Journal of Pediatric Surgery (2007) 42, 12–18

ELSEVIER

Journal of Pediatric Surgery

www.elsevier.com/locate/jpedsurg

Robert E. Gross Lecture

Fetomaternal cell trafficking: a story that begins with prenatal diagnosis and may end with stem cell therapy

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The focus of my research laboratory is the bidirectional trafficking of cells and nucleic acids between the pregnant woman and her fetus. For many years we have been studying both intact cells [1], and more recently, cell-free DNA and messenger RNA sequences that are detectable in various maternal body fluids [2], such as plasma and serum [3], cerebrospinal fluid [4], and amniotic fluid [5,6]. This is translational research, which concentrates on the development of novel prenatal diagnostic tests to improve detection of fetal abnormalities or complications of pregnancy. An unexpected outcome of this work has been the observation that the stem cells persist in the mother's blood and organs for decades postpartum [7]. This is the work that will be reviewed in this lecture.

This unanticipated aspect of our research originated as part of global efforts to improve noninvasive prenatal diagnosis for Down syndrome. It is currently recommended by the American College of Obstetricians and Gynecologists to offer maternal serum screening for both the detection of Down syndrome as well as open neural tube defects [8]. Approximately 76% of cases of Down syndrome are detected at a false-positive rate of 5% using the current quadruple serum screening test (alpha-fetoprotein, β-human chorionic gonadotropin, unconjugated estriol, inhibin A). More recent results from large-scale clinical trials, such as the National Institutes of Health–sponsored FASTER trial [9], have shown that first-trimester ultrasound measurement of the nuchal translucency space, in combination with first-and second-trimester serum screening, result in a 96% detection rate of Down syndrome. Our laboratory has studied ways to isolate and analyze intact fetal nucleated erythrocytes from maternal blood [10]. Although this work has been challenging because of the rarity of fetal cells in maternal blood, progress continues to be made through a variety of innovative approaches [11].

1. Initial disappointment leads to novel observation

In the mid-1990s, our goal was to identify monoclonal antibodies that would recognize uniquely fetal cell surface antigens that would allow us to separate intact fetal cells from the blood of pregnant women. At that time we hypothesized that monoclonal antibody to CD34, an antigen expressed on the surface of hematopoietic stem cells (HSCs), would be useful in physically isolating fetal HSC. Using fluorescent-activated cell sorting and polymerase chain reaction (PCR) amplification of a Y-chromosome sequence, we sorted male $CD34^+$ cells from the blood of 6 pregnant women who were later shown to be carrying female fetuses [7]. Experiments were then repeated in 8 healthy nonpregnant women, and again, male DNA was detected in the $CD34^+$- and $CD34^+CD38^+$-positive cells of 6 of 8 women studied. This was remarkable because the oldest woman had given birth to her son 27 years earlier.

Presented at the 37th Annual Meeting of the American Pediatric Surgical Association, May 20–24, 2006, Hilton Head, SC.

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0022-3468/$ – see front matter
doi:10.1016/j.jpedsurg.2006.09.047

These results implied that male cells circulated in their mother's blood for a long time after pregnancy. These women were entirely healthy and had never been transfused or had an organ transplant. The data suggested that these women with sons became chimeras. This subsequently established the field of fetal cell microchimerism.

Microchimerism is the presence of a small number of cells derived from more than one person in a given individual. Microchimerism can develop from twinning [12], blood transfusion [13], solid organ transplant [14], and bidirectional trafficking during pregnancy [15]. Since our first report of the long-term persistence of fetal cells in the mother, several investigators have identified factors that influence the development of fetal cell microchimerism. For example, histocompatibility between the mother and fetus may affect the frequency and extent of fetal cell microchimerism [16]. In addition, the presence of specific tissue-type antigens, such as maternal HLA-DQA1*0501, influences microchimerism [17]. There is more fetomaternal cell trafficking when the placenta is abnormal and in certain complications of pregnancy, such as with some fetal aneuploidies and preeclampsia. In addition, the time that has elapsed since pregnancy is important, as it appears to take some time to establish microchimerism [7,18].

We performed a meta-analysis that showed that a maternal history of pregnancy loss, either through miscarriage, or elective termination, increased the chance of fetal cell microchimerism [19]. Unfortunately, the published literature did not differentiate between natural and voluntary interruption of pregnancy. Elective termination causes a large fetomaternal transfusion of viable cells at an early developmental stage [20].

2. Fetomaternal trafficking and maternal health

The phenomenon of fetomaternal cell trafficking is unique to women. In 1996, Nelson [21] hypothesized that diseases that are more common in women than men could result from fetal cell microchimerism, and that fetal cells could go awry, causing disease similar to a graft-versus-host disease. In collaboration with Dr Nelson we tested this hypothesis by analyzing peripheral blood from women with systemic sclerosis for the presence of male cells [22]. It is important to recognize that male fetal cells are used for proof of principle. It is believed that male and female fetal cells cross the placenta in equal numbers. However, it is quicker and easier to detect male cells in the maternal blood or organs because of the universal nature of the Y chromosome, which the normal fertile woman lacks. It is possible to detect female fetal cells in the mother by using family-specific polymorphisms or uniquely paternally inherited genes. Our more recent experiments in the mouse use paternally inherited non–sex-dependent transgenes to track the presence of fetal cells in the mother [23].

Data from a large number of studies have shown that fetal cells tend to concentrate in the clinically affected tissues. For example, Johnson et al [24] studied a number of tissues, including spleen, thyroid, kidney, large intestine, lung, heart, skin, and small intestine, from a young mother who died of intestinal perforation as a complication of systemic lupus erythematosus. The study was performed using fluorescence in situ hybridization (FISH) probes for the X and Y chromosomes. More than 500 male cells were seen in the small intestine, the organ that had the greatest clinical pathology.

Subsequently, a number of conditions that affect women more than men have been analyzed for the association between fetal cell microchimerism and the specific pathology; this has been summarized in previous articles [25,26]. The most common diseases studied to date include systemic sclerosis, polymorphic eruption of pregnancy, systemic lupus erythematosus, primary biliary cirrhosis, Sjögren syndrome, Hashimoto disease, Graves disease, and cutaneous lichen planus. Using both FISH analysis and PCR amplification of Y-chromosome sequences, as well as PCR amplification of specific HLA sequences, multiple investigators have shown that fetal cell microchimerism is significantly increased in the blood and affected tissues of women with systemic sclerosis [22,27]. Similarly, Hashimoto disease appears to be significantly associated with fetal cell microchimerism [28]. Other conditions that are more common in or even exclusive to women, such as primary biliary sclerosis, are not associated with fetal cell microchimerism [29]. Sjögren syndrome by itself is not associated with increased numbers of fetal cells, although when it presents with systemic sclerosis, there is significantly increased microchimerism [30].

It is important to recognize that although it has been consistently shown that in some conditions fetal cells migrate specifically to clinically affected organs, the available evidence does not yet conclusively indicate that fetal cells cause disease. Furthermore, it has been demonstrated that fetal cells are present in maternal organs affected by both autoimmune and non-autoimmune conditions, such as hepatitis C [31] and cervical cancer [32]. Therefore, it is premature to conclude that fetal cell microchimerism results in a graft-versus-host phenomenon.

3. The "aha!" moment

The "aha!" moment occurred when under the microscope we were analyzing surgical specimens from a 48-year-old woman who had a progressively enlarging nodular goiter for 10 years, which resulted in a partial thyroidectomy. Her pathologic studies showed that she had adenomatous hyperplasia, but no inflammation. Her thyroid specimen was used as a control for another experiment in which we were studying the relationship between fetal cell microchimerism and autoimmune thyroiditis. The woman's

reproductive history revealed that she delivered a male in 1967 and a female in 1971, but had no other sources of microchimerism, including blood transfusion, organ transplant, or a history of a twin sibling. Using X and Y chromosome–specific probes, we demonstrated that a section of her thyroid was composed of entirely male cells. These cells had the appearance of normal thyroid follicular cells and surrounded colloid [33]. The implication of this finding was that somehow, this woman had acquired a male stem cell during her pregnancy, and that that stem cell migrated to her thyroid, a clinically diseased organ, to form new thyroid cells. At this point we began to consider the possibility that as a result of pregnancy, women acquired pregnancy-associated progenitor cells, or PAPCs. The cellular origin of the PAPCs was unknown.

To further address this question, we obtained archived paraffin-embedded tissue from 10 women who had at least one son. We performed a new set of FISH experiments using X and Y chromosome–specific probes and combined these studies with histology and immunohistochemistry [34]. In epithelial tissues, 14% to 60% of XY^+ cells expressed cytokeratin, an epithelial marker. In lymph nodes and spleen, 90% of the XY^+ cells expressed CD45, a common leukocyte marker. Furthermore, in one liver sample, 40% of the XY^+ cells expressed heppar-1, a hepatocyte marker. Interestingly, in thyroid tissue in which we were able to examine both healthy as well as diseased parts of the organ, there was a nonrandom distribution of fetal cell type. For example, in the abnormal section of tissue, the XY^+ fetal cells expressed cytokeratin. In the healthy part of the thyroid, the XY^+ fetal cells expressed CD45. This suggested that the fetal cells migrated to the thyroid as $CD45^+$ hematopoietic cells, but differentiated at the site of tissue injury [34]. At approximately the same time that this article was published, O'Donoghue et al [35], working in London, isolated bone marrow from the ribs of British grandmothers who were undergoing clinically indicated thoracotomies. They cultured male mesenchymal stem cells (MSCs) from every woman who had given birth to a son (13-51 years earlier). They subsequently induced the MSC to differentiate into fat, muscle, nerves, and bone. Importantly, they demonstrated the long-term persistence of an actual population of stem cells, MSC, in the postpartum woman.

4. Stem cells: is it only embryonic or adult?

The fact that 2 groups could independently derive data that suggested that a new population or populations of stem cells were acquired via pregnancy was unexpected and challenged established paradigms. The prevailing wisdom is that there are predominantly 2 types of stem cells, embryonic and adult. In the human, embryonic stem cells derive from the 5-day-old human embryo and are pluripotent. Adult stem cells derive from mature tissue and respond to individual needs for growth or repair, but they have low plasticity. We hypothesize that during pregnancy, women acquire cells from their fetuses that are retained for decades. Some of these fetal cells are stem or progenitor cells that have the capacity to repair maternal organs. Therefore, sex matters in any discussion regarding the pluripotency of adult stem cells.

5. The importance of knowing reproductive history for evaluation of transdifferentiation studies

Bianchi and Fisk [36] tested the hypothesis that clinical studies of adult stem cell differentiation largely ignore reproductive history of women who are donors or recipients of sex-mismatched transplants. Using the PubMed database, they searched the literature using the filter January 1, 2000, through September 30, 2005, for articles that described human studies written in the English language. The search terms included *cardiac/heart*, *hepatic/liver*, *neuron/brain*, *lung/pulmonary*, *renal/kidney*, *bone marrow* and *stem cell or transplant*, and *gender or FISH or Y-chromosome*. We identified 54 articles that studied the fate of sex-mismatched transplants. None contained complete reproductive history information. Only 4 (7%) of 54 of the studies stated that the women had no history of male children.

Why is the lack of reproductive history important? The 54 articles concluded that male cells in the women who had received bone marrow transplants from male donors were the result of transdifferentiation of the transplanted cells. There was no mention of fetal cells as a possible origin of the male cells. Chimerism is a frequent phenomenon in normal women [37]. In one study in the Netherlands, Koopmans and colleagues performed Y-chromosome FISH analyses on the histologically normal organs of 7 women who underwent autopsy, and whose child and transfusion status were known. They demonstrated cells with a Y chromosome present in 13 of 51 kidneys, 10 of 51 livers, and 4 of 61 hearts studied. Furthermore, they showed that the male cells had differentiated. They observed male tubular epithelial cells in the kidney, hepatocytes in the liver, and cardiomyocytes in the heart. They concluded that, "theoretically these organs could have been used for transplant" and would therefore give the misleading impression that the donor's cells had differentiated in the recipient. The limitations of this study were the following: some women with no known history of male children had male cells present. However, there was no information available about elective abortion or a history of miscarriage. In addition, 45 of 75 women had a prior history of blood transfusions, so theoretically they could have acquired male cells via that route.

Our group has performed a meta-analysis of all studies of microchimerism in which a reproductive history was given [19]. A maternal history of pregnancy loss, either through

miscarriage or elective termination, carried an increased risk of developing microchimerism. Furthermore, in prior studies using quantitative PCR amplification of the Y chromosome, we showed that elective termination of pregnancy causes a large fetomaternal transfusion of viable cells at an early developmental stage (a median of 1552 nucleated cells per 16 mL of whole blood) [20].

Conclusive evidence that cells from a terminated fetus can persist in the mother and differentiate into cells in the mature organ came from the following study by Johnson et al [31], published in the journal *Hepatology* in 2002. In this study we received liver biopsy material from a woman with hepatitis C. She was a control subject for a study in which we were analyzing the association between fetal cell microchimerism and primary biliary cirrhosis. This woman had a history of having had one son, who was then 18 years old. Using X and Y chromosome–specific probes, we demonstrated that part of her liver contained entirely female (XX) cells, yet another part of her liver contained thousands of cells that were male (XY). We were able to obtain enough cells to isolate DNA and perform PCR amplification of short tandem repeat (STR) sequences. The DNA in the male part of the liver and the female part of the liver appeared related to each other, in that they each shared an allele at each locus tested. However, the STRs in the woman's son's DNA did not match the STRs in the male DNA in the liver. We were disappointed to conclude that the male cells in her liver could not originate from her son. However, after requesting and receiving a more detailed reproductive history, we realized that this woman had had 4 additional pregnancies, including 2 elective terminations and 2 miscarriages. We were subsequently able to obtain genomic DNA from 2 of the fathers of her noncompleted pregnancies. One of the fathers appeared to be a biological match for the male cells in the liver. We hypothesized, but can never prove, that these fetal cells originated from an elective termination of pregnancy that had been fathered by the man whose DNA we tested. Thus, in one woman, cells from a fetus that was terminated 17 to 19 years earlier remained for a long time in her body. We hypothesize that these fetal cells survived, migrated to her liver, which was a clinically diseased organ, and repopulated a significant portion of her liver. Interestingly, she did well clinically despite not taking her medications and not complying with medical care [31].

Therefore, conclusions from a large body of human work examining microchimerism demonstrate that fetuses transfer cells with multilineage potential to their mothers. Furthermore, microchimeric fetal cells appear to acquire tissue-specific markers in the environment of a diseased or injured maternal organ [34].

6. Animal models of microchimerism

Approximately 3 years ago we turned to using mouse models of disease. This was because in humans we had great difficulty simultaneously getting accurate pregnancy histories and appropriate clinical tissue to study. The mouse, with its 3-week gestation time and the opportunity to control breeding, was an attractive animal model. In addition, with the availability of many transgenic lines, we were able to use specific markers to track fetal cells in the mother mouse. The current animal models that we use include the C57Bl6 and the CD1 wild-type female mated with a syngeneic male that carries the green fluorescent protein (GFP) transgene. An additional model is the FVB wild-type female mated to a transgenic male that is carrying a luciferase gene that is expressed under a variety of different promoters. Our initial experiments used both the cytomegalovirus (CMV) promoter, which is reportedly ubiquitously expressed, as well as the vascular endothelial growth factor receptor 2 (VEGFR2) promoter, which is expressed in the setting of wound healing and inflammation.

Our laboratory has developed a number of different methods to detect the relatively rare fetal cells. We have determined that to detect fetal cells, a sensitivity of 1 fetal in 10^5 maternal cells is necessary. We have achieved this sensitivity with real-time quantitative PCR amplification of fetal transgenes [23] as well as with FISH analysis using chromosome-specific probes. Using the MOFLO flow cytometer (Dako, Fort Collins, CO), we can detect fetal cells expressing GFP at a level of 1 GFP^+ cell in 1×10^6 GFP^- cells. We are also using in vivo imaging, although at the present time we are trying to determine if we can achieve the sensitivity needed to detect fetal cells in the living intact mother mouse.

There are advantages and disadvantages of the different mouse breeding models. Using GFP, we achieve the best sensitivity of detection of rare fetal cells by real-time quantitative PCR amplification. Polymerase chain reaction is also the most accurate for quantitation of fetal cells pre- and postinjury. We can also correlate morphology and immunohistochemical staining with GFP. In general, however, using GFP, we need to kill the mouse, and this precludes studying the same mouse over time. By using the in vivo imaging system we can study an individual mouse before, during, and after pregnancy, and use specific genetic constructs to study gene expression in the fetal cells.

Initially we needed to determine whether the natural history of fetal cell microchimerism in the mouse was equivalent to the human. Although both animals have hemochorial placentation, the anatomy of the placenta differs between the mouse and the human [38]. In a study of 9 wild-type females bred to syngeneic GFP transgenic males, we detected fetal cells in all pregnant mice using real-time PCR amplification of the GFP sequence [39]. There were specific organ-to-organ differences. The number of fetal cells was significantly higher in the murine lungs during pregnancy. We also showed that the frequency of fetal cells decreases after delivery, such that by 3 weeks after delivery, fetal cells cannot be detected. However, multiple pregnancies seem to increase the burden of fetal cell

microchimerism. After a third pregnancy, 30% of mice have detectable fetal cells. These data not only showed that mice develop microchimerism as a consequence of pregnancy, but also defined baseline values for healthy mice.

As in humans, there are increasing data to show that fetal cell microchimerism increases in diseased or injured rodents. For example, Christner and Jimenez [40] increased the number of fetal cells in the peripheral blood of mice after injection of vinyl chloride, a model for systemic sclerosis. Imaizumi et al [41] showed that fetal cells accumulate in the thyroid glands of mouse with peripartum autoimmune thyroiditis. Other investigators have shown that fetal GFP^+ cells migrate to liver damaged by alcohol and to kidneys damaged by exposure to gentamicin [42]. Furthermore, Tan et al [43] demonstrated that fetal GFP^+ cells cross the blood-brain barrier, migrate to an induced central nervous system lesion, and differentiate into neural cells.

7. Fetal cell response to maternal chemical and surgical injury

In our laboratory, we have been studying a variety of injuries in the liver and skin. The liver injuries include a chemical injury (carbon tetrachloride vs vegetable oil injection), a surgical injury (partial hepatectomy), and a genetic model (tyrosinemia). In the skin we have been examining punch skin biopsies as well as sutured skin wounds. We decided to focus our initial efforts on the liver because preliminary results in humans suggested that the liver is the site of fetal cell microchimerism in both healthy and diseased states [29,31]. Furthermore, there is a low level of cellular turnover in the liver. Data exist on hepatic stem cell reconstitution of the liver, and there are many adult models of liver disease. In preliminary experiments, Dr Richard Freeman performed partial hepatectomy on postpartum mice. Recovery from this injury induces a rapid regeneration process based on healthy hepatocyte division. This regeneration process does not involve HSCs or oval cell recruitment. We studied 7 mice that underwent partial hepatectomy 3 weeks after delivery [44]. Animals were then killed at 7 days postsurgery when histology showed active regeneration. Results showed that no GFP^+ microchimeric cells were found by PCR amplification or microscopy in the baseline liver removed at surgery or in the regenerated liver. Therefore, liver regeneration after partial hepatectomy does not appear to involve fetal cells.

For our chemical injury we injected 1 mL of 20% carbon tetrachloride into postpartum mice that had been bred once to GFP^+ males. The control animals were injected with vegetable oil and also bred to GFP^+ males. Mice were killed at either 4 or 8 weeks after injection. Carbon tetrachloride was selected as a chemical toxin because it induces a rapid injury with a prolonged regeneration process that involves the recruitment of HSCs and/or oval cells. The results showed that all chemically injured livers had fetal GFP^+ cells present by 8 weeks after the injury. By real-time PCR amplification, maternal liver had significantly more fetal cells present at 8 weeks than at 4 weeks ($P = .006$). Therefore, we concluded that fetal cells could persist postpartum and home to chemically injured liver. Fetal cell response appears to be dynamic over time. In addition, the spleens of chemically injured animals were grossly enlarged; by PCR the spleen was determined to be a site of fetal cell migration.

We have also chosen to study skin injury because of the human data showing that fetal cells migrate into the skin of women who have clinical manifestations of polymorphic eruption of pregnancy [45]. The skin is easily accessible and we can injure the mice without causing lethality. Furthermore, one can see the physical results of healing through morphometric analysis. In our initial experiments, we wished to determine how early in gestation one could observe a luciferase signal in pregnant mice. In wild-type females that are carrying CMV-luciferase–positive fetuses or vascular endothelial growth factor receptor 2-luciferase–positive fetuses, the luciferase signal appears in pups between 12 and 14 days of gestation (Fig. 1). Using in vivo imaging we studied CMV-luciferase–positive fetal cells and their potential migration to the wound. Our preliminary experiments appeared to have promising results in that it appeared that fetal cells migrated specifically to the wound. However, in subsequent experiments with additional controls, we did not observe a statistically significant increased signal-to-noise ratio. This may be because of the fact that

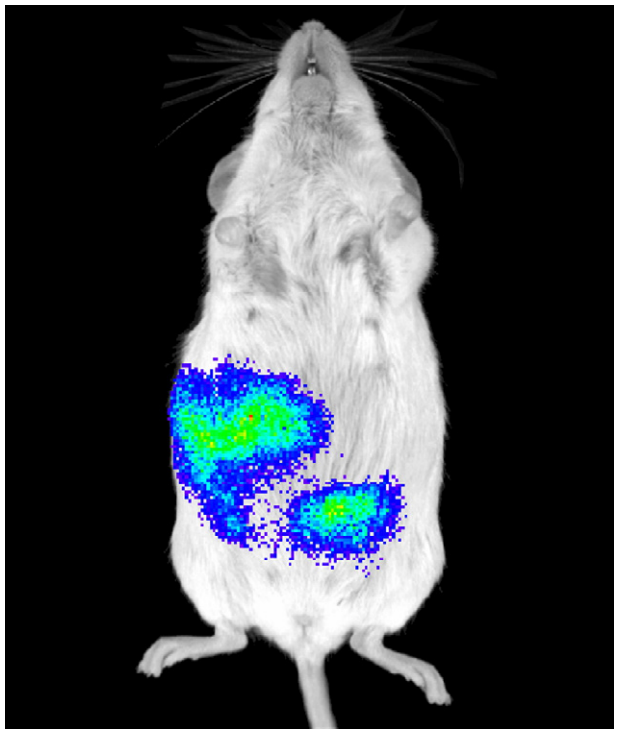

Fig. 1 Bioluminescence image of a living wild-type (FVB) pregnant mouse that has been bred to a transgenic male mouse that expresses the luciferase transgene. The colored areas indicate sites of transgenic pups and their placentas.

the CMV promoter is not truly ubiquitous and, therefore, the luciferase signal may not be expressed in HSC. Alternatively, it may be that we cannot achieve the sensitivity needed to detect fetal cells by in vivo imaging because there are too few of them present in the wound.

8. What are the pregnancy-associated progenitor cells?

At the present time the cell type of origin of the PAPCs is unknown. This is an area of active investigation in our laboratory. The PAPCs could be HSCs, given the prior data on the presence of $CD34^+$ fetal cells in maternal blood. The data of O'Donoghue et al [35] suggest that some of the cells are MSCs. They could also be endothelial cells, hemangioblasts, leukocytes, or placental stem cells [46]. We are examining the origin of these cells through fluorescence-activated cell sorting experiments in which we will perform double staining to examine subsets of the GFP^+ cells.

9. Summary and future directions

In summary, results in murine and human pregnancies are similar despite differences in placentation. They both demonstrate the low-grade transfer and long-term persistence of fetal cells in the healthy mother. After some, but not all injuries, fetal cells home to sites of tissue damage both during and after pregnancy and increase locally in the diseased or injured organ. In both the human and the mouse the retained fetal cells appear to have multilineage capacity and express differentiation markers that are appropriate at the site of the affected maternal tissue. Therefore, studies of adult stem cell differentiation in females need to consider the possibility that some of the adult stem cells are actually fetal.

Our future directions include a desire to fully characterize the fetal cell types in the mother. We wish to determine if fetal cells create functional improvement in response to maternal injury. We need to isolate and culture the fetal stem cells and prove that they differentiate. We also need to determine if fetal stem cells in the adult have a competitive advantage over endogenous adult stem cells. If we can prove this to be true, this would provide an intriguing explanation as to why women live longer than men.

Finally, it is important to recognize that microchimerism is multigenerational and bidirectional so that men have stem cells from their mothers (maternal cell microchimerism) and women have stem cells from both their mothers and their fetuses and children. The long-term consequences of this phenomenon are currently unknown. In an editorial that accompanied an article that we wrote on maternal cell microchimerism [47], Hall [48] suggested that "your mother may not be looking over your shoulder; she may be in your shoulder!"

Despite initial disappointment over not identifying appropriate cell surface antibodies for prenatal diagnosis, the data have directed us to findings of potentially broader significance. It would be truly remarkable if fetal stem cells that are present in the woman postpartum represent a novel population of stem cells with therapeutic potential.

Acknowledgments

The author acknowledges the financial support of the National Institutes of Health, Bethesda, MD, for this work, specifically R01HD49469, T32HD049341, and R25GM066567. In addition, she acknowledges the various members of her research laboratory, without whom the work on microchimerism would not be possible. These include Kirby Johnson, PhD; Kiarash Khosrotehrani, MD, PhD; Eric Su, MD; Yutaka Fujiki, MD, PhD; Helene Stroh; Kai Tao; Raquel Reyes; Ellen Miller; Bharath Srivatsa, MD; Osamu Samura, MD; Dong Yun Cha, MD; Osamu Samura, MD; and Tom Wataganara, MD.

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