

Transfer of Fetal Cells With Multilineage Potential to Maternal Tissue

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FETAL CELLS ENTER THE MATERNAL circulation during all pregnancies.^{1,2} They can persist in maternal blood or tissues for decades, creating a state of physiologic microchimerism in the parous woman.³ Recent studies detected male cells of presumed fetal origin in 30% to 50% of healthy women who had prior male pregnancies.⁴ The long-term consequences of fetal cell microchimerism for maternal health are only beginning to be appreciated. Fetal microchimeric cells are present in higher numbers in women with some autoimmune diseases, such as systemic sclerosis, than in control groups.^{5,6} We have also observed fetal cells in the tissues of women with nonautoimmune disorders, such as hepatitis C⁷ and cervical cancer.⁸ Thus, we developed an alternate hypothesis in which fetal cells were associated with the maternal response to injury as opposed to causing disease.

During pregnancy, the fetal cells that enter the maternal circulation are predominantly of hematopoietic origin, such as nucleated red blood cells, lymphocytes, or hematopoietic stem cells.^{9,10} Trophoblasts and mesenchymal stem cells also circulate within maternal blood.^{11,12} Following pregnancy, male fetal cells have been demonstrated in the

Context During pregnancy, fetal CD34⁺ cells enter the maternal circulation, persist for decades, and create a state of physiologic microchimerism. Many studies have confirmed the residual presence of fetal cells in maternal blood and tissues following pregnancy. Fetal cells may respond to maternal injury by developing multilineage capacity in maternal organs.

Objective To verify that fetal microchimeric cells express markers of epithelial, leukocyte, and hepatocyte differentiation within maternal organs.

Design, Setting, and Patients Archived paraffin-embedded tissue section specimens from 10 women who had male offspring and were previously found to have high numbers of microchimeric cells, and 11 control women who had no prior male pregnancies. Male cells were identified by fluorescence in situ hybridization, using X and Y chromosome-specific probes, followed by histologic and immunochemical studies using anticytokeratin (AE1/AE3) as a marker of epithelial cells, anti-CD45 as a leukocyte marker, and hepar-1 as a hepatocyte marker.

Main Outcome Measure Percentage of microchimeric cells expressing nonhematopoietic markers.

Results A total of 701 male (XY+) microchimeric cells were identified (mean [SD], 227 [128] XY+ cells per million maternal cells). In maternal epithelial tissues (thyroid, cervix, intestine, and gallbladder), 14% to 60% of XY+ cells expressed cytokeratin. Conversely, in hematopoietic tissues, such as lymph nodes and spleen, 90% of XY+ cells expressed CD45. In 1 liver sample, 4% of XY+ cells expressed hepar-1. Histologic and immunochemical evidence of differentiation, as assessed by independent observers, was highly concordant ($\kappa=0.72$).

Conclusion The detection of microchimeric male cells, bearing epithelial, leukocyte, or hepatocyte markers, in a variety of maternal tissue specimens suggests the presence of fetal cells that may have multilineage capacity.

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CD34⁺ compartment.^{3,13,14} They have also been found in various sorted subsets of maternal peripheral mononuclear blood cells, such as T, B, and natural killer cells, or cells that express the CD4 or CD8 antigens,^{15,16} suggesting that fetal microchimeric cells may be capable of engraftment and differentiation along the hematopoietic pathway.

Little information is available on the phenotype of fetal microchimeric cells in nonhematopoietic tissues and most published studies suggest that fetal cells express hematopoietic markers.^{17,18} In contrast, we previously reported that the male cells of presumably fetal origin observed in the thyroid of a woman

affected with a multinodular goiter had a follicular morphology.¹⁹ We therefore tested our hypothesis by examining tissue specimens from women, affected with a variety of diseases, who had male offspring to determine the morphology, cell surface, and intracellular phenotype of fetal cells within maternal organs.

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See also pp 81 and 104.

METHODS

Study Design

Individual male (XY+) microchimeric cells were evaluated for their cell surface and intracellular phenotype. We selected tissue samples from 10 women, previously studied by our laboratory group that had significant and easily detectable male cell microchimerism.^{7,8,19-21} All of the initial studies documenting the presence of microchimerism included appropriate control patients, including women who had no prior male pregnancies. In the present study, we contemporaneously analyzed skin and cervical tissue from 11 women with no known history of a male pregnancy. We obtained approval from the institutional review board and written informed consent from all patients who underwent surgery or biopsies. To the extent possible, we obtained complete pregnancy histories from study participants, including the number of sons, daughters, and abortions (spontaneous and elective), as well as the possibility of other sources of microchimeric cells. None of the women had a twin brother or had received an organ transplant at the time of tissue collection. One woman had a history of blood transfusion from a donor of unknown sex.

Fluorescence In Situ Hybridization and Immunolabeling

We performed fluorescence in situ hybridization (FISH) analysis of the tissue sections, as previously described,^{21,22} with simultaneous immunolabeling.²³ We tested 3 different mouse monoclonal IgG1 antibodies: AE1/AE3 anticytokeratin (Chemicon International, Temecula, Calif) was used to identify epithelial cells, anti-CD45 (Dako, Carpinteria, Calif) to identify leukocytes, and heppar-1 (Dako) to identify hepatocytes. In all experiments, a mouse IgG1 (BD Bioscience, San Diego, Calif) was used as an isotypic control.

Scoring

Following hybridization and immunostaining, we included tissue sections for subsequent analysis if the following criteria were met: FISH, immunostaining, and morphologic.

FISH Criteria. During the hybridization procedure, there was minimal loss of cells and more than 75% of nuclei contained FISH signals. Male cells had 2 different-colored FISH signals, representing both the X and Y chromosomes, and an intact nuclear border. We recorded the coordinates of microchimeric cells, which allowed us to retrieve 701 (97.9%) of 716 cells on the

slide. We also estimated the total number of nuclei in each section by counting them in 10 fields at 400× magnification and counting the number of fields to cover the whole tissue section. We then extrapolated the frequency of male cells among a million maternal cells for each tissue section.

Immunostaining Criteria. We considered the immunostaining results to be positive if target areas were stained and nontarget areas were not stained. For CD45, the target areas were defined as nucleated cells inside blood vessels and nontarget areas were defined as any epithelial tissue. For heppar-1 and cytokeratin, the target area was defined as liver parenchyma or epithelial area, respectively, and nontarget areas were defined as cells inside blood vessels. In addition, to further prove the specificity of our antibodies, we performed immunostaining with the anticytokeratin antibody on liver, lymph node, and spleen tissue, and with heppar-1 on skin, spleen, heart, and thyroid tissue. We also performed 2 series of immunostaining experiments on a cord blood sample obtained during a full-term cesarean delivery with all the antibodies described above to determine if circulating fetal cells express hepatocyte or epithelial cell markers.

Table 1. Characteristics and Results of Immunolabeling Studies on Microchimeric Cells of Women (n = 10)

Tissue Type	Woman	Age, y	No. of Children		No. of Fetal Losses*	History of Transfusion	Disease	Frequency of Male Cells†	% of Male Cells (No. of Male Cells/Total No. of Cells)		
			Male	Female					CD45+	Cytokeratin+	Heppar-1+
Thyroid	A	48	1	2	0	0	Goiter	500	0 (0/85)	60 (48/93)	NA
Thyroid	B	37	2	1	0	0	Goiter	30	0 (0/60)	32 (19/60)	NA
Thyroid	C	74	2	2	0	0	Goiter	30	67 (43/64)	14 (8/59)	NA
Cervix	D	57	1	3	5	0	Cancer	40	37 (12/32)	37 (12/32)	NA
Cervix	E	50	1	1	2	0	Cancer	30	33 (2/6)	50 (5/10)	NA
Cervix	F	63	1	0	3	0	Cancer	10	NA	0 (0/14)	NA
Gallbladder	G‡	46	1	1	0	1	Autoimmune hepatitis	NA	36 (8/22)	19 (4/21)	NA
Intestine	H	34	2	0	0	0	SLE	30	55 (11/20)	40 (6/15)	NA
Liver	G‡	46	1	1	0	1	Autoimmune hepatitis	1300	64 (30/47)	NA	4 (10/234)
Liver	I	41	2	1	0	0	Systemic sclerosis	50	72 (13/18)	0 (0/7)	0 (0/13)
Spleen	G‡	46	1	1	0	1	Autoimmune hepatitis	NA	86 (43/50)	NA	NA
Lymph node	J	67	1	NA	0	0	Systemic sclerosis	250	100 (17/17)	0 (0/17)	NA

Abbreviations: NA, experiment not performed or information not available; SLE, systemic lupus erythematosus.

*Fetal loss due to either elective abortion or spontaneous miscarriage.

†Per million maternal cells.

‡History of blood transfusion from a donor of unknown sex.

Morphologic Criteria. After evaluating both FISH and immunostaining results, we stained tissue sections with hematoxylin and eosin. We then relocated microchimeric male cells based on their slide coordinates and assessed morphology and relative location within a section using a light microscope. Cells that were not part of the section were excluded. Morphology and immunostaining were independently evaluated by 2 investigators (K.K. and R.N.S.).

Statistical Analyses

Each microchimeric cell received a score of 0 if hematopoietic or 1 if epithelial or hepatocyte. The concordance between the morphology and immunohistochemical assessments were compared for all cells that had both criteria scored by estimating the κ value. In thyroid specimens, the cells were also evaluated as being inside or outside the diseased area of the tissue section. Thyroid samples were the only specimens in which the pathologic area (adenomatous tissue) could be clearly distinguished from healthy surrounding tissue. All other specimens contained exclusively diseased tissue. We compared the frequency of cytokeratin-positive microchimeric cells inside and outside the diseased area by using the Kruskal-Wallis test.²⁴

RESULTS

We performed FISH analyses and identified a total number of 701 XY+ cells (mean [SD], 227 [128] XY+ microchimeric per million maternal cells) in archived paraffin-embedded tissue section specimens from 10 women (mean age, 51.7 years; range, 34-74 years) who had male offspring. We subsequently evaluated the cell surface and intracellular phenotype of the XY+ cells by immunolabeling, morphology, and relative location within the sample (TABLE 1). We also performed FISH analysis on tissue biopsies from women who had no history of a male pregnancy (n=11) and found no XY+ cells (TABLE 2).

Anticytokeratin did not stain hematopoietic tissues, such as lymph node

Table 2. Fluorescence In Situ Hybridization on Tissues From Control Women Without a Known History of a Male Pregnancy (n = 11)

Woman	No. of Female Children	No. of Fetal Losses*	Tissue Type	Disease	No. of Male Cells
1	5	0	Uterus	Myoma	0
2	2	0	Uterus	Myoma	0
3	0	0	Uterus	Cancer	0
4	2	0	Uterus	Cancer	0
5	2	0	Uterus	Cancer	0
6	ND	ND	Skin	Lupus	0
7	ND	ND	Skin	Dermatomyositis	0
8	ND	ND	Skin	Dermatomyositis	0
9	ND	ND	Skin	Mycosis fungoides	0
10	ND	ND	Skin	Mycosis fungoides	0
11	ND	ND	Skin	Mycosis fungoides	0

Abbreviation: ND, not determined because women were only asked about male deliveries.
*Fetal loss due to either elective abortion or spontaneous miscarriage.

or spleen, but did stain biliary epithelium as expected. Antihepatocyte antibody (hepar-1) was specific for liver and did not stain any of the additional tissues tested (skin, heart, thyroid, and spleen). In addition, unlike anti-CD45, anticytokeratin and hepar-1 antibodies did not stain cord blood cells. In 90% of cases in which there was positive immunochemical staining of a cell, independent histological assessment of that cell after hematoxylin and eosin staining was substantially concordant with regard to morphology ($\kappa=0.72$).

We found a mean (SD) frequency of 190 (157) XY+ microchimeric cells per million maternal cells among 3 women with multinodular goiters who underwent partial thyroidectomy. In each of the 3 women, 14% to 60% of the XY+ cells stained positively with cytokeratin, a marker of epithelial differentiation (FIGURE 1). In 1 case, some of the XY+ fetal cells that expressed cytokeratin were integrated into a thyroid follicle. In 2 of 3 thyroid specimens, none of the microchimeric cells expressed CD45, a common leukocyte antigen. A large inflammatory infiltrate was observed in the third woman's thyroid; 67% of the XY+ cells expressed CD45.

We also analyzed the differentiation pattern of XY+ cells, according to their physical location within a pathologic or healthy area. The 3 thyroid specimens studied included a mac-

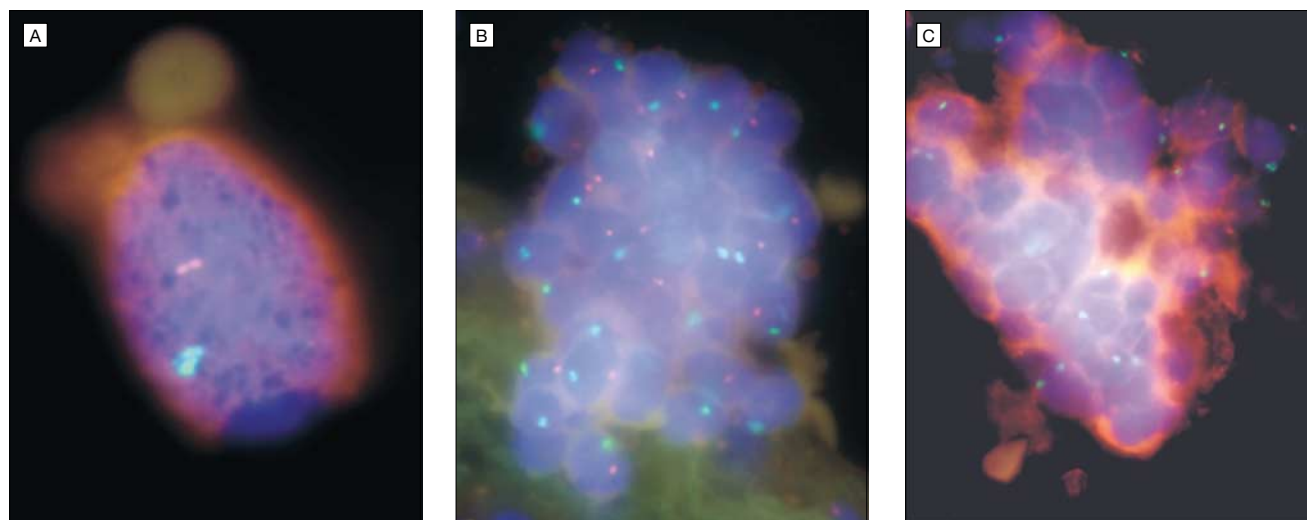
roscopically visible adenoma surrounded by healthy thyroid tissue. Histological examination of these 3 specimens revealed that most of the microchimeric cells (114 of 150 cells successfully relocated) were not part of the adenomatous tissue but were in the surrounding healthy thyroid tissue. Interestingly, fetal cells inside the adenoma (36 of 150) had a significantly higher percentage of cytokeratin expression than cells outside the adenoma (92% vs 17%, respectively; $P<.001$). The reverse situation was found for CD45: XY+ cells outside the adenoma more frequently expressed CD45 than cells inside the adenoma (32% vs 3%, respectively; $P<.001$).

We also analyzed other epithelial tissues, such as cervical epithelium specimens from 3 women, and digestive epithelial (gallbladder, intestine) tissues from 2 women. We found a comparable pattern of differentiation, 20% to 56% of the XY+ cells expressed cytokeratin and 30% to 55% expressed CD45.

In hematopoietic tissues, such as lymph nodes and spleen from 2 women, 90% of the XY+ cells expressed CD45. None of the cells expressed cytokeratin. We also performed double staining (CD45 and cytokeratin) in most tissues; microchimeric cells never stained positively with both antibodies.

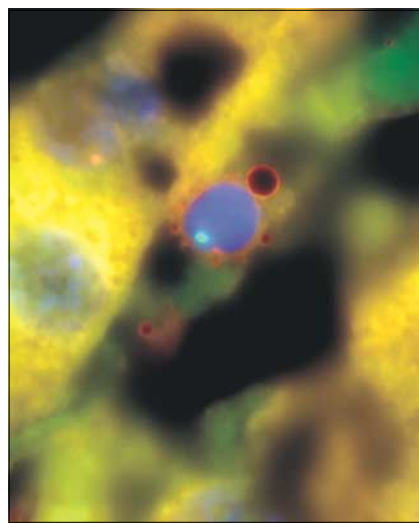
In liver specimens of 2 women (patients G and I), most of the XY+ cells

Figure 1. Cytokeratin Expression in Microchimeric Cells in Thyroid



Photomicrographs show fluorescence in situ hybridization (FISH) using Cy3-labeled X (orange) and fluorescein isothiocyanate conjugated-labeled Y (green) chromosome probes and immunofluorescence staining for cytokeratin using mouse monoclonal AE1/AE3 anticytokeratin antibody and fluorochrome Texas Red (red). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue). A, Male microchimeric cell with 1 Y chromosome (green), 1 X chromosome (orange), and stained with anticytokeratin antibody (red) (patient A; magnification $\times 1000$). B, Interphase FISH of thyroid tissue showing a group of microchimeric cells identified by the presence of X and Y chromosomes (orange and green, respectively). The X or Y chromosome may not be observed in each nucleus, as they may not be in the same plane of focus (patient C; magnification $\times 400$). This group of cells did not stain positively for cytokeratin. C, Combined FISH and immunofluorescence staining of a group of microchimeric cells with 1 X and 1 Y chromosome. This group of cells expresses cytokeratin (red). The X or Y chromosome may not be observed in each cell, as they may not be in the same plane of focus (patient A; magnification $\times 400$).

Figure 2. CD45 Expression in Microchimeric Cells in Liver (Patient I)



Photomicrograph showing a male microchimeric cell with Cy3-labeled X (orange) and fluorescein isothiocyanate conjugated-labeled Y (green) chromosome probes and immunoperoxidase staining for CD45 using mouse monoclonal anti-CD45 antibody and 3-amino-9-ethylcarbazole (AEC) as the chromogen (red). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue). The microchimeric cell is in a sinusoid area. Surrounding AEC stain (red) reveals CD45 expression (magnification $\times 1000$).

expressed CD45 (FIGURE 2). In 1 woman, 4% of the fetal microchimeric cells stained with the hepatocyte marker heppar-1. These cells had a morphology compatible with that of hepatocytes (FIGURE 3).

COMMENT

The use of stem cells as a novel treatment for repair of diseased organs in the human is an area of intense interest for the worldwide scientific community, as well as the lay public and many governments. In this study, we show that XY+ microchimeric cells in maternal tissues, acquired most likely through pregnancy, express leukocyte, hepatocyte, and epithelial markers. These data suggest that pregnancy may result in the physiologic acquisition of a fetal cell population with the capacity for multilineage differentiation. We have coined the term *pregnancy-associated progenitor cells* to describe this population.

Our study was based on a small number of patients already selected for having high numbers of microchimeric cells. We recognize that there is an inherent selection bias in the study

women, but to perform the study, adequate numbers of fetal microchimeric cells must be present in the tissue to be further analyzed. Therefore, the conclusions drawn from our study may only apply to women with high numbers of microchimeric cells.

Most of the women did not have any additional sources of microchimerism, such as solid organ transplantation. One of the 10 patients had a history of blood transfusion. Transfusion-associated microchimerism is highly unlikely to develop unless large quantities of blood are transfused in the setting of trauma.²⁵ Therefore, it is most likely that the XY+ cells in this study are fetal in origin.

In almost all tissues, XY+ cells bearing CD45, the common leukocyte antigen, were observed at variable frequencies. These results are consistent with previous findings that suggest that fetal microchimeric cells are originally blood cells, including hematopoietic progenitor cells.^{3,10,14,15}

XY+ microchimeric cells that expressed cytokeratin, a marker of epithelial cell differentiation, were never ob-

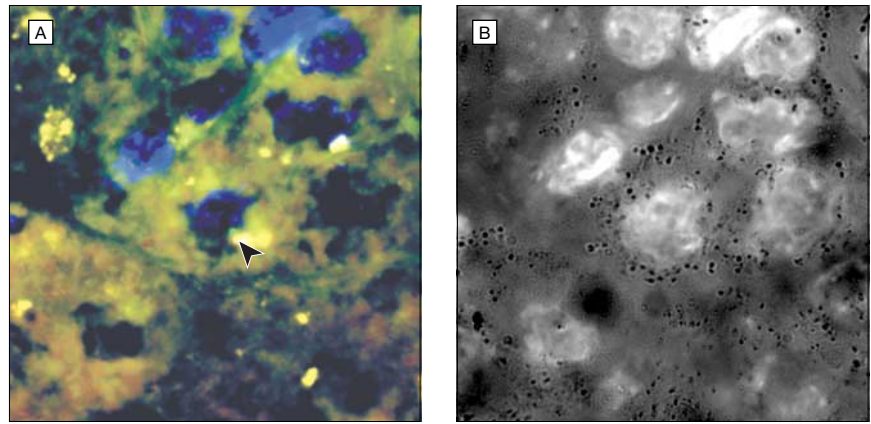
served in hematopoietic tissues (eg, lymph node). The concordance of morphological and immunohistochemical findings supports the idea that some fetal cells may have an epithelial phenotype. In 1 woman, in whom higher numbers of microchimeric cells were present, we were also able to detect cells with evidence of a hepatocyte marker. We also show that fetal cord blood cells do not express epithelial or hepatocyte markers, suggesting that the microchimeric fetal cells acquire these markers in the environment of maternal tissues.

Our study did not determine the type of fetal progenitor cells originally transferred during the pregnancies of the women. Fetal blood contains a variety of stem cell types, including mesenchymal stem cells and hematopoietic stem cells.²⁶ During pregnancy, fetal hematopoietic and mesenchymal progenitor cells circulate within maternal blood and can be cultured in maternal peripheral blood for up to 6 months after delivery.^{10,12,14}

Fetomaternal transfusion may be even higher after an elective termination of pregnancy.²⁷ We have shown previously by meta-analysis that a reproductive history that includes an elective termination or an early fetal loss is associated with a higher incidence of microchimerism in maternal tissues.²⁸ The CD34⁺ fetal cells are present in maternal blood for decades after delivery in 75% of women studied,³ as well as in the CD34⁺-enriched cell fraction of women undergoing granulocyte colony-stimulating factor bone marrow stimulation.¹³ Our results imply but do not prove that fetal CD34⁺ hematopoietic stem cells that persist post partum may have multilineage capacity. Another possibility is that pregnancy results in the acquisition of a different type of circulating stem cell, perhaps from the placenta, which has epithelial characteristics.

The nonhematopoietic morphology and phenotypes of the fetal cells that we observed may result from different mechanisms. Fetal progenitor cells could transdifferentiate into he-

Figure 3. Heppar-1 (a Hepatocyte Marker) Expression in Microchimeric Cells in Liver Parenchyma (Patient G)



Photomicrographs of interphase fluorescence in situ hybridization using Cy3-labeled X (orange) and fluorescein isothiocyanate conjugated-labeled Y (green) chromosome probes and immunoperoxidase staining for heppar-1 using mouse monoclonal anti-heppar-1 antibody and 3-amino-9-ethylcarbazole as the chromogen (red). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue). A, Photomicrograph using ultraviolet light of liver parenchyma showing a microchimeric cell with a Y chromosome (green, arrowhead). The morphology is similar to surrounding hepatocytes (magnification $\times 1000$). B, Photomicrograph using bright light with immunoperoxidase staining of the microchimeric cell in A showing that the cell is stained with heppar-1 as demonstrated by dark immunoprecipitate (magnification $\times 1000$).

matopoietic, hepatic, or epithelial cells. They could also adopt the host tissue phenotype by fusing with hepatocytes or epithelial cells.²⁹ In our identification of microchimeric XY⁺ cells based on X and Y chromosome FISH signals, we never detected an XY⁺ cell with an interphase karyotype suggestive of a fused nucleus (XXXXY) or having 2 separate nuclei. However, we cannot exclude the possibility that some fetal and maternal cells fuse their cytoplasm, especially in dense tissues, such as liver, in which the outer limits of each cell are hard to distinguish. Whatever the mechanism involved, we believe that the idea of fetal cells expressing non-hematopoietic markers is novel and may have important long-term health implications for the woman who has undergone pregnancy by providing her with a younger population of cells that may have different capabilities in the response to tissue injury.

In conclusion, we have shown that fetal cells, in a variety of maternal tissues, have morphologic and protein expression characteristics of not only hematopoietic but also epithelial and hepatic cells. These data suggest that, at least in some women after preg-

nancy, fetal cells transferred during pregnancy develop multilineage capacity either by cell fusion or transdifferentiation. Further study of naturally occurring fetal cell microchimerism may be useful in determining the characteristics of the specific progenitor cell population and the exact mechanisms involved in its apparent differentiation.

Author Contributions: Dr Bianchi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Khosrotehrani, Johnson, Bianchi.

Acquisition of data: Khosrotehrani, Cha, Salomon.

Analysis and interpretation of data: Khosrotehrani, Johnson, Salomon, Bianchi.

Drafting of the manuscript: Khosrotehrani, Cha.

Critical revision of the manuscript for important intellectual content: Khosrotehrani, Johnson, Salomon, Bianchi.

Statistical expertise: Khosrotehrani.

Obtained funding: Bianchi.

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It is unwise to be too sure of one's own wisdom. It is healthy to be reminded that the strongest might weaken and the wisest might err.

—Mahatma Gandhi (1869-1948)