Fetal Cells in the Pregnant Mouse Are Diverse and Express a Variety of Progenitor and Differentiated Cell Markers¹

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ABSTRACT

To better understand fetomaternal cell trafficking during pregnancy, we used a mouse model to determine the cell surface markers expressed on fetal cells, based on the hypothesis that fetal progenitor cells have the capacity to repair maternal organs, whereas more differentiated cells might initiate graft versus host disease. Wild-type females were mated to either homozygous or hemizygous transgenic males and euthanized in the peripartum period. Using dual color flow cytometry, we analyzed fetal transgene positive cells for the presence of nine markers (ITGAM, ITGB1, PECAM, CD34, CD44, PTPRC, ENG, SLAMF1, and CXCR4) to begin to identify the phenotype and degree of differentiation of fetal cells in nine maternal organs (lung, liver, spleen, blood, bone marrow, kidney, heart, thymus, and brain). Fetal cells were found in all maternal organs following either type of mating, albeit always at a higher frequency following mating with homozygous males. Some organs (e.g., lung and liver) had a wide variety of fetal cell markers present, while other organs (e.g., bone marrow and spleen) had a skewed distribution of fetal cell markers. Fetal cells in the murine pregnant female are diverse. Our results suggest that the fetal cells comprise a mixed population of progenitor and differentiated cells, with different relative proportions in different maternal organs. Future studies will address whether fetal cells cross the placental barrier in a differentiated state or as a homogenous population and subsequently differentiate in target maternal organs.

differentiation, fetal cell microchimerism, phenotype, pregnancy, trafficking

INTRODUCTION

The clinical significance of fetal cell microchimerism, resulting from fetomaternal cell trafficking during pregnancy, labor, and delivery, is still largely unknown. However, an increasing body of literature links it to both the cause and cure

of maternal diseases [1, 2]. In the human, fetal cell trafficking has been described in healthy women [3, 4], women with various diseases [5–7], and at autopsy [8, 9]. Two aspects of fetal cell identity are critical toward understanding the relevant mechanisms: first, whether fetal cells arrive in maternal tissue as terminally differentiated cells or as progenitor cells capable of differentiation within maternal tissue, and second, if fetal cells in general, and specific cell types in particular, are randomly distributed among various maternal organs. It is important to determine if fetal progenitor cells, which may be capable of differentiation and therefore potentially involved in tissue repair, are present in maternal tissue. Conversely, if more mature fetal cells are present in maternal tissues, they may induce a maternal immune response, thereby participating in a graft-versus-host reaction that has been hypothesized to be associated with the prevalence of autoimmune disease in postpartum women [10].

Major limitations of studying microchimerism in the human include inadequate pregnancy histories and a lack of healthy and diseased tissues for analysis. Due to these limitations, we developed a mouse model for further study [11, 12] based on past demonstrations of fetal cell trafficking in animals with hemochorial placentas [13–16]. In the mouse model, we used wild-type females mated to a syngeneic hemizygous male strain that carries a single transgene for enhanced green fluorescence protein (GFP); approximately 50% of offspring (and approximately 50% of fetal cells) are therefore GFP+ [12]. This model permits us to track fetal cells in the wild-type mother's body during pregnancy and postpartum using a variety of techniques. We have recently applied the model to determine the frequency, organ-specific location, and gestational trafficking dynamics of fetal cells in healthy female mice during their first pregnancy [17]. This study showed that fetal cells were first observed at Day e11 (Embryonic Day 11) and increased in number with advancing gestational age. The maximum number of fetal cells was found just prior to term and decreased rapidly after delivery, becoming nearly undetectable by Days 5–6 postpartum. In addition, there was a nonrandom pattern of distribution of fetal cells within maternal organs, with the lung having the greatest concentration, followed by the spleen and liver. We also found that flow cytometry is the optimal method to quantify fetal cell microchimerism, compared to *in vivo* imaging and real-time PCR.

For this study, we added a new transgenic strain that is homozygous for the *Gfp* transgene [18]. Using this strain as the paternal line in the matings, 100% of offspring and 100% of fetal cells inherit the *Gfp* sequence. With dual-color flow cytometry, we analyzed transgene positive fetal cells for the presence of a variety of cluster of differentiation (CD) markers

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to identify the degree of differentiation of fetal cells present in maternal organs. We used a panel of nine individual monoclonal antibodies on single cell suspensions from nine different maternal organs. The antibodies were selected to give a broad overview of the possible different fetal cell types present and to answer the question as to whether the fetal cell population was homo- or heterogenous.

The antibodies in the study recognize antigens expressed on hematopoietic cells, including progenitor cells, such as CD34, because these cells have been shown to persist in human and rhesus monkey maternal blood for decades after pregnancy [3, 19–21]. A subset of adherent fetal CD34⁺ cells, suggested to be a primitive stem cell population, have been observed at a high frequency in maternal blood [22]. Other markers studied included hematopoietic cells that express CXCR4 (chemokine (C–X–C motif) receptor 4 or CD184), because hematopoietic stem cells that are positive for both CD34 and CXCR4 are a more primitive cell type than CD34 positive/CXCR4 negative cells [23], and SLAM family antigens, such as SLAMF1 (signaling lymphocytic activation molecule family member 1 or CD150). SLAMF1⁺/CD244[–]/CD48[–] cells are suggested to be among the most primitive of hematopoietic stem cells [24], although SLAMF1 is also expressed on mature cells such as lymphocytes and dendritic cells.

In addition to hematopoietic cells, mesenchymal stem cells (MSC) were studied [25, 26]. Mesenchymal stem cells are positive for CD44 [27], ITGB1 (integrin beta 1 (fibronectin receptor beta) or CD29), and ENG (endoglin or CD105), and negative for PECAM1 (platelet/endothelial cell adhesion molecule 1, PECAM1 or CD31), CD34, and PTPRC (protein tyrosine phosphatase, receptor type, C or CD45) [28]. The panel also included an antibody to an endothelial cell marker (PECAM1). While PECAM1 is a marker of terminally differentiated lung cells and endothelial cells in the adult, PECAM1⁺ cells have also been reported to be involved in maternal neoangiogenesis during pregnancy [29]. To determine if fetal white cells are present in maternal tissue, antibodies to the pan-leukocyte marker PTPRC (CD45) and ITGAM (integrin alpha M or CD11b), a marker of integrin- α positive myeloid cells (specifically macrophages and natural killer cells), were also included [30].

MATERIALS AND METHODS

Mice

The Institutional Animal Care and Use Committee (IACUC) of the Tufts University School of Medicine Division of Laboratory Animal Medicine (DLAM) approved the protocol described here. All institutional guidelines regarding the ethical use of experimental animals were followed. We mated wild-type C57BL/6J female mice (stock no. 664; Jackson Laboratories, Bar Harbor, ME) to one of two different enhanced green fluorescent protein (EGFP) transgenic strains of male mice. One is the hemizygous transgenic strain C57BL/6-Tg(ACTB-EGFP)10sb/J (stock no. 3291; Jackson Laboratories) and the other is the homozygous transgenic strain C57BL/6-Tg(CAG-EGFP)C14-Y01-FM1310sb (stock no. 267; provided by Dr. Masaru Okabe, Riken BioResource Center, Ibaraki, Japan), which has a copy of the *Gfp* transgene on each homolog of chromosome 14. Both transgenic strains share a C57BL/6J genetic background, with the *Gfp* transgene under the control of a ubiquitous chicken beta-actin promoter and a cytomegalovirus enhancer [18, 31].

Eight- to ten-wk-old C57BL/6J virgin female mice were mated to either hemizygous or homozygous transgenic males. Parameters of mating, including duration and gestational age determination, were previously described [17].

Tissue Collection and Single Cell Suspension

Euthanasia was performed in a carbon dioxide gas chamber followed by cervical dislocation. Blood collection, erythrocyte removal, mononuclear cell preparation, bone marrow cell isolation, solid tissue dissection, and preparation of single-cell suspensions were performed as previously described [17].

Comparison of GFP Fluorescence Between Transgenic Strains

Because this was the first study in which we used the homozygous *Gfp* strain, we first compared GFP fluorescence between the strains. Mononuclear cells from blood and single cell suspensions of organs from 10-wk-old wild-type, hemizygous transgenic and homozygous transgenic male mice were used to compare their fluorescent intensity by flow cytometry. Blood mononuclear cells and single-cell suspensions from all the other organs were washed once with flow cytometry buffer and resuspended in 200 μ l of flow cytometry buffer (PBS with 2% fetal bovine serum and 0.1% sodium azide) with 1 μ g/ml 7-amino-actinomycin D (7-AAD) (BD Pharmingen, San Diego, CA) to stain the dead cells. Cells were analyzed by flow cytometry using the flow cytometry system FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). 7-Amino-actinomycin D, phycoerythrin, and EGFP were excited at 488 nm and measured at 647 nm (FL-3 LP670 filter), 575 nm (FL-2 585/42 filter), and 510 nm (FL-1 530/30 filter), respectively. Data were collected from 50 000 live cells according to forward scatter (FSC), side scatter (SSC) and 7-AAD gating. Cells positive for GFP expression, based on FL-1 fluorescence, were identified and mononuclear cells from blood were further identified based on 7-AAD-negative and PTPRC-positive staining and quantified. FlowJo software version 8.5.3 (Tree Star, Ashland, OR) was used for the data analysis.

Fetal Cell Phenotyping

Pregnant wild-type female mice ($n = 43$) were analyzed for fetal cell marker analysis. Females were mated with hemizygous ($n = 24$) or homozygous ($n = 19$) transgenic males and euthanized at Days 17–20 postpartum. This time was selected because our prior research indicated that fetomaternal trafficking is maximal late in pregnancy [17]. Single cell suspensions from solid organs and blood were washed once with flow cytometry buffer. After washing, cells were stained for 60–90 min with monoclonal antibody. Monoclonal antibodies used were allophycocyanin-conjugated rat anti-mouse CD44 (eBioscience, San Diego, CA), CD150 (eBioscience), and CXCR4 (BD Pharmingen), and biotin-conjugated rat anti-mouse ITGAM (BD Pharmingen), ITGB1 (Abcam, Cambridge, MA), PECAM1 (BD Pharmingen), CD34 (eBioscience), PTPRC (BD Pharmingen), and SLAMF1 (eBioscience). All the antibody concentrations were optimized by fluorescence-activated cell sorting using serial dilutions of each antibody with a constant number of cells from each target organ prior to analysis. Streptavidin-allophycocyanin (BD Pharmingen) was used as a developing reagent for biotinylated antibodies.

After antibody staining, the cells were washed twice with flow cytometry buffer and resuspended in 200 μ l of flow cytometry buffer with 1 μ g/ml 7-AAD. Allophycocyanin was excited at 633 nm and measured at 660 nm (680/30 FL-4 filter). High-speed flow cytometric analysis was conducted with MoFlo (DAKO, Fort Collins, CO). Data were collected from 7 to 15 million mononuclear cells that were counted using the MoFlo hardware counter. Abnormally shaped cells were excluded by FSC and SSC gating. Autofluorescent noise, which distributes in a linear pattern on FL-2 (570/40 filter) and FL-3 (670/40 filter), was gated out. 7-AAD-positive dead cells were also excluded using FL-3 gating. High-speed flow sorting (20 000–80 000 cells/sec) was conducted for each sample. Summit 4.3 software (DAKO) was used for data analysis. Fetal GFP⁺ cell counts (FL-1 [530/40 filter]) were determined and shown as the number per 10 million maternal cells. Fetal cell phenotypes were shown as the number of antibody positive cells among the number of GFP-positive cells. At least four mice (two hemizygous and two homozygous transgenic matings) were analyzed for each antibody.

Statistics

The nonparametric Kruskal-Wallis test was used to compare the proportions of antibody-positive cells among different organs. The frequency of fetal cells in maternal organs following hemizygous and homozygous transgenic mating was examined using the Wilcoxon rank sum test. Statistical significance was assigned at $P < 0.05$. All statistical analyses were performed using SAS/STAT software (SAS Institute, Inc., Cary, NC).

RESULTS

Comparison of GFP Fluorescence Between Transgenic Strains

The fluorescence intensities of GFP⁺ cells isolated from various organs from wild-type (negative control) and both hemizygous and homozygous transgenic strains of mice

FIG. 1. Flow cytometry results showing fluorescence intensity of cells isolated from various organs from three strains of male mice. Red: wild type; blue: hemizygous transgenic; green: homozygous transgenic.

(positive controls), as determined by flow cytometry, are shown in Figure 1. Peak GFP intensity of cells isolated from blood (i.e., PTPRC+), bone marrow, spleen, and thymus is greater in the homozygous compared to the hemizygous transgenic strain, while peak intensity of cells isolated from liver is greater in the hemizygous strain. Peak GFP fluorescence intensity was roughly equivalent in cells isolated from heart, lung, kidney, and brain between the two transgenic strains.

Comparison of Fetal Cell Microchimerism Between Transgenic Strains

The observed proportion of fetal transgenic phenotype, as determined by detection of green fluorescence by UV excitation, was as expected (demonstrating Mendelian inheritance), i.e., 48.6% from hemizygous mating and 100% from homozygous mating (chi-square test, $P = 0.992$).

Fetal cells were found in all the maternal organs following mating with either homozygous or hemizygous transgenic males. The frequency of fetal cells varied among organs (Supplemental Table S1, available online at www.biolreprod.org), with the organs coalescing into three groups based on these frequencies. The first group consisted of those organs in which the median number of total GFP+ cells was above 10 per 10 million maternal cells and that of antibody-positive fetal cells was above 1 per 10 million maternal cells. These organs included lung, liver, spleen, blood, and bone marrow. The second group consisted of organs (i.e., kidney and heart) in which GFP+ cells were consistently observed, yet the median

number of antibody-positive fetal cells was 0 (i.e., below the limit of sensitivity by flow cytometry). The third group consisted of the brain and thymus, in which GFP+ cells were only occasionally detected and the count of antibody-positive cells was 0 (Supplemental Table S1). The observed median frequency of fetal cell microchimerism was higher in all the organs from female mice mated to homozygous transgenic males compared to matings with hemizygous transgenic males (Fig. 2). This difference was statistically significantly different in the lung, liver, spleen, bone marrow, kidney, and heart ($P < 0.05$).

Among the maternal organs in which there were significant differences in the frequency of fetal cells between the two strains and median values > 0 for both transgenic strains (i.e., lung, liver, spleen, bone marrow, and kidney) (Fig. 2), the ratio of fetal cells found after mating with homozygous transgenic males compared to that of hemizygous males was higher than expected based on the difference in transgene inheritance (i.e., a ratio of 2:1). These five maternal organs had ratios of 2.3:1, 3.2:1, 2.9:1, 3.3:1, and 3.6:1; respectively. The only organ with median values > 0 for both transgenic strains and a nonsignificant difference in the median number of fetal cells between the two transgenic strains was blood, with a ratio of 1.6:1.

Fetal Cell Phenotyping

Although there was a difference in transgene copy number between hemizygous and homozygous transgenic mice, there were no significant differences in the observed relative median frequency of all cellular phenotypes combined among all the

FIG. 2. Statistical comparison of median fetal cell numbers in maternal organs at Day e18 following mating with hemizygous (CAG) and homozygous (Riken) males. The median number of fetal cells is statistically significantly higher in most maternal organs (i.e., lung, liver, spleen, bone marrow, kidney, and heart) following mating with homozygous males compared to mating with hemizygous males ($P < 0.05$). Lines represent median values, filled circles represent mean values, boxes represent 25th and 75th percentiles, whiskers represent 10th and 90th percentiles, and open circles represent outliers.

maternal organs that were analyzed. Therefore, we combined results from both types of matings into a single data set (Table 1 and Fig. 3).

Maternal lung tissue, which contained the highest number of fetal cells, had a wide variety of cell markers present (Fig. 3). The varied distribution of cell markers was also apparent in the liver. Conversely, there appeared to be a skewed distribution of specific cellular markers in other organs, such as blood, bone marrow, and spleen. In each of these organs, a single cell marker was found more frequently than all the others. In blood, these were cells, possibly MSC, that express ITGB1. In bone marrow and spleen, PTPRC⁺ cells predominated. In the

kidney, endothelial cells (PECAM1⁺) as well as ITGB1⁺ cells were more commonly found. In the heart, only ENG cells were found at a significant median frequency. Thymus and brain were included for completeness, but in each organ, only two GFP⁺ fetal cells were found. Therefore, only limited conclusions can be drawn on the differential phenotype of these cells. Nonetheless, the antigens expressed by these cells were organ specific (e.g., SLAM family receptors in the thymus). Among the five maternal organs with the most fetal cells (i.e., lung, liver, spleen, blood, and bone marrow), there was a statistically significant difference in the distribution of fetal cell markers (Table 1).

TABLE 1. Median percentage of specific CD-positive fetal cells among all fetal cells present in maternal organs.^a

Antibody	Lung	Liver	Spleen	Blood	Bone marrow	<i>P</i> value
ITGAM	20.8	16.5	8.0	8.3	33.3	0.2
ITGB1	16.7	2.6	0.0	40.0	0.0	0.008
PECAM1	40.7	6.9	13.0	0.0	0.0	0.002
CD34	24.4	15.9	0.0	0.0	0.0	0.0003
CD44	29.5	11.1	15.4	25.0	0.0	0.008
PTPRC	39.8	28.8	45.9	0.0	100.0	<0.0001
ENG	17.0	6.0	0.0	0.0	5.6	0.2
SLAMF1	0.6	0.0	0.0	0.0	0.0	0.1
CXCR4	5.3	0.0	0.0	0.0	0.0	<0.0001
<i>P</i> value	0.003	0.003	0.0002	0.02	0.005	

^a Data cannot be combined among organ or antibody, as each organ was evaluated separately for each antibody analyzed.

FIG. 3. Median percentage of fetal cells positive for each antibody out of all GFP+ fetal cells isolated from each organ. The y-axis indicates percent of GFP+ cells that express each antigen. Cells from each organ were evaluated separately for each antibody analyzed. Therefore, data cannot be combined across the x-axis.

DISCUSSION

In this study, we applied transgenic mouse models of fetal cell trafficking to study nine different fetal cell markers within nine maternal organs. We also compared the results obtained from matings between wild-type females and two different transgenic strains of male mice, one hemizygous and one homozygous for the *Gfp* transgene. Importantly, we demonstrated that the fetal cell population present within various maternal organs is diverse, comprising cells with a variety of progenitor and differentiated cell markers. Furthermore, we showed that different maternal organs contain fetal cells with different relative proportions of cell surface markers.

Among organs with significant differences in the frequency of fetal cells following homozygous and hemizygous transgenic mating, the ratio of fetal cells was greater than the expected ratio of 2:1 (i.e., from 2.3:1 to 3.6:1). These results suggest that the greater number of transgenic pups resulting from homozygous transgenic matings leads to a greater number of observed fetal cells over that following hemizygous matings. This difference may also be due to the increased brightness of GFP fetal cells that possess the homozygous transgenic construct compared to those with the hemizygous construct. While there were differences due to unknown cause(s) in the peak fluorescent intensities of cells isolated from various organs between the two transgenic strains, these differences were not sufficiently large enough to eliminate the overall benefit of using the homozygous transgenic strain to detect fetal cells in wild-type maternal organs.

As an initial approach to investigate the phenotype of fetal cells present among maternal organs, we selected a broad panel of antibodies that recognize immature (progenitor) cell types

(CD34), predominantly mature (terminally differentiated) cell types (ITGAM and PTPRC), and a mixture of mature and immature cells (ITGB1, PECAM1, CD44, ENG, SLAMF1, and CXCR4). We found that the fetal cell populations among maternal organs consisted of two major subsets, hematopoietic cells (e.g., CD34, PTPRC, SLAMF1, and CXCR4 positive) and nonhematopoietic (e.g., mesenchymal and endothelial) cells. The fetal hematopoietic cells were present in maternal hematopoietic tissue, such as bone marrow and spleen, although surprisingly, they did not predominate in maternal blood. Further work is needed to determine if the lack of hematopoietic markers in blood is due to the relatively small number of total cells that are isolated compared to solid organs, to technical artifact (e.g., staining intensity), or to trafficking dynamics. Nonhematopoietic fetal cells were found in other organs, such as lung and kidney.

In maternal lung tissue, there are a variety of fetal cell markers present at approximately equivalent frequencies. This somewhat random distribution of cell markers suggests a passive mechanism of cell accumulation, perhaps related to blood flowing from uterine veins that drain into lymph nodes and the portal circulation [15]. It appears that most, if not all, cell types that pass through the lung are retained in that organ. However, in other organs, specific cell markers are over- or underrepresented. For example, PTPRC+ cells predominate in bone marrow, while ITGB1+ cells comprise a significant proportion of fetal cells in blood. The nonrandom distribution of fetal cell markers in these organs suggests either an active mechanism of fetal cell recruitment or retention of specific fetal cell markers due to local or regional differences in the microenvironment (i.e., cell-cell or cell-matrix adhesion

interactions). It is also possible that the lower prevalence of fetal cells outside the lung may reduce the potential diversity of cell types. Nevertheless, while some of the antibodies used in this study recognize both mature (i.e., terminally differentiated) and immature (i.e., progenitor) cell types, these data suggest that pregnancy-associated progenitor cell populations as well as terminally differentiated fetal cells are present in a variety of maternal organs. These progenitor fetal cell types may be capable of participating in maternal tissue repair, suggesting that fetal cells may contribute in a positive way to maternal health, as we have previously demonstrated in a mouse model of liver injury [32].

Clearly, these results suggest the need for further investigation to more definitively distinguish between progenitor and more mature microchimeric fetal cell types to further the understanding of the phenotypic profile of fetal cells among murine maternal organs. Because we have analyzed fetal cells only for single markers at a time and there is no single marker that identifies certain cell types (e.g., MSC), further assessment of fetal cells using panels of markers will be necessary to more definitively identify specific phenotypes. This includes, for example, the demonstration of MSC through the analysis of fetal cells for the presence of markers such as ITGB1 and CD44 [33] combined with the absence of markers such as THY1 (CD90), KIT (CD117), and PTPRC [34] or through the combined analysis of ENG, THY1, NT5E (CD73), and STRO-1 (STRO-1 is a cell surface protein [antigen] expressed by bone marrow stromal cells and erythroid precursors). A wide variety of markers must be selected using a robust experimental design, as the defining characteristics of MSC are inconsistent among investigators [35]. Other potential analyses include the assessment of early vs. late hematopoietic fetal cells through combined staining for PECAM1, CD34, and/or CD38. These analyses, in conjunction with analysis of cell proliferation markers such as 5-bromo-2-deoxyuridine, would allow progenitor cells to be distinguished from more mature cells through the demonstration of proliferation, self-renewal, and association with a niche [33].

The results shown in this study are significant in that they demonstrate for the first time that the fetal cell population in maternal organs is diverse and complex. Previously, microchimeric fetal cells have been postulated to have somewhat uniform biological characteristics that are “betwixt and between” those of embryonic and adult stem cells [36]. In contrast, our data suggest that they are not uniform, and that some of the fetal cells may be more stem cell-like than others. Our results also demonstrate that with the increased detection of fetal cells using the paternal homozygous transgenic strain, we have significantly improved signal to noise ratios in the detection of fetal cells. This resolves a technical issue that limited past studies. The paternal transgene is a gender-independent fetal marker.

Taken together, these results should allow us to answer important biological questions, such as whether fetal cells cross the placenta as a uniform population, with subsequent local differentiation in maternal organs, or whether they are transferred into the mother as a mixed population that reflects the contents of whole fetal blood. This will be accomplished through the continued development and application of this type of animal model of fetal cell trafficking, particularly with the use of panels of antibodies to more definitively identify the phenotypes of the fetal cells in maternal organs throughout gestation and after delivery. These questions also have biological significance, as they will help us to better understand the differentiation capabilities of fetal microchimeric cells. Further investigation is necessary to determine if naturally

acquired fetal stem cells can be harvested in sufficient numbers and/or expanded in culture to reach a number that would be necessary for therapeutic applications in regenerative medicine.

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