Mesenchymal stromal cell turnover in the normal adult lung revisited

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Seyed-Razavi Y, Williams B, Winkler DA, Bertoncello I. Mesenchymal stromal cell turnover in the normal adult lung revisited. Am J Physiol Lung Cell Mol Physiol 305: L635–L641, 2013. First published September 6, 2013; doi:10.1152/ajplung.00092.2013.—We have employed a simple and robust noninvasive method of continuous in vivo long-term bromodeoxyuridine (BrdU) labeling to analyze lung mesenchymal stromal cell turnover in adult mice in the steady state. Mathematical modeling of BrdU uptake in flow cytometrically sorted CD45negCD31negSca-1pos lung cells following long-term feeding of BrdU to mice in their drinking water reveals that lung mesenchymal stromal cells cycle continuously throughout life. Analysis of BrdU incorporation during long-term feeding and during chasing (delabeling) following replacement of BrdU-water with normal water shows that the CD45negCD31negSca-1pos lung mesenchymal stromal cell compartment turns over at a rate of ~2.26% per day with a time to half-cycled of 44 days, an estimated cell proliferation rate of 0.004/day, and a cell death rate of 0.018/day.

lung mesenchymal stromal cells; bromodeoxyuridine; cell cycling; cell turnover

IN A BRIEF REVIEW PUBLISHED in 1983, documenting information about cell kinetics and cell turnover in the lung, Bowden (7) pertinently stated that “the study of cellular kinetics in the normal lung is prerequisite to an understanding of the pulmonary responses to injury and of the processes that initiate and control cellular regeneration and repair.” It is therefore surprising that, despite the burgeoning interest in lung regeneration, inflammation, and repair in recent years, the field largely continues to rely on estimates of lung cell cycling and turnover in the steady state that predate that review (23, 44).

Those studies employed classical autoradiographic techniques and histomorphometry (17, 47) to analyze the cell kinetics of often ill-defined cell populations in tracheal, bronchiolar, and alveolar regions of the lung, mostly in neonatal and young animals. They concluded that adult mouse lung bronchiolar and alveolar epithelial cell lineages turned over at a rate of no more than 1% per day in the steady state (7, 13, 23, 44, 45). More recent studies measuring bromodeoxyuridine (BrdU) incorporation in airway and alveolar epithelial cells by flow cytometry (42, 46) or immunohistological analysis (38) are in broad agreement with this estimate. Analysis of genetically engineered mice has revealed that terminally differentiated ciliated airway epithelial cells are especially long lived, having an average half-life of 17 mo (37). Consequently, it is generally accepted that the adult lung epithelium is mitotically quiescent in the absence of injury (11, 17, 43) and that the very low basal rate of lung epithelial cell proliferation in the steady state is sufficient to account for replacement of airway and alveolar epithelial cells damaged by normal wear and tear.

By comparison, little is known about the cell kinetic status of adult lung mesenchymal stromal cells in the steady state. Early studies reviewed by Kauffman (23) and by Evans and Shami (17) showed that the labeling index of lung interstitial fibroblasts in situ increased sharply to levels up to 8–10 times higher than that of juxtaposed epithelial cells during perinatal lung growth and alveolar septation and then rapidly declined. Recent analysis of lung regeneration in adult mice postpneumonectomy also reveals a rapid but transient increase in the proliferative activity of lung mesenchymal stromal cells in the days immediately following surgery, predominantly associated with the alveolar parenchyma (31, 32). However, values for the labeling index or the level of BrdU incorporation in adult lung interstitial cells in the steady state are rarely reported and then typically range from undetectable to less than 0.2% (20, 31, 38).

Resident lung mesenchymal stromal cells play a critical role during fetal and neonatal lung development and in lung remodeling, regeneration, and repair throughout life (16, 26, 40). They manufacture, assemble, and maintain the biomatrix scaffold that holds the lung together (12). They are an indispensable source of soluble and insoluble growth factors, cytokines, chemokines, and proteases, which orchestrate airway arborization and the maintenance of airway and alveolar epithelial cell lineages, and they play an integral role in the recruitment and regulation inflammatory cells in the injured and diseased lung (22, 24). Consequently, precise information about the cell kinetic behavior of lung mesenchymal cells is essential in devising optimal strategies to harness lung epithelial-mesenchymal trophic units to effect repair following insult or injury and to attenuate or arrest disease progression.

The paucity of information about the cell kinetic status and turnover of the lung mesenchymal stromal cells in adult mice has previously been attributed to a lack of definitive biomarkers and robust protocols for their prospective isolation and characterization (41). Our laboratory has recently developed a robust multiparameter cell separative strategy that has enabled us to resolve EpCAMposcx6-integrinposβ4-integrinposCD24low epithelial stem/progenitor cells (EpiSPC) and CD45negCD31negSca-1pos mesenchymal stromal progenitor cells in the adult mouse lung on the basis of their differential expression of EpCAM and the Sca-1 cell surface antigen (28, 29). We have shown that CD45negCD31negSca-1pos lung cells express a mesenchymal stem cell immunophenotypic and transcriptional signature profile and that they are highly enriched for fibroblast colony-forming cells.
with a lipofibroblastic and myofibroblastic differentiation potential. Importantly, we have also shown that the growth of EpiSPC in an organotypic clonogenic cell assay is dependent on coculture of EpiSPC with CD45negCD31negSca-1pos lung mesenchymal cells and mesenchyme-derived growth factors (19, 20).

In this study, we have employed a noninvasive, nontoxic in vivo BrdU-label protocol that we have previously used to analyze the kinetic behavior of the murine hematopoietic stem cells (8, 9) to analyze the cycling and turnover of lung mesenchymal stromal cells in adult mice. The mathematical modeling of BrdU incorporation in prospectively isolated CD45negCD31negSca-1pos lung cells in unperturbed adult C57BL/6 mice held under specific pathogen-free conditions during long-term feeding and chasing of BrdU in their drinking water revealed that adult lung mesenchymal stromal cells cycle continuously in the steady state with a time to half-cycle of ~44 days, equivalent to a turnover rate of 2.26% per day.

MATERIALS AND METHODS

Mice. Specific pathogen-free adult C57BL/6 mice aged between 8 and 12 wk were purchased from the Animal Resources Centre (ARC, Perth, Western Australia) and maintained in a clean conventional animal house. Mice were fed sterile mouse pellets (Barastoc, St. Arnaud, Victoria, Australia) and acidified water ad libitum.

Animal experimentation was conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition), and experimental protocols were approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee.

BrdU administration. A stock solution of BrdU (Sigma, St. Louis, MO) was made up in sterile water (10 mg/ml) and stored frozen or at 4°C and further diluted (0.5 mg/ml) for administration to mice in their drinking water ad libitum for periods of up to 14 wk exactly as previously described (8, 9). Long-term administration of BrdU at this dose was shown to be noninvasive and nontoxic to mice in our previous analysis of the cycling and turnover of hematopoietic stem cells (8). Preparation of lung cell suspensions. Freshly excised adult mouse lungs were rinsed twice in 20 ml of sterile PBS (310 mosM) to remove contaminating blood and tissue debris prior to removing the trachea and finely mincing the lung lobes with sterile scissors. Lungs were not perfused prior to preparation of cell suspensions for analysis of endogenous mesenchymal cell turnover because it has previously been shown that mesenchymal cells are not detected in the circulating peripheral blood of unperturbed mice under normal physiological conditions (14). The finely minced lung tissue was resuspended and washed in excess PBS and then resuspended and digested in 3 mg/ml collagenase Type I (Roche, Basel, Switzerland) in PBS in a volume of 2 ml per lung (60 min; 37°C) on a shaking incubator. The resulting cell suspension was further disaggregated by trituration through an 18-gauge needle, diluted in PBS, filtered through a 40-μm nylon cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ), and washed twice in PBS supplemented with 2% newborn calf serum (PBS-2%Se) by centrifugation (1,300 rpm; 5 min; 10°C). Discontinuous density gradient centrifugation was employed to remove contaminating erythrocytes, neutrophils, and cell debris; 20 ml aliquots of lung cell suspension (2–3 lungs per gradient) were layered on 10 ml Nycodren 1.077A (1.077 g/ml; 265 mosM; Nycomed Pharma, Oslo, Norway) (600 g; 20 min; room temperature, brake off) in 50-ml sterile polypropylene tubes. Low-density cells at the PBS-Nycoprep interface were collected and washed twice in excess PBS-2%Se and resuspended in PBS-2%Se and held on ice prior to further manipulation.

Flow cytometric analysis and sorting. Biotinylated or directly conjugated rat anti-mouse CD31, CD45, Sca-1, or Thy-1 and relevant second antibody reagents and isotype controls tagged with fluorescence including FITC, phycoerythrin (PE), or allophycocyanin (APC) were purchased from Becton Dickinson. 7-Amino-actinomycin D (2 μg/ml Sigma) or Fluoro-Gold (5) (FG; 1 μg/ml, Molecular Probes, Eugene, OR), were used for flow cytometric detection and exclusion of nonviable cells. Low-density lung cells were resuspended in PBS-2%Se (5 × 107 cells/ml) in an optimally pretitrated cocktail of antibodies and incubated for 20 min on ice. The labeled cells were washed in excess PBS-2%Se and resuspended at 5–10 × 106 cells/ml and held on ice for flow cytometric analysis and sorting.

Analysis of BrdU incorporation. Following predetermined periods of BrdU administration, single cell suspensions were prepared for flow cytometric analysis and sorting of CD45negCD31negSca-1pos cells from the pooled lungs of 3–5 mice. Cytospins (3–5 per cell aliquot) were prepared by centrifuging sorted cells onto polylysine-coated slides (350 rpm; 10 min; Shandon Cytospin 2 centrifuge, Thermo Scientific, Waltham, MA) such that 5–7.5 × 104 cells were deposited per slide. Cytospins were air dried overnight and stored at 4°C until labeling for the determination of BrdU incorporation using the BrdU in situ detection kit (BD Biosciences) as described by the manufacturer.

BrdU-labeled cytospins were scored (×200 magnification) using a microscope fitted with a calibrated eyepiece grid. At least 3, and up to 10, sequential fields were scored on each of three replicate slides per sorted cell fraction at each time point to acquire a minimum of 1,000 events. The proportion of BrdU-positive and -negative cells in each fraction was then calculated, taking account of the variability in scoring BrdU-positive cells in each field. The turnover of the adult lung mesenchymal cell compartment was calculated by employing an elegant mathematical algorithm developed for the kinetic analysis of BrdU labeling of the T lymphocyte compartment of rhesus macaque monkeys (6).

RESULTS

The cell kinetic behavior of the lung mesenchymal stromal cell compartment in the steady state was determined by analysis of BrdU incorporation in CD45negCD31negSca-1pos cells prospectively isolated from the lungs of mice fed BrdU in their drinking water for predetermined intervals of up to 14 wk (Fig. 1). In another series of experiments, BrdU incorporation in CD45negCD31negSca-1pos lung mesenchymal cells was also analyzed in chase (delabelling) experiments in which mice fed BrdU in drinking water for 56 days were then provided normal drinking water for up to 9 wk. The average recovery of flow cytometrically sorted CD45negCD31negSca-1pos cells in BrdU fed mice was comparable to historical control values for normal mice (3.1 ± 0.5 × 107 cells vs. 2.4 ± 0.3 × 107 cells), representing ~1% of the initial lung cell suspension. Analysis
of the incidence and recovery of CD45<sup>−</sup>CD31<sup>−</sup>Sca-1<sup>+</sup> lung mesenchymal stromal cells revealed no significant differences in these parameters as a consequence of long-term BrdU administration (Table 1; \( P = 0.2842 \), one-way ANOVA).

The mathematical modeling of the cycling, turnover, proliferation rate, and death rate of CD45<sup>−</sup>CD31<sup>−</sup>Sca-1<sup>+</sup> cells employed an elegant mathematical algorithm previously developed by Bonhoeffer et al. (6) for the cell kinetic analysis of BrdU labeling of the T lymphocyte compartment of rhesus macaque monkeys. Briefly, this simple compartment model relates the fraction of BrdU-labeled cells, \( f_L(t) \), to the rate of entry (s), proliferation (p), and death (d) of cells in the compartment. The fraction of BrdU-labeled cells as a function of time, \( f_L(t) \), can be written as

\[
f_L(t) = \begin{cases} 
    A_1(1 - e^{-(d+p)t}), t \leq t_e \\
    A_2 + (f_L(t_e) - A_2)e^{-(d+p)(t-t_e)}, t_e \leq t \leq t_d 
\end{cases}
\]

The two regimes in Eq. 1 represent the feeding and chase phases of the experiment, where \( t_e \) is the time at the end of BrdU labeling and \( t_d \) is the time at the end of the chase. The parameters \( A_1 \) and \( A_2 \) are functions of the rates of entry, death, and proliferation

\[
A_1 = 1 - \alpha = 1 - \frac{s_U(d-p)}{s(d+p)} \\
A_2 = s'L/s
\]

where \( \alpha = s_U/[d+P]T_0 \), \( T_0 \) is the total size of the population at time \( t = 0 \), and the subscripts L and U denote the number of BrdU-labeled and unlabeled cells. \( A_1 \) and \( A_2 \) have intuitive interpretations as the asymptotic level that the fraction of labeled cells approach during long-term labeling (feeding) and delabeling (chase) periods, respectively. Bonhoeffer et al. (6) extended the kinetic model to include imperfect labeling, label dilution of cell division, nonconstant source terms, and cell turnover, but the form of the equations was similar to Eq. 1. The interpretation of the parameters in the model needed to be modified in this case. Given the difficulty in carrying out and analyzing these labeling experiments, we considered the use of a more complex model was not justified by the data.

The two rate expressions in Eq. 1 can be rearranged to enable the Sca-1<sup>+</sup> BrdU-labeling experiments to yield rate information. For the feeding regime \((t < t_e)\), Eq. 1 can be rearranged simply to yield

\[
\ln[A_1 - f_L(t)] - \lnA_1 = -(d+p)t
\]

Table 1. Long-term BrdU labeling does not affect the incidence or recovery of Sca-1<sup>+</sup> lung mesenchymal stromal cells

<table>
<thead>
<tr>
<th>BrdU Uptake, Days</th>
<th>Mice per Group</th>
<th>Age at Sampling, Days</th>
<th>Viable Low-Density Cells/Lung, ( \times 10^6 )</th>
<th>Incidence of Viable Low-Density CD45&lt;sup&gt;−&lt;/sup&gt;CD31&lt;sup&gt;−&lt;/sup&gt;Sca-1&lt;sup&gt;+&lt;/sup&gt; Cells/Lung, %</th>
<th>Viable Low-Density CD45&lt;sup&gt;−&lt;/sup&gt;CD31&lt;sup&gt;−&lt;/sup&gt;Sca-1&lt;sup&gt;+&lt;/sup&gt; Cells/Lung, ( \times 10^5 )</th>
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BrdU, bromodeoxyuridine.
A plot of ln[A$_1$ - f$_1$(t)] vs. t should yield a straight line with slope (d + p) and intercept lnA$_1$. The plot of this relationship for the BrdU feeding regime is shown in Fig. 2A. The slope of the least squares fit to the data (Fig. 2A) allows estimation of the death plus proliferation values (d + p) (- slope of feeding graph) of 0.0226/day for CD45$^{pos}$CD31$^{neg}$Sca-1$^{pos}$ cells, giving a half d + p time of 44 days for the CD45$^{pos}$CD31$^{neg}$Sca-1$^{pos}$ cells.

For the BrdU chase experiments (t > t$_c$), Eq. 1 can be rearranged simply to yield

$$\ln(f_1(t) - A_2) - \ln(f_1(t_c) - A_2) = -(d + p)(t - t_c)$$ (5)

A plot of ln[f$_1$(t) - A$_2$] vs. t - t$_c$ should yield a straight line (Fig. 2B) with slope -(d + p) and intercept ln[f$_1$(t$_c$) - A$_2$]. The data for the chase are less numerous than for the feeding experiments; consequently the experimental uncertainties are greater. We used a value of 10% for CD45$^{neg}$CD31$^{neg}$Sca-1$^{pos}$ as the asymptote (A$_2$) and omitted the 98-day CD45$^{neg}$CD31$^{neg}$Sca-1$^{pos}$ chase data point, which was a large outlier. The slopes of the transformed chase experimental data provides an estimate of the death-proliferation rate (d + p) (- slope of chase graph). Using these assumptions and removing outliers allowed an estimation of the death-proliferation values (d + p) of 0.0138/day (half-life of 72 days) for CD45$^{neg}$CD31$^{neg}$Sca-1$^{pos}$ cells.

Taken together, the transformed feeding and chase experiments enable the calculation of estimated proliferation and death rates knowing the values for (d + p) from the feeding experiments and (d + p) from the chase experiments. This gives the proliferation rate of CD45$^{neg}$CD31$^{neg}$Sca-1$^{pos}$ cells as 0.004 per day and the death rate as 0.018 per day.

A plot of ln[(f$_1$(t) - A$_2$)] with slope 0.0226/day for CD45$^{neg}$CD31$^{neg}$Sca-1$^{pos}$ cells. These estimates average the cell kinetic behavior of the CD45$^{neg}$CD31$^{neg}$Sca-1$^{pos}$ lung mesenchymal stromal cell compartment, which is known to comprise cells with different potentialities (27). In particular, lipofibroblastic (Thyhi) and myofibroblastic (Thylo) first resolved on the basis of their differential expression of the Thy-1 antigen by Phipps et al. (36). To quantify potential differences in the cell kinetic behavior of different lung mesenchymal stromal cell lineages, we analyzed the rate of BrdU incorporation by Thy$^{hi}$ and Thy$^{lo}$ subpopulations of CD45$^{neg}$CD31$^{neg}$Sca-1$^{pos}$ cells over 14 days of continuous BrdU labeling (Fig. 3). Although this revealed a significant difference in the rate of BrdU incorporation by Thy$^{hi}$ and Thy$^{lo}$ stromal cell subpopulations (9.1 ± 0.4 vs. 7.2 ± 0.5%, respectively; P = 0.002 Mann-Whitney test), the cell kinetic behavior of these subpopulations did not differ significantly from that of the CD45$^{neg}$CD31$^{neg}$Sca-1$^{pos}$ lung mesenchymal stromal cell compartment as a whole.

**DISCUSSION**

Previous studies that have attempted to analyze the cycling and turnover of lung stromal cells in situ in rodents have predominantly employed autoradiographic techniques to measure the labeling indexes of cells of interest. Many of these early studies relied on imprecise histomorphological identification of parenchymal and interstitial lung cell types resulting in estimates of cell cycling and turnover, which effectively averaged the kinetic behavior of diverse fibroblastoid, stromal, inflammatory, and also endothelial cell elements. Where the cell kinetic status of lung stromal cell subsets was more rigorously documented this was mostly confined to analysis of perinatal and neonatal mice at a time of high proliferative activity in the lung. In fact, a typical study observed that too few autoradiographically labeled alveolar lipofibroblastic and nonlipofibroblastic cells could be detected in rats beyond 11 days of age to reliably estimate their proliferative behavior (10). Consequently, we set out to systematically analyze the long-term cycling and turnover of mesenchymal stromal cells in the adult mouse lung to answer two simple but important questions: does the mesenchymal stromal cell compartment in the normal adult lung actively cycle and turn over in the steady state and, if so, how rapidly? To minimize the impact of confounding environmental factors we chose to do so in mice bred and maintained in specific pathogen-free conditions for the duration of the study.

By employing a simple and robust noninvasive method of continuous in vivo long-term BrdU labeling (8, 9) to mathematically model the cell kinetic behavior of well-characterized
flow cytometrically isolated lung mesenchymal stromal cells we have demonstrated that the mesenchymal stromal cells in the mouse lung are not quiescent in the steady state but cycle continuously to maintain lung tissue homeostasis in response to normal wear and tear and participate in reactivation of the epithelial-mesenchymal trophic unit in remodeling the lung in response to insult or injury (2, 22, 31, 32, 39, 40). In normal unperturbed C57Bl/6 adult mice, CD45negCD31negSca-1pos mesenchymal stromal cells turn over at a rate of 2.26% per day with a time to half-cycled for the compartment of 44 days.

The predicted rate of mesenchymal stromal cell proliferation and cell death in the steady state also provides an intriguing insight into the organization and cell cycle kinetics of the adult lung mesenchymal stromal cell compartment. Homeostasis is predicated on a fine balance between the rate of cell proliferation and the rate of cell death. Paradoxically, our modeling yields an estimated cell death rate (0.018 per day) that is far higher than the cell proliferation rate (0.004 per day) of mesenchymal stromal cells. However, this disparity, also observed in previous modeling of adult murine and human T-lymphocyte kinetics (6, 30), is consistent with the behavior of a compartment comprising kinetically heterogeneous sub-populations where the estimated death rate is biased toward that of the more actively dividing subpopulations (3).

The model predicts that the CD45negCD31negSca-1pos lung mesenchymal stromal cell compartment comprises multiple cell subpopulations with differing proliferative histories and probabilities of cycling, and consequently differing propensities to be recruited into cycle in response to perturbation or injury. The observed difference in the turnover of CD45neg CD31negSca-1pos cells with differing propensities for myofibroblastic (Thy-1neg) or lipofibroblastic (Thy-1pos) differentiation (Fig. 3) is consistent with this interpretation. So too is the heterogeneous proliferative behavior of multiple lung mesenchymal stromal cell subpopulations revealed by in vivo cell lineage tracing in transgenic reporter mice following bleomycin-induced lung fibrosis (38). However, with the exception of mesenchymal cells resolved on the basis of the differential expression of Thy-1 and few if any other biomarkers, immunophenotypically discrete mesenchymal stromal cell subpopulations have only been detected in the injured lung (18, 34–36).

Attempts to histomorphometrically localize BrdU-labeled cells to determine whether lung mesenchymal stromal cell subpopulations in the steady state turn over at different rates in different regions of the airway and alveolar parenchyma were confounded by the rare and sparse distribution of labeled cells in lung tissue sections. Others have also noted the relative absence of BrdU-incorporating lung stromal cells on microscopic examination of the airway and alveolar parenchyma of the uninjured lung (31). Even then, unambiguous histomorphometric identification of BrdU-labeled mesenchymal stromal cells (CD45posCD31posSca-1pos) in situ would require their precise immunophenotypic discrimination from BrdU-labeled endothelial cells (CD45posCD31posSca-1pos) and hematopoietic cells (CD45posCD31negSca-1pos) differentially expressing common markers. Increased BrdU incorporation has been observed in lung mesenchymal stromal cell compartments following injury (32, 38), but immunohistomorphometric discrimination of BrdU-labeled mesenchymal stromal cells in those settings would be confounded by autofluorescence and by the emergence of mesenchymal phenotypes whose relationship to those found in the steady state is unclear (34, 35).

Consequently, the extent to which the discordant cell proliferation and death rates measured in this study reflect intrinsic differences in cell kinetic status, proliferative potential, or responsiveness to perturbation, as opposed to the heterogeneous behavior of a common mesenchymal progenitor cell responding to cues provided by region-specific microenvironments along the proximal-distal axis of the lung, remains unclear (34, 35).

Importantly, biological variables in inbred mouse strains including stem and progenitor cell pool size, cell kinetic status, sensitivity to cytokines, and perturbation are both genetically determined (4, 15, 21) and context dependent. Consequently, precise baseline values for mesenchymal stromal cell kinetic parameters in specific mouse strains will need to be established under the specific environmental conditions in which experimental animals are maintained.

Mouse strain differences have been documented in the incidence, proliferative, and differentiative potential of bone marrow-derived mesenchymal cells (33) but have not been systematically analyzed in mesenchymal cells isolated from other organs includ-
ing the lung. However, it is well known that different mouse strains exhibit differences in profibrotic responses of the lung to irradiation (1), to cytotoxic drugs including bleomycin (19, 25), and to TGF-β (25). Although these strain differences invoke complex multifactorial fibrotic mechanisms, it is also likely that differences in the cell kinetic status of the lung mesenchymal stromal cell compartment is a contributory factor.

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