

Fetal cell microchimerism in the maternal mouse spinal cord

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ABSTRACT

Fetal cell microchimerism refers to the persistence of fetal cells in the maternal tissues following pregnancy. It has been detected in peripheral organs and the brain, but its existence in the spinal cord has not been reported. Our aim was to detect fetal cell microchimerism in the spinal cord of maternal mice. C57BL/6 female mice were crossed with GFP transgenic male mice and sacrificed after their first or third delivery. GFP-positive cells, which were presumably from fetuses whose fathers were GFP transgenic, were detected in the spinal cord by fluorescence microscopy and immunohistochemistry. PCR was also performed to detect GFP DNA, which must come from GFP hemizygous fetuses. We found GFP-positive cells and detectable GFP DNA in most of the maternal spinal cords. Twenty percent (1/5) of the mice that were only pregnant once had detectable fetal cells, while 80% (4/5) of those that were pregnant three times had detectable fetal cells. Some fetal cells, which not only emitted green fluorescence but also expressed NeuN, were detected in the spinal cords from maternal mice. These results indicate that fetal cells migrate into the spinal cord of a maternal mouse during and/or after the gestational period, and the fetal cells may differentiate into neurons in the spinal cord.

Keywords: fetal cell microchimerism; green fluorescent protein; spinal cord; mouse

INTRODUCTION

Fetal cells have been observed in the maternal circulation after human pregnancy^[1] and persist there for decades^[2]. In addition to the peripheral circulation, fetal cells have also been found to engraft in almost all peripheral tissues in both humans and animal models^[3–10]. These engrafted cells appear to have the ability of multi-directional differentiation and express differentiation markers. For example, fetal microchimeric cells are capable of engraftment and differentiation along the hematopoietic pathway^[11–13]. Studies from Dr. Nelson's lab^[14] showed that fetal cells found in the liver of women who had given birth to sons included cells that expressed hepatocyte antigens (cytokeratins); studies by Wang *et al.*^[15] showed that, after tissue injury in the liver and kidneys, engrafted fetal cells transformed into hepatocytes and tubular cells. In view of these findings, it is believed that persistent fetal cells provide a rejuvenating source of fetal progenitor cells that may have the capacity to participate in maternal tissue repair^[16].

Furthermore, Tan *et al.*^[17] demonstrated that fetal cells can even pass through the blood-brain barrier, respond to brain tissue injury, and adopt the location, morphology, and expression of immunocytochemical markers indicative of perivascular macrophage-, neuron-, astrocyte-, and oligodendrocyte-like cell types. This finding has raised the hope that exogenously-engrafted fetal cells contribute to adult neurogenesis. Although it is uncertain whether fetal stem-cells in the adult brain have a competitive advantage over endogenous neuronal stem-cells, it seems that fetal stem-cells potentially possess therapeutic value for spinal cord injury^[18],

and diseases such as amyotrophic lateral sclerosis^[19], multiple sclerosis^[20], and cervical spondylotic myelopathy^[21]. Here, we report fetal microchimerism in the maternal mouse spinal cord using GFP-based detection, together with morphological and immunocytochemical evidence.

MATERIALS AND METHODS

Mice

All animal protocols were approved by the University of Manitoba Animal Care Ethics Committee. Twelve- to 16-week-old male homozygous C57BL/6 Cr Slc TgN (act-EGFP) OsbC14-Y01-FM131 mice (green mice) were crossed with young adult female wild-type C57BL/6 mice (6–8 weeks old). All the offspring were hemizygous green mice. Control wild-type young adult female C57BL/6 mice remained virgins. Young adult green mouse pups provided positive controls. Ex-breeder wild-type female C57BL/6 mice and male green mice were purchased from the Central Animal Care Breeding Facility, University of Manitoba, Winnipeg, Canada. These mice had been held as breeding stock since the age of 6–8 weeks. Three weeks after the first delivery, the mothers underwent a second or third breeding. One week or four weeks after delivering their last litter, the mothers were euthanized with an overdose of isoflurane and perfused with 0.9% ice-cold saline followed by 4% paraformaldehyde in phosphate buffer (4°C, pH 7.4). Thirty minutes later, the spinal cords were removed and fixed again in cold 4% paraformaldehyde for 12 h. After fixation, the cords were immersed in cold 30% sucrose solution. One day later, the cords were removed and stored at –80°C until analyzed.

Visualization of Fetal Green Mouse Cells

The spinal cords were serially sectioned (20 µm) on a Shandon SME Cryotome Cryostat (Ramsey, MN). Selected sections were first washed three times (10 min each) in phosphate-buffered saline (PBS). Then, they were dipped briefly in distilled H₂O, treated with 1 mmol/L CuSO₄ (Fisher Scientific, Ottawa, CA) in ammonium acetate buffer (50 mmol/L CH₃COONH₄, pH 5.0) for 20 min, rinsed briefly in distilled H₂O, and returned to PBS^[22]. Then they were mounted with FluoromountTM aqueous mounting medium (Sigma) and viewed under a Nikon TE2000-E microscope equipped with a RETIGA camera (QImaging).

Immunohistochemistry

The spinal cords were serially sectioned (20 µm) on a cryostat and selected sections were immunostained. The primary antibodies were mouse monoclonal anti-GFP (1:250, Abcam, Cambridge, MA), rabbit polyclonal anti-GFP (1:250, Abcam), and mouse monoclonal anti-NeuN (1:200, Chemicon, Temecula, CA). The secondary antibodies were Alexa Fluor 594-labelled goat anti-mouse IgG (Invitrogen, Burlington, CA), and Alexa Fluor 488-labelled goat anti-rabbit IgG (Invitrogen). Both of the secondary antibodies were used at 1:200 dilution.

Briefly, sections were washed three times in PBS (10 min each), transferred to PBS containing 0.3% Triton X-100 (PBST) for 30 min, and blocked with 5% goat serum in PBST for 1 h at room temperature. Then they were incubated with appropriate primary antibody overnight at 4°C. After washing three times in PBS, the sections were incubated with the appropriate secondary antibody for 1 h at room temperature in the dark, followed by three washes (10 min each) in 0.1% PBS-Tween20. After staining with Hoechst 33342 (Calbiocam, San Diego, CA) for 3 min, the sections were treated with 1 mmol/L CuSO₄ in ammonium acetate buffer for 20 min, dipped briefly in distilled H₂O, and returned to PBS. The sections were finally mounted with FluoromountTM aqueous mounting medium and viewed under the TE2000-E microscope.

Detection of GFP-specific DNA by PCR

The spinal cord sections prepared above were washed three times (20 min each) in PBS, then they were incubated in lysis buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% SDS, 1 mg/mL proteinase K, pH ~6.0) at 56°C overnight. After centrifugation at 12 000 g for 10 min, DNA was extracted from the supernatant with the GenEluteTM Mammalian Genomic DNA Miniprep kit (Sigma) and resuspended in distilled water. The amount of sample DNA was quantified by fluorometry, and the concentration was adjusted to 500 ng/µL.

The following GFP-specific PCR primers were used: forward: 5'-GTAAACGGCCACAAGTTCAGC-3'; reverse: 5'-CATGCCGAGAGTGATCCCG-3'. PCR amplification was carried out by adding 1 ng DNA to 50 µL amplification mixture [0.2 mmol/L dNTP, 2 mmol/L MgCl₂, 0.2 µmol/L of each primer, 50 mmol/L Tris-HCl, 10 mmol/L KCl and 1 U of Taq Platinum DNA polymerase (Gibco)]. The thermal

profile was 94°C for 3 min, followed by 35 cycles at 94°C for 45 s, 58°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. To check for variability in DNA extraction and the absence of inhibition during the PCR, the housekeeping gene β -actin was analyzed in parallel for each sample using the following primers: forward: 5'-GCCTGTGGTACGACCAGAGGCATACAG-3'; reverse: 5'-GATGACGATATCGCTGCGCTGGTCG-3'. The thermal profile was 94°C for 3 min, followed by 25 cycles at 94°C for 45 s, 60°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min.

Serial dilutions of DNA were prepared with GFP cells diluted in wild-type cells (ranging from 0.001% to 1%) and the samples were amplified in order to compare their DNA concentrations. PCR products were separated on 1.5% agarose gel, visualized by ethidium bromide (Sigma) staining, and analyzed using a FluorChem 8900 imager (Alpha Innotech, San Leandro, CA). The optical density multiplied by the area of the band was used to semi-quantify the PCR product. The value of GFP PCR product was normalized against the amount of PCR product for actin obtained from the same sample. A standard curve was prepared between the number of GFP cells and the ratio of PCR product optical density, from which unknown samples were interpolated.

Statistical Analysis

Statistical differences between two groups of pregnant mice were assessed by the χ^2 test. Statistical significance was assumed when $P < 0.05$.

RESULTS

Cupric Sulfate Reduces Natural Fluorescence without Effects on GFP Fluorescence and Immunofluorescent Labeling

Naturally-fluorescent pigments (i.e., lipofuscin, flavins, and porphyrins) accumulate in the cytoplasm of many cell types and can often complicate fluorescence microscopy because of their broad excitation and emission spectra^[23, 24]. These pigments often emit green (Fig. 1A) and red light (Fig. 1C), making it difficult to distinguish specific from nonspecific autofluorescence^[25]. Based on the literature^[22, 26], we tested the effect of CuSO₄ on reducing the natural fluorescence and found that 1 mmol/L CuSO₄ almost eliminated the

background autofluorescence (Fig. 1B, D) while retaining the GFP fluorescence (Fig. 1E, F).

Fetal Cells Are Engrafted into the Maternal Spinal Cord

Engrafted fetal cells were identified through their expression of GFP, which emits green light under fluorescence microscopy. They were also identified by immunocytochemistry using the anti-GFP primary antibody and fluorescence-labeled secondary antibodies. If the secondary antibody was labeled with Alexa Fluor 594, fetal cells were marked as red. In this study, both methods were applied simultaneously. Interestingly, we found that not all green cells were labeled with anti-GFP antibody and emitted red light (Fig. 2A–C), while some GFP-immunopositive (red) cells did not emit green light (Fig. 2D–F). Some cells were positive for both GFP fluorescence (green) and immune-labeling (red) (Fig. 3). Although in negative controls we found a few cells that only emitted either green or red light, none emitted green and red together. We therefore decided to define cells that were positive for both GFP fluorescence (green) and immune-labeling (red) as engrafted fetal cells. Meanwhile, we found that most of these engrafted cells had a larger nucleus than other cells on the same section.

We also determined whether the engrafted fetal cells differentiated into neuron-, astrocyte-, and oligodendrocyte-like cell types. We chose NeuN, GFAP (1:100, Abcam, Toronto, ON, Canada), and Olig2 (1:100, Millipore, Billerica, MA) as immunocytochemical markers of neurons, astrocytes, and oligodendrocytes, and did triple staining. Finally, we found that engrafted fetal cells, in 3 samples, did differentiate into neuron-like cells (Fig. 4) but not into astrocyte- and oligodendrocyte-like cells (data not shown).

Semi-quantification of Fetal Cells in the Maternal Spinal Cord

Semi-quantification of fetal cells in the maternal spinal cord was carried out by counting the fetal cells on each section and by PCR amplification for GFP-specific DNA. To enhance the semi-quantification, we compared several doses of DNA and various numbers of PCR cycles in several preliminary experiments, and optimized a condition that yielded a coefficient of ~ 0.9 (Fig. 5B). Because the linear regression was not perfect, variable-interval values instead of precise values were used to describe the

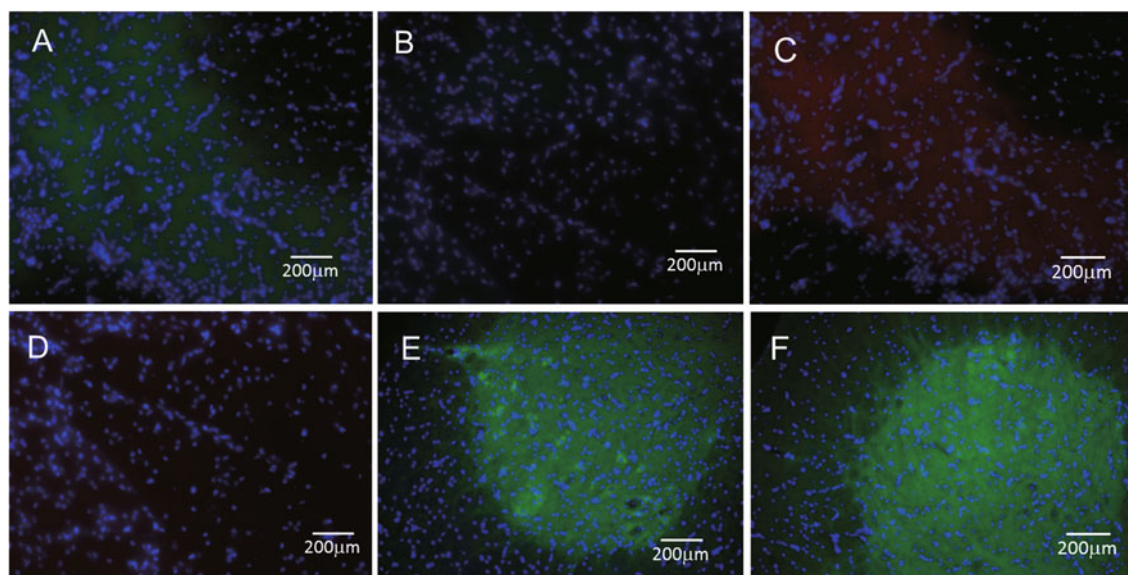


Fig. 1. Cupric sulfate reduces natural fluorescence while retaining GFP fluorescence ($\times 10$ magnification). Natural fluorescent pigments often emit green (A) and red light (C). CuSO_4 (1 mmol/L) evidently eliminated the fluorescent pigment-induced autofluorescence (B, D). Regarding GFP fluorescence from GFP transgenic mice (E), 1 mmol/L CuSO_4 did not have any effects (F).

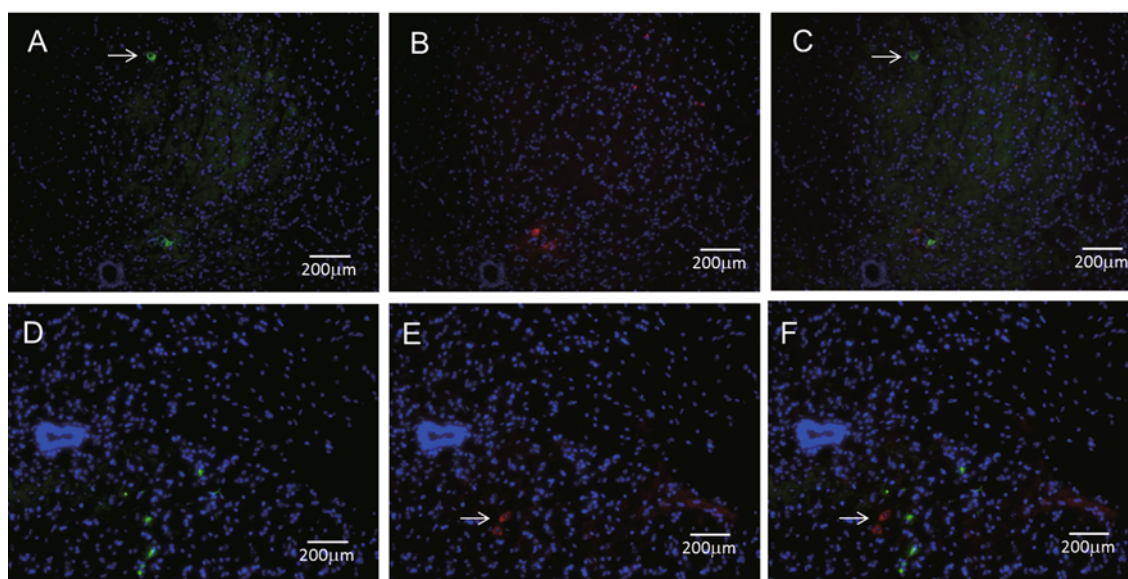


Fig. 2. Fetal cells can be identified through the combination of GFP fluorescence (green) and GFP immunofluorescence (red). Not all green cells (A, C), however, were immune-labeled with the anti-GFP antibody (B, C), and some GFP-positive (red) cells (D, F) did not emit green light (E, F, arrows). C is the overlay of A and B, and F is of D and E.

amounts of fetal cells (Table 1). Our results showed that fetal cells were undetectable in the negative control group. Some mice that had either one or three gestations had detectable fetal cells in their spinal cords three weeks after

their last delivery. However, the frequency of fetal cells was generally low. The morphologic method revealed that only 1–3 cells per 40 sections were GFP-positive green mouse fetal cells in the spinal cord. Meanwhile, the semi-

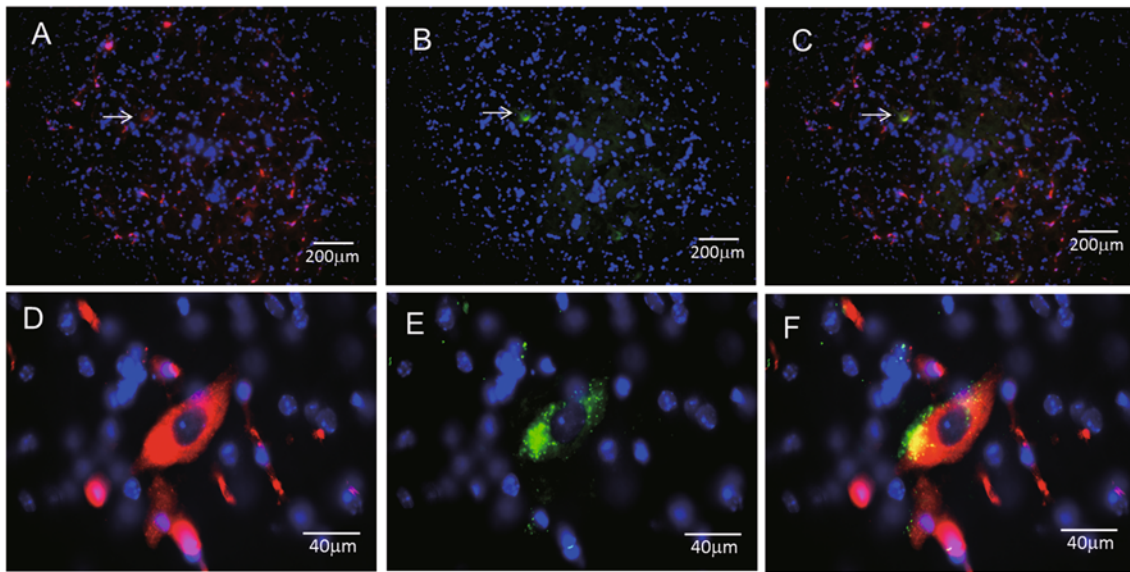


Fig. 3. Fetal cells that had GFP were identified through fluorescence microscopy directly (green, B, E), and by immunofluorescence staining (red, A, D) using monoclonal anti-GFP as primary antibody and Alexa Fluor 594 as secondary antibody. C and F are merged images of A and B, and D and E, respectively.

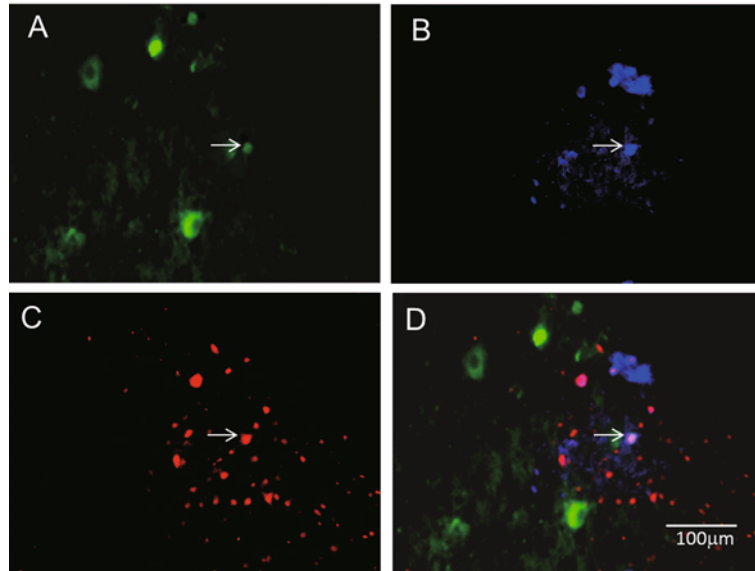


Fig. 4. Fetal cells can differentiate into a neuron-like cell type. Fetal cells with a combination of GFP fluorescence (A, green) and GFP immunofluorescence (B, blue) were identified. The section was immunostained with an antibody to NeuN, a neuronal marker (C, red). The merged picture indicates that a fetal cell (arrow) expressed NeuN in the nucleus (D).

quantified PCR of genomic DNA showed that fewer than 1 fetal cell per 10^5 maternal cells were GFP-positive green mouse fetal cells (Table 1). Interestingly, 20% of maternal mice with one pregnancy had detectable fetal cells, while

80% of those with three pregnancies had them, indicating that the number of pregnancies has a positive effect on the engrafting of fetal cells ($\chi^2 = 3.6$, $\chi^2_{0.05(1)} = 3.84$, $0.05 < P < 0.1$) (Table 1).

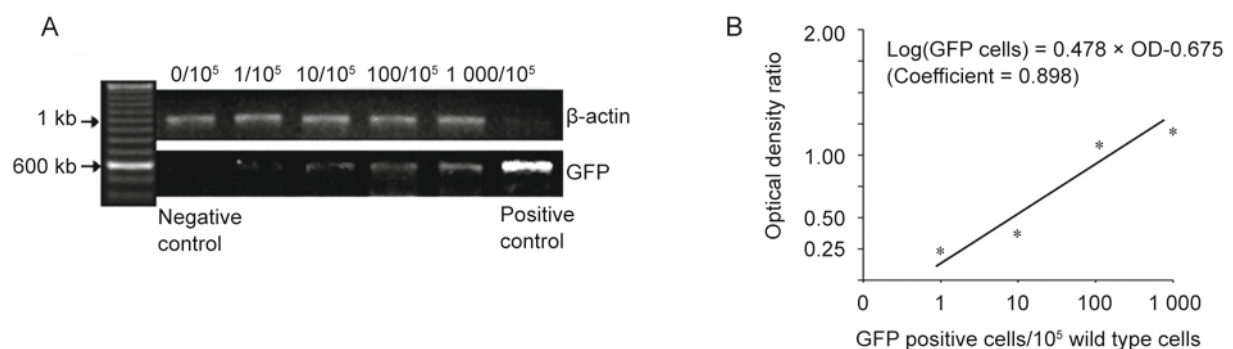


Fig. 5. Semi-quantification of fetal cells in maternal spinal cord through PCR amplification for GFP-specific DNA. DNA dilution series prepared from GFP cells diluted in wild-type cells (ranging from 0.001% to 1%) were amplified (A). PCR products of GFP and β -actin were separated on 1.5% agarose gels, visualized by ethidium bromide staining, and analyzed using a FluorChem 8900 imager. The values of the GFP PCR product were normalized against the amount of PCR product for actin obtained from the same sample. The standard curve was prepared between the number of GFP cells and the ratio of PCR product optical density, from which samples were interpolated (B).

Table 1 Semi-quantification analysis of fetal cells in maternal spinal cord

Number of pregnancies	Microscopy (fetal cells/40 sections)	PCR amplification (fetal cells/wild-type cells)
0	-	-
0	-	-
0	-	-
0	-	-
1	-	-
1	-	-
1	2	0–1/10 ⁵
1	-	-
1	-	-
3	-	-
3	1	0–1/10 ⁵
3	2	0–1/10 ⁵
3	3	1/10 ⁵ –10/10 ⁵
3	1	0–1/10 ⁵

Mice were assigned to three groups: controls, mice with one gestation, and mice with three gestations. Semi-quantification of fetal cells in the maternal spinal cord was carried out by counting the fetal cells in each section, in combination with PCR amplification for GFP-specific DNA. The sign ‘-’ indicates that fetal cells were not found in the sections or there was no amplification band on 1.5% agarose gels. We obtained the same positive results with two types of semi-quantification. Five of 10 pregnant mice were found to have engrafted fetal cells in the cord. The χ^2 test showed that an increased number of pregnancies had a positive effect on the chance of detecting fetal cells ($\chi^2 = 3.6$, $\chi^2_{0.05(1)} = 3.84$, $0.05 < P < 0.1$).

DISCUSSION

The first molecular biological techniques used to detect fetal cells in the maternal body were the PCR amplification and fluorescence *in situ* hybridization (FISH) of a

Y-chromosome-specific sequence. In 1989, Lo *et al.*^[27] were the first to show that the Y-chromosome-specific sequence from a male fetus could be amplified from blood samples of pregnant women by PCR amplification based on male

DNA. Subsequently, this technique was used to determine when fetal-cell DNA first appeared in the peripheral blood of pregnant women, how long fetal cells survived in the host, and the frequency of microchimerism in women with a male fetus^[28, 29]. FISH allows for direct visualization of nuclei bearing X and Y chromosomes, which identifies male cells with specificity and allows for the localization and morphologic assessment of the microchimeric cells^[30]. Therefore, investigators now usually use both PCR amplification and FISH based on male DNA to make their results more reliable, informative, and comprehensive^[4].

However, the male-DNA-based detection technique is limited to visual counting of male cells in a female host, which means that it cannot be used to determine female fetal microchimerism in mothers. Meanwhile, the exchange of chimeric cells between human leukocyte antigen (HLA) identical twins is a common event. Approximately 8% of human twins and 21% of triplets have chimeras from their siblings in their blood cell populations^[31], showing that male cells in the maternal circulation are not always derived from the fetus. This is further supported by the observation that both women with sons and women without children have many organs containing Y-chromosome-positive cells. Similarly, many of the organs of women with and without a history of blood transfusion have Y-chromosome-positive cells^[4]. These disadvantages indicate that the male-DNA-based detection technique may not be sufficient, particularly in rodent models, which are polyembryonic and have an extremely low fetal microchimeric rate in certain tissues. Considering that a very low microchimeric rate might appear in the maternal mouse spinal cord, we adopted the GFP-based detection technique, by which we could achieve the best sensitivity of detection of rare fetal cells. We crossed wild-type female mice with GFP transgenic male mice^[16]. Engrafted fetal cells were identifiable by PCR amplification of GFP or by observing the morphology and immunohistochemical staining for GFP^[15, 32].

At the beginning of this study, we found autofluorescent pigments in the spinal cords of mice, especially in those that had been pregnant three times. These autofluorescent pigments emit green light and red light, making distinctions between specific labeling and nonspecific autofluorescence difficult. That is, some cells filled with these autofluorescent pigments in the cytoplasm might be counted as GFP cells, which would give a false-positive result. After

pretreatment with 1 mmol/L CuSO₄, the pigment-induced autofluorescence was markedly reduced while the GFP fluorescence was retained. Therefore, we suggest that pretreatment with 1 mmol/L CuSO₄ should be a routine step in the detection of rare GFP cells.

Morphology^[15] and immunohistochemical staining for GFP^[17, 33], which both allow the direct visualization of GFP cells, have been used to identify fetal cells in previous studies. However, we found that neither morphology nor immunohistochemistry alone could be used to reliably recognize fetal cells, due to the fact that cells that only emit green light or red light were found in some negative samples. However, we found that morphology in combination with immunohistochemical staining for GFP actually resolved this problem. Thus, cells that emitted both green and red light, which were not found in negative sections, were finally identified as fetal cells.

We visualized fetal cells in the spinal cord, suggesting that they pass through the blood-spinal-cord barrier. It is unlikely that the cells simply attached to capillary blood vessels, because at least some of the engrafted cells differentiated into a neuron-like cell type. Meanwhile, this finding also supports the previous hypothesis that engrafted fetal cells appear to have multi-directional differentiation capacity and can provide a rejuvenating source of fetal progenitor cells to participate in maternal tissue repair.

Regarding the number of engrafted fetal cells, our results showed that fewer than 1 fetal cell per 10⁵ maternal cells (4 of 5 mice) or 1–3 fetal cells per 40 sections were found in the spinal cord of maternal mice. This number is far less than that in the brain (~1 000 fetal cells/10⁵ maternal cells)^[17], but similar to that in the liver and spleen (1–10 fetal cells/10⁵ maternal cells)^[15, 33]. Due to the innate disadvantages of our quantification method, these values may not be very accurate but they still reflect the phenomenon that the rate of microchimerism in the spinal cord of maternal mice was extremely low. Meanwhile, an increasing number of pregnancies seems to have a positive effect on the chance of detecting fetal cells, but has no impact on the innately low microchimeric rate in the spinal cord. It is not clear how the number of fetal cells in the mother's spinal cord is positively related to the number of pregnancies. This may be caused either by the accumulation of total fetal cells in the spinal cord from each pregnancy, or by the increased migration ability of the fetal

cell with repetitive pregnancies.

The results of this study demonstrate that fetal microchimeric cells can pass through the blood-spinal-cord barrier and enter maternal spinal cord tissue. Although the microchimeric rate is very low, engrafted fetal cells have a multi-lineage capacity and can differentiate into a neuron-like cell type. Further studies are needed to address what factors or methods can promote the engraftment of fetal cells in the maternal spinal cord and increase the microchimeric rate, which will lay a foundation for the hypothesis that engrafted fetal cells can functionally improve a variety of degenerative disorders of the spinal cord.

ACKNOWLEDGEMENTS

This work was supported by the Manitoba Health Research Council (MHRC) and the Canadian Institutes for Health Research (CIHR). We thank Jacqueline Hogue for her assistance in preparing the manuscript.

Received date: 2013-01-07; Accepted date: 2013-03-11

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