FGFR2 is required for airway basal cell self-renewal and terminal differentiation

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Key words: Cre-Lox; Lung; Mouse; Trachea; Progenitor

Summary Statement: During adult airway epithelial homeostasis FGFR2 signalling is required for self-renewing divisions of the basal stem cells and to maintain expression of the key transcription factor SOX2.
SUMMARY
Airway stem cells slowly self-renew and produce differentiated progeny to maintain homeostasis throughout the life-span of an individual. Mutations in the molecular regulators of these processes may drive cancer or degenerative disease, but are also potential therapeutic targets. Conditionally deleting one copy of FGF Receptor 2 in adult mouse airway basal cells results in self-renewal and differentiation phenotypes. We show that FGFR2 signalling correlates with maintenance of expression of a key transcription factor for basal cell self-renewal and differentiation, SOX2. This heterozygous phenotype illustrates that subtle changes in Receptor Tyrosine Kinase signalling can have significant effects, perhaps providing an explanation for the numerous changes seen in cancer.

INTRODUCTION
Like human airways, the mouse trachea contains three major epithelial lineages (Rock et al., 2010; Teixeira et al., 2013). Basal cells (BCs) are stem cell population and include slowly-dividing stem cells and committed luminal precursors (Mori et al., 2015; Rock et al., 2009; Watson et al., 2015). Luminal secretory cells self-renew and produce terminally-differentiated ciliated cells (Rawlins and Hogan, 2008; Rawlins et al., 2009; Rawlins et al., 2007). Multiple studies have shown that SOX2 is a key transcription factor (TF) for the development and maintenance of all airway epithelial cells (Gontan et al., 2008; Hashimoto et al., 2012; Ochieng et al., 2014; Que et al., 2009; Tompkins et al., 2011; Tompkins et al., 2009). Deletion of Sox2 in adult mouse tracheal epithelium caused loss of differentiated cells. Moreover, the Sox2Δ/Δ BCs were less able to proliferate in vitro, or in vivo following injury (Que et al., 2009). SOX2 is thus required for BC self-renewal and luminal differentiation. SOX2 over-expression can be a driver of squamous cell carcinoma which has a predominantly basal cell phenotype (Correia et al., 2017; Ferone et al., 2016).

FGFR2 function has been extensively studied during lung branching where one of its roles is to maintain undifferentiated epithelial progenitors by inhibiting SOX2 expression (Abler et al., 2009; Que et al., 2007; Volckaert et al., 2013). However, at later stages of embryonic development ectopic FGF10 can promote BC differentiation in SOX2+ airway progenitors (Volckaert et al., 2013). The same study expressed a secreted dominant-negative FGFR2 in the late stages of embryogenesis and suggested that there could be a role for FGFR2 signalling in maintenance of airway BCs. We have now specifically tested this hypothesis in
the steady-state adult mouse trachea and show that FGFR2 is required for BC self-renewal and terminal differentiation. Moreover, FGFR2 signalling maintains SOX2 expression.

RESULTS AND DISCUSSION

FGFR2 is required for normal tracheal homeostasis

We detected FGFR2 protein in airway basal cells and at the apical surface of secretory cells (Fig. 1A,B), confirming previous results (Watson et al., 2015). To determine the role of FGFR2 in BCs, we conditionally deleted one copy of Fgfr2 and activated a GFP reporter in adult tracheal BCs using Tg(KRT5-CreER); Rosa26R^{GFP/+}; Fgfr2^{fl/+} (Fgfr2 conditional heterozygous, cHet) and control Tg(KRT5-CreER); Rosa26R^{GFP/+} mice (Fig. 1C). To test for co-recombination between Fgfr2^{fl} and the reporter we isolated GFP+ BCs by flow cytometry as GFP+, GSIβ4-lectin+ cells at 3 weeks post-Tamoxifen (tmx) induction and performed RT-qPCR for Fgfr2 (Fig. 1D). This confirmed that cHet BCs had approximately 50% of the control Fgfr2 mRNA level. Hence, we use GFP+ cells as a surrogate marker for Fgfr2Δ/+ cells, being aware that co-recombination will not be 100%. Tracheae were harvested at intervals to assess the contribution of GFP+, Fgfr2Δ/+ BCs to the epithelium during homeostatic turn-over (Fig. 1E). At 1.5 weeks post-tmx, ~30% of total BCs were GFP+ in Fgfr2cHet and control mice. In controls this percentage increased to ~60% at 5 weeks post-tmx, before dropping to initial levels by 24 weeks. By contrast, in the Fgfr2 cHet tracheae, the percentage of GFP+ BCs remained approximately constant at 5 weeks, but decreased to <5% of total basal cells by 24 weeks (Fig. 1F). In both genotypes, labelled BCs produced labelled luminal cells. Luminal differentiation initially appeared more rapid in the Fgfr2cHets. However, luminal cell production was not sustained over time, likely due to the loss of GFP+ BCs, and by 24 weeks the percentage of labelled luminal cells was significantly lower in the Fgfr2cHet tracheae (Fig. 1G).

This showed that Fgfr2cHet BCs can produce luminal cells, but that mutant basal and luminal cells are gradually lost. One possible reason for the loss of Fgfr2cHet cells is differential fitness and competition with neighbouring wild-type cells (Vivarelli et al., 2012). To test this we mixed pure populations of Rosa26R^{tdTomato+}; Fgfr2^{fl/+} with unlabelled Fgfr2^{fl/+} BCs (1:2 ratio) and assessed their ability to compete in vitro at steady-state and following injury. We were unable to find evidence for differential proliferation or survival in the mixed cultures and conclude that it is unlikely that cell competition contributes to the observed loss of mutant cells (Fig. S1; movies 1-5).
**Fgfr2 cHet BCs do not differentiate into fully mature luminal cells**

We asked if the loss of Fgfr2cHet cells was due to a decrease in cell division. As expected proliferation rates were low in all tracheae, but dividing GFP⁺ cells were observed (Fig. S2A). We noted an increase in proliferation of the Fgfr2cHet GFP⁺ cells at 1.5 weeks post-tmx, although this was not statistically significant and the change was not sustained over time (Fig. S2B). Thus altered proliferation does not explain the phenotype. We also assessed apoptosis using Cleaved-Caspase-3 staining, but did not identify Caspase-3⁺ cells (665 GFP⁺ cells scored in 4 independent 5 week samples; Fig. S2C,D).

We assessed the ability of Fgfr2cHet cells to differentiate by analysing the luminal (KRT8) and basal (KRT5) cytokeratins at 5 weeks post-tmx (Fig. 2A). A higher percentage of the total GFP⁺ cells co-stained with KRT8 in the mutants, indicating that more cells had begun differentiation to a luminal fate (Fig. 2B). Similarly, plotting the GFP/T1α staining (Fig. 1D) as percentage of GFP⁺ cells which are (GFP⁺, T1α⁻) showed more differentiating cells in the mutants (Fig. 2B). Thus Fgfr2cHet cells exit the basal layer at a greater rate than controls and their descendants take on a luminal KRT8⁺, T1α⁻ fate, suggesting a self-renewal defect.

At steady-state BCs initially differentiate into secretory cells which later produce ciliated cells (Watson et al., 2015). Cell fate analysis at 5 weeks post-tmx showed that both control and Fgfr2cHet BCs produce secretory SCGB1A1⁺ cells (Fig. 2C,D). Moreover, there were no signs of goblet cell production in the mutants (Fig. 2C; n=4 MUC5AClo cells observed from 859 cells counted in 5 Fgfr2cHet individuals). However, analysis of acetylated tubulin-positive cilia (marker of terminal luminal differentiation) at 24 weeks post-tmx showed that the Fgfr2cHet cells never took on a ciliated cell identity (Fig. 2E).

**Fgfr2 cHet BCs have high levels of β-galactosidase activity in vitro**

We tested the ability of Fgfr2 cHet cells to proliferate and differentiate in vitro using a high dose of an adenovirus containing CMV-Cre (Ad-Cre) to recombine Rosa26R(GFP)⁺; Fgfr2Δ/+ and control Rosa26R(GFP)⁺GFP BCs grown in self-renewing conditions (Fig. 3A). When analysed by genomic PCR this resulted in an almost-pure population of Fgfr2Δ/+ cells (Fig. S3A,B). Four days after Ad-Cre-mediated deletion we observed an increased proportion of KRT8⁺ cells in the Fgfr2cHet cultures (Fig. 3A-C). This recapitulates the in vivo phenotype.
and supports the conclusion that *Fgfr2cHet* BCs have a self-renewal defect. Additional cultures were passaged and grown to confluence before differentiation at air-liquid interface (Fig. S4A-D). The *Fgfr2cHet* cells survived passaging but did not reach confluence and failed to express markers of ciliated or basal cell differentiation. Moreover, passaged cells were unable to grow in sphere-forming assays (Fig. S4E-H). The passaged *Fgfr2cHet* cells were somewhat enlarged and flattened, possibly indicating a senescent phenotype (Rodier and Campisi, 2011). We therefore tested for senescence-associated β-galactosidase activity in primary cultures of *Fgfr2cHet* cells. β-galactosidase activity was detected in 3/3 *Fgfr2cHet* cultures and 0/3 controls (Fig. 3D). Senescence of the *Fgfr2cHet* cells in vivo could potentially explain why the luminal GFP+ cells can express secretory markers, but do not later produce ciliated cells. However, we cannot absolutely exclude a luminal fate choice defect in *Fgfr2cHet* BCs.

**Lower levels of SOX2 expression in the *Fgfr2* conditional heterozygous cells**

We determined the effects of decreasing FGFR2 signalling on down-stream pathways using immunoblotting. There was a 1.5-fold decrease in phosphorylated AKT in the *Fgfr2Δ/+* cells (Fig. 3E,F), but no change in phosphorylated ERK1/2 (Fig. S3C,D). These changes are consistent with a decrease in FGFR2 signalling via the PI3K-AKT pathway, which was implicated as the main pathway downstream of FGFR2 in adult small airway secretory cells and the developing trachea (Volckaert et al., 2013; Volckaert et al., 2011).

Most strikingly, there was a 2-fold decrease in SOX2 in the *Fgfr2Δ/+* cells (Fig. 3E,F; S3C,D). We confirmed the decrease in SOX2 protein at a cellular level by in vitro immunostaining (Fig. 3G). Similarly, there was consistently lower SOX2 expression in GFP+ cells in the *Fgfr2cHet* tracheae in vivo (Fig. 3H, arrows). As expected from the genetic strategy, in the mutants we also observed GFP+,SOX2+ cells (Fig. 3H, arrowheads) and GFP-,SOX2- cells (Fig. 3H, yellow arrows), both are likely to have recombined only one floxed allele. Co-immunostaining with FGFR2 confirmed that the GFP+,SOX2+ cells observed in the mutants retained high levels of FGFR2 protein (Fig. 3I).
FGF7 and 10 can promote BC colony expansion in vitro

We predicted that if decrease in Fgfr2 results in loss of BC self-renewal, then activation of FGFR2 in vitro should promote the growth of BC colonies. FGF7 and 10 are expressed in homeostatic tracheae (Balasooriya et al., 2016) and known to activate FGFR2 preferentially in vitro and in vivo (Ornitz et al., 1996). We plated wild-type BCs at low density and added FGF7 or 10 on culture day 2 after colonies were established (Fig. 4A). Addition of FGF7 or 10 had the opposite effect to decreasing Fgfr2 and significantly increased colony size (Fig. 4B,C). Interestingly, FGF7 and 10 had no effect on the level of Sox2 mRNA (Fig. 4D).

In conclusion, our data suggest that a normal function of FGFR2 signalling in adult airway BCs is to promote asymmetric self-renewing divisions (Fig. 4E). This is consistent with work in the embryonic trachea where ectopic FGF10 was observed to promote BC fate (Volckaert et al., 2013). By contrast, our previous work on FGFR1 in adult BCs showed that FGFR1 is required to inhibit steady-state proliferation and does not change the ability of BCs to self-renew (Balasooriya et al., 2016). Thus FGFR1 and FGFR2 have independent functions in airway BCs. We cannot exclude the possibility that they also have other over-lapping functions.

We also show that steady-state FGFR2 signalling is required, directly or indirectly, to maintain SOX2 protein levels in the adult airway. This is in contrast to the branching lung where FGFR2 inhibits SOX2 expression at the tips. Interestingly, an FGFR2-SOX2 inductive relationship has been observed in other cell types (Mansukhani et al., 2005). An FGFR2-SOX2 relationship may be maintained in some squamous lung cancers where FGFR2 and SOX2 transcript levels are often correlated (Kim et al., 2016).

Haploinsufficiency of Fgfr2 in conditionally-deleted adult cells

We were surprised that our Fgfr2cHet BCs displayed striking phenotypes when germline Fgfr2Δ/+ animals are viable and fertile (Yu et al., 2003). We therefore looked for subtle epithelial defects in germline deleted Fgfr2Δ/+ tracheae compared to wild-type siblings, but were unable to find any abnormalities (Fig. S5). Fgfr2 is haploinsufficient in several organs including the lacrimal and salivary glands (Shams et al., 2007). We suggest that in mouse embryos heterozygous for Fgfr2 genetic compensation operates in most tissues. However, conditional heterozygous deletion in the adult by-passes such mechanisms. This is very
similar to recent findings from zebrafish genetics where genetic compensation has been found to operate in germline mutants, but not in acute knock-downs (Rossi et al., 2015). It raises the possibility that many genes which the mouse developmental community assume are uninteresting/redundant based on lack of germline knock-out phenotypes do play important roles in development/homeostasis.

MATERIALS AND METHODS

Mice
Experiments approved by local ethical review committees and conducted according to Home Office project licenses PPL80/2326 and 70/812. Fgfr2fx (Yu et al., 2003), Tg(KRT5-CreER) (Rock et al., 2009), Rosa26R-fGFP (Rawlins et al., 2009), Gt(ROSA)26Sor tm1(CAG-tdTomato*,EGFP*)Ees (Prigge et al., 2013). Fgfr2Δ/+ animals were generated by crossing Fgfr2fx to Zp3-Cre (de Vries et al., 2000). Genetic background: C57Bl/6J. Males and females >8 weeks old were used. Wild-types: C57Bl/6J.

Tamoxifen
Adult (>8 week) animals were injected intraperitoneally four times, every other day, with 0.2 mg / gram body weight tamoxifen.

Tracheal epithelial cell culture
Tracheal cells were isolated following published methods (Rock et al., 2009). Briefly, incubation in Dispase II (Gibco, 16 U/ml) 20 minutes room temperature. Epithelial sheets dissociated using 0.1% trypsin/EDTA. Unless otherwise stated, 5x10⁴ cells in 0.5ml MTEC/+ media (You et al., 2002) were plated on collagen-coated 12-well tissue culture inserts (BD Falcon, 353180). For tracheospheres cells were passaged into 50% matrigel (Becton Dickinson). Adeno-Cre (University of Iowa, Gene Transfer Vector Core) was incubated at MOI 2500; vector pfu 1x10⁶ for 8 hours. Recombinant mouse FGF7 and 10 (R and D Systems) were used at 100 ng/ml. For competition assays, mixed populations of cells were grown to confluence and then imaged every 4 hours for 10 days in a Nikon Biostation. Alternatively, confluent cultures were scratched and imaged every 2 hours for 5 days. In vitro experiments were preformed in triplicate.
**Immunostaining**

Tracheae were fixed in 4% paraformaldehyde, 4°C, 4 hours; washed PBS, sucrose protected, embedded in OCT (Optimum Cutting Temperature Compound, Tissue Tek) and sectioned at 6 μm. Airway culture inserts were washed in PBS, fixed 10 minute 4% paraformaldehyde, room temperature and permeabilized 0.3% triton X-100. Primary antibodies (supplemental table). Alexa Fluor conjugated secondary antibodies (1:2000 Life Technologies). DAPI, fluoromount (Sigma). X-gal staining was performed using Senescence β-galactosidase staining kit (Cell Signalling, 9860).

**Microscopy and Image scoring**

Slides were imaged on a Zeiss AxioImager compound, or Leica Sp8/Sp5 confocal, microscope. Cells were scored manually in Fiji. For cryosections every epithelial cell along the entire proximal to distal length of a longitudinal section from the centre of the trachea was scored. For cultured cells at least three random fields of view from each insert were scored. Raw cell counts are available as a supplemental file.

**RT-qPCR**

Primary tracheal epithelial cells were isolated and sorted using a fluorescence-activated cell sorting MoFlo flow cytometer. GFP+ basal cells from control and Fgfr2 cHet tracheae were sorted as GFP+, GSLβ4 lectin+ (Balasooriya et al., 2016). Total RNA was extracted using Qiagen RNEasy Mini Kit. Taqman gene expression assays for Ppia (Mm02342429_g1); Fgfr2 (Mm01269930_m1); Sox2 (Mm03053810_s1) (Life Technologies) were used.

**Immunoblots**

Cells were collected in Cell Extraction Buffer (Invitrogen, FNN0011) with protease inhibitor (Roche 04693116001) and PMSF (Sigma, P7626). Proteins separated on 10 or 12% SDS-PAGE gels before transfer onto Millipore Immobilon-P PVDF Membrane (Merck Millipore, IPVH00010). Primary antibodies (supplemental table). Detection with HRP-conjugated secondaries (Abcam, 1:10000) and enhanced chemiluminescence (Thermo Scientific, PI-32109). Quantitation is based on protein from three biological replicates separated on the same polyacrylamide gel. Band intensity was analysed in Fiji normalised to the loading control.
Statistics
P-values were obtained using an unpaired two-tailed student’s t-test with unequal variance.

COMPETING INTERESTS
No competing interests declared.

AUTHOR CONTRIBUTIONS
G.B. designed and performed experiments, analysed data and edited the manuscript. M.G. designed, performed and analysed experiments. E.P. designed experiments and edited the manuscript. E.R. conceived and led the project, designed and performed experiments, analysed data and wrote and edited the manuscript.

FUNDING
This study was supported by the Medical Research Council (G0900424 to E.R.). Core grants: Gurdon Institute: Wellcome Trust (092096), Cancer Research UK (C6946/A14492); Stem Cell Initiative: Wellcome Trust/MRC.
REFERENCES


Figure 1. Decreasing Fgfr2 levels in basal cells results in altered tracheal homeostasis. (A, B) Adult tracheal sections. (A) Green: FGFR2; red: T1α (basal cells). (B) Green: FGFR2; red: SCGB1A1 (secretory cells). FGFR2⁺ secretory cells (arrow heads); rare SCGB1A1⁺,FGFR2⁻ cells (arrow). (C) Experimental schematic. (D) Relative expression of Fgfr2 mRNA in GFP⁺ basal cells from control and Fgfr2 cHet mice 3 weeks post-tmx (E) Representative sections from control Tg(KRT5-CreER); Rosa26R<sup>GFP</sup>/⁺ and cHet Tg(KRT5-CreER); Rosa26R<sup>GFP</sup>/⁺; Fgfr2<sup>fx/+</sup> tracheae. Green: GFP (Rosa reporter); red: T1α (basal cells). Arrowheads = GFP⁺ basal cells. (F, G) Graphs to show percentage of the total T1α⁺ BCs that are also GFP⁺ (F) and percentage of the total T1α⁻ luminal cells that are also GFP⁺ (G). Blue: DAPI. Error bars = sem. Scale bars = 50 μm.
Figure 2. *Fgfr2* conditional heterozygous basal cells do not produce terminally differentiated luminal cells. (A) Confocal projections from control and *Fgfr2* cHet tracheae 5 weeks post-tmx. Green: GFP (*Rosa* reporter); red: KRT5 (basal cells); white: KRT8: (luminal cells) blue: DAPI (nuclei). Arrowheads: GFP\(^+\) luminal cells. Arrows: GFP\(^+\) basal cells. (B) Percentage of all GFP\(^+\) cells 5 weeks post-tmx which are GFP\(^+\), T1\(\alpha\) (see 1D) or...
GFP⁺, KRT8⁺ (see 2A). (C) Sections from control and and Fgfr2 cHet tracheae 5 weeks post-tmx. Green: GFP (Rosa reporter); red: SCGB1A1 (club cells); white: MUC5AC (mucous). Arrows: club cells containing a low level of MUC5AC protein. (D) Percentage of all GFP⁺ cells 5 weeks post-tmx which are GFP⁺, SCGB1A1⁺. (E) Confocal sections from control and Fgfr2 cHet tracheae at 24 weeks post-tmx. Green: GFP (Rosa reporter); red: acetylated tubulin (cilia). Error bars = sem. Scale bars = 20 μm (A, C); 25 μm (E).
A. Epithelial cell seeding (D0) → Ad-Cre infection (High titer) → Analysis (D2) → Analysis (D6)

B. % cells expressing KRT5 and/or 8

- Control: p=0.04
- Fgfr2β+ : p=0.05
- Fgfr2α+ : p=0.02

C. KRT5, KRT8, DAPI
- Control
- Fgfr2β+ :

D. β-galactosidase assay

E. SOX2, pAKT, AKT, Histone H3, β-Actin
- Control
- Fgfr2β+ :

F. Relative levels SOX2 (A.U.)
- Control: p=0.001
- Fgfr2β+ :

G. E-cad, SOX2, DAPI
- Control
- Fgfr2β+ :

H. GFP SOX2, DAPI
- Control
- Fgfr2β+ :

I. GFP, SOX2, FGFR2, DAPI
- Control
- Fgfr2β+ :
Figure 3. *Fgfr2* conditional heterozygous basal cells have high levels of β-galactosidase and low levels of SOX2. (A) Experimental schematic for B-G. (B) Percentage tracheal epithelial cells at day 6 post-seeding expressing KRT5 and/or KRT8. (C,D) Control and *Fgfr2* cHet tracheal cells day 6 post-seeding. (C) Green: KRT5 (basal cells); red: KRT8 (luminal cells). (D) X-gal assay for β-galactosidase activity (blue pigment). (E) Representative western blots from control and *Fgfr2* cHet BCs. (F) Quantification of protein levels in (E). (G) SOX2 in cHet BCs day 6 post-seeding. Green: E-cadherin (lateral cell membranes); red: SOX2. (H, I) Confocal images of control and *Fgfr2* cHet tracheal sections 5 weeks post-tmx. Green: GFP (*Rosa* reporter); red: SOX2. Magenta: FGFR2 (I only). Arrows: lineage-labelled cells with decreased levels of SOX2. Arrow heads: lineage-labelled cells with no change in SOX2. Yellow arrows: unlabelled cells with decreased SOX2. Bracket in I: patch of GFP+ cells that have decreased FGFR2 and no SOX2. Blue: DAPI. Error bars = sem. Scale bars = 100 μm (C); 250 μm (D); 50 μm (G); 25 μm (H,I).
Figure 4. FGF7 and FGF10 increase colony size of wild-type basal cells. (A) Experimental schematic. Epithelial cells plated at low density, 3x10^4 cells/insert. (B) Colonies formed by control, FGF7, or 10-treated wild-type cells. Red: E-cadherin; blue: DAPI. Bar = 100 μm. (C) Number of cells per colony in B. (D) Level of Sox2 mRNA relative to control (normalized to 1) in cells treated with FGF7 or 10 for 1 or 2 days. Bars = sem. (E) Model: Fgfr2 cHet BCs rarely make self-renewing divisions in which a new BC is produced. Mutant BCs are more likely to produce descendants with luminal morphology/markers which are unable to completely differentiate, possibly because they senesce. The result is that GFP^+ Fgfr2 cHet cells are gradually diluted out from both the basal and luminal populations and the epithelium is sustained by GFP^- wild-type BCs.
A. Confluent mosaic culture → 10 days → Competition

B. 0 hours, 240 hours
   - Control: red: Fgfr2Δ/+, unlabelled: Fgfr2Δ/+
   - Competition: red: Fgfr2Δ/+, unlabelled: +/-

C. Confluent mosaic culture → Scratch wound → ~5 days
   - No competition
   - Competition

D. Scratch, 0 hours, 114 hours
   - Control: red: Fgfr2Δ/+, unlabelled: Fgfr2Δ/+
   - Competition: red: Fgfr2Δ/+, unlabelled: +/-
Figure S1. No evidence for cell competition between $Fgfr2^{d/+}$ and $Fgfr2^{+/+}$ basal cells in vitro. (A) Experimental set-up in B. Freshly isolated basal cells were mixed at a 1:2 ratio, grown to confluence on cell culture inserts and imaged at intervals for 10 days. In cultures with no competition both cell populations will continue at the same ratio, whereas in cultures with competition the patch size of the “loser” cell population will decrease over time. (B) 0 and 240 hour frames from phase contrast/red channel time-lapse experiments. Upper panel: control experiment, red cells: $Fgfr2^{d/+}$, unlabelled cells: $Fgfr2^{d/+}$. Lower panel: competition experiment, red: $Fgfr2^{d/+}$, unlabelled: $Fgfr2^{+/+}$. No evidence for competition was observed. (C) Experimental set-up in D. Freshly isolated basal cells were mixed at a 1:2 ratio, grown to confluence on cell culture inserts, mechanically wounded using a pipette tip and imaged at intervals for 5 days. In cultures with no competition labelled and unlabelled cells will contribute approximately equally to wound closure. In cultures with competition, the “loser” cell population will contribute less to wound closure. (D) 0 hour and 114 hour frames from phase contrast/red channel time-lapse experiments. Upper panel: control experiment, red cells: $Fgfr2^{d/+}$, unlabelled cells: $Fgfr2^{d/+}$. Lower panels: competition experiment, red cells: $Fgfr2^{d/+}$, unlabelled cells: $Fgfr2^{+/+}$. No evidence for competition was observed. Bar = 0.5 mm in all panels. See also movies 1-5.
**A**

- Tg(KRT5-CreER); R26RfGFP/+ (Control)
- Tg(KRT5-CreER); R26RfGFP/+; Fgfr2<sup>−/−</sup> (Fgfr2 cHet)

**B**

- Bar graph showing the percentage of GFP+ cells coexpressing GFP/KI67 over weeks post-tmx.
- Data points show:
  - p = 0.08
  - p = 0.16
  - p = 0.36

**C**

- Tg(KRT5-CreER); R26RfGFP/+ (Control)
- Tg(KRT5-CreER); R26RfGFP/+; Fgfr2<sup>−/−</sup> (Fgfr2 cHet)

**D**

- E18.5 GR<sup>+</sup> lung (Positive control for Caspase-3 staining)
Figure S2. Fgfr2 conditional heterozygous basal cells can proliferate and show no evidence of apoptosis. (A) Sections from control Tg(KRT5-CreER); Rosa26R<sup>GFP/+</sup> and cHet Tg(KRT5-CreER); Rosa26R<sup>GFP/+</sup>; Fgfr2<sup>fx/+</sup> tracheae at 1.5 and 24 weeks post-tmx. Green: GFP (Rosa reporter); red: KI67 (proliferating cells); blue: DAPI (nuclei). Arrowheads mark KI67 positive cells. (B) Quantitation of the percentage of GFP<sup>+</sup> cells that co-express KI67 throughout the experimental timecourse. Error bars = sem. (C) Sections from control Tg(KRT5-CreER); Rosa26R<sup>GFP/+</sup> and cHet Tg(KRT5-CreER); Rosa26R<sup>GFP/+</sup>; Fgfr2<sup>fx/+</sup> tracheae at 5 weeks post-tmx. Green: GFP (Rosa reporter); red: Cleaved Caspase-3 (apoptotic cells); blue: DAPI (nuclei). (D) Section of E18.5 Glucocorticoid receptor null lung (GR<sup>−/−</sup>, also known as Nr3c1) as a positive control for Cleaved Caspase-3 staining. Green: E-cadherin (lateral membranes); red: Cleaved Caspase-3 (apoptotic cells); blue: DAPI (nuclei). Scale bar = 50 µm in all panels.
Figure S3. Fgfr2 conditional heterozygous tracheal cells fail to terminally differentiate and self-renew in vitro

(A) Experimental schematic. Control (Rosa26R<sup>GFP</sup>/<sup>GFP</sup>) and cHet (Rosa26R<sup>GFP</sup>/<sup>+</sup>; Fgfr2<sup>fx/+</sup>) tracheal epithelial cells were seeded in BC expansion conditions and infected with Ad-Cre at day 2. On day 4 BCs were passaged onto new collagen-coated inserts for further expansion and ALI differentiation. (B) cHet BCs attach and proliferate post-passaging on collagen-coated inserts. (C, D) Control cultures form fully-differentiated monolayers containing multiciliated cells (C) and differentiated BCs (D) by 12 days post-seeding, but cHet BCs do not reach confluence and do not express differentiated markers in vitro. Arrows: fragmented nuclei, or multi-nucleate cells, seen in cHet cultures, but not controls. (E) Experimental schematic. Control (Rosa26R<sup>GFP</sup>/<sup>GFP</sup>) and cHet (Rosa26R<sup>GFP</sup>/<sup>+</sup>; Fgfr2<sup>fx/+</sup>) tracheal epithelial cells were seeded in BC expansion conditions and infected with Ad-Cre at day 2. On day 4 BCs were passaged into matrigel for sphere-forming assays. (F) Representative confocal sections of control and Fgfr2 cHet cultures 2 days post-seeding in matrigel. Green: KRT8; red: KRT5. (G) Images of control and Fgfr2 cHet tracheospheres 9 days post-seeding in matrigel. (H) Tracheosphere diameter, arbitrary units. Scale bars = 100 µm (B-D, G); 5 µm (F).
A. Epithelial cell seeding (D0) followed by Ad-Cre infection (High titer) (D2) results in BC (D4) analysis (E) at D2 post-seeding and analysis (F, G) at D12 post-seeding.

B. D2 post-seeding: DAPI and GFP images showing R26RfGFP/R26RfGFP and R26RfGFP/+; Fgfr2fx/+.

C. D12 post-seeding: DAPI, GFP, and ACT images showing R26RfGFP/R26RfGFP and R26RfGFP/+; Fgfr2fx/+.

D. D12 post-seeding: DAPI and KRT5 images showing R26RfGFP/R26RfGFP and R26RfGFP/+; Fgfr2fx/+.

E. Epithelial cell seeding (D0) followed by Ad-Cre infection (High titer) (D2) results in BC (D4) analysis (E) at D2 post-seeding and Matrigel (D9) spheres analysis at D9 post-seeding.

F. R26RfGFP/R26RfGFP and R26RfGFP/+; Fgfr2fx/+ images at D2 post-seeding showing KRT8, KRT5, and DAPI.

G. D9 post-seeding (matrigel) images showing R26RfGFP/R26RfGFP and R26RfGFP/+; Fgfr2fx/+.

H. Graph showing the diameter of tracheospheres (Day 9 post-seeding) with p=0.0001.
Figure S4. Decrease in FGFR2 signalling in vitro does not affect levels of MEK-ERK signalling. (A) Schematic of in vitro experimental time-course. (B) Representative genotyping (gDNA) PCR from Rosa26R^{GFP/GFP} and Rosa26R^{GFP/+}, Fgfr2^{fx/+} viral-infected cells at day 6. Note that the cHet cells have efficient amplification of the wild-type (wt) and deleted (Δ) alleles, but very little amplification of the floxed (fx) allele indicating high levels of recombination in vitro. (C) Representative western blots from control and Fgfr2 cHet day 6 basal cells showing levels of SOX2, pERK1/2, total ERK and Histone H3. (F) Quantification of protein levels in (E).
Figure S5. *Fgfr2*Δ/+ adult mice have a normal tracheal epithelium. (A) Representative sections from control *Rosa26R*\(^{GFP/GFP}\) and sibling *Rosa26R*\(^{GFP/GFP}; Fgfr2*\(^{Δ/+}\) tracheae. Green: SCGB1A1 (secretory cells); red: T1α (basal cells); white: acetylated tubulin (cilia); blue: DAPI. (B) Quantitation of the percentage of epithelial cells which are basal, and luminal cells which are ciliated in the two genotypes. Error bars = sem. Scale bar = 20 µm.
### Figure S1: Development of K5+\, K8+ cells

<table>
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<tr>
<th>Treatment</th>
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<th>1 week</th>
<th>2 weeks</th>
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<tr>
<td>KRT5-CreER/+; R26-fGFP/+; Fgfr2 fx/+</td>
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### Figure S2: Analysis of cell populations

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### Figure S3: Quantification of cell populations

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### Figure S4: Comparison of cell populations

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### Figure S5: Development of germline and conditional heterozygous cell counts

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