Fetal Cells in the Murine Maternal Lung Have Well-Defined Characteristics and Are Preferentially Located in Alveolar Septum

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The transfer of fetal cells to maternal organs occurs in mouse and human pregnancy. Techniques such as polymerase chain reaction and flow cytometry do not permit study of fetal cell morphology or anatomic location. Using a green fluorescent protein (GFP) transgenic mouse model, our objective was to determine whether GFP+ signal emanates from intact or degraded fetal cells, and whether they have a characteristic appearance and location within maternal lung. Four wild-type female mice were mated to males homozygous for the *Gfp* transgene and studied at days e16–18. Controls were 2 females mated to wild-type males. Morphologic appearance and anatomic position of each GFP+ object within maternal lung was recorded. GFP signals were sufficiently bright to be visualized without anti-GFP antibody and were confirmed by confocal microscopy to be separate from fluorescent artifact. Of 438 GFP+ objects detected, 375 (85.6%) were from intact cells, and 63 (14.4%) were acellular. Four distinct categories of intact cells were observed. Of these, 23.2% had mononuclear morphology with a relatively large nucleus and GFP+ cytoplasm (Group A). An additional group of cells (10.1%) had mononuclear morphology and podocyte extensions (Group B). The remainder of cells had fragmented nuclei or cytoplasm. Both intact cells and acellular fragments were predominantly localized to the maternal alveolar septum ($P < 0.0001$). This study demonstrates that fetal GFP+ cells are predominantly located in the alveolar septum and have characteristic morphologies, although it remains unclear whether these represent distinct categories of cells or degrading cells. Nevertheless, this naturally acquired population of fetal cells in maternal lung should be considered in studies of lung biology and repair.

Introduction

THE TRANSFER OF FETAL CELLS to maternal organs occurs in mouse and human pregnancies [1,2]; these cells have been shown to persist after delivery in both species as well [2,3]. By applying quantitative techniques, such as polymerase chain reaction (PCR) amplification and fluorescence-activated cell sorting (FACS), it is now known that fetal cell microchimerism is common and persists postpartum [4,5]. In addition, fetal cells are found in specific maternal organs [3,6]. However, both PCR and FACS do not permit either the study of cell morphology or determination of the anatomic location of the fetal cells within the maternal tissue of interest.

In human organs, localization of male, presumably fetal, cells has been generally achieved microscopically by using fluorescence in situ hybridization (FISH) analysis with a Y

chromosome probe [7]. An obvious limitation of this approach is that only ~50% of fetal cells can be detected. Although FISH analysis has been successfully used to quantify fetal cells [8,9], the protease digestion step needed for successful probe hybridization can compromise the integrity of the tissue sections and the morphological characteristics of the fetal cells [7]. Despite these technical challenges, FISH analysis in conjunction with immunohistochemistry [10] has successfully located and phenotypically characterized human microchimeric fetal cells [11,12].

To better control reproductive histories, tissue processing, and other technical variables, we developed a mouse model to further study fetomaternal cell trafficking [5] using animals transgenic for the green fluorescent protein (GFP) [13]. In our model, wild-type females are mated to males transgenic for GFP; fetal cells are then detectable in maternal

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tissue by the presence of either the *Gfp* transgene sequence or the fluorescent protein product. With the use of a male that is homozygous for the *Gfp* transgene, 100% of fetal cells can be located in maternal tissue [14]. This is an improvement over a previous model in which a hemizygous transgenic male was utilized, and only ~50% of pups inherited the transgene [5,15,16]. With these animal models, we showed that fetomaternal cell trafficking increases as gestation advances, with a peak frequency before delivery at approximately day e18 [16], and that fetal cells are nonrandomly distributed in maternal organs, with the greatest number found in maternal lung [5,16].

This study, therefore, was performed to determine whether the fetal GFP positive signals we previously detected in maternal organs emanate from intact or degraded fetal cells. Our approach was to systematically evaluate the fetal cells by fluorescence and confocal microscopy. Wide-field fluorescence microscopy was used to locate and characterize the fetal cells. Confocal microscopy was performed to determine the spatial distribution of the fetal cells within the maternal tissue architecture, as well as to confirm that the findings did not reflect fluorescent artifact. We tested the hypothesis that fetal GFP positive cells in the maternal lung (a) have a characteristic morphology and (b) are typically and reproducibly found in nonrandom anatomic locations.

Materials and Methods

Mice

This investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science, ©1996). The Institutional Animal Care and Use Committee of the Tufts University School of Medicine Division of Laboratory Animal Medicine approved the protocol described here. All institutional guidelines regarding the ethical use and care of experimental animals were followed. Eight- to 10 week-old virgin wild-type C57BL/6J females ($n=4$) (stock no. 000664; Jackson Laboratories, Bar Harbor, ME) were mated to enhanced GFP homozygous transgenic male mice C57BL/6-Tg (CAG-EGFP) C14-Y01-FM131Osb (stock no. 267; provided by Dr. Masaru Okabe, Riken BioResource Center, Ibaraki, Japan), which have a copy of the *Gfp* transgene on each homolog of chromosome 14. The *Gfp* transgene is under the control of a ubiquitous chicken beta-actin promoter and a cytomegalovirus enhancer. Pregnant females were euthanized just before delivery between days e16 and e18. Control matings of wild-type C57BL/6J females ($n=2$) to wild-type C57BL/6J males were also performed and processed by using the same parameters used for the experimental animals.

Tissue collection and processing

Mice were sacrificed, and their tracheas were exposed. Inflation of the lung was accomplished by using a needle attached to a syringe filled with 10% neutral buffered formalin directed into the trachea. The lungs and trachea were then removed en bloc, fixed in 10% neutral buffered formalin for 5 h, briefly rinsed in phosphate-buffered saline (PBS), and placed into 30% sucrose in PBS overnight at 4°C. Lungs were then injected with a small amount of 1:1 optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc.,

Torrance, CA): 30% sucrose in PBS. After injection, tracheal tissue was removed. The lungs were then placed in 100% OCT compound and frozen over liquid nitrogen.

Slide preparation

Lung cryosections were taken in an antero-posterior direction. Sections were first cut from the most anterior surface of the lung (level 1) and then at increments of 2 mm (levels 2 through 4). The full thickness of the frozen, OCT-embedded whole lung was ~10 mm. Therefore, levels 2, 3, and 4 represented ~20%, 40%, and 60% of the lung. Frozen slides were thawed at room temperature for 2 min and gently dipped in PBS, followed by staining with 0.3 µg/mL of 4'-6-diamidino-2-phenylindole (DAPI) for 5 min. Sections were air dried, mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA), and stored at 4°C, protected from light until microscopic analysis.

Wide-field fluorescence microscopy

Three slides from each experimental and control animal (total of 18 slides) were manually examined by one individual using a Zeiss Axioskop fluorescent microscope. Each slide contained 4 sections (one from each of the levels). Slides were blindly scored with regard to mating history. An object was considered to be GFP positive and included in the analysis if it was visible through a single band pass FITC/GFP filter (excitation: 480 nm, emission: 535 nm) with no signal bleed through additional single band pass filters [ie, DAPI (excitation: 365 nm, emission: 400 nm) and Cy3 (excitation: 560 nm, emission: 630 nm)]. Each GFP positive object was categorized into 1 of 4 groups; Group A: mononuclear cells with regular, homogenous, nongranular GFP positive cytoplasm, Group B: mononuclear cells with podocyte-like extensions and nongranular GFP positive cytoplasm, Group C1: regular or irregular shaped cells with nonhomogenous (eg, fragmented) nuclei and homogeneous nongranular GFP staining, Group C2: regular or irregular shaped cells with granular GFP positive cytoplasm, and Group D: GFP positive fragments without a nucleus. The anatomic location of each GFP positive cell or object within the lung was also recorded.

Confocal microscopy

After the examination of lung sections under wide-field fluorescence microscopy, separate 5 µm thick lung sections were counterstained with Topro-3 and examined by z-section confocal microscopy, using spectral unmixing to establish spectral separation, for the presence of GFP positive objects, including both intact cells and acellular fragments (ie, group D). Images were captured using a Zeiss Axiovert 200M confocal spinning disk microscope with 63× and 100× magnification. GFP was excited at 488 nm with an argon laser, and fluorescence was detected at 500 to 540 nm with no signal bleed through additional single band pass filters [eg, DAPI (excitation: 365 nm, emission: 400 nm)]. Sequential images of each cell or fragment of interest were captured by a CoolsnapHQ/ICX 285 CCD camera (Photometrics, Tucson, AZ) at intervals of 0.245 µm size from top to bottom of the lung, resulting in "slice" images. The upper

and lower focal planes, which comprise the top and bottom optical slices of the lung, were set with an inter-slice distance of 0.245 μm . After image capture, out-of-focus blur was removed from the set of image slices by using Autodeblur software (Autoquant, Watervliet, NY). This software applies nearest-neighbor algorithms to individual images or to the entire image set, in a process termed *deconvolution*. Stack images were obtained from the series of slices taken from the z-section and were deconvoluted by using the conservative approach with 15 iterations and a smoothing function of 0.5, with an image size of 512 $\times$ 512 pixels and a bin of 1 $\times$ 1 (Autodeblur).

Statistical analysis

Student's *t*-test was used to compare the number of GFP positive objects (cells and acellular fragments) within the lung parenchyma to those in alveolar air spaces. The Kruskal-Wallis test was used to compare the frequency of GFP positive objects across morphological groups and across levels of maternal lung in an antero-posterior direction.

Results

The GFP signals were sufficiently bright such that they could be directly visualized with minimal processing and without the need for anti-GFP antibody. No GFP positive objects were seen in the control mice mated to wild-type males. Using wide-field fluorescent microscopy, a total of

438 GFP positive objects were found in the 4 mice mated to GFP positive males; 375 of these (85.6%) were in the form of intact cells, and 63 (14.4%) were acellular fragments. Although the numbers of GFP positive cells and acellular fragments varied slightly between animals (Table 1), the numbers have been grouped to permit statistical analysis.

Among all GFP positive cells and acellular fragments ($n=438$), 89.5% ($n=392$) were localized to the lung parenchyma, whereas 10.5% ($n=46$) were found in air spaces (Table 1). The frequency of all GFP objects combined (ie, Groups A, B, C1, C2, and D) and the frequency of each individual group of cells were statistically significantly higher within the lung parenchyma compared with air spaces ($P<0.0001$). Overall, fetal cells appeared to be predominantly located within the alveolar septum and near capillaries (Figs. 1–3).

Group A cells, which were characterized by mononuclear morphology, a relatively large nucleus, and regular, homogeneous, nongranular GFP positive cytoplasm (Fig. 1), comprised 23.2% ($n=87$) of the total number of intact fetal cells. Within this group, 75 cells (86.2%) localized to the lung parenchyma, and 12 cells (13.8%) were in air spaces (Table 1).

Group B cells also had mononuclear morphology and nongranular GFP positive cytoplasm but had podocyte-like extensions (Fig. 2). They were the least frequent type of cells observed, comprising 10.1% ($n=38$) of the total number of intact cells. Of these cells, 34 (89.5%) were in the parenchyma, and 4 (10.5%) were in air spaces (Table 1).

TABLE 1. DISTRIBUTION AND CATEGORIZATION OF FETAL CELLS IN MATERNAL LUNG BY FLUORESCENCE MICROSCOPY

Mouse	Lung level ^a	Group A			Group B			Group C1			Group C2			Group D			All groups		
		Total	P	A	Total	P	A	Total	P	A	Total	P	A	Total	P	A	Total	P	A
1	1	15	14	1	7	5	2	11	11	0	20	19	1	1	1	0	54	50	4
	2	21	12	9	1	1	0	14	12	2	8	7	1	4	4	0	48	36	12
	3	7	7	0	3	2	1	14	13	1	16	14	2	1	0	1	41	36	5
	4	7	7	0	3	3	0	6	6	0	13	8	5	8	8	0	37	32	5
	All	50	40	10	14	11	3	45	42	3	57	48	9	14	13	1	180	154	26
2	1	0	0	0	1	0	1	16	15	1	3	3	0	10	10	0	30	28	2
	2	5	5	0	1	1	0	5	5	0	1	1	0	0	0	0	12	12	0
	3	2	2	0	2	2	0	6	6	0	2	2	0	0	0	0	12	12	0
	4	0	0	0	1	1	0	4	4	0	1	1	0	0	0	0	6	6	0
	All	7	7	0	5	4	1	31	30	1	7	7	0	10	10	0	60	58	2
3	1	1	0	1	5	5	0	10	10	0	0	0	0	4	3	1	20	18	2
	2	1	1	0	2	2	0	4	4	0	3	3	0	0	0	0	10	10	0
	3	3	3	0	3	3	0	11	9	2	4	4	0	4	3	1	25	22	3
	4	2	1	1	1	1	0	9	6	3	3	1	2	6	4	2	21	13	8
	All	7	5	2	11	11	0	34	29	5	10	8	2	14	10	4	76	63	13
4	1	16	16	0	1	1	0	27	27	0	2	2	0	4	3	1	50	49	1
	2	4	4	0	5	5	0	13	12	1	6	6	0	6	5	1	34	32	2
	3	2	2	0	1	1	0	6	6	0	1	1	0	8	7	1	18	17	1
	4	1	1	0	1	1	0	6	6	0	5	5	0	7	6	1	20	19	1
	All	23	23	0	8	8	0	52	51	1	14	14	0	25	21	4	122	117	5
Total	1	32	30	2	14	11	3	64	63	1	25	24	1	19	17	2	154	145	9
	2	31	22	9	9	9	0	36	33	3	18	17	1	10	9	1	104	90	14
	3	14	14	0	9	8	1	37	34	3	23	21	2	13	10	3	96	87	9
	4	10	9	1	6	6	0	25	22	3	22	15	7	21	18	3	84	70	14
	All	87	75	12	38	34	4	162	152	10	88	77	11	63	54	9	438	392	46

^aLevel 1 was at the anterior surface of the lungs; levels 2–4 were sectioned 2 mm posteriorly from the previous level.

A, air space; P, parenchyma.

FIG. 1. Photomicrographs captured after wide-field fluorescence microscopy showing examples of Group A cells, which are mononuclear cells with regular, homogeneous, nongranular GFP positive cytoplasm. *Upper left panel:* 400 $\times$ magnification, *other panels:* 1,000 $\times$ magnification. GFP, green fluorescent protein. Color images available online at www.liebertonline.com/scd

FIG. 2. Photomicrographs captured after wide-field fluorescence microscopy showing examples of Group B cells, which are mononuclear cells with regular, homogeneous, nongranular GFP positive cytoplasm but also possessing podocyte-like extensions. *Upper right panel:* 400 $\times$ magnification, *other panels:* 1,000 $\times$ magnification. Color images available online at www.liebertonline.com/scd

FIG. 3. Photomicrographs captured after wide-field fluorescence microscopy showing examples of Group C1 (*upper panels*) and C2 (*lower panels*) cells, which are regular or irregular shaped cells with nonhomogeneous (eg, fragmented) nuclei and homogeneous nongranular GFP staining (C1) or regular or irregular shaped cells with granular GFP positive cytoplasm (C2), and Group D: GFP positive fragments without a nucleus. *Lower right panel:* 400 $\times$ magnification, *other panels:* 1,000 $\times$ magnification. Color images available online at www.liebertonline.com/scd

Group C1 cells (regular or irregular shaped cells with nonhomogenous [eg, fragmented] nuclei and homogeneous nongranular GFP staining) (Fig. 3) were seen at the highest frequency, with 162 intact cells observed. This represents 43.2% of the total population of the intact fetal cells. Within this group, 152 (93.8%) were in the lung parenchyma, whereas only 10 (6.2%) were in air spaces (Table 1).

Group C2 cells, which were characterized as regular or irregular shaped cells with granular GFP positive cytoplasm (Fig. 3), were found at a frequency similar to that of Group A cells, comprising 23.5% ($n=88$) of the total number. Of these, 77 cells (87.5%) were in the parenchyma, and 9 cells (12.5%) were in air spaces (Table 1).

A total of 63 acellular fragments (Fig. 4), represented as Group D (Table 1), were found among all specimens examined, with 85.7% ($n=54$) in the lung parenchyma and 14.3% ($n=9$) in air spaces (Table 1).

There was a statistically significant difference in the frequency of morphological groups within maternal lung, with the order in frequency being Group C1 ($n=162$), C2 ($n=88$), A ($n=87$), D ($n=63$), and B ($n=38$) ($P=0.010$). There was also a statistically significant decrease in the frequency of all GFP positive cells and acellular fragments in an antero-posterior direction in the lung ($P=0.034$), with the total numbers decreasing from 154 (level 1), to 104 (level 2), to 96 (level 3) to 84 (level 4). This decrease was driven by GFP positive objects within parenchyma, as the number of objects in the air spaces was fairly consistent from anterior to posterior (Table 1).

To confirm that the GFP positive objects visualized using wide-field fluorescence microscopy did not originate from artifact, we also performed z-section confocal microscopy. The

spectral unmixing property of confocal microscopy combined with deconvolution allowed for the identification of the same cell types that were observed by using wide-field fluorescence microscopy, such as Group A and B cells (Fig. 5). These results confirm that fetal cells in maternal lung are a heterogeneous group of cells with various morphologies. Further, with the

FIG. 4. Photomicrographs captured after wide-field fluorescence microscopy showing examples of acellular GFP positive fragments (Group D). 400 $\times$ magnification. Color images available online at www.liebertonline.com/scd

FIG. 5. Photomicrographs captured after z-section confocal microscopy using spectral unmixing, showing examples of Group A and B cells. *Upper panel:* stacked images representing Topro-3 counterstained nuclei. *Middle panel:* stacked images representing GFP positive signals. *Lower panel:* combination of TOPRO-3 stained and GFP positive images after deconvolution (*upper arrow:* Group A cell, *lower arrow:* Group B cell). 400 $\times$ magnification. Color images available online at www.liebertonline.com/scd

use of confocal microscopy and spectral unmixing, we were able to confirm that GFP positive fragments (Group D) did not originate from fluorescent artifact (Fig. 6).

Discussion

This is the first demonstration of intact fetal cells in the maternal alveolar septum (or near the alveolar capillary) in a murine model. Since no GFP antibodies were used, it is unlikely that the findings of this study using wide-field fluorescent microscopy represent artifact or autofluorescence. Nevertheless, we also performed z-section confocal microscopy to confirm that the GFP signals, including those representing fragments, do not originate from fluorescent artifact. The number of GFP positive objects observed in each

FIG. 6. Photomicrographs captured after z-section confocal microscopy using spectral unmixing, showing examples of Group D fragments. *Upper panel:* stacked images representing Topro-3 counterstained nuclei. *Middle panel:* stacked images representing GFP positive signals. *Lower panel:* combination of TOPRO-3 stained and GFP positive images after deconvolution. 400 $\times$ magnification. Color images available online at www.liebertonline.com/scd

lung approximates those from our previous studies [5,16]. The advantage of microscopic evaluation of microchimeric cells used in the current study over flow cytometry is that intact cells, degrading cells undergoing apoptosis or necrosis, and acellular fragments can be distinguished from each other.

Four distinct categories of cells were observed. It is not clear whether these 4 unique cell groups represent a continuum of evolution/destruction of a single cell type, or whether they represent multiple cell types. Based on morphology and location, there is no evidence that the fetal cells are a part of the ciliated cell population or the Clara cell population, both of which are associated with airways. Although the specific phenotype of the fetal cells that were observed is unclear, it is intriguing that the vast majority of cells were found within the lung parenchyma, and a

significant number of these (Group A cells) had intact nuclear and cell membranes. One limitation of this study is its small sample size; additional analyses of larger numbers of cells in more animals are necessary to clarify whether these groups represent distinct categories of cells or whether they represent a continuum of degrading cells.

Our results suggest that about one-fourth of the total GFP positive objects are Group A cells, which we suggest may be immature (eg, progenitor) cells, although further characterization of these cells is necessary to support this contention. However, this is intriguing in the context of earlier studies that have suggested the presence of pregnancy-associated progenitor cells with multilineage capacity [12,17,18]. It is also possible that these Group A cells may be in an earlier stage of degradation than the other types. For example, Groups C and D appear to be undergoing apoptosis/necrosis. Indeed, although it has been shown that the peak frequency of fetal cell microchimerism occurs just before delivery, the number of fetal cells in maternal organs rapidly decreases after delivery [5,16]. Therefore, most cells are rapidly cleared from maternal tissues.

Even with the presence of cells that are likely in the process of apoptosis or necrosis, perhaps initiated by the maternal immune response, the intact cells observed both in this study and previous studies demonstrate the long-term persistence of fetal cells after pregnancy. This indicates that further study is needed to more fully characterize morphological cell types observed here, such as the Group A cells. An additional intriguing aspect of our results is the Group B cells, which possess irregular shapes that suggest movement or migration. It is possible that these are dendritic cells, which are heterogenous, have large surface to volume ratios, are present in tissues that are in contact with the external environment, and are capable of migration [19,20]. Further immunophenotyping studies of Group A and B cells are warranted. These studies should include microscopy and/or flow cytometry combined with antibody staining to provide more definitive phenotypic characterization of fetal cells in maternal lung. For example, CD45 or cytokeratin staining could be used to help determine whether these cells are leukocytes or trophoblasts, and stem cell antibody panels could be used to determine whether they are progenitor cells that may play a role in maternal tissue repair. In addition, since fetal cells have been hypothesized to play a role in maternal tumorigenesis [21], future experiments could be designed as well to test this hypothesis with regard to lung cancer.

Fetal GFP positive cells were predominantly located within the alveolar septum, and near capillaries, rather than in the air spaces, bronchioles, or large blood vessels. The fetal cells also appeared to be nonrandomly distributed through the thickness of the lung, as shown by the decreased numbers of cells in an antero-posterior direction. Whether this represents a concentration of fetal cells near the cell surface of the lungs or an artifact of tissue preparation or microscopy is at present unclear. Definitive localization requires 3-dimensional studies (eg, confocal imaging). A previous study also demonstrated the presence of numerous GFP positive fetal cells near the surface using stereomicroscopy in whole maternal lung [16].

In the lung stem field, most studies have focused on proof of principle that bone-marrow-derived stem cells can home

to the lung and differentiate into various lung cell phenotypes [22–24]. There has been great interest in whether these exogenously derived stem cells prevent or ameliorate various types of lung injuries. None of these studies, however, consider or indeed even acknowledge the potential presence of fetal cells in the adult lung [25]. The results presented here, in conjunction with those of previous studies that demonstrate the possible involvement of pregnancy-associated progenitor cells in maternal tissue repair [9,12,17], suggest that the naturally acquired population of fetal cells should be considered in any studies investigating the role of stem cells in the lung.

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Author Disclosure Statement

No competing financial interests exist.

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