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Single-cell Wnt signaling niches maintain stemness of alveolar type 2 cells

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Alveoli, the lung's respiratory units, are tiny sacs where oxygen enters the bloodstream. They are lined by flat AT1 cells, which mediate gas exchange, and AT2 cells, which secret surfactant. Rare AT2s also function as alveolar stem cells. We show that AT2 lung stem cells display active Wnt signaling and many of them are near single, Wnt-expressing fibroblasts. Blocking Wnt secretion depletes these stem cells. Daughter cells leaving the Wnt niche transdifferentiate into AT1s: maintaining Wnt signaling prevents transdifferentiation whereas abrogating Wnt signaling promotes it. Injury induces AT2 autocrine Wnts, recruiting 'bulk' AT2s as progenitors. Thus, individual AT2 stem cells reside in single cell fibroblast niches providing juxtacrine Wnts that maintain them, whereas injury induces autocrine Wnts that transiently expand the progenitor pool. This simple niche maintains the gas exchange surface, and is coopted in cancer.

Although there has been great progress identifying tissue stem cells, much less is known about their niches and how niche signals control stem cell function and influence daughter cell fate (*1*, *2*). The best understood examples come from genetic systems (*3*) such as the Drosophila testis niche, where 10-15 cells ("the hub") provide three short-range signals to the 5-10 stem cells they contact (*4*). These signals promote stem cell adhesion to the niche and inhibit differentiation, but following polarized division, a daughter cell leaves the niche, escaping the inhibitory signals and initiating sperm differentiation. In mammalian systems, stem cells and their niches are typically more complex, with more cells and more complex cell dynamics. Even in the best-studied tissues (*5*–*8*), there is incomplete understanding of niche cells, signals, and the specific aspects of stem cell behavior each signal controls. Here we describe an exquisitely simple stem cell niche and control program that maintains the lung's gas exchange surface.

Mouse genetic studies have identified a hierarchy of stem cells that replenish the alveolar surface (*9*), some active only following massive injury (*10*, *11*). Normally, the epithelium is maintained by rare 'bifunctional' alveolar type 2 (AT2) cells, cuboidal epithelial cells that retain the surfactant biosynthetic function of standard ("bulk") AT2 cells (*12*) but also serve as stem cells (*13*, *14*). Their intermittent activation gives rise to AT1 cells, exquisitely thin epithelial cells that mediate gas exchange, and generates slowly expanding clonal 'renewal foci' that together create ~7% new alveoli per year (*13*). Dying cells are proposed to provide a mitogenic signal transduced by the EGFR-KRAS pathway that triggers stem cell division (*13*). However, it is unclear how stem cells are selected from bulk AT2 cells, how they are maintained, and how the fate of daughter cells–stem cell renewal vs. reprogramming to AT1 identity–is controlled.

Here we molecularly identify alveolar stem cells as a rare subpopulation of AT2 cells with constitutive Wnt pathway activity, and show that a single fibroblast near each stem cell comprises a Wnt signaling niche that maintains the stem cell and controls daughter cell fate. Severe injury recruits ancillary stem cells, by transiently inducing autocrine Wnt signaling in 'bulk' AT2 cells.

Results

Wnt pathway gene Axin2 *is expressed in a rare subpopulation of AT2 cells*

Canonical Wnt signaling activity marks stem cells in various tissues (*8*), and the Wnt pathway is active in developing alveolar progenitors (*15*–*17*). To determine if AT2 cells in adult mice show Wnt activity, we examined expression of Wnt target *Axin2* (*18*) using an *Axin2-Cre-ERT2* 'knock-in' allele crossed to Cre reporter *Rosa26mTmG* . After three daily tamoxifen injections to induce Cre-ERT2 at age 2 months, FACS showed 1% of purified AT2 cells expressed the GFP reporter (Fig. 1D). Immunostaining for GFP and canonical AT2 marker SftpC showed labeled AT2 cells distributed sporadically throughout the alveolar region (Fig. 1, A to C). Multiplex single molecule FISH [Proximity Ligation in situ Hybridization, PLISH (*19*)] confirmed a distributed population of

Axin2-expressing AT2 cells (fig. S1). Axin2$^+$ AT2 cells represent a stable subpopulation because the percentage of labeled AT2 cells did not increase when tamoxifen injections were repeated one and two weeks after the initial induction, and the percentage was similar among animals induced at different ages (Fig. 1G). This subpopulation expressed all AT2 markers, including surfactant proteins and lipids (fig. S2), suggesting the cells are physiologically functional. No AT1 or airway epithelial cells were marked under these "pulse-labeling" conditions (>1000 AT1 cells scored in each of 3 mice), although other (non-epithelial) alveolar cells were (see below). Thus, Axin2$^+$ AT2 cells represent a rare, stable subpopulation of mature AT2 cells.

Axin2$^+$ AT2 cells have alveolar stem cell activity

The fate of the labeled AT2 cells was examined a half or one year later (half or full year "chase") (Fig. 1, E to I). Labeled cells exhibited three features of stem cells. First, unlike most AT2 cells, which are quiescent (*20*), 79% of Axin2+ AT2 cells generated small clones of labeled cells (Fig. 1, H to J). Daughter cells remained local (Fig. 1I) with some found as doublets (fig. S3B) indicating recent division; on occasion an Axin2$^+$ AT2 cell was seen dividing (fig. S3A), an intermediate we never observed for bulk AT2 cells in normal lungs. Second, lineage-labeled AT2 cells expanded six-fold relative to unlabeled cells during a one-year chase (Fig. 1G). Third, labeled cells gave rise to another alveolar cell type, shown by appearance of AT1 cells expressing the lineage label (Fig. 1F). Like AT2 daughter cells, daughter AT1 cells were typically found in close association with the presumed founder Axin2$^+$ AT2 cell. Thus, Axin2$^+$ cells constitute a rare AT2 subpopulation with stem cell activity, which slowly (about once every 4 months) self-renew and produce new AT2 and AT1 cells.

Fibroblasts provide short-range Wnt signals to neighboring AT2 stem cells

Wnts are local signals with typical range of just 1-2 cells (*21*). Fibroblasts were an excellent candidate for the Wnt source because some contact AT2 cells (*22*), such as Pdgfrα-expressing fibroblasts that support surfactant production and formation of alveolospheres in culture (*14*, *23*, *24*). Transmembrane protein Porcupine (Porcn), which acylates and promotes secretion of Wnts (*25*) and marks Wnt signaling centers (*26*), was expressed in rare alveolar stromal cells (fig. S4A), most of which were *Pdgfrα*-expressing fibroblasts (fig. S4C) and some were closely associated with AT2 cells (fig. S4B). Serial dosing of Porcupine inhibitor C59 reduced the pool of Axin2$^+$ AT2 cells by 68% (Fig. 2A). Targeted deletion in lung mesenchyme (using *TBX^{LME}-Cre*) or fibroblasts (*Pdgfrα-Cre-ER*) of *Wntless*, another transmembrane protein required for Wnt secretion (*27*), also reduced the pool (Fig. 2, B and C). The remaining Axin2$^+$ AT2 cells could be due to incomplete deletion or perdurance of *Wntless* in PDGFRα$^+$ fibroblasts or to another Wnt source (see below).

Single cell RNA sequencing of alveolar fibroblasts revealed a subset expressing *Wnt5a*, most of which (74%) also expressed *Pdgfrα* (Fig. 2D). Many *Wnt5a*$^+$ fibroblasts also expressed low levels of one or two other *Wnts*, including *Wnt2*, *Wnt2b*, *Wnt4*, and *Wnt9a*, as did other smaller subpopulations of fibroblasts (Fig. 2D). AT2 cells did not express *Porcupine* (fig. S4) or any *Wnt* genes (fig. S5) under normal conditions. *Wnt5a*-expressing fibroblasts (fig. S6) were scattered throughout the alveolar region, most near an Axin2$^+$ AT2 cell (Fig. 2, E to H). Although Wnt5a is sufficient to induce *Axin2* in AT2 cells (Fig. 2H), it is not the only Wnt operative in vivo because others can also induce *Axin2* (Fig. 2I), and deletion of *Wnt5a* with *Tbx4^{LME}-Cre* reduced Axin2$^+$ AT2 cells in vivo by 15% and the effect did not reach significance (P = 0.12). We conclude that Wnt5a, and other Wnts expressed by the fibroblasts, activate the canonical Wnt pathway in neighboring AT2 cells. This signal is short range, because AT1 cells derived from Axin2$^+$ AT2 cells do not express *Axin2* (n > 1000 cells scored in 3 lungs at age 4 months), implying they do not maintain *Axin2* expression once they move away from the Wnt source. Some *Wnt*-expressing fibroblasts themselves expressed *Axin2* (Fig. 2D and fig. S6), suggesting they can also provide an autocrine signal.

Wnt signaling prevents reprogramming of alveolar stem cells into AT1 cells

To investigate the function of Wnt signaling, we deleted *β-catenin*, a transducer of canonical Wnt pathway activity, in mature AT2 cells using *Lyz2-Cre* or *SftpC-CreERT2* while simultaneously marking recombined cells using *Rosa26mTmG*. We reasoned that only cells with active Wnt signaling (Axin2$^+$ AT2 cells) would be affected. The number of AT1 cells expressing the AT2 lineage mark tripled, while preserving the percentage of lineage-labeled AT2 cells (85 ± 3% of AT2 cells vs 82 ± 3% in wild-type *β-catenin* controls, n = 500 AT2 scored in 3 biological replicates) and alveolar structure (Fig. 3, A, B, and D; and fig. S7, A, B, and D). Interestingly, 27% of AT2-lineage-marked AT1 cells in this experiment (48 of 172 scored cells in 3 animals) were not physically associated with a marked founder AT2 cell (Fig. 3, E and F), implying the stem cell had directly converted into an AT1 cell, rare in control lungs (4%, n = 145 scored cells in 3 biological replicates). Thus, abrogation of constitutive Wnt signaling promotes transdifferentiation of Axin2$^+$ AT2 cells into AT1 cells.

We also prevented AT2 cells from downregulating Wnt signaling by expressing a stabilized *β-catenin* (*β-catenin^{Ex3}*). This did not induce proliferation (fig. S8) or other obvious effects on AT2 cells, but reduced lineage-marked AT1 cells 2.7-fold (Fig. 3, C and D, and fig. S7, C and D).

Under culture conditions that maintain AT2 identity (*28*), Wnt antagonist Dickkopf 3 (*29*) increased the percentage of cells that reprogrammed to AT1 fate 3.8-fold (2.5 ± 1.5% vs. 9.5 ± 0.1%) (Fig. 3, G and H). Conversely, under conditions that promote differentiation to AT1 fate (*28*), Wnt5a inhibited this transdifferentiation 2.6-fold (21 ± 1% vs 8 ± 1%) (Fig. 3I). CHIR99021, a pharmacological activator of canonical Wnt signaling, had a similar effect (Fig. 3I).

Thus, canonical Wnt signaling maintains the AT2 stem cell pool by preventing their reprogramming to AT1 identity, both in vivo and in vitro. Although Wnt signaling alone had little effect on AT2 proliferation (Fig. 3, J and K, and fig. S8), it enhanced EGF's mitogenic activity (Fig. 3, J and K).

Wnt signaling is induced in 'ancillary' AT2 stem cells following epithelial injury

To investigate stem cell activity following injury, we established a genetic system (*30*) to ablate alveolar epithelial cells. Diphtheria toxin receptor was expressed throughout the lung epithelium of adult mice using *Shh-Cre*. Diphtheria toxin (DT, 150 ng) triggered apoptosis in ~40% of alveolar epithelial cells (fig. S9, A and B) but spared enough for repair (Fig. S9C) and survival. Nearly all remaining AT2 cells (85%) began proliferating after injury (Fig. 4, A and C), indicating 'bulk' AT2 cells are recruited as 'ancillary' progenitors during repair; similar recruitment of bulk AT2 cells is observed following hyperoxic injury (see below) (*31*, *32*). Most AT2 cells (73%) expressed *Axin2* following DT-triggered injury, indicating canonical Wnt signaling is broadly induced in ancillary stem cells (Fig. 4, B and C, and fig. S10C). Inhibition of Wnt signaling with C59 abrogated AT2 proliferation and blocked repair (Fig. 4C and fig. S9D). Thus, Wnt signaling recruits ancillary AT2 cells with progenitor capacity following severe injury.

Injury induces autocrine signaling in AT2 cells

There was no change in *Wnt5a* expression (fig. S10c) or stromal expression of Porcupine (fig. S11, A and B), following DT-triggered injury. By contrast, Porcupine was broadly induced in AT2 cells (figs. S10C and S11, A to C), suggesting injury activates autocrine Wnt secretion. Indeed, we found *Wnt7b*, expressed in alveolar progenitors during development (*16*) but not healthy adult AT2 cells (fig. S5), was broadly induced in AT2 cells following DT-triggered injury (Fig. 4, D and E). AT2 expression of *Wnt7b* and Porcupine, and activation of canonical Wnt signaling, were induced within 24 hours of injury (Fig. 4, D and E, and fig. S10C). AT2 proliferation initiated over the next two days, peaking at day 5 as epithelial integrity was restored, after which AT2 proliferation and gene expression returned toward baseline and new AT1 cells appeared (fig. S10, B and C).

To explore the generality of injury-induced autocrine Wnt signaling, we used hyperoxic injury (75% oxygen) to induce alveolar repair (fig. S12A) (*31*). This allowed us to mark and genetically manipulate alveolar cells by endotracheal delivery of an adeno-associated virus encoding Cre (AAV9-Cre) into lungs of mice carrying a Cre-dependent reporter and conditional Wnt pathway alleles. qPCR analysis of FACS-purified, lineage-labeled AT2 cells (fig. S12, B and C) showed hyperoxic injury induced AT2 expression of *Wnt7b* and six other *Wnt* genes by 3 to 12-fold, and similarly induced *Axin2* (5-fold) and *Lef1* (7-fold), indicating autocrine activation of the canonical Wnt pathway (Fig. 5A). The suite of induced *Wnts* did not include most *Wnts* expressed by the fibroblast niche including *Wnt5a* (Fig. 5A). AAV9-Cre-mediated mosaic deletion of *Wntless* in ~50% of alveolar epithelial cells (fig. S12, D to F) decreased AT2 proliferation following injury (Fig. 5, B and C). The effect was cell autonomous because AT2 cells expressing Cre-GFP, but not neighboring AT2 cells, showed diminished proliferation (Fig. 5, B and C). This autocrine effect is mediated by multiple Wnts because deletion of just one induced *Wnt* (*Wnt7b*) did not diminish proliferation. Thus, epithelial injury induces AT2 expression of a suite of autocrine *Wnts*, which transiently endow bulk AT2 cells with progenitor function and proliferative capacity.

Discussion

We molecularly identified a rare subset of AT2 cells with stem cell function ($AT2^{stem}$) scattered throughout the mouse lung in specialized niches that renew the alveolar epithelium throughout adult life. $AT2^{stem}$ express Wnt target *Axin2*, and many lie near single fibroblasts expressing *Wnt5a* and other *Wnt* genes that serve as a signaling niche (Fig. 6). $AT2^{stem}$ divide intermittently, self-renewing and giving rise to daughter AT1 cells that lose Wnt activity when they exit the niche. Maintaining canonical Wnt signaling blocked transdifferentiation to AT1 identity, whereas loss of Wnt signaling promoted it.

Our results support a model in which each Wnt-expressing fibroblast defines a niche accommodating one $AT2^{stem}$, and this short-range ("juxtacrine") signal selects and maintains $AT2^{stem}$ identity (Fig. 6). When $AT2^{stem}$ divides, daughter cells compete for the niche. The one that remains in the niche retains $AT2^{stem}$ identity; the other leaves the Wnt niche, escaping the signal and reprogramming to AT1 fate. Cells leaving the niche can also become standard AT2 cells, presumably if they land near a signaling center that selects bulk AT2 fate. This streamlined niche, comprising just a single Wnt-expressing fibroblast and stem cell, minimizes niche impact on alveolar gas exchange. It also explains why each expanding focus of new alveoli is clonal, derived from a single $AT2^{stem}$ that typically remains associated with the growing focus (*13*). Although our model posits that the niche cell selects the stem cell, it remains uncertain how the scattered niche cells are

selected. Some niche cells themselves are Axin2^{+} (Fig. 2D and fig. S6) (*24*), suggesting that autocrine Wnt signaling might maintain the niche as juxtacrine signaling maintains the stem cell within it.

Our results also reveal a transient expansion of the alveolar progenitor population following epithelial injury, when the rare AT2^{stem} are insufficient. Many normally quiescent 'bulk' AT2 cells turn on *Axin2* and serve as ancillary progenitors that rapidly proliferate and regenerate lost alveolar cells (Fig. 6). This widespread recruitment of AT2 cells to AT2^{stem} function is not achieved by expansion of the fibroblast Wnt niche. Rather, injury induces Porcupine and another suite of *Wnts,* including *Wnt7b,* in AT2 cells. This switch to autocrine control of stem cell identity obviates dependence on the stromal niche. As the epithelium is restored, *Wnt* expression subsides in ancillary AT2^{stem} and they cease proliferating and begin differentiating into AT1 cells or returning to bulk AT2 identity. The Wnt pathway is broadly active during alveolar development (*16*, *17*) and cancer (see below), where most cells proliferate, so it may have a general role in maintaining alveolar stem/progenitor states.

Our data suggest that Wnt signaling, whether juxtacrine Wnts from a fibroblast or autocrine Wnts induced by injury, endows AT2 cells with two stem cell properties. One is AT2^{stem} gene expression and identity, preventing reprogramming to AT1 (and presumably bulk AT2) fate (Fig. 3). The other is an ability to proliferate extensively, as observed following injury when ancillary AT2^{stem} divide rapidly to restore the epithelium (Fig. 6). Thus, Wnt signaling confers stem cell identity ("stemness") on AT2 cells, but does not itself activate the stem cells (Fig. 3, J and K, and fig. S8). Proliferation is controlled by EGFR/KRAS signaling (Fig. 3, J and K) (*13*), presumably activated by EGF ligand(s) from dying cells (*33*). We propose that Wnt and EGFR/KRAS pathways function in parallel to select and activate alveolar stem cells, respectively, explaining their synergy (Fig. 3, J and K).

The above findings have implications for lung adenocarcinoma, the leading cancer killer (*34*) initiated by oncogenic mutations that activate EGFR/KRAS signaling in AT2 cells (*13*, *35*, *36*). Although most AT2 cells show a limited proliferative response, a rare subset proliferates indefinitely forming deadly tumors (*13*): the tumor-initiating cells could be AT2^{stem}. Indeed, a subpopulation of adenocarcinoma cells was recently found to have Wnt pathway activity and function as tumor stem cells, with associated cells serving as their Wnt signaling niche (*26*). As oncogenic EGFR/KRAS drives stem cell proliferation, Wnt signaling would maintain their identity; this explains why Wnt signaling has little proliferative effect on its own but potentiates KRAS^{G12D} and BRAF^{V600E} mouse models of lung carcinogenesis, and why Wnt inhibition induces tumor senescence (*26*, *37*, *38*). Wnt antagonists might thus be powerful adjuvants in adenocarcinoma therapy, attacking stem cell identity while EGFR antagonists target stem cell activity. One reason stem cell identity may have restricted during evolution to rare AT2 cells is it minimizes cells susceptible to transformation.

There is growing appreciation that some mature cells in other tissues can also provide stem cell function (*8*, *9*, *39*). Like AT2^{stem}, their clinical utility has been overlooked as more classical "undifferentiated" and pluripotent stem cells have been sought. Our study shows that stem cells and their niche cells can each represent minor, solitary subsets of mature cell types. By molecularly identifying such rare subpopulations and niche signals, it should be possible to isolate and expand them for regenerative medicine.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S13
References (*40*–*52*)

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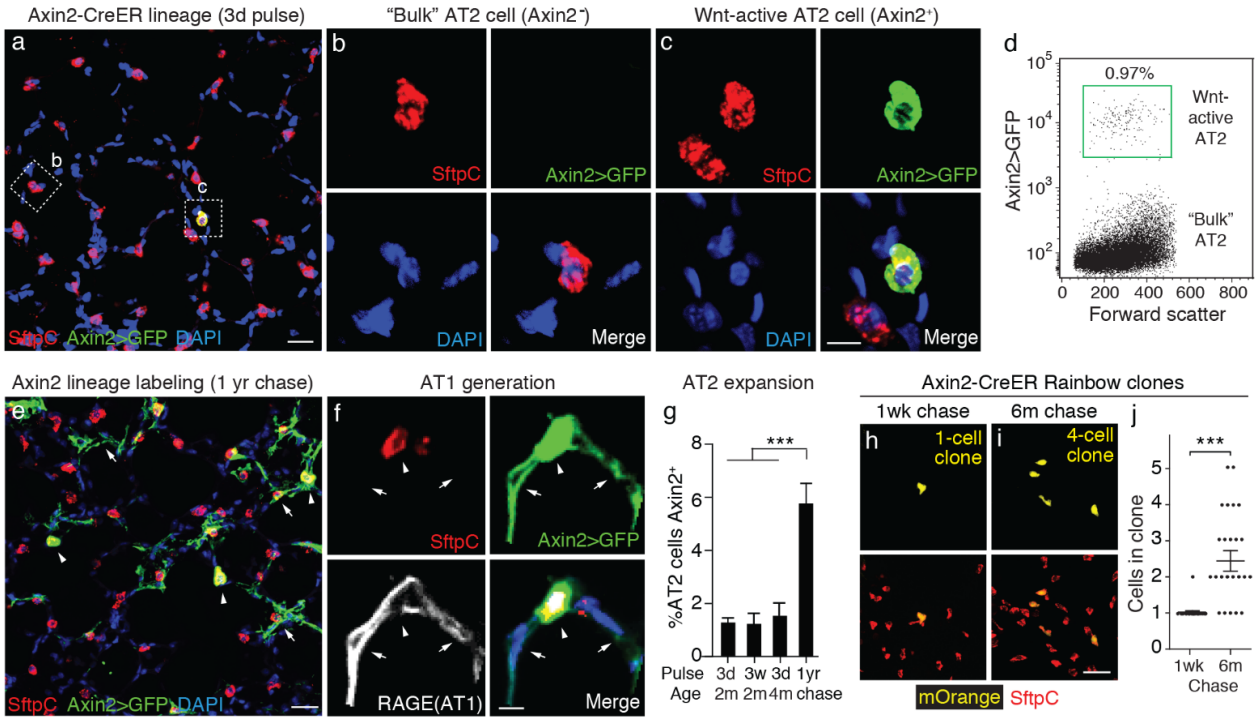

Fig. 1. ***Axin2*** **marks rare AT2 cells with stem cell activity.** (**A** to **C**) Alveoli of adult (2 months) *Axin2-CreERT2;Rosa26mTmG* mouse lung immunostained for Cre reporter mGFP, AT2 marker pro-surfactant protein C (SftpC), and DAPI 5 days after 3 daily injections of 3 mg tamoxifen (3d "pulse") to lineage label *Axin2*-expressing (Axin2$^+$) cells (Axin2>GFP). Close-ups show Axin2$^-$ "bulk" AT2 cell (B) and rare Axin2$^+$ AT2 (C) indicating Wnt pathway activation. (**D**) FACS of AT2 cells above shows 1.0 ±0.5% (n = 3 biological replicates) express Axin2>GFP. (**E**) Alveoli labeled as above harvested one year later ("1 yr chase"). Note increased Axin2-lineage AT2 cells (arrowheads) and labeled AT1 cells and fibroblasts (arrows), the latter from another Axin2$^+$ lineage. (**F**) Close-up of lung as in (E) showing Axin2$^+$ lineage-labeled AT1 cells (arrows). RAGE, AT1 membrane marker; arrowhead, AT2 cell. (**G**) Quantification of Axin2-lineage labeled AT2 cells immediately following 3d or 3 week pulse (3d pulse each week) at age 2 months, 3d pulse at 4 months, or after 3d pulse at 2 months plus 1 year chase. Mean ±SD (n = 3500 AT2 cells scored in 2-4 biological replicates). (**H** and **I**) Alveoli of *Axin2-CreERT2; Rosa26Rainbow* mice given limiting dose of tamoxifen (2 mg) at 2 months to sparsely label Axin2+ cells (H) with different fluorescent clone markers (mOrange in H,I), and immunostained for SftpC 1 week (H) or 6 months (I) later to detect AT2 clones. (**J**) Quantification of AT2 clone sizes 1 week and 6 months after labeling. ***P < 0.001 (t test). Scale bars: 25 μm (A) and (E), 5μm (C) and (F), 20 μm (I).

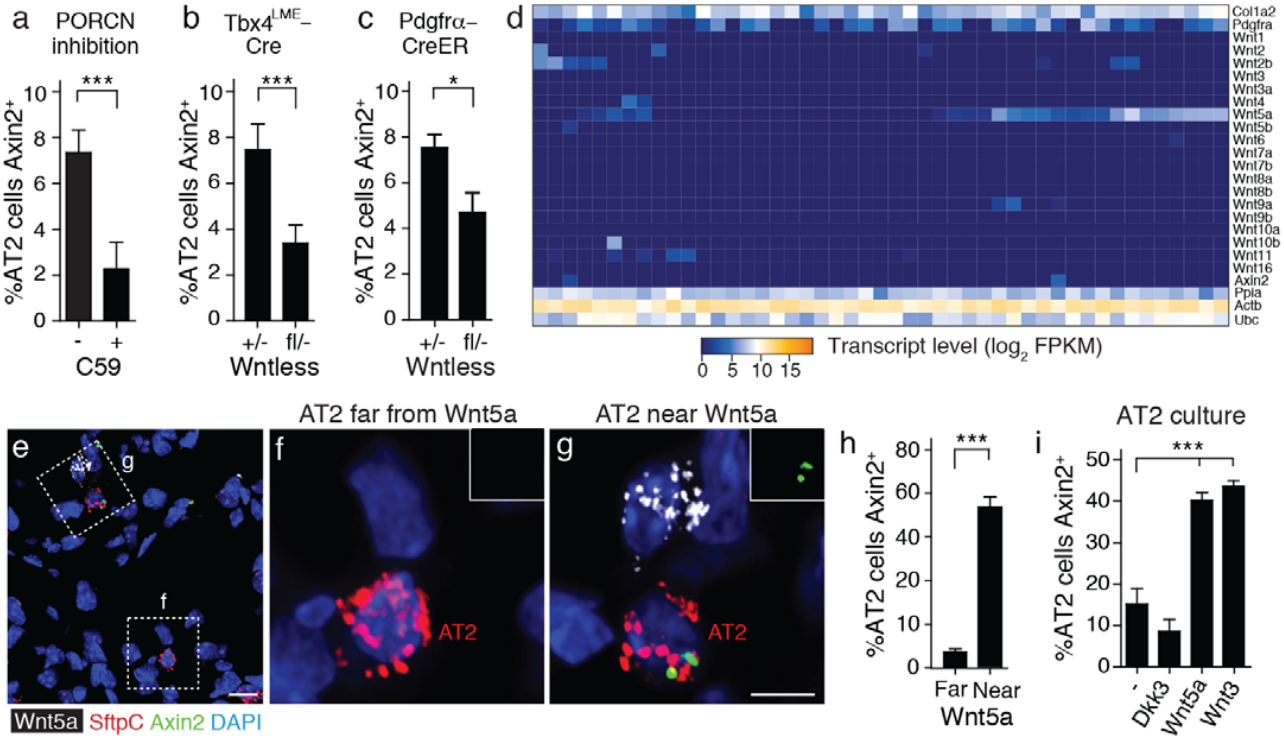

Fig. 2. Single, Wnt-secreting fibroblasts comprise the stem cell niche. (**A**) Effect of 5 daily doses of Wnt secretion (Porcupine, PORCN) inhibitor C59 (+) or vehicle control (−) on *Axin2* expression in AT2 cells at age 2 months, measured by PLISH for *Axin2* and *Sftpc*. Mean ±SD, (*n* = 900 AT2 cells scored in 3 biological replicates). ***$P = 0.001$ (t test). (**B** and **C**) Effect on *Axin2*-expresssion in AT2 cells of inhibiting fibroblast Wnt secretion by deleting *Wntless* with *Tbx4^{LME}-Cre* (B) or *Pdgfrα-CreERT2* induced with 3 mg tamoxifen 3 days before analysis (C). *$P = 0.02$; ***P = 0.003 (*t* test). (**D**) Expression of *Wnts*, fibroblast markers (*Pdgfrα*, *Col1a2*), *Axin2*, and ubiquitous controls (*Ubc, Ppla, Actb*) in 47 alveolar fibroblasts (columns) from B6 adult lungs analyzed by single cell RNA sequencing. (**E**) *Wnt5a*, *Axin2*, and *SftpC* mRNA detected by PLISH of adult (2 months) alveoli. Blue, DAPI. Note rare *Wnt5a*-expressing cell (box g). (**F** and **G**) Close-ups showing AT2 cells far from (F) or near (G) a *Wnt5a*-expressing cell. Insets, *Axin2* channel of AT2 cell. AT2 near *Wnt5a* source expresses *Axin2*. (**H**) Quantification showing percent (mean ±SD) of AT2 cells, located far (>15 µm, *n* = 132 cells from 3 biological replicates) or near (<15µm, n=150 cells) a *Wnt5a* source, that express *Axin2*. ***$P < 0.0001$ (*t* test). (**I**) *Axin2* expression in AT2 cells isolated from adult *Axin2-lacZ* mice and cultured (5 days) with indicated Wnts or antagonist Dickkopf3 at 1ug/mL, then assayed (Spider-gal) for LacZ. ***$P < 0.0001$ (*t* test). Scale bars: 10 µm (E), 5 µm (G).

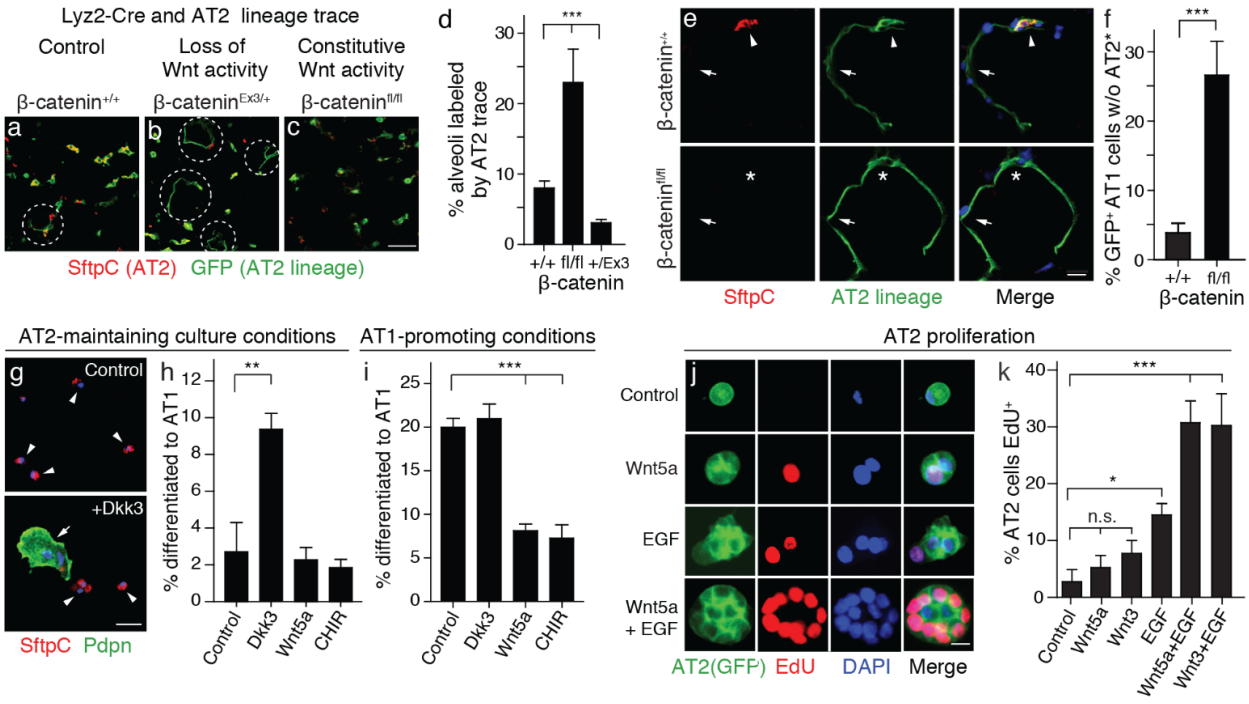

Fig. 3. Wnt signaling prevents reprogramming to AT1 fate. (**A** to **D**) Alveoli of adult (8 months) *Lyz2-Cre;Rosa26mTmG* (A), *Lyz2-Cre;Rosa26mTmG β-catenin$^{fl/fl}$* (B) and *Lyz2-Cre;Rosa26mTmG; β-catenin$^{Ex3/+}$* (C) mice immunostained for SftpC and Lyz2-Cre (AT2) lineage trace (mGFP). Dashed circles, alveolar renewal foci identified by squamous AT1 expressing AT2 lineage trace. Bar, 50μm. Quantification (D) showing percent (mean ±SD) alveoli with AT2 lineage-labeled AT1 cells (n=25 100μm z-stacks scored in 2 or 3 biological replicates). ***P = 0.002 (Kruskal-Wallis). (**E** and **F**) Close up of renewal foci (E) as above in control (*Lyz2-Cre;β-catenin$^{+/+}$*, upper) or *β-catenin* conditional deletion (*Lyz2-Cre;β-catenin$^{fl/fl}$*, bottom). Note AT1 (arrow) and its AT2 parent (arrowhead) in control, but absence of AT2 parent (*) in *β-catenin* deletion, implying loss of stem cell by reprogramming to AT1 fate. Quantification (F) shows percent (mean ± SD) AT1 cells from AT2 lineage (GFP$^+$) lacking AT2 parent. ***P = 0.0004 (*t* test). (**G** to **I**) AT2 cells from B6 adult mouse lungs cultured in Matrigel [AT2-maintaining conditions, (G) and (H)] or on poly-lysine coated glass [AT1-promoting conditions (I)] without (control) or with indicated Wnt pathway antagonist (150ng/ml Dkk3) or agonists (100ng/ml Wnt5a or 10nM CHIR99201). After 4 days, cells were immunostained for SftpC and Podoplanin (G), and percent (mean ±SD) of AT2 (cuboidal SftpC$^+$; arrowheads) and AT1 cells (large, squamous, Podoplanin$^+$; arrow) quantified (n=500 cells from 3 biological replicates) (H and I) **P = 0.002; ***P < 0.001 (t test). (**J**) AT2 cells isolated from adult (2 months) *Sftpc-CreER; Rosa26mTmG* mice were cultured in Matrigel as above without (control) or with indicated Wnts (100ng/ml) and EGF (50ng/ml), then proliferation assayed by EdU incorporation.· (**K**) Quantification (n = 400 cells scored, 4 biological replicates). *P = 0.007; ***P < 0.001 (*t* test). n.s., not significant. Scale bars: 50 μm (C) and (G), 10 μm (E), 5 μm (J).

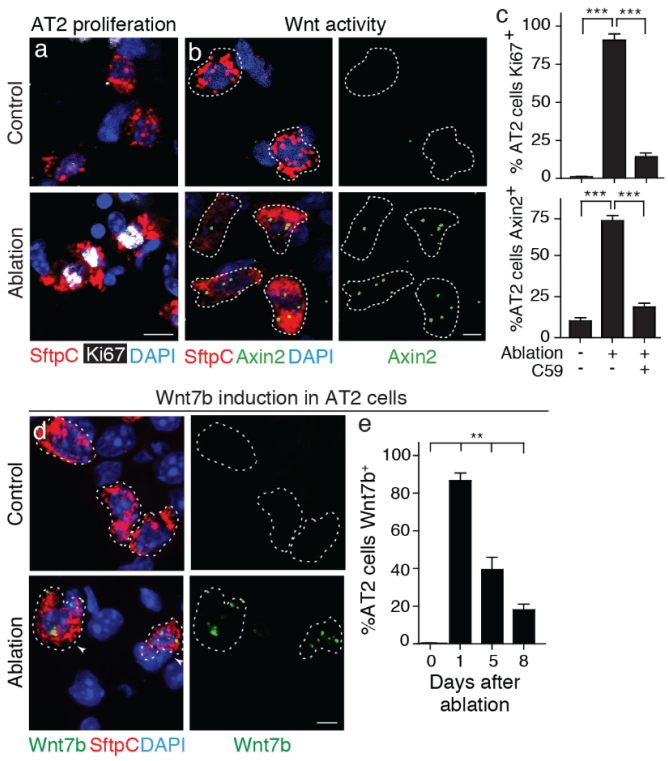

Fig. 4. Genetically-targeted epithelial injury induces Wnt signaling and proliferation of bulk AT2 cells. (**A** to **C**) Alveoli of *Shh-Cre; Rosa26LSL-DTR* (Diphtheria toxin receptor) animals injected with vehicle (control, upper panels) or limiting dose (10 ng) of Diphtheria toxin (DT) to induce sporadic epithelial cell ablation (lower panels), then immunostained five days later for SftpC and Ki67 (A), or probed by PLISH for *SftpC* and *Axin2* expression (B). Quantification (C) (n = 250 cells in 4 animals) of percent AT2 cells expressing Ki67 (mean ± S.D) or *Axin2* (mean ± S.D). Ablation induces proliferation and Wnt signaling in most AT2 cells, both abrogated by Wnt secretion inhibitor C59. ***P < 0.001 (t test). (**D**) Alveoli as above probed by PLISH for *Wnt7b* and *SftpC* mRNA 1 day after vehicle (control) or DT injection (ablation). (**E**) Kinetics of *Wnt7b* induction following ablation (n = 300 AT2 cells scored per animal, 4 biological replicates per time point, mean ± S.D). **P = 0.005 (Kruskal-Wallis). Scale bars: 10 μm (A), 5 μm (B) and (D).

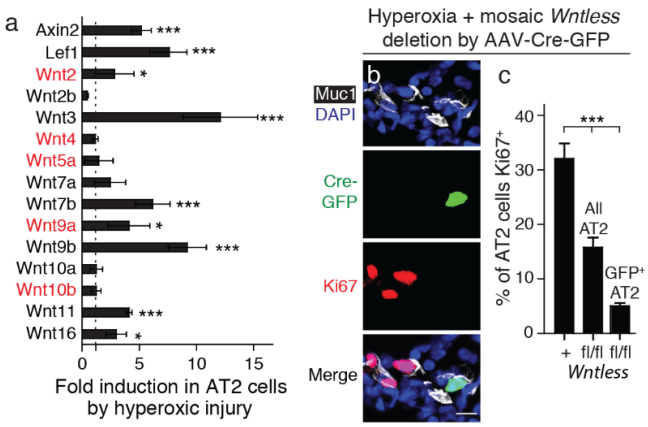

Fig. 5. Hyperoxic injury induces autocrine Wnt signaling in bulk AT2 cells. (**A**) Expression of *Wnt* genes and targets (*Axin2*, *Lef1*) measured by qRT-PCR of FACS-sorted, lineage-labeled bulk AT2 cells from mice 2d after hyperoxic alveolar injury (5d at 75% O_2) (fig. S12b), normalized to values before hyperoxic injury (mean ±SD, $n = 3$ mice per condition). Red, *Wnts* expressed by fibroblast niche (Fig. 2D). $^*P < 0.05$, $^{***}P < 0.001$ (t test). (**B** and **C**) Alveoli immunostained as indicated from adult (age 2 months) *Wntless*$^{fl/fl}$ mouse with alveolar epithelium infected with *AAV9-GFP-Cre* virus (Fig. S12d-f) to mosaically delete *Wntless* 1 week before hyperoxic injury. Note injury-induced proliferation (Ki67 staining) of AT2 cells (Muc1 apical marker) (fig. S12A) except AT2 infected with AAV-Cre-GFP to delete *Wntless*. (C) Quantification showing percent AT2 cells Ki67$^+$ following hyperoxic injury of AAV9-Cre-GFP-infected control (*Wntless*$^{+/+}$) and *Wntless*$^{fl/fl}$ mice, scored for all or just GFP$^+$ (AAV9-Cre-GFP-infected) AT2 cells. Mean ±SD (n = 500 total AT2 cells scored per mouse, 3 mice per condition, including 52 GFP$^+$ cells). Scale bar, 10 μm.

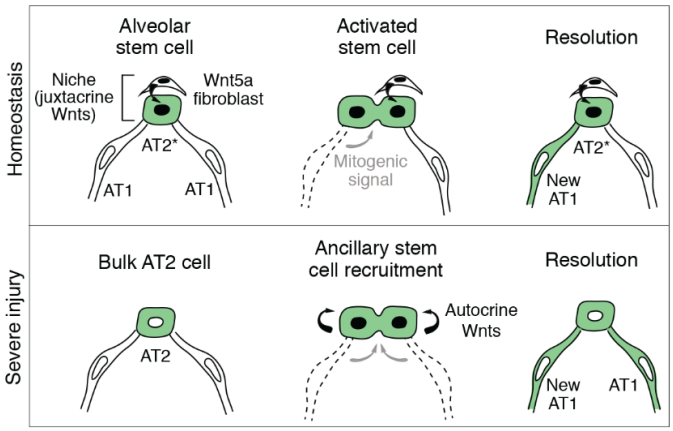

Fig. 6. Model of alveolar stem cells and their niches. (**Top**) During homeostasis, the niche is a single fibroblast constitutively expressing *Wnt5a* and/or other *Wnts* that provide "juxtacrine" signal (arrow) to the neighboring AT2 cell (green cytoplasm, lineage trace; black nucleus, Wnt pathway active), selecting/maintaining it as a stem cell. Upon receiving a mitogenic signal from the dying AT1 cell (middle), the activated stem cell proliferates. Daughter cells (green) compete for the niche. One remains in the niche as a stem cell. The other leaves the niche, losing Wnt signal and transdifferentiating into a new AT1 cell (right). (**Bottom**) Following severe injury, bulk AT2 are recruited as "ancillary" stem cells by induction of autocrine Wnts, allowing unlimited proliferation in response to mitogens. Autocrine Wnts diminish as injury resolves.

Single-cell Wnt signaling niches maintain stemness of alveolar type 2 cells

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