

157

158 **Lung compliance measurement**

159 Mice were deeply anaesthetized with a mixture of 1.2 µl/g Ketamine 10% (100 mg/mL, Bela
160 Pharm, Germany), 0.6 µl/g Domitor 10% (0.5 mg/ml; Orion Finland), 1:4 parts heparin, and
161 dissolved in saline. Lung function was measured using the SCIREQ Flexi-Vent forced
162 oscillation plethysmograph to give an overall readout of lung function. Mice were intubated
163 trans-tracheally and ventilated at a rate of 150 breaths per minute with a positive end-
164 expiratory pressure (PEEP) between 1 and 3 cmH₂O. PEEP was calculated automatically by
165 FlexiVent 7 software and dependent on the weight of the animal. After stable ventilation was
166 achieved (spontaneous breathing ceased as the heart continued to beat) a 3 second, weight
167 dependent, fixed volume waveform was initiated every 15 – 20 seconds, eight times. During
168 this perturbation, “Snapshots” of respiratory compliance were taken and the average of eight
169 measurements represented the value of one biological sample.

170 **Left lobe perfusion and isolation**

171 The left lobe was perfused from 22cm–24cm above the mouse for 1 min with PBS followed by
172 2min with 4% PFA. The trachea was tied off with a string, and the lung was removed and

173 placed in 4% PFA for at least 24hours at room temperature or up to one week at 4°C. Lungs
174 were then embedded with a Leica embedding machine (EG 1150C). Paraffin blocks were
175 kept cold and 3–4 µm sections were cut.

176

177 **Hematoxylin and Eosin (H/E)**

178 3–4 µm sections were deparaffinized, dipped in water and stained in Mayer's Hematoxylin
179 solution for 1–3 min and washed under running tap water for up to 10 min. Slides were
180 monitored under the microscope for staining progression. Slides were then incubated for 2min
181 in Eosin dye and brought back through increasing gradients of EtOH and xylene, then
182 coverslipped with Pertex mounting media.

183

184 **Masson's Trichrome Stain**

185 3–4 µm sections were deparaffinized, and stained with Gomori's Green Trichrome Stain Kit
186 (Dako AR166) according to manufacturers protocol.

187

188 **Lung morphometry**

189 For alveolar morphometry, lungs were flushed with PBS at a vascular pressure of 20 cm
190 H₂O. Then PBS was infused via the trachea at a pressure of 20cm H₂O and fixed with 4%
191 paraformaldehyde in phosphate-buffered saline (pH 7.0) via the trachea at a pressure of 20
192 cm H₂O. Investigations were performed using 5 µm sections of paraffin-embedded left lobe of
193 the lungs. The mean linear intercept, mean air space, and mean septal wall thickness were
194 measured after staining with hematoxylin and eosin (H/E). Total scans from the left lobe were
195 analyzed using a Leica DM6000B microscope with an automated stage according to the
196 procedure previously described (25, 42) which was implemented into the Qwin V3 software
197 (Leica, Wetzlar, Germany). Horizontal lines (distance 40 µm) were placed across each lung
198 section. The number of times the lines cross alveolar walls was calculated by multiplying the
199 length of the horizontal lines and the number of lines per section then dividing by the number
200 of intercepts. Bronchi and vessels above 50 µm in diameter were excluded prior to the
201 computerized measurement. The airspace was determined as the non-parenchyma, non-
202 stained area. The septal wall thickness was measured as the length of the line
203 perpendicularly crossing a septum. From the respective measurements, mean values were
204 calculated.

205

206 **Fibrosis quantification on histological sections**

207 Ashcroft scoring was performed blinded using a modified Ashcroft scoring protocol as
208 described by (14) on H/E stained sections of murine left lobes. Traditionally, multiple 20X
209 images are scored, however we imaged stained left lobes with light-microscopy at the lowest
210 objective (1.25X), which allowed for visualization of the entire section. ImageJ software was
211 used to measure the area of the lung that was covered in confluent fibrotic mass (data are

212 presented as % confluent fibrosis per total area of the section). Sections were measured
213 blindly, a total of 3 times, and scores were averaged.

214

215 **Hydroxyproline assay**

216 QuickZyme total collagen assay was performed according to manufacturer's instructions.
217 Briefly, either cranial and accessory or caudal and medial lobes were extracted, rinsed briefly
218 in PBS and dried overnight in a ventilated hood. Lungs were weighed and 6 M HCl was
219 added for a final concentration of 50 mg tissue per ml. Lungs and collagen standards were
220 then incubated for 20 hours overnight at 95°C and cooled to room temperature. Next tubes
221 were centrifuged at 13,000 x g for 10 min. Hydrolyzed supernatant was diluted 10-fold with 4
222 M HCl and used for the assay. A microplate reader (Tecan Infinite 200 PRO) was used for
223 color detection. A standard curve was calculated from the collagen standards and the total
224 hydroxyproline content was assessed.

225

226 **RNA extraction**

227 After lung function measurements were taken, the right bronchus was clamped and either
228 cranial and accessory or caudal and medial lobes were removed, placed in TRIZOL,
229 homogenized in GentleMACs and frozen in liquid nitrogen for RNA extraction. Next,
230 transcardiac perfusion of the left lobe was performed with a 20 G needle and 15 ml PBS.

231

232 **Western blot**

233 Loading buffer was added to protein samples from cell extracts (5% SDS in bromophenol
234 blue and β -mercaptoethanol) denatured for 5 min at 95°C and cooled on ice. At least 10 μ g of
235 sample was loaded on a 10% polyacrylamide gel and run at 25 mA per gel for approximately
236 2 hrs. Samples were then electrically transferred to a polyvinylidene fluoride (PVDF)
237 membrane (Amersham) by semi-dry electro blotting (70 mA per Gel; gel size: 7 x 9 cm) for 90
238 min. The membrane was blocked with 5% milk in TBS blocking buffer at RT on shaker for 1 h
239 followed by incubation with primary antibody: COL1a1 (Meridian #T47770R) FGF1 (Abcam
240 #ab9588 1:2000), FGF7 (Santa Cruz #sc27126 1:200), FGF10 (Abcam #ab71794, 1:200),
241 Col1a1 (Meridian #T40777R, 1:1000, 8% gel), SPRY2 (Santa Cruz #sc10082, 1:200), SPRY4
242 (Santa Cruz #sc30051, 1:200), FGFR1 (Santa Cruz #sc-121, 1:200), FGFR2 (Santa Cruz
243 #sc-122, 1:200), p-ERK1/2 (Cell Signaling #4370S; 1:1000), total ERK1/2 (Cell Signaling
244 #9102S; 1:1000), p-AKT total (Cell Signaling #4060S, 1:1000), total-AKT (Cell Signaling
245 #4691S, 1:3000) β -Actin (Abcam #ab8227; 1:30000), GAPDH (Cell Signaling #cs2118,
246 1:1000) overnight at 4°C. After washing with 1X TBS-T four times for 15 min each, the
247 membrane was incubated with swine anti-Rabbit HRP (Dako #P0217) secondary antibody
248 (dilution 1:2000) at RT for 1hr followed by four times washing with 1X TBS-T buffer for 15 min
249 each. The protein bands were detected by ECL (Enhanced Chemi-luminescence, Amersham,
250 Germany) treatment, followed by exposure of the membrane.

251

252 **Quantitative PCR**

253 RNA was reverse-transcribed (Qiagen QuantiTect Reverse Transcription Kit (205313). cDNA
254 was diluted to a concentration between 20 ng/μl. Primers were designed using Roche Applied
255 Sciences online Assay Design Tool. All primers were designed to span introns and blasted
256 using NCBI software for specificity. Sybr Green Master Mix (Applied Biosciences 4309155)
257 was used for RT-PCR with a Roche LightCycler 480 machine. Samples were run in triplicates
258 using *Hprt* as reference genes for mouse samples.

259

260 **FACS**

261 Accessory and caudal lobes were isolated in ice-cold Hank's balanced salt solution (HBSS).
262 Next, lobes were chopped finely using sterile razor blades and transferred to a 10ml solution
263 of 0.5% collagenase in HBSS. Then, solution was heated to 37°C on a hot plate stirring on
264 high for 60 min. Next the dissociated homogenate was passed through 18G, 20G, 24G
265 needles respectively, then filtered through 70 μm and 40 μm filter. One volume HBSS was
266 added to dilute collagenase and homogenates were centrifuged at 1500 rpm for 5 min to
267 remove the enzyme solution. Cells were then resuspended in 500 μl 0.5% FCS in PBS and
268 stained with Anti-RFP pAb Rabbit, Life Technologies, R10367 (1:200) for 20 minutes at 4°C,
269 followed by washing and flow cytometric analysis with LSR Fortessa equipped with
270 FACSDiva™ software (BD Bioscience).

271

272 **Statistical analyses**

273 One-way ANOVA was performed on densitometry plots of western blots followed by a
274 Dunnett's Test of significance. A Student's t-Test was performed on the log-transformed value
275 of the qPCR fold changes as well as compliance, hydroxyproline, and confluent areas of
276 fibrosis measurements. For FACS analyses, t-tests were performed on the probit values. A
277 binomial significance test was used to determine the statistical significance of soluble Fgfr2b
278 detection. Conformity of the data with the assumptions of the tests was checked with residual
279 analysis.

280

281

282

283

284

285

286

287 **Results**

288 **Modest recruitment of the Fgf-signaling pathway during spontaneous repair initiated**
289 **by bleomycin-induced lung injury in mice**

290 To investigate whether the endogenous Fgf-signaling pathway is recruited during bleomycin-
291 induced lung injury, CD1 mice were given 1U/kg bleomycin intratracheally (i.t.) or saline. This

292 bleomycin dose generates robust fibrosis and gives a survival rate of 100% at day 28 in CD1
293 mice (Figure 1A). Mice were sacrificed at given time points according to weight loss to ensure
294 maximal survival to day 28 (Figure 1B). Injury was confirmed in each mouse by lung
295 compliance measurements (Figure 1C), calculation of confluent fibrotic areas of H/E stained
296 left lobes (Figure 1D), hydroxyproline deposition (Figure 1E), Masson's Trichrome staining
297 (Figure F-J), and Col1a1 western blotting of whole lung homogenate (Figure 1K, L). In this
298 model, bleomycin-induced lung injury peaked between 14 and 21 dpi. An additional model
299 using historical controls (5U/kg, C57bl/6 females), was also used to comparatively evaluate
300 Fgf-signaling targets and mRNA expression and similar results were obtained (data not
301 shown).

302

303 Next, western blots were performed on Fgfr2b ligands, receptors, and downstream signaling
304 targets on lung homogenate lysates isolated from animals 7, 14, 21 and 28 days after
305 bleomycin injury or 2 weeks after saline administration. Col1a1 expression was used as an
306 indicator of fibrotic injury and most strongly expressed at 14 dpi and slightly reduced
307 thereafter (Figure 1K, L). Fgf ligands were decreased for the most part following injury with
308 the exception of Fgf10, which was slightly elevated compared to Fgf1 and Fgf7 at 14 dpi
309 (Figure 1K, L). Fgfr1 and Fgfr2 expression increased significantly following injury. Importantly,
310 qPCR analyses indicated that Fgfr11b, Fgfr2c, and Fgfr1c isoforms were increased while
311 Fgfr2b remained decreased (Figure 1M). Spry2, a negative regulator of Mapk-signaling in the
312 epithelium was decreased following injury, though expression was increased at 28 dpi (Figure
313 1K, L). Spry4, a negative regulator of Mapk-signaling in the cells of mesenchymal origin (lung
314 fibroblasts) was slightly elevated at 21 and 28 dpi (Figure 1K, L). Etv-4, a downstream target
315 of Fgf10 signaling, also known as Pea-3, was strongly induced following injury (Figure 1K, L).
316 Lastly, general Mapk-signaling targets, which are also targets of Fgf-signaling were
317 evaluated. At 7 dpi, p-Akt was slightly increased and again strongly activated at 28 dpi (Figure
318 1K,L). The marker p-Erk1 peaked at 14 dpi and decreased at 28 dpi (Figure 1K, L), while p-
319 Erk2 was not significantly regulated (Figure 1K,L). Compared to mice injured with a higher
320 dose of bleomycin (5U/kg, data not shown), p-Akt signaling was similarly regulated. It was
321 strongly induced 3–4 weeks following injury (Figure 1K,L). In addition, p-Erk1 and p-Erk2
322 were moderately regulated in both models.

323

324 In summary, during spontaneous repair after bleomycin injury in wild type mice, Fgf10
325 expression was moderately increased along with receptor Fgfr1b, and downstream targets
326 Spry2, Etv4, and p-Akt at 28 dpi. The endogenous, epithelial expressed Fgfr2b receptor, was
327 drastically reduced following bleomycin-injury (Figure 1M) and began to recover expression at
328 21 dpi while c-isoform expression remained significantly elevated. The significance and
329 contribution of the Fgf10/Fgfr1b-signaling axis to lung repair is still elusive and will require
330 further investigation.

331

332 **Validation of the *Rosa26^{rtTA/+};tet(O)sFgfr2b/+* (DTG) transgenic line**

333 To test whether the attenuation of all Fgfr2b ligands would result in increased fibrosis, the
334 *Rosa26^{rtTA/+};tet(O)sFgfr2b/+* ; in this study referred to as the double transgenic (DTG) mouse
335 line was used (28) (Figure 2A). The *Rosa26* promoter drives ubiquitous expression of reverse
336 tetracycline transactivator, which in the presence of doxycycline (dox), binds a tetracycline
337 response sequence. Binding results in the activation of a CMV promoter, which drives
338 expression of a chimeric transgene containing the extracellular binding domain of Fgfr2b
339 fused with the heavy chain domain of IgG (Figure 2A). The line was previously used in the
340 context of naphthalene and hyperoxia lung injury to demonstrate the critical role of Fgfr2b
341 ligands in the lung repair process (12, 13, 39). In addition, this line was also used to define
342 the role of Fgfr2b ligands during early lung embryonic and late lung development (22), limb
343 development (5) postnatal mammary gland development (29) incisor homeostasis (28) and
344 gut homeostasis (6).

345
346 In this study, DTG littermates lacking the *Rosa26^{rtTA/+}* construct (*Rosa26^{+/+};tet(O)sFgfr2b/+*)
347 were used as controls, and referred to as single transgenics or (STG). To confirm the
348 functionality of soluble Fgfr2b in our experimental conditions, validation was also performed in
349 adult female animals. First, the lung-specific efficiency of the driver was tested using
350 *Rosa26^{rtTA/+};tet(O)Cre/+;Tomato^{fl/+}* mice fed +dox-food for 7 days (Figure 2E-F). RFP
351 expression was detected in approximately 25% of total cells by FACS and in none of the cells
352 in mice lacking the *tet(O)Cre* transgene as illustrated by fluorescence stereomicroscopy
353 (Figure 2B-E”).

354
355 In order to confirm the function of the chimeric receptor in adult lungs, a study was performed
356 to test the ability of induced DTG lungs (+dox food ad libitum) to attenuate exogenous FGF7
357 signaling. Female STG mice were used as controls and received PBS with 0.1% BSA, the
358 same solution in which the recombinant FGF7 was resuspended. DTG mice were fed either
359 normal food or +dox food for one week. Next, females from each group received an intra-
360 tracheal dose of 10 µg of rFGF7. Then, 30 minutes later, the whole lung was collected, and
361 lysates were blotted for p-Akt and p-Erk1/2 signals. While +dox DTG mice blocked the FGF7-
362 mediated elevation in p-Akt and p-Erk1-signals to levels of PBS-treated controls, p-Erk2
363 remained elevated. Failure to block p-Erk2 was possibly due to overabundance of FGF7 and
364 resultant signaling via endogenous Fgfr2 receptors (Figure 2F-H’).

365
366 Morphometric analyses were performed on STG and DTG mice fed +dox food from PN1 to
367 PN105 (Figure 2I-L). The chimeric transcript was detected only in DTG mice (Figure 2I) and
368 lung compliance remained unaffected (Figure 2J). In concurrence with previous studies (12),
369 morphometric analyses revealed no significant differences in mean linear intercept, airspace,
370 or septal wall thickness in DTG mice (Figure 2K-M). While no lung defects were present, DTG

371 mice fed +dox food from postnatal days (PN) 28-88 showed characteristic inhibition of
372 maxillary incisor regeneration (Figure 4H-H') (5).

373

374 **Attenuation of *Fgfr2b* ligands during fibrosis formation post-bleomycin injury did not**
375 **result in increased fibrosis**

376 To determine whether endogenous *Fgfr2b* ligands expedite fibrosis resolution, a relatively low
377 dose of bleomycin (1U/kg i.t.), which generated between 80% and 100% survival and mild
378 fibrosis at day 28 was used. First, *Fgfr2b* ligands were attenuated from (d6-d14) (Figure 3)
379 and next during fibrotic resolution (d14-d28) (Figure 4). *Fgfr2b* ligands attenuation had no
380 impact on relative survival (Figure 3A) though a trend towards delayed weight recovery in
381 DTG mice was observed (Figure 3B). Specific soluble *Fgfr2b* expression in DTG was
382 confirmed (Figure 3C). A slight increase in lung compliance was measured at day 28 in DTG
383 vs. STG (Figure 3D). Upon histological examination both STG and DTG lungs showed areas
384 of fibrosis (Figure 3E-F). However, quantification of the confluent areas of fibrosis in the left
385 lobe did not reveal any difference between DTG and STG (Figure 3G). In agreement with
386 these results, no differences were observed for total hydroxyproline (Figure 3H). Together,
387 these results indicate that attenuation of *Fgfr2b* ligands post bleomycin injury (d6-d28) had no
388 impact on fibrosis resolution by 28 dpi.

389

390 Although downstream targets were just moderately engaged in wild type mice injured with
391 either 1.0U/kg or 5.0U/kg bleomycin doses, a slightly higher bleomycin dose was performed
392 (2U/kg i.t.) in order to test whether endogenous *Fgfr2b* ligands would play an important role in
393 lung repair following more severe injury. To focus on the contribution of *Fgfr2b* ligands to the
394 resolution phase, ligands were attenuated from 14 through 28 dpi. No difference in survival
395 rate (Figure 4A) was observed. The relative weight change between DTG and STG were
396 similar (Figure 4B). The expression of soluble *Fgfr2b* was detected only in DTG as previously
397 reported (Figure 4C). No difference in lung function was observed (Figure 4D). However, no
398 difference was observed for hydroxyproline deposition between DTG and STG (Figure 4H)
399 was observed. In summary, attenuation of *Fgfr2b* ligands post bleomycin injury following the
400 peak of fibrosis (d14-d28) does not have any impact on the extent of fibrosis at day 28.

401

402 **Attenuation of *Fgfr2b* ligands immediately following bleomycin injury did not result in**
403 **increased fibrosis**

404 Attenuating *Fgfr2b* ligands at later stages following injury (d6-d28 and d14-d28) had no effect
405 on the level of bleomycin induced lung injury incurred. As *Fgfr2b* ligands are known to have a
406 protective effect on lung epithelium, next, it was tested whether attenuation of endogenous
407 *Fgfr2b* ligands signaling immediately afterwards and throughout injury impacts the extent of
408 fibrosis incurred at 28 dpi. *Fgfr2b* ligands were attenuated throughout injury (d0-d28). With
409 the exception of one STG animal, all animals survived until sacrificed at day 28 (Figure 5A).
410 No significant differences in relative weight change were observed, although as in Figure 5B,

411 a trend towards delayed weight recovery in DTG mice was observed (Figure 5B). Specific
412 soluble *Fgfr2b* expression in DTG was confirmed (Figure 5C). No significant change in lung
413 compliance was measured at day 28 in DTG vs. STG (Figure 5D). Upon histological
414 examination both STG and DTG had areas of fibrosis (Figure 5E-F). However, quantification
415 of the confluent fibrosis areas did not reveal any difference between DTG and STG lungs
416 (Figure 5G). In agreement with these results, no differences were observed for total
417 hydroxyproline (Figure 5H). Attenuation of *Fgfr2b* ligands in DTG mice for the duration of the
418 injury did not lessen fibrosis incurred at 28 dpi.

419

420 In an attempt to further engage endogenous repair mechanisms, 3.0U/kg i.t of bleomycin was
421 used in the final experiment (Figure 6). The soluble receptor was induced from the day of
422 bleomycin-injury until the day of sacrifice; between 6 and 11 dpi (Figure 6). While the initial
423 aim of this study was to analyze the extent of fibrosis at 28 dpi, 3.0U/kg i.t of bleomycin was
424 too severe for CD1 mice. Therefore mice were sacrificed and analyzed at earlier time points.
425 5 mice from both the STG and the DTG groups were removed at day 6 for analyses based on
426 weight-loss criteria (< 20% of initial weight, data not shown). The other mice (STG; n=3 and
427 DTG; n=4) were analyzed at day 11. Induction of the soluble receptor was detected in DTG
428 mice (Figure 6A). A trend towards increased weight loss for DTG mice that survived to day 11
429 was observed (Figure 6B). While some variability in measurements occurred due to
430 differences in the day of sacrifice (d6-d11), no differences between STGs and DTGs were
431 detected at any time point in regards to compliance (Figure 6C), hydroxyproline (Figure 6D)
432 and the confluent fibrotic areas (Figure 6G). In wild type mice, bleomycin injury triggered
433 decreased *Sftpc* expression at day 7 and *Scgb1a1* at day 14, (data not shown). Therefore,
434 expression of AECII cell specific transcript *Sftpc*, *Scgb1a1* for Clara cells, and the general
435 epithelial marker *EpCam* were measured in animals sacrificed at 6 dpi. However, no
436 differences in marker expression were observed between DTG and STG lungs (Figure 6H-J).

437

438

439

440

441 **Discussion**

442 ***Fgfr2b* ligand signaling is important for lung development and repair**

443 Fgfs play pleiotropic roles both during organogenesis and homeostasis (15). *Fgfr2b* ligands are
444 part of a family of 22 identified members. *Fgf10* is required for lung formation and loss of
445 *Fgf10* leads to lung agenesis while loss of *Fgf1* and *Fgf7* during development does not result
446 in a lung phenotype in mice. The cellular and molecular basis for differences between *Fgf7*
447 and *Fgf10* signaling are still unclear. Recently, mass spectrometry-based proteomics revealed
448 that *Fgf7* and *Fgf10* elicit distinct biochemical response downstream of the *Fgfr2b* receptor. In
449 particular, *Fgf7* leads to rapid but transient phosphorylation of Akt and Shc while *Fgf10* leads
450 to the progressive and sustained phosphorylation of these mediators. In addition, *Fgf10*

451 triggers phosphorylation of tyrosine 734 and the associated recruitment of SH3bp4, a
452 relatively novel adaptor protein. It has been proposed that Fgf10 leads to increased recycling
453 of Fgfr2b at the cell surface while Fgf7 results in transient signaling (8). Unlike Fgf7 and
454 Fgf10, Fgf1, not only signals via Fgfr2b, but via all Fgf receptor isoforms.

455

456 Fgf10 appears to be not only critical for the survival and proliferation of the distal epithelial
457 lung progenitors (35) but also for the repair of the bronchiolar epithelium following
458 naphthalene exposure (39). Lung epithelial cell specific overexpression of *Fgf10* in mice
459 during the first, second or third week post-bleomycin injury was both protective and
460 therapeutic as characterized by increased survival and attenuated lung fibrosis (11).
461 Administration of Fgf7 or Palifermin, a pharmacological agent composed of a truncated form
462 of Fgf7, also reduced fibrosis and increased survival both in both rats and mice (7, 10, 22,
463 32). In humans, haploinsufficiency for *FGF10* is associated with COPD (18). Likewise, in
464 humans, SNPs in *FGF7* correlate with increased risk for developing COPD (4).

465

466 In contrast to previous studies which used exogenous Fgfr2b ligands to attenuate
467 bleomycin-induced lung injury, this study first assessed the activation of endogenous Fgfr2b
468 ligands in injured wild type mice, and then attenuated Fgfr2b ligands following bleomycin
469 injury in order to assess their contribution to lung repair. Given the significant reduction in
470 Fgfr2b receptor expression following bleomycin injury, it was not surprising that attenuation of
471 endogenous Fgfr2b ligands had no effect on fibrosis outcome.

472

473

474 **DTG mice efficiently attenuate endogenous Fgfr2b ligands**

475 The *Rosa26^{rtTA/+};tet(O)sFgfr2b/+* (DTG) mouse line was used to ubiquitously trap all Fgfr2b-
476 ligands during injury. Fgf1, in addition to ligands of the Fgf7 subfamily: Fgf3, Fgf7, Fgf10, and
477 Fgf22, bind most strongly to Fgfr2b (43). Though Fgf10 contains a nuclear localization signal
478 and may in some contexts act in an intracrine fashion (20), the *Rosa26^{rtTA/+};tet(o)sFgfr2b/+*
479 model has been demonstrated to mimic the loss of *Fgf10* expression during development (35)
480 demonstrating that Fgf10's activity in the lung is mainly via secreted, paracrine signaling. The
481 impact of trapping Fgfr2b ligands during lung injury has been previously reported. Induction of
482 soluble Fgfr2b under the *SpC-rtTA/+* promoter in adult mice subjected to hyperoxia injury led
483 to abnormal expression of surfactant during injury which contributed to increased lethality
484 (12). A similar result was obtained using *Rosa26^{rtTA/+};tet(o)sFgfr2b/+* neonates exposed to
485 hyperoxia (Chao and Bellusci, unpublished results). Furthermore, in the context of
486 naphthalene injury (39) as well as H1N1 influenza virus infection (Quantius et. al., manuscript
487 in preparation) lung injury was increased in DTG animals. Mice used in this study were
488 thoroughly validated and demonstrated both the embryonic and adult phenotypes
489 characteristic of this line.

490

491 **Attenuation of endogenous Fgfr2b ligands during bleomycin-injury did not result in**
492 **increased fibrosis**

493 Doxycycline fed bleomycin-injured *Rosa26^{rtTA/+};tet(o)sFgfr2b/+* (DTG) mice did not incur
494 increased fibrosis compared to injured STG mice. There are several explanations for the lack
495 of increased injury in DTG mice following bleomycin injury. First, the weak recruitment of Fgf
496 ligands in this injury model indicated an insignificant contribution of endogenous Fgfr2b-
497 ligands to the repair process. Second, while solFgfr2b traps both Fgf7 and Fgf10 ligands,
498 which have been shown to convey protective signals to the injured epithelium, it also traps
499 Fgf1, which binds to all Fgfrs. Though its effects are not well characterized, Fgf1-signaling is
500 potentially ambivalent in the context of bleomycin lung injury. As Fgf1 signals to cells of both
501 epithelial and mesenchymal origins, blocking an Fgf1-mediated survival signal to fibroblasts
502 following bleomycin injury, especially following 14 dpi, when fibroblasts are most abundant,
503 may be beneficial. Moreover, in a study using a soluble, dominant negative Fgfr2c-isoform
504 construct, it was demonstrated that blocking mesenchymal Fgfr2c mediated signaling
505 following bleomycin injury attenuated fibrosis (17). Third, although FACS analyses of
506 *Rosa26^{rtTA/+};Tomato^{fl/+}* mice revealed that doxycycline induced *rtTA* expression in 25% of the
507 lung cells, for maximal ligand attenuation during bleomycin injury, a lung specific driver may
508 be required. Lastly, compensation by other endogenous repair pathways such as Wnt (37),
509 may adequately compensate for the attenuation of Fgf-signaling. In the future, lung cell type
510 specific models targeting specific ligands and receptors are needed to more accurately
511 dissect the role of Fgf-signaling in lung repair.

512

513 **Tyrosine kinase inhibitors demonstrate therapeutic effects in bleomycin-treated mice**
514 **further suggesting endogenous Fgfr2b ligand signaling is dispensable for repair**

515 Tyrosine kinase receptors mediate a variety of growth factor signaling pathways. High levels
516 of MAPK and ERK phosphorylation are associated with IPF. Three major tyrosine kinase
517 pathways relevant for lung disease have been described: the VEGFR and PDGFR and the
518 FGFR pathways. The tyrosine kinase inhibitor BIBF1120 blocks these pathways and
519 demonstrated a protective and therapeutic in the bleomycin model (40). In addition, phase III
520 clinical trials demonstrated that BIBF1120 treatment both decreased the rate of decline in
521 forced vital capacity and reduced the number of acute exacerbations in IPF patients (41). In
522 mouse models, just as enhanced activation of the epithelial receptor tyrosine kinase Fgfr2b-
523 isoform via exogenous Fgfr2b ligands leads to epithelial protection and lessens fibrosis,
524 attenuation of Fgfr2c-isoform signaling also attenuates fibrosis. These results suggest that
525 enhanced Fgfr2b-isoform signaling expedites lung protection and repair, while Fgfr2c-isoform
526 ligand signaling (via Fgf1, Fgf2, Fgf9) may fuel the fibrosis fire by relaying survival signals to
527 fibroblasts. In conclusion, although global tyrosine kinase inhibitors such as BIBF1120 likely
528 inhibit FGFR2b-signaling, the contribution of VEGF, PDGF, and FGFR2(c) to fibrosis
529 formation may be far greater. Whether exogenously stimulating Fgfr2b-signaling following

530 treatment with tyrosine kinase inhibitors further attenuates bleomycin-induced fibrosis injury
531 remains to be investigated.

532

533

534

535

536

537

538

539

540

541

542

543

544

545

546

547

548

549

550 **Acknowledgements**

551 We would like to thank Jonathan Branch, Clarence Wigfall, and Soula Danopoulos for their
552 technical support at CHLA. AAR and SB acknowledge the support of the Russian
553 Government Program of Competitive growth of Kazan Federal University.

554

555

556

References

- 557 1. **Antoniou SA.** Nintedanib (BIBF 1120) for IPF: a tomorrow therapy?
558 *Multidiscip Respir Med* 7: 41, 2012.
- 559 2. **Bellusci S, Grindley J, Emoto H, Itoh N, Hogan B.** Fibroblast growth
560 factor 10 (FGF10) and branching morphogenesis in the embryonic mouse
561 lung [Online]. *Development* 124: 4867–4878, 1997.
562 [http://dev.biologists.org/content/124/23/4867.abstract?ijkey=9bab527d](http://dev.biologists.org/content/124/23/4867.abstract?ijkey=9bab527d4f955e41d0a7ebcc3c868d143209da1a&keytype2=tf_ipsecsha)
563 [4f955e41d0a7ebcc3c868d143209da1a&keytype2=tf_ipsecsha](http://dev.biologists.org/content/124/23/4867.abstract?ijkey=9bab527d4f955e41d0a7ebcc3c868d143209da1a&keytype2=tf_ipsecsha) [5 Jul.
564 2013].
- 565 3. **Belteki G, Haigh J, Kabacs N, Haigh K, Sison K, Costantini F, Whitsett J,**
566 **Quaggin SE, Nagy A.** Conditional and inducible transgene expression in
567 mice through the combinatorial use of Cre-mediated recombination and
568 tetracycline induction. *Nucleic Acids Res* 33: e51, 2005.
- 569 4. **Brehm JM, Hagiwara K, Tesfaigzi Y, Bruse S, Mariani TJ, Bhattacharya**
570 **S, Boutaoui N, Ziniti JP, Soto-Quiros ME, Avila L, Cho MH, Himes B,**
571 **Litonjua AA, Jacobson F, Bakke P, Gulsvik A, Anderson WH, Lomas DA,**
572 **Forno E, Datta S, Silverman EK, Celedón JC.** Identification of FGF7 as a
573 novel susceptibility locus for chronic obstructive pulmonary disease.
574 *Thorax* 66: 1085–90, 2011.
- 575 5. **Danopoulos S, Parsa S, Al Alam D, Tabatabai R, Baptista S, Tiozzo C,**
576 **Carraro G, Wheeler M, Barreto G, Braun T, Li X, Hajihosseini MK,**
577 **Bellusci S.** Transient Inhibition of FGFR2b-ligands signaling leads to
578 irreversible loss of cellular β -catenin organization and signaling in AER
579 during mouse limb development. *PLoS One* 8: e76248, 2013.
- 580 6. **Denise Al Alam, Soula Danopoulos, Frederic G Sala, Dana Almohazey,**
581 **G. Esteban Fernandez, Mark R Frey, Henri R Ford, Tracy Grikscheit**
582 **SB.** Fgf10 alters the balance between Goblet cells and Paneth cells in the
583 adult mouse small intestine. *Am. J. Physiol. - Gastrointest. Liver Physiol.* in
584 *press*.
- 585 7. **Deterding RR, Havill AM, Yano T, Middleton SC, Jacoby CR, Shannon**
586 **JM, Simonet WS, Mason RJ.** Prevention of bleomycin-induced lung injury
587 in rats by keratinocyte growth factor. [Online]. *Proc Assoc Am Physicians*
588 109: 254–68, 1997. <http://www.ncbi.nlm.nih.gov/pubmed/9154642> [10
589 Mar. 2013].
- 590 8. **Francavilla C, Rigbolt KTG, Emdal KB, Carraro G, Vernet E, Bekker-**
591 **Jensen DB, Streicher W, Wikström M, Sundström M, Bellusci S,**
592 **Cavallaro U, Blagoev B, Olsen J V.** Functional proteomics defines the
593 molecular switch underlying FGF receptor trafficking and cellular outputs.
594 *Mol Cell* 51: 707–22, 2013.
- 595 9. **Gossen M, Bujard H.** Tight control of gene expression in mammalian cells
596 by tetracycline-responsive promoters. [Online]. *Proc Natl Acad Sci U S A*
597 89: 5547–51, 1992.

- 598 <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=49329&tool=pmcentrez&rendertype=abstract>.
- 599
- 600 10. **Guo J, Yi ES, Havill AM, Sarosi I, Whitcomb L, Yin S, Middleton SC,**
- 601 **Piguet P, Ulich TR.** Intravenous keratinocyte growth factor protects
- 602 against experimental pulmonary injury. [Online]. *Am J Physiol* 275: L800–
- 603 5, 1998. <http://www.ncbi.nlm.nih.gov/pubmed/9755113> [14 May 2013].
- 604 11. **Gupte V V, Ramasamy SK, Reddy R, Lee J, Weinreb PH, Violette SM,**
- 605 **Guenther A, Warburton D, Driscoll B, Minoo P, Bellusci S.**
- 606 Overexpression of fibroblast growth factor-10 during both inflammatory
- 607 and fibrotic phases attenuates bleomycin-induced pulmonary fibrosis in
- 608 mice. *Am J Respir Crit Care Med* 180: 424–36, 2009.
- 609 12. **Hokuto I, Perl A-KT, Whitsett JA.** Prenatal, but not postnatal, inhibition
- 610 of fibroblast growth factor receptor signaling causes emphysema. *J Biol*
- 611 *Chem* 278: 415–21, 2003.
- 612 13. **Hokuto I, Perl A-KT, Whitsett JA.** FGF signaling is required for
- 613 pulmonary homeostasis following hyperoxia. *Am J Physiol Lung Cell Mol*
- 614 *Physiol* 286: L580–7, 2004.
- 615 14. **Hübner R-H, Gitter W, El Mokhtari NE, Mathiak M, Both M, Bolte H,**
- 616 **Freitag-Wolf S, Bewig B.** Standardized quantification of pulmonary
- 617 fibrosis in histological samples. *Biotechniques* 44: 507–11, 514–7, 2008.
- 618 15. **Itoh N, Ornitz DM.** Fibroblast growth factors: from molecular evolution to
- 619 roles in development, metabolism and disease. *J Biochem* 149: 121–30,
- 620 2011.
- 621 16. **Izvolzsky KI, Shoykhet D, Yang Y, Yu Q, Nugent MA, Cardoso W V.**
- 622 Heparan sulfate-FGF10 interactions during lung morphogenesis. [Online].
- 623 *Dev Biol* 258: 185–200, 2003.
- 624 <http://www.ncbi.nlm.nih.gov/pubmed/12781692> [7 Jul. 2013].
- 625 17. **Ju W, Zhihong Y, Zhiyou Z, Qin H, Dingding W, Li S, Baowei Z, Xing W,**
- 626 **Ying H, An H.** Inhibition of α -SMA by the ectodomain of FGFR2c attenuates
- 627 lung fibrosis. *Mol Med* 18: 992–1002, 2012.
- 628 18. **Klar J, Blomstrand P, Brunmark C, Badhai J, Håkansson HF, Brange CS,**
- 629 **Bergendal B, Dahl N.** Fibroblast growth factor 10 haploinsufficiency
- 630 causes chronic obstructive pulmonary disease. *J Med Genet* 48: 705–9,
- 631 2011.
- 632 19. **Korfei M, Ruppert C, Mahavadi P, Henneke I, Markart P, Koch M, Lang**
- 633 **G, Fink L, Bohle R-M, Seeger W, Weaver TE, Guenther A.** Epithelial
- 634 endoplasmic reticulum stress and apoptosis in sporadic idiopathic
- 635 pulmonary fibrosis. *Am J Respir Crit Care Med* 178: 838–46, 2008.
- 636 20. **Kosman J, Carmean N, Leaf EM, Dyamenahalli K, Bassuk JA.**
- 637 Translocation of fibroblast growth factor-10 and its receptor into nuclei of
- 638 human urothelial cells. *J Cell Biochem* 102: 769–85, 2007.
- 639 21. **Lewis D, Scullion J.** Palliative and end-of-life care for patients with
- 640 idiopathic pulmonary fibrosis: challenges and dilemmas. [Online]. *Int J*
- 641 *Palliat Nurs* 18: 331–7, 2012.
- 642 <http://www.ncbi.nlm.nih.gov/pubmed/22885965> [10 Mar. 2013].
- 643 22. **Liu C-J, Ha X-Q, Jiang J-J, Lv T-D, Wu C.** Keratinocyte growth factor (KGF)
- 644 gene therapy mediated by an attenuated form of *Salmonella typhimurium*
- 645 ameliorates radiation induced pulmonary injury in rats. [Online]. *J Radiat*

- 646 *Res* 52: 176–84, 2011. <http://www.ncbi.nlm.nih.gov/pubmed/21436609>
647 [26 Mar. 2013].
- 648 23. **Lü J, Izvolsky KI, Qian J, Cardoso W V.** Identification of FGF10 targets in
649 the embryonic lung epithelium during bud morphogenesis. *J Biol Chem*
650 280: 4834–41, 2005.
- 651 24. **Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng**
652 **LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H.** A robust and
653 high-throughput Cre reporting and characterization system for the whole
654 mouse brain. *Nat Neurosci* 13: 133–40, 2010.
- 655 25. **McGrath-Morrow SA, Cho C, Soutiere S, Mitzner W, Tudor R.** The effect
656 of neonatal hyperoxia on the lung of p21Waf1/Cip1/Sdi1-deficient mice.
657 *Am J Respir Cell Mol Biol* 30: 635–40, 2004.
- 658 26. **Nelson WJ, Nusse R.** Convergence of Wnt, beta-catenin, and cadherin
659 pathways. *Science* 303: 1483–7, 2004.
- 660 27. **Ohuchi H, Hori Y, Yamasaki M, Harada H, Sekine K, Kato S, Itoh N.**
661 FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ
662 development. *Biochem Biophys Res Commun* 277: 643–9, 2000.
- 663 28. **Parsa S, Kuremoto K-I, Seidel K, Tabatabai R, Mackenzie B, Yamaza T,**
664 **Akiyama K, Branch J, Koh CJ, Al Alam D, Klein OD, Bellusci S.** Signaling
665 by FGFR2b controls the regenerative capacity of adult mouse incisors.
666 *Development* 137: 3743–52, 2010.
- 667 29. **Parsa S, Ramasamy SK, De Langhe S, Gupte V V, Haigh JJ, Medina D,**
668 **Bellusci S.** Terminal end bud maintenance in mammary gland is
669 dependent upon FGFR2b signaling. *Dev Biol* 317: 121–31, 2008.
- 670 30. **Perl A-KT, Tichelaar JW, Whitsett JA.** Conditional gene expression in the
671 respiratory epithelium of the mouse. [Online]. *Transgenic Res* 11: 21–9,
672 2002. <http://www.ncbi.nlm.nih.gov/pubmed/11874100> [8 Oct. 2014].
- 673 31. **Rafii R, Juarez MM, Albertson TE, Chan AL.** A review of current and
674 novel therapies for idiopathic pulmonary fibrosis [Online]. *J. Thorac. Dis.* 5:
675 48–73, [date unknown].
676 <http://www.jthoracdis.com/article/view/843/html> [15 May 2014].
- 677 32. **Sakamoto S, Yazawa T, Baba Y, Sato H, Kanegae Y, Hirai T, Saito I, Goto**
678 **T, Kurahashi K.** Keratinocyte growth factor gene transduction
679 ameliorates pulmonary fibrosis induced by bleomycin in mice. *Am J Respir*
680 *Cell Mol Biol* 45: 489–97, 2011.
- 681 33. **Sauer B.** Inducible gene targeting in mice using the Cre/lox system.
682 *Methods* 14: 381–92, 1998.
- 683 34. **Schwenk F, Baron U, Rajewsky K.** A cre-transgenic mouse strain for the
684 ubiquitous deletion of loxP-flanked gene segments including deletion in
685 germ cells. [Online]. *Nucleic Acids Res* 23: 5080–1, 1995.
686 [http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=307516&too](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=307516&tool=pmcentrez&rendertype=abstract)
687 [l=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=307516&tool=pmcentrez&rendertype=abstract) [8 May 2013].
- 688 35. **Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T,**
689 **Yagishita N, Matsui D, Koga Y, Itoh N, Kato S.** Fgf10 is essential for limb
690 and lung formation. *Nat Genet* 21: 138–41, 1999.
- 691 36. **Sugahara K, Iyama K, Kuroda MJ, Sano K.** Double intratracheal
692 instillation of keratinocyte growth factor prevents bleomycin-induced lung
693 fibrosis in rats. *J Pathol* 186: 90–8, 1998.

- 694 37. **Tanjore H, Degryse AL, Crossno PF, Xu XC, McConaha ME, Jones BR,**
695 **Polosukhin V V, Bryant AJ, Cheng D-S, Newcomb DC, McMahon FB,**
696 **Gleaves LA, Blackwell TS, Lawson WE.** β -catenin in the alveolar
697 epithelium protects from lung fibrosis after intratracheal bleomycin. *Am J*
698 *Respir Crit Care Med* 187: 630–9, 2013.
- 699 38. **Verkaar F, Zaman GJR.** New avenues to target Wnt/ β -catenin signaling.
700 *Drug Discov Today* 16: 35–41, 2011.
- 701 39. **Volckaert T, Dill E, Campbell A, Tiozzo C, Majka S, Bellusci S, De**
702 **Langhe SP.** Parabronchial smooth muscle constitutes an airway epithelial
703 stem cell niche in the mouse lung after injury. *J Clin Invest* 121: 4409–19,
704 2011.
- 705 40. **Wollin L, Maillet I, Quesniaux V, Holweg A, Ryffel B.** Anti-fibrotic and
706 anti-inflammatory activity of the tyrosine kinase inhibitor, nintedanib, in
707 experimental models of lung fibrosis. *J. Pharmacol. Exp. Ther.* (February
708 20, 2014). doi: 10.1124/jpet.113.208223.
- 709 41. **Woodcock H V, Molyneaux PL, Maher TM.** Reducing lung function
710 decline in patients with idiopathic pulmonary fibrosis: potential of
711 nintedanib. *Drug Des Devel Ther* 7: 503–10, 2013.
- 712 42. **Woyda K, Koeblich S, Reiss I, Rudloff S, Pullamsetti SS, Rühlmann A,**
713 **Weissmann N, Ghofrani HA, Günther A, Seeger W, Grimminger F,**
714 **Morty RE, Schermuly RT.** Inhibition of phosphodiesterase 4 enhances
715 lung alveolarisation in neonatal mice exposed to hyperoxia. *Eur Respir J*
716 33: 861–70, 2009.
- 717 43. **Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M, Ornitz**
718 **DM.** Receptor specificity of the fibroblast growth factor family. The
719 complete mammalian FGF family. *J Biol Chem* 281: 15694–700, 2006.
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748

749
750
751
752
753
754
755

Figure Legends

Figure 1: Fibrosis injury (1U/kg) peaked between 14 and 21 dpi in CD1 female, wild type mice, moderate recruitment of Fgfr2b-signaling following injury (Fgfs)

756
757 Survival curve (A). Relative weight change (B). Compliance (C). Quantification of confluent
758 fibrotic areas in H/E stains of left lobes (D). Hydroxyproline content of medial lobes (E).
759 Massons trichrome stain of representative time points following injury (F-J) Western blots for
760 Fgf-signaling pathway members of saline or bleomycin treated mice (K) Quantification of blot
761 densities normalized to saline controls (gene of interest divided by control gene) and
762 represented as percent of saline expression (L). Since the western blot antibodies used do
763 not distinguish between receptor isoforms, qPCR was performed on epithelial (b-isoforms)
764 and mesenchymal c-isoforms of Fgfr1 and Fgfr2 receptor. Already at 7 dpi, *Fgfr2b* was
765 significantly reduced, and remained so until 21 dpi. At 14 dpi, c-isoforms of both receptors
766 were increased, as well as Fgfr1b. C-isoforms remained elevated at 21 dpi, while *Fgfr1b*
767 returned to saline levels (M). One-way ANOVA was performed against control values and
768 error bars represent 95% confidence intervals; § (p<0.05) †(p<0.005) ★(p<0.0001). Scale
769 bars: 100 µm.

770
771

Figure 2. Validation of *Rosa26^{rtTA/+};tet(O)sFgfr2b/+* (DTG) mice

772 The *Rosa26* promoter drives ubiquitous expression of reverse tetracycline transactivator
773 (rtTA), which in the presence of doxycycline (dox), binds a tetracycline response sequence.
774 Binding results in the activation of a CMV promoter, which drives expression of a chimeric
775 transgene containing the extracellular binding domain of Fgfr2b fused with the heavy chain
776 domain of IgG (A). Lung-specific efficiency of *Rosa26* promoter was tested using
777 *Rosa26^{rtTA/+};tet(O)Cre/+;Tomato^{fl/+}* mice fed +dox-food for 7 days (B-C). RFP expression was
778 detected in approximately 25% of total cells by FACS and in none of the cells in mice lacking
779 the *tet(O)Cre* transgene as illustrated by fluorescence stereomicroscopy (D-E’). STG mice
780 (n=3) received 50ul of PBS with 0.1% BSA, DTG mice fed normal food (n=3) or +dox food
781 (n=4) for 1 week and were given an intra-tracheal dose of 10 µg of FGF7. 30 minutes later,
782 the lysates were collected and blotted for p-Akt and p-Erk1/2 signals (F). +dox DTG mice
783 blocked FGF7-mediated p-Akt (G) and p-Erk1-signals (H), p-ERK2 (H’) remained elevated,
784 (Figure 2F–H’). The chimeric transcript was detected only in DTG mice (I) and lung
785 compliance was not changed in DTG mice fed +dox food from post-natal (PN) PN1 to PN105
786 (J). Morphometric analyses were performed on these mice and (K-O) and in concurrence with
787 previous studies no significant differences in mean linear intercept, airspace, or septal wall
788 thickness were observed in DTG mice. Adult DTG mice fed +dox food from postnatal day
789 (PN28-88) failed to regenerate maxillary incisors (H-H’).

790

791 **Figure 3: Lack of endogenous *Fgfr2b* ligands signaling during injury (6–28 dpi) did not**
792 **lead to increased bleomycin-induced lung injury (28 dpi).**

793 Survival curve (A). Relative weight change (B). All DTG mice tested positive for *solFgfr2b*
794 transcript at 28 dpi (C). Compliance (D). Low and high magnification of H/E staining of STG
795 control mice (E and E') and DTGs fed doxycycline food from 6 to 28 dpi (F and F').
796 Quantification of confluent fibrotic areas in H/E stains of left lobes (G). Hydroxyproline content
797 of accessory and medial lobes (H). Scale bars E and F: 2 mm; E' and F': 200 µm.

798

799

800 **Figure 4: Lack of endogenous *Fgfr2b* ligands signaling during injury (14–28 dpi) did**
801 **not lead to increased bleomycin-induced lung injury (28 dpi).**

802 Survival curve (A). Relative weight change (B). All DTG mice tested positive for *solFgfr2b*
803 transcript at 28 dpi (C). Compliance (D). Low and high magnification of H/E staining of STG
804 control mice (E and E') and DTGs fed doxycycline food from 14 to 28 dpi (F and F').
805 Quantification of confluent fibrotic areas in H/E stains of left lobes (G). Hydroxyproline content
806 of accessory and medial lobes (H). *Col1a1* expression (I). Scale bars E and F: 2 mm; E' and
807 F': 200 µm.

808

809 **Figure 5: Lack of endogenous *Fgfr2b* ligands signaling during injury (0–28 dpi) did not**
810 **lead to increased bleomycin-induced lung injury (28 dpi).**

811 Survival curve (A). Relative weight change (B). All DTG mice tested positive for *solFgfr2b*
812 transcript at 28 dpi (C). Compliance (D). Low and high magnification of H/E staining of STG
813 control mice (E and E') and DTGs fed doxycycline food from 0 to 28 dpi (F and F').
814 Quantification of confluent fibrotic areas in H/E stains of left lobes (G). Hydroxyproline content
815 of accessory and medial lobes (H). Scale bars E and F: 2 mm; E' and F': 200 µm.

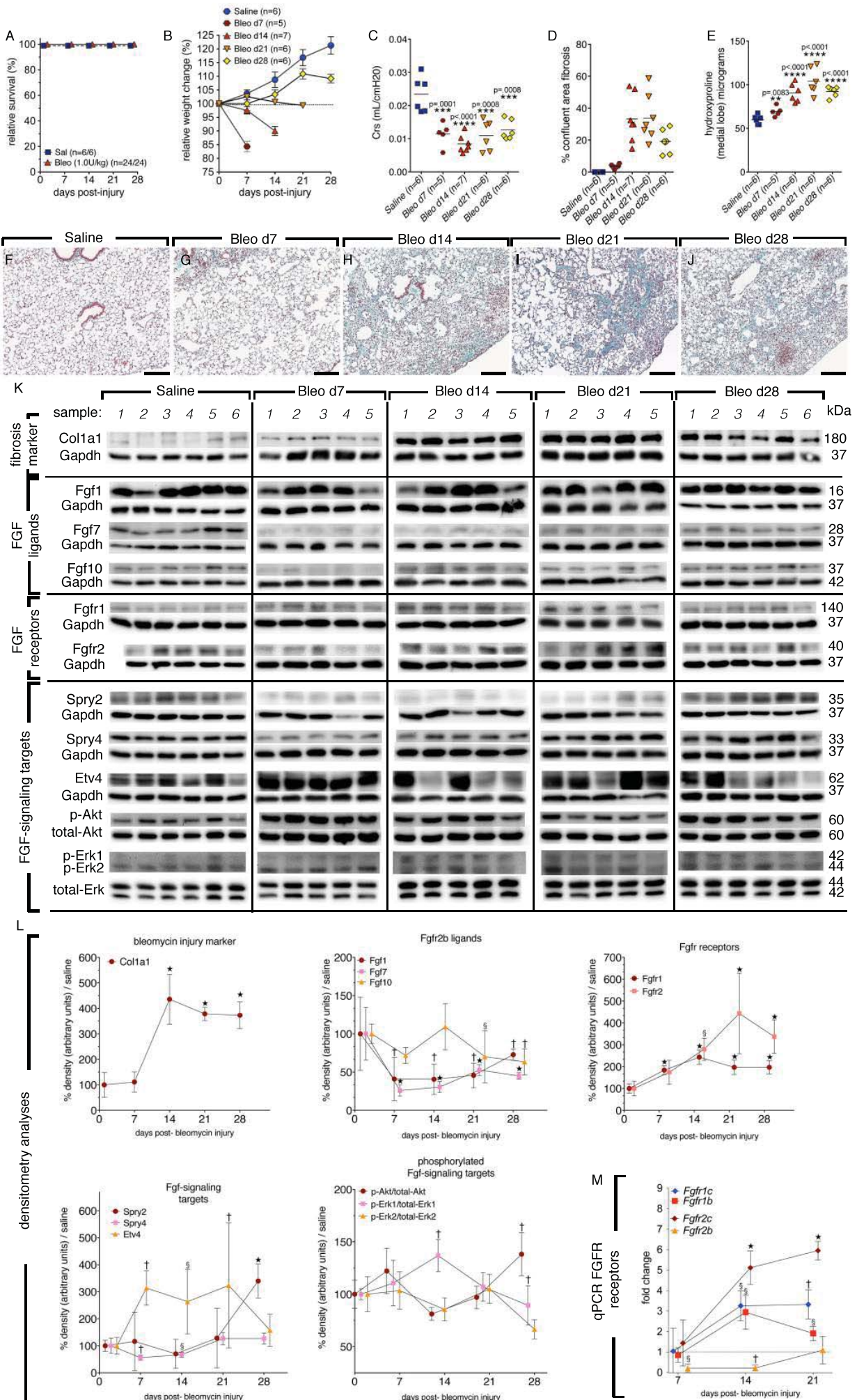
816

817 **Figure 6: Lack of endogenous *Fgfr2b* ligands signaling during injury (6–11 dpi) did not**
818 **lead to increased bleomycin-induced lung injury (6-11 dpi).**

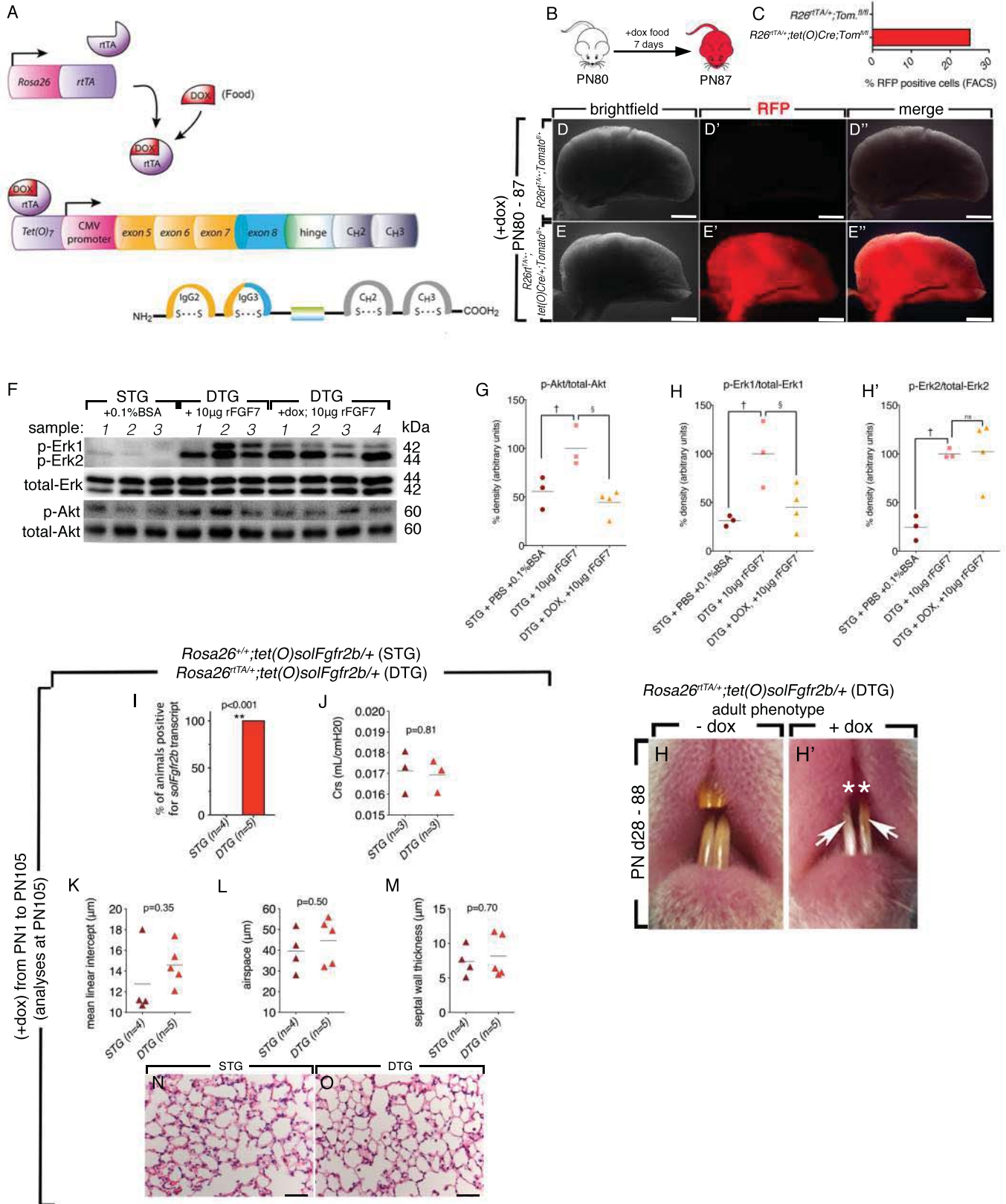
819 All DTG mice tested positive for *solFgfr2b* transcript at 28 dpi (A). Relative weight change in
820 mice that survived until 11 dpi (3/8 STG) and (4/9 DTG) (B). Compliance (C). Hydroxyproline
821 content of accessory and medial lobes (D). Low and high magnification of H/E staining of
822 STG control mice at 11 dpi (E and E') and DTGs fed doxycycline food from 0 to 11 dpi (F and
823 F'). Quantification of confluent fibrotic areas in H/E stains of left lobes (G). No decreases in
824 epithelial marker expression were detected in injured 6 dpi DTG mice vs. STG; qPCR for
825 *Sftpc*, *Scgb1a1*, *EpCam*; corresponding saline controls normalized to one (data not shown)
826 (H–J). Scale bars E and F: 2 mm; E' and F': 200 µm.

827

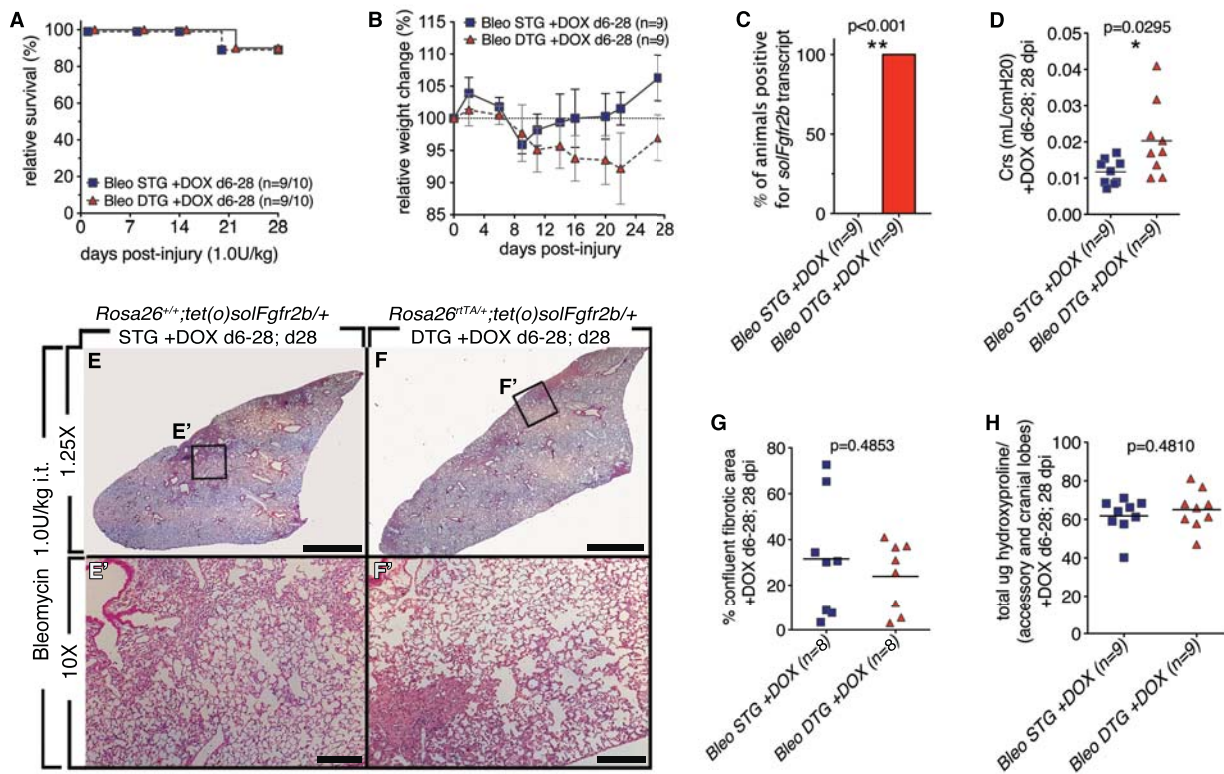
828



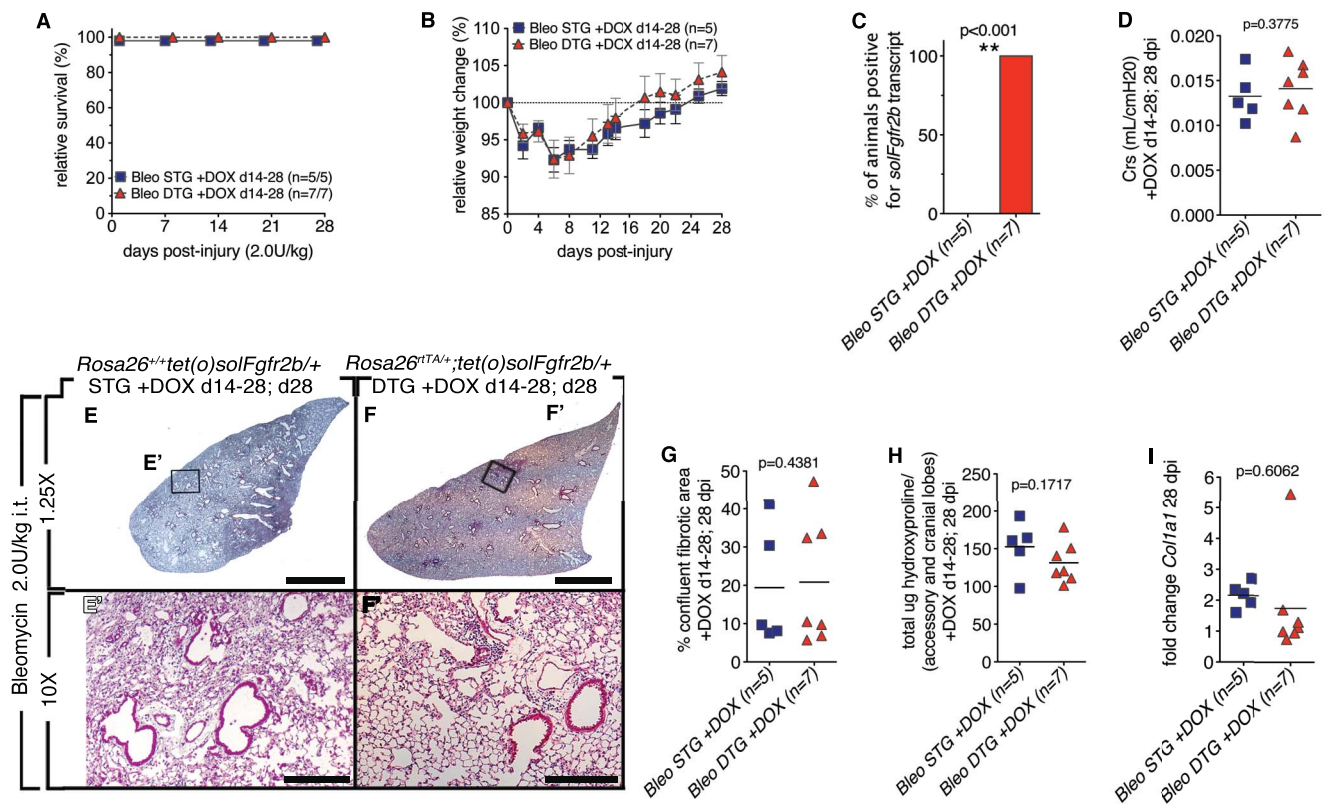
MacKenzie et. al, AJP Lung Figure 2



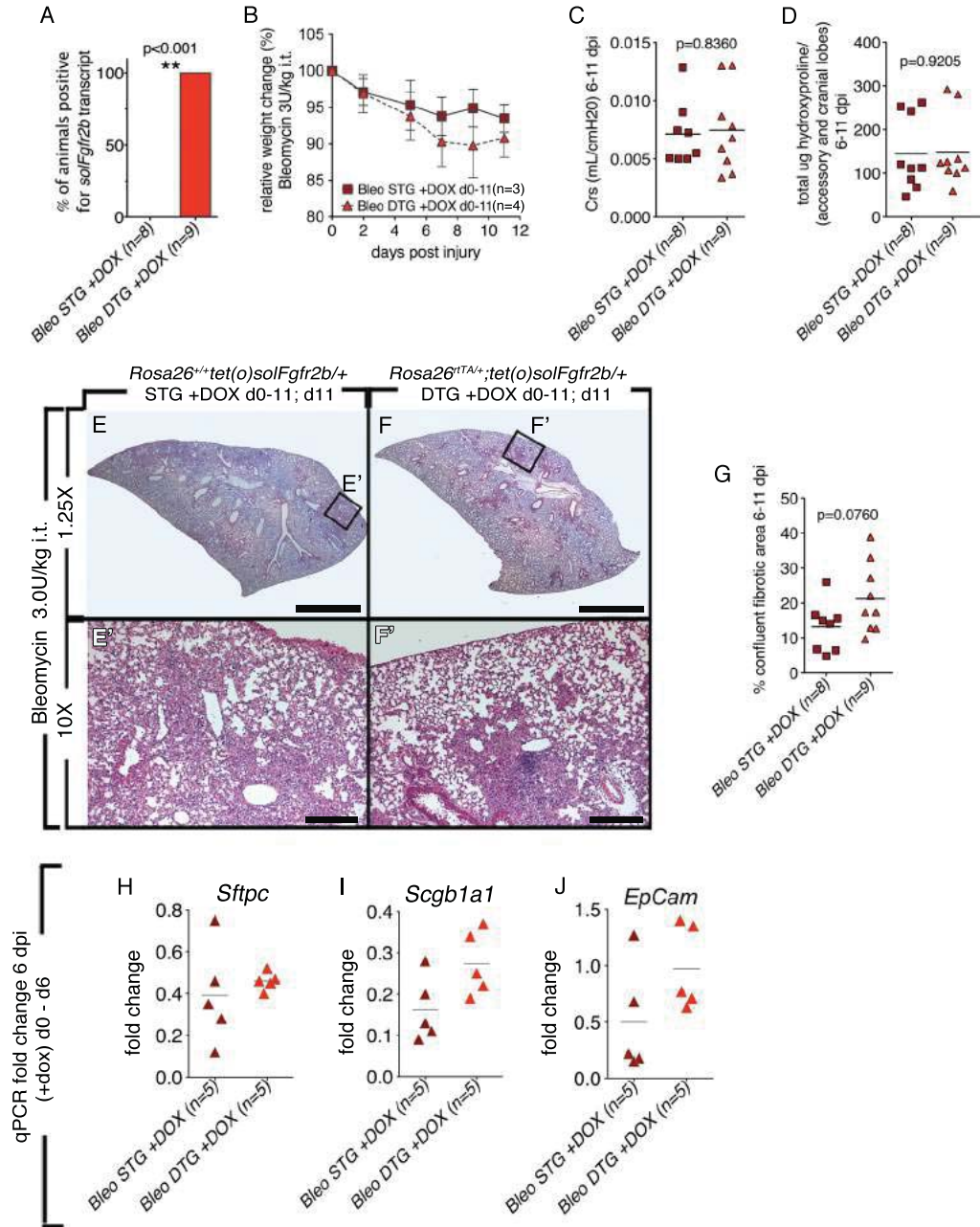
MacKenzie et. al, AJP Lung Figure 3



Mackenzie et. al, AJP Lung Figure 4



MacKenzie et. al, AJP Lung Figure 5



Mackenzie et. al, AJP Lung Figure 6

