***Ager-CreER^{T2}*: A New Genetic Tool for Studying Lung Alveolar Development, Homeostasis, and Repair**

Mei-I Chung and Brigid L. M. Hogan

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina

Abstract

The alveolar region of the lung is composed of two major epithelial cell types: cuboidal alveolar type 2 cells (AT2 cells), which produce surfactant proteins, and large, thin, alveolar type 1 cells (AT1 cells), specialized for efficient gas exchange. AT1 cells cover more than 95% of the alveolar surface and constitute a major barrier to the entry of pathogenic agents. Relatively few genetic tools are available for studying the development of AT1 cells, the function of genes expressed in them, and the effect of specifically killing them *in vivo* in the adult lung. One distinguishing feature of AT1 cells is the high level of expression of the gene *Ager*, encoding the advanced glycation endproduct-specific receptor, a member of the immunoglobulin superfamily of cell surface receptors. In this paper, we report the

generation of a novel *Ager-CreER^{T2}* allele in which Cre recombinase is inserted into the first coding exon of the endogenous gene. After treatment with tamoxifen the allele enables *Ager*⁺ progenitor cells to be efficiently lineage labeled during late embryonic development and AT1 cells to be killed in the adult lung using a Rosa26-diphtheria toxin A allele. Significantly, adult mice in which approximately 50% of the AT1 cells are killed survive the loss; repair is associated with increased proliferation of SFTPC⁺ (surfactant protein C-positive) AT2 cells and the upregulation of *Ager* expression. The *Ager-CreER^{T2}* allele thus expands the repertoire of genetic tools for studying AT1 turnover, physiology, and repair.

Keywords: *Ager*; Cre recombinase; lung; type 1 alveolar epithelial cells; type 2 alveolar stem cells

The distal region of the mammalian lung is composed of millions of tiny air sacs, known as alveoli, that are made up of epithelial cells, capillary vasculature, stromal cells, and resident immune cells. There are two epithelial cell types: cuboidal alveolar type 2 cells (AT2 cells), specialized for the production, storage, and secretion of surfactant proteins; and large, thin, alveolar type 1 cells (AT1 cells) that are the major sites of gas exchange between air and blood (1–6). Each AT1 cell has an area of around 5,100 μm^2 , and together they cover approximately 95% of the surface of the distal lung. They are connected to each other, and to AT2 cells, by apical tight junctions, and the basal surface lies on a

basal lamina, which is fused with that of the closely apposed capillary endothelial cells. Reconstruction of tissue sections shows that individual AT1 cells can have a quite complex “branched” three-dimensional structure, with cytoplasmic “plates” extending between adjacent alveoli (3). The cytoplasm itself contains relatively few specialized organelles apart from microvesicles involved in transcellular transport.

Given that AT1 cells provide the major air–blood barrier of the distal lung, it is important that they are replaced quickly if they are damaged, such as by exposure to toxic agents. New AT1 cells also have to be generated during the

compensatory regrowth and alveologenesis that follows the surgical removal of a lung lobe by pneumonectomy (2, 7, 8). In all injury/repair models that have been studied, mature AT1 cells do not self-renew but are replaced by the proliferation, differentiation, and spreading of AT2 cells that, as a population, function as alveolar stem cells (9–11). There is, however, evidence that during remodeling after pneumonectomy, a small percentage of AT1 cells, which may be immature, can give rise to AT2 cells (2, 7).

To better understand the biology, function, and regeneration of AT1 cells, researchers have used a number of strategies

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Correspondence and requests for reprints should be addressed to Brigid L. M. Hogan, Ph.D., F.R.S., George Barth Geller Professor and Chair, Department of Cell Biology, Duke Medicine, 307 Research Drive, Durham, NC 27710. E-mail: brigid.hogan@duke.edu.

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to purify the cells from the postnatal lung and to catalog genes preferentially expressed in them compared with other lung epithelial cell types (2, 12). For example, *Hopx* (HOP homeobox) is a transcription factor expressed in all AT1 cells and podoplanin (also known as T1 α), Ager (advanced glycation endproduct receptor), and *Igfbp2* (insulin growth factor-binding protein) are membrane-associated proteins preferentially expressed on the cell surface. Ager belongs to the immunoglobulin superfamily of transmembrane receptors and has a broad repertoire of ligands, including proteins that accumulate extracellularly during inflammation, aging, and infection (13). Ager lies upstream of NF- κ B as well as mitogen-activated protein kinases, and there is some evidence that it promotes cell adhesion and spreading of AT1 cells, but its precise *in vivo* function is still not fully understood; several isoforms are generated by proteolytic cleavage and alternative splicing, including a soluble form that may function as a decoy receptor. Ager-null mice are fully viable but show physiological defects related to inflammation and vascular function (13).

Experimental insights into the molecular mechanisms driving lung regeneration and repair *in vivo* depend to a large degree on the availability of genetic tools for manipulating genes in specific cell types. Given that there may be different subpopulations of AT2 and AT1 cells expressing different amounts or subsets of marker genes (2, 10, 11), it is advantageous for the field to have available a “toolkit” of different reporter and Cre recombinase alleles driven by different endogenous genes. In previous studies on the role of AT1 cells in lung biology, researchers have used *Hopx-ERCre* (7) and *Hopx-CreER* and *Igfbp-CreER* alleles (2). In this paper, we report the generation of a new *Ager-CreER* “knock-in” allele that is very efficient in driving recombination in Ager-expressing cells after administration of tamoxifen (Tmx). The allele can be used to lineage label Ager⁺ progenitor cells in the embryonic lung and to kill adult AT1 cells after recombination of *Rosa26-DTA*. Surprisingly, even after death of about 50% of the AT1 cells, adult mice are viable, and lung architecture is minimally affected, likely owing to the rapid replacement of AT1 cells by the proliferation and differentiation of AT2 cells.

Methods

Mice

To generate the *Ager-CreER^{T2}* knock-in allele, the previously described strategy was used (14). Briefly, 8 kb of 5' untranslated region and exons 1–7 was retrieved from a BAC (bacterial artificial chromosome) clone (bMQ174; Source BioScience) and recombined into the vector pL25B. A CreER^{T2}-polyA cassette and an FRT-flanked neomycin resistance cassette were recombined into the start codon to obtain the targeting construct. The construct was then electroporated into G4 (C57BL/6Ncr $\times$ 129S6/SvEvTac) hybrid embryonic stem cells. CreER^{T2} recombination into the *Ager* locus was confirmed using Southern blot analysis. Four correct clones were injected into C57BL/6 blastocysts. To remove the neomycin resistance cassette, mice were bred to 129S4-Gt(ROSA)26Sor^{tm2(FLP*)Sor/J}. *Ager-CreER^{T2}* mice were maintained on a C57BL/6J background. *Rosa26-CAG-lsl-tdTomato* (hereafter *Rosa26-tdTm*) (15) and B6.129P2-Gt(ROSA)26Sor^{tm1(DTA)Lky/J} (*Rosa26-DTA*; JAX 009669; The Jackson Laboratory) (16, 17) and *Ager-H2B:Venus* (in which H2B:Venus fusion protein is expressed under control of the endogenous *Ager* locus [14]) mice were maintained on a C57BL/6J background. All experiments were performed according to institutional animal care and use committee-approved protocols.

Tmx Administration

For embryonic experiments, pregnant female mice were gavaged with one dose of 50 or 100 μ g/g body weight Tmx dissolved in corn oil. To label or kill adult AT1 cells, four doses of 200 μ g/g body weight Tmx were given every other day, unless otherwise stated.

5-Ethynyl-2'-Deoxyuridine Labeling

Mice were injected intraperitoneally with 50 μ g/g body weight 5-ethynyl-2'-deoxyuridine (EdU) dissolved in PBS, and lungs were fixed after 3 hours. EdU incorporation was detected using the Click-iT Plus EdU Alexa Fluor 647 kit (C10640; Thermo Fisher Scientific).

Histology and Immunofluorescence Analysis

Lungs were fixed and processed as described previously (14). For immunofluorescence analysis, tissue sections were blocked in 3%

BSA, 10% donkey serum, and 0.1% Triton X-100 for 1 hour at room temperature. Primary antibodies were diluted in block solution and were applied and incubated overnight at 4°C. All fluorophore-conjugated secondary antibodies were diluted at 1:500 and incubated for 1 hour at room temperature. Primary antibodies used were as follows: CD11b (101201, 1:100 dilution; BioLegend), LAMP-3/CD208 (DDX0191, 1:200 dilution; Dendritics), GFP (GFP-1020, 1:500 dilution; Aves Labs), HOPX (sc-398703, 1:50 dilution; Santa Cruz Biotechnology), red fluorescent protein (600401379, 1:250 dilution; Rockland Immunochemicals), receptor for advanced glycation endproducts/AGER (MAB1179, 1:200 dilution; R&D Systems), and SFTPC (surfactant protein C) (ab3786, 1:500 dilution; Merck Millipore; and sc-7706, 1:100 dilution; Santa Cruz Biotechnology). Images were captured using AxioCam imager and LSM 710 and LSM 780 microscopes (Carl Zeiss Microscopy).

Quantification and Statistics

For quantification, one longitudinal section per sample was imaged and analyzed using ImageJ software (NIH) ($n \geq 3$ animals/experiment). Prism software (GraphPad Software) was used to plot the quantification values. Statistical analysis was performed using unpaired two-tailed Student's *t* tests between groups. Values on graphs are shown as mean $\pm$ SD.

qRT-PCR

Total RNA and cDNA were prepared as described previously (14). qPCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories), and reactions were run on a StepOnePlus system (Applied Biosystems). Expression levels were normalized to *Gapdh*. Primer sequences were as follows: *Ager* forward: 5'-CCATCCAACTCCGAGTCAG-3'; *Ager* reverse: 5'-CACAGGTCAAGGTCA CAGTC-3'; *Hopx* forward: 5'-GCTGTGCCTCATCGCAG-3'; *Hopx* reverse: 5'-CAAGCCTTCTGACCGCC-3'.

Results

Generation of *Ager-CreER^{T2}* Mice

The generation of the *Ager-CreER^{T2}* “knock-in” allele, which uses the ATG of the first coding exon of the endogenous

gene, is shown in Figures 1A and 1B. Evidence that it is a null allele comes from immunohistochemical analysis of sections of adult wild-type, heterozygous, and homozygous mutant lungs (Figure 1C) and from qPCR analysis of RNA extracted from them (Figure 1D). Previous studies have shown that *Ager*-null mutant mice are viable (13). Likewise, homozygous

Ager-CreER^{T2} mice were apparently normal and fully fertile.

***Ager-CreER^{T2}* Drives Recombination in *Ager*⁺ Progenitor Cells of the Embryonic Mouse Lung**

Previous studies have shown that *Ager* is expressed in epithelial progenitor cells in the developing mouse lung (7, 12, 18).

Specifically, immunohistochemistry shows that at Embryonic Day (E) 15.5, it is coexpressed with HOPX in a population of cells in the “stalks” of distal tips (7). To test the ability of the *Ager-CreER^{T2}* allele to drive recombination in the embryonic lung, we administered Tmx to pregnant *Ager-CreER^{T2}; Rosa26-tdTm* mice at E16.5 and harvested embryonic lungs at E17.5 and

Figure 1. Generation of *Ager-CreER^{T2}* knock-in mice. (A) Schematic diagram of the strategy for generating the *Ager-CreER^{T2}* allele. (B) Correct targeting of embryonic stem cells was confirmed using Southern blot analysis. AflIII enzyme was used to generate 4.8-kb wild-type (WT) and 3.5-kb mutant DNA fragments. (C) Immunohistochemistry shows that the AT1 (alveolar type 1) markers AGER (advanced glycation endproduct-specific receptor) and HOPX (HOP homeobox) label *Ager-CreER^{T2}* adult lung tissue. However, AGER is not detected in the homozygous *Ager-CreER^{T2}/CreER^{T2}* lung. Scale bars = 100 μ m. (D) qPCR analysis reveals that *Ager* transcript levels are significantly reduced in homozygous mouse lung, whereas levels of *Hopx* transcript are not affected. The mRNA expression levels of *Ager-CreER^{T2}/+* and *Ager-CreER^{T2}/CreER^{T2}* samples were normalized to *Ager^{+/+}*. $n = 3$ animals. Data shown as mean $\pm$ SD. * $P < 0.05$. HSV-TK = herpes simplex virus thymidine kinase; n.s. = not significant; polyA = polynucleotide adenylyltransferase; UTR = untranslated region.

E18.5. As shown in Figures 2A and 2B, $18.32 \pm 6.70\%$ of $AGER^{+}$ cells are lineage labeled at E17.5, whereas $52.37 \pm 4.14\%$ of $HOPX^{+}$ cells are labeled at E18.5. The majority of cells that were lineage labeled with tdTomato are AT1 cells ($HOPX^{+}$ or $AGER^{+}$) ($96.16 \pm 1.03\%$ at E17.5; $97.78 \pm 1.16\%$ at E18.5), and only a few Tomato⁺ cells express SFTPC ($3.84 \pm 1.03\%$ at E17.5; $2.19 \pm 1.13\%$ at E18.5) (Figure 2B).

Use of *Ager-CreER*^{T2} to Lineage Label AT1 Cells in the Adult Lung

To establish the efficiency and specificity of the *Ager-CreER*^{T2} allele in the adult mouse lung, we treated mice carrying the *Rosa26-tdTm* allele with different concentrations of Tmx (four doses of $10 \mu\text{g/g}$ and either one or four doses of 0.2 mg/g) (Figure 3). This resulted in labeling of $9.52 \pm 0.98\%$, $21.85 \pm 1.18\%$, and $56.87 \pm 3.63\%$ AT1 cells, respectively, as judged by coexpression of the lineage trace with the marker protein HOPX (Figures 3D and 3F–3H). Only a small percentage of SFTPC⁺ AT2 cells were labeled ($1.35 \pm 0.35\%$, $1.52 \pm 0.06\%$, and $1.04 \pm 0.57\%$,

respectively). In control lungs not exposed to Tmx, only $0.12 \pm 0.03\%$ of AT1 cells were labeled (Figure 3E). Epithelial cells lining the bronchiolar airways and in the mesothelium were not labeled.

Use of *Ager-CreER*^{T2} to Specifically Kill AT1 Cells In Vivo

In previous studies, we have successfully used a combination of *Rosa26-DTA* and *Sftpc-CreER*^{T2} alleles to specifically ablate approximately 50% of the AT2 cells of the adult lung, with little effect on overall tissue architecture (18). After recombination, the *Rosa26-DTA* allele results in expression of the A subunit of diphtheria toxin, which kills cells even if they do not express diphtheria toxin surface receptor. To use the same strategy for killing AT1 cells, we bred mice carrying *Ager-CreER*^{T2}, *Rosa26-tdTm* and *Rosa26-DTA* alleles. As shown in Figure 4, 4 days after the administration of four doses of Tmx (0.2 mg/g), there are approximately 50% less $HOPX^{+}$ AT1 cells in the lung than in lungs of control mice (Figures 4A and 4F), suggesting that approximately 50% of the AT1 cells were

killed. Significantly, all of the mice ($n = 4$) survived the Tmx administration. In agreement with earlier studies in which about half of the AT2 cells were killed with DTA (18), there was no major alteration in tissue architecture associated with loss of AT1 cells, apart from the appearance of more $CD11b^{+}$ cells (monocytes, neutrophils, and macrophages) in the alveoli (Figure 4B). Some lineage-labeled AT1 cells survived the treatment, presumably because they recombined the *Rosa26-tdTm* but not the *Rosa26-DTA* allele. Significantly, no EdU incorporation was seen in any of these AT1 cells 4 days after Tmx treatment. EdU incorporation was, however, seen in $21.15 \pm 3.98\%$ of the AT2 cells, suggesting that they proliferate in response to loss of AT1 cells (Figures 4D and 4F). In addition, some of the SFTPC⁺ AT2 cells show a more elongated morphology compared with their normal cuboidal shape seen in the control lung (Figure 4D, yellow arrowheads). To further investigate changes in AT2 cells after ablation of AT1 cells, we generated *Ager-CreER*^{T2} mice carrying the *Ager-H2b:Venus* allele in which an H2b:Venus fusion protein is expressed in the nucleus under the control of the endogenous *Ager* locus (14). *Ager-CreER*^{T2}/*H2b:Venus*; *Rosa26-DTA* mice were treated with four doses of Tmx (0.2 mg/g), and their lungs were fixed and analyzed 4 days later. In the *Ager-CreER*^{T2}/*H2b:Venus* lungs, approximately 5% of SFTPC⁺ AT2 cells are Venus⁺. By contrast, approximately 50% of SFTPC⁺ cells are Venus⁺ in *Ager-CreER*^{T2}/*H2b:Venus*; *Rosa26-DTA* lung (Figure 4E).

Discussion

In the present paper, we report the generation of a novel *Ager-CreER*^{T2} “knock-in” allele that augments the repertoire of genetic tools available for studying AT1 development, function, and regeneration in the mouse lung. We have shown in the present study that this allele is efficient in lineage labeling *Ager*⁺ progenitor cells in the developing lung during the very earliest stages of alveologenesis (E16.5) (Figure 2). This result suggests that the allele can, in the future, be useful for following the fate of individual *Ager*⁺ cells, using low doses of Tmx and Rosa-Confetti lineage-tracing tools.

Previous studies have indicated that AT2 cells in the postnatal lung express low

Figure 2. *Ager-CreER*^{T2}; *Rosa26-tdTm* lineage labels *Ager*⁺ epithelial progenitors. (A) *Ager-CreER*^{T2}; *Rosa26-tdTm* embryos were exposed to tamoxifen ($50 \mu\text{g/g}$) at Embryonic Day (E)16.5, and lungs were fixed and analyzed by immunohistochemistry at E17.5 ($n = 3$). All panels are images from the same section that was stained with antibodies to RFP (red fluorescent protein), AGER, and SFTPC (surfactant protein C). Of $AGER^{+}$ AT1 cells, $18.32 \pm 6.70\%$ were lineage labeled. Of the lineage-positive cells, $96.16 \pm 1.03\%$ are $AGER^{+}$, and $3.84 \pm 1.03\%$ are SFTPC⁺. (B) A higher dose of tamoxifen ($100 \mu\text{g/g}$) was given at E16.5, and lungs were fixed and analyzed at E18.5. Of $HOPX^{+}$ AT1 cells, $52.37 \pm 4.14\%$ were labeled. At this dose, the majority of Tomato⁺ (tdTomato-positive) cells are still $HOPX^{+}$ ($97.78 \pm 1.16\%$). However, a few Tomato⁺ cells are SFTPC⁺ ($2.19 \pm 1.13\%$) (arrow). The two left panels are images of the same section stained with antibodies to RFP and HOPX. The far right panel is an adjacent section stained with antibodies to RFP and SFTPC. Scale bars = $100 \mu\text{m}$.

Figure 3. *Ager-CreER^{T2}* efficiently and specifically lineage labels adult AT1 (alveolar type 1) cells using the *Rosa26-tdTm* reporter. (A) Adult *Ager-CreER^{T2}; Rosa26-tdTm* mice ($n = 3$) were treated with four doses of tamoxifen (Tmx; 0.2 mg/g) to lineage label *Ager⁺* cells. After 4 days, Tomato⁺ cells were widely distributed in the alveolar region but not in the bronchioles (br). (B) Higher magnification shows that tdTomato signal colocalizes with AGER. (C) A small percentage ($1.04 \pm 0.57\%$) of SFTPC⁺ AT2 cells were also labeled (arrow). The inset shows the boxed region with SFTPC signal only. (D and D') Immunohistochemistry of the same section with antibodies to RFP/tdTomato and HOPX. Quantification based on colocalization of tdTomato signal with HOPX nuclear staining (arrowheads) shows that $56.87 \pm 3.63\%$ of HOPX⁺ AT1 cells were lineage labeled. All non-lineage-labeled HOPX⁺ cells are marked with arrows. (E–H) Lineage labeling with different concentrations of Tmx. (E) Of AT1 cells, $0.12 \pm 0.03\%$ were tdTomato⁺ in lungs without Tmx. (F) Four doses of 10 $\mu\text{g/g}$ Tmx labels $9.52 \pm 0.98\%$ of AT1 cells. (G) One dose of 0.2 mg/g Tmx labels $21.85 \pm 1.18\%$ of AT1 cells. (H) Four doses of 0.2 mg/g Tmx label $56.87 \pm 3.63\%$ of AT1 cells. Scale bars = 100 μm in A and D, 20 μm in B, and 50 μm in C and E–H.

levels of *Ager* transcript, as judged by RNA-sequencing analysis of isolated cells (2, 12). However, we found that in the adult mouse, the *Ager-CreER^{T2}* allele labels only very few AT2 cells, even after four doses of Tmx (Figure 3). This suggests that the *Ager* expression level in most AT2 cells is too low to drive sufficient amounts of CreER to support recombination of *Rosa26-tdTm*. Although it is possible that recombination will occur with other

Rosa26 reporter alleles, we conclude that the *Ager-CreER* allele will likely be a useful tool for studying gene function specifically in AT1 cells of the adult lung. This is backed up by the finding that recombination of the *Rosa26-tdTm* allele is low in the absence of Tmx (Figure 3E), indicating that expression of *Ager-CreER^{T2}* is not leaky.

A significant finding in this study is that no obvious pathology such as fibrosis

was seen after 4 days or 2 weeks in the lungs of *Ager-CreER^{T2}; Rosa26-tdTm/Rosa26-DTA* lungs after ablating approximately 50% of the AT1 cells. This result suggests that the surviving AT1 cells and/or AT2 cells are able to rapidly spread to cover denuded basal lamina while AT2 cells proliferate and differentiate into AT1 cells to complete the repair. In our studies, we found evidence that approximately 21% of the AT2 cells proliferate after ablation of

Figure 4. Specific *in vivo* ablation of AT1 cells. (A) Immunofluorescence reveals lineage-labeled HOPX⁺ AT1 cells in *Ager-CreER^{T2}; Rosa26-tdTm* (control) and *Ager-CreER^{T2}; Rosa26-tdTm/Rosa26-DTA* (DTA) ($n = 4$) lungs 4 days after Tmx treatment. Quantification in F shows that HOPX⁺ cells make up $8.35 \pm 0.66\%$ of total DAPI⁺ cells in the control lung, whereas this proportion is reduced to $3.78 \pm 0.26\%$ four days after Tmx treatment. By 14 days, the proportion is restored to normal ($6.75 \pm 1.15\%$ in control and $6.53 \pm 0.46\%$ in DTA lungs). (B) Immunofluorescence shows increased numbers of CD11b⁺ cells after ablation of about half of the AT1 cells (4 d after last dose of Tmx). (C) Histochemistry also shows more immune cells present in the DTA lung at this time, but no grossly abnormal phenotype. (D and F) Control and DTA mice were exposed to 5-ethynyl-2'-deoxyuridine (EdU) for 3 hours before being killed, 4 days after last Tmx treatment. Boxed region in upper panels is shown at higher magnification below. In *Ager-CreER^{T2}; Rosa26-tdTm* control lungs (left panels), only about $1.53 \pm 0.45\%$ of AT2 cells proliferate (EdU⁺). By contrast, the number significantly increases to $21.15 \pm 3.98\%$ in *Ager-CreER^{T2}; Rosa26-tdTm/Rosa26-DTA* lungs (white arrowheads). SFTPC⁺ cells showing elongated morphology are labeled with yellow arrowheads. (E) Analysis of lungs from *Ager-CreER^{T2}/H2b:Venus* and *Ager-CreER^{T2}/H2b:Venus; Rosa26-DTA* mice 4 days after Tmx treatment reveals that $50.17 \pm 2.13\%$ of SFTPC⁺ cells express *Ager-H2B:Venus* following loss of AT1 cells, compared with $5.70 \pm 1.87\%$ in control. SFTPC⁺ *Ager-H2B:Venus*⁺ cells are labeled with white arrowheads. Scale bars = 100 μm in A and C and 50 μm in B, D, and E. Data shown are mean ± SD. *P < 0.05; **P < 0.01.

AT1 cells, whereas more of them upregulate expression of *Ager*, as judged by expression of an H2B:Venus fusion protein inserted into the *Ager* endogenous locus. In the future, it will be interesting to ask whether a specific subpopulation of AT2 cells preferentially responds to the loss of AT1 cells, as suggested by studies

using other injury/repair models in the adult mouse (10, 11). In addition, the *Ager-CreER^{T2}* allele will be a useful tool for testing the function of genes specifically expressed in AT1 cells, such as their potential roles in providing a major barrier between the alveolus and the vasculature, supporting the integrity of

closely associated endothelial cells, and contributing to the niche of AT2 stem cells. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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