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Identification of fetal mesenchymal stem cells in maternal blood: implications for non-invasive prenatal diagnosis

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Strategies for genetic prenatal diagnosis on fetal cells in the maternal circulation have been limited by lack of a cell type present only in fetal blood. However, the recent identification of mesenchymal stem cells (MSC) in first trimester fetal blood offers the prospect of targeting MSC for non-invasive prenatal diagnosis. We developed protocols for fetal MSC enrichment from maternal blood and determined sensitivity and specificity in mixing experiments of male fetal MSC added to female blood, in dilutions from 1 in 10^5 to 10^8. We then used the optimal protocol to isolate fetal MSC from maternal blood in the first trimester, using blood taken after surgical termination of pregnancy as a model of increased feto-maternal haemorrhage. In model mixtures, we could amplify one male fetal MSC in 2.5×10^7 adult female nucleated cells, yielding a 100% pure population of fetal cells, but not one fetal MSC in 10^8 nucleated cells. Fetal MSC were identified in one of 20 post-termination maternal blood samples and confirmed as fetal MSC by XY fluorescence in-situ hybridization (FISH), immunophenotyping and osteogenic and adipogenic differentiation. We report the isolation of fetal MSC from maternal blood; however, their rarity in post-termination blood suggests they are unlikely to have a role in non-invasive prenatal diagnosis. Failure to locate these cells routinely may be attributed to their low frequency in maternal blood, to sensitivity limitations of enrichment technology, and/or to their engraftment in maternal tissues soon after transplacental passage. We speculate that gender microchimerism in post-reproductive maternal tissues might result from feto-maternal trafficking of MSC in early pregnancy.

Key words: fetal cells/maternal blood/mesenchymal stem cells/microchimerism/non-invasive prenatal diagnosis

Introduction

Invasive procedures limit the uptake of prenatal diagnosis for chromosomal and monogenic disorders, because of their associated risk of fetal loss. Over the last 20 years there has been much interest in the development of non-invasive techniques. Of these, isolation of fetal cells from the maternal circulation early in pregnancy could replace existing methods such serum and nuchal screening, since it should allow exact genetic diagnosis without risk to the fetus. Despite the variety of fetal cells recognized in the maternal circulation, including trophoblast cells, nucleated erythrocytes, leukocytes, platelets and progenitors, non-invasive prenatal diagnosis has been hampered by technical challenges in enrichment, identification and diagnosis. In particular, current strategies for prenatal diagnosis on fetal cells in the maternal circulation are limited by the lack of a cell type present only in fetal blood and by the low frequency of the trafficking fetal cells (Bianchi, 1999; Scheuler *et al*., 2001; Bianchi *et al*., 2002; Bohmer *et al*., 2002).

In non-invasive prenatal diagnosis, fetal cells must be distinguished from maternal cells, usually by enriching them to an acceptable level of purity before identifying them as uniquely of fetal origin. Many approaches have been designed to recover fetal cells from maternal blood, but all are problematic: enrichment procedures result in significant loss of rare fetal cells, and most known fetal cell types are also found in adult blood, rendering identification difficult. Overcoming the rarity of fetal cells, while maintaining fetal cell recovery, could be achieved by amplifying fetal cells *in vitro* post-enrichment, allowing fetal karyotyping to be carried out on dividing cells. However, cells suitable for enrichment are not usually those that expand successfully in culture. Many groups, including our own, have attempted to expand fetal erythroid progenitors or fetal CD34+ haemopoietic progenitors in culture, with disappointing results (Chen *et al*., 1998; Campagnoli *et al*., 2000; Jansen *et al*., 2000; Campagnoli *et al*., 2002; Manotaya *et al*., 2002) and, with few exceptions, fetal haematopoietic progenitors have not been found in maternal blood before 16 weeks gestation.

The recent identification of mesenchymal stem cells (MSC) in first trimester fetal blood (Campagnoli *et al*., 2001) offers the prospect of targeting fetal MSC for non-invasive prenatal diagnosis, since MSC are not known to circulate in healthy adults (Fernandez *et al*., 1997; Lazarus *et al*., 1997). MSC are found in fetal blood from 7 to 13 weeks gestation; 11–13 weeks is recognized as the optimal time for prenatal diagnosis, as most of the risk of spontaneous miscarriage has passed and late first trimester diagnosis is still possible. In addition, unlike other candidate fetal cells for non-invasive prenatal diagnosis, fetal MSC have a characteristic morphology and immunophenotype (Campagnoli *et al*., 2001), are readily expandable *in vitro* and, like their counterparts in adult bone marrow (Pittenger *et al*., 1999; Reyes *et al*., 2001; Devine, 2002), they have the capacity to differentiate into

a number of mesenchymal lineages (osteogenic, chondrogenic, myogenic and adipogenic).

It is now recognized that fetal cells normally pass into the maternal circulation (Bianchi and Lo, 2001). The principal mechanism is feto-maternal haemorrhage, and trafficking appears to occur as a result of damage to trophoblast villi (Charnock-Jones and Burton, 2000). Placentae associated with chorionic villous haemorrhage show the villous stroma filled with fetal erythrocytes and leakage of fetal cells through the disrupted trophoblast layer (Soma *et al*., 1998). Physiological first trimester feto-maternal haemorrhage therefore should result in the passage of all fetal cell types, including fetal MSC, into maternal blood, from where even a single MSC isolated could be clonally expanded into a pure source of fetal cells.

The aim of this study was to investigate fetal MSC as potential targets for non-invasive prenatal diagnosis in first trimester maternal blood. We established a protocol for fetal MSC enrichment from maternal blood and determined sensitivity and specificity in mixing experiments with adult nucleated cells. We then applied this method to the isolation of fetal MSC from maternal blood after first trimester termination of pregnancy, which is a biological model of increased feto-maternal haemorrhage (Bianchi *et al*., 2001).

Materials and methods

Ethics

Blood and fetal tissue collection was approved by the institutional Research Ethics Committee in compliance with national guidelines regarding the use of fetal tissue for research purposes. All women gave written informed consent for collection and use of human tissues.

Sample collection

First trimester fetal blood samples (50–500 µl) were obtained by ultrasound-guided cardiac aspiration between 7 and 14 weeks gestation before clinically indicated surgical termination of pregnancy. Fetal gestational age was determined by crown–rump length measurement. A sample of trophoblast was obtained from the products for gender determination. Maternal peripheral blood samples (20–40 ml) were collected under general anaesthesia either before and/or after termination of pregnancy, or in the antenatal clinic at the time of clinically indicated venepuncture. We excluded multiple pregnancies, as well as those complicated by threatened miscarriage and fetal structural or chromosomal abnormalities, since these conditions might involve increased feto-maternal haemorrhage. Female blood used in the in-vitro model mixtures was obtained from two sources: the majority were late second/third trimester blood samples from the antenatal clinic, and in a few cases pre- or post-termination blood was taken from women undergoing first trimester termination of pregnancy, where the fetus was shown to be female by fluorescence in-situ hybridization (FISH).

Culture of MSC from fetal blood

Fetal blood was plated in 100 mm dishes at 10^5 nucleated cells per ml (Campagnoli *et al*., 2001) and cultured in 10% fetal bovine serum (FBS; Stem Cell Technologies, Canada) in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich Company Ltd, UK) supplemented with 2 mmol/l L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin (Gibco BRL® Life Technologies Ltd, UK) at 37°C in 5% CO_2. After 3 days, non-adherent cells were removed and the medium replaced. Adherent cell colonies were detached with 0.25% trypsin EDTA (Stem Cell Technologies), expanded, cultured to confluence in 25 cm^2 flasks, trypsinized and stored in liquid nitrogen. The MSC used in the model mixtures were derived from four male fetuses and were established in culture at passages ranging from 2 to 7.

Fluorescence in-situ hybridization (FISH)

Fetal gender was determined in fetal blood or trophoblast by XY FISH (Choolani *et al*., 2001) using commercial probes (Vysis, Abbott Laboratories Ltd, UK). So that identification of the Y chromosome by FISH in adherent cells could be used as proof of principle, male fetal cells were used in mixing experiments and clinical samples were confined to women carrying a male fetus. Conversely, blood used in the model mixtures was taken from women known to be carrying a female fetus.

For XY FISH, cytospun slides with 10 000–50 000 cells were fixed with either 100 µl of 3:1 v/v methanol: glacial acetic acid (Carnoy's fixative) or in 1:1 methanol: acetone. Chromosome-specific centromeric repeat probes DXZ1, labelled with SpectrumOrange™, and DYZ3, labelled with SpectrumGreen™ (Vysis) were used. Slides were analysed by epifluorescence microscopy (fluorescence microscope; Zeiss Axioskope, Germany). Images were captured using a cooled charge-coupled device camera and reviewed in Quipps m-FISH software (Vysis). Nuclei with two red signals were classified as female (XX) and those with one red signal and one green signal as male (XY). Each slide was scanned for hybridization efficiency and analysed only if ⩾75% of nuclei contained both signals. These were counted when the intensity and diameter of the fluorescent signals was approximately equal and inside a distinct nucleus with an intact border, as indicated by DAPI staining. At least 5000 nuclei per FISH slide were counted and FISH was later repeated on re-expanding cells.

Enrichment protocol

All blood samples were processed immediately after collection. Female blood was diluted 1:1 with Roswell Park Memorial Institute 1640 medium (RPMI; Sigma-Aldrich Co. Ltd), layered over Ficoll 1077 g/ml (Histopaque-1077; Sigma–Aldrich Co. Ltd), centrifuged at 454 *g* for 30 min at 20°C and the mononuclear cell (MNC) layer at the gradient interface collected, washed twice in RPMI and resuspended in 10 ml of washing buffer: containing 200 µl 0.5 mol/l EDTA, 2.5 ml 10% bovine serum albumin and made up to 50 ml with phosphate-buffered saline (Gibco BRL® Life Technologies Ltd). To enrich fetal MSC, red (100% glycophorin A+) and white (100% CD45+) blood cells were depleted using the MidiMACS immunomagnetic separation system (MACS, magnetic activated cell sorting; Miltenyi Biotec, UK), according to the manufacturer's instructions. MNC were resuspended in 80 µl of washing buffer per 10^7 cells, and incubated with 20 µl of MACS CD45 microbeads (anti-human murine leukocyte common antigen; Miltenyi Biotec) per 10^7 cells, and 20 µl GPA Microbeads (anti-human murine Glycophorin A; Miltenyi Biotec) for 20 min at 4°C. Washed cells were resuspended in 2 ml and applied to LD separation columns (Miltenyi Biotec), precalibrated with 2 ml of buffer. Unlabelled cells, which passed through the column and were collected as the CD45– fraction, were pooled, washed and resuspended in 10% FBS/DMEM, plated and incubated at 37°C in 5%CO_2 for 2–4 weeks. The duration of culture, which is a trade-off between achieving confluence and maintaining cell viability, was chosen to make sure no adherent cells were missed. All experiments were carried out using consistent materials, equipment and methodology.

Characterization of adherent cells

Adherent cells were identified as mesenchymal by their morphology and immunophenotype (CD45–, CD14–, CD68–, CD34–, SH2+, Vimentin+ and type I collagen-) using alkaline phosphatase immunocytochemistry (Polak and Van Noorden, 1997) and as fetal by XY FISH. Stem cell properties were confirmed by expansion maintaining the mesenchymal phenotype and by osteogenic and adipogenic differentiation, as previously described (Campagnoli *et al*., 2001).

Statistics

Statistics were calculated using Microsoft Excel software (Version 9.0© 1985–2000. Microsoft Corporation, USA). Unless otherwise stated, all values are expressed as median (range).

Results

Enrichment of fetal MSC from adult blood: in-vitro model mixtures

To establish optimal cell purification and culture systems for detection of fetal MSC in maternal blood, cultured male fetal MSC were mixed with 20–40 ml adult female whole blood, to achieve dilutions of 1 in 10^5 to 1 in 10^8 nucleated cells (n = 6 at each dilution). Numbers of MSC >100 were obtained by serial dilution from a larger known

Table I. Enrichment of fetal mesenchymal stem cells (MSC) from adult female blood: model mixtures of a range of concentrations of fetal MSC diluted in adult blood

Dilution of fetal MSC in adult female blood	1 in 10^5	1 in 10^6	1 in 10^7	1 in 2.5×10^7	1 in 10^8
No. of experiments	6	6	6	8	6
Volume of maternal blood analysed (ml)	19.5 (18–26)	22.5 (17–36)	31 (25–34)	32 (32–34)	39 (32–46)
No. of XY MSC added	1422 (1250–2200)	240 (67–398)	30.5 (25–34)	10 (10–10)	4 (3–8)
Mononuclear cell count ($\times10^6$/ml)	2.1 (1.3–6.2)	3.2 (2–6)	5.1 (3.8–12.2)	5.7 (3.8 – 7.5)	7.6 (4.6–8.2)
Days in culture	11.5 (10–14)	19 (13–21)	19 (13–21)	23.5 (17–27)	29 (24–37)
Total no. of MSC recovered	45 000 (40 000–84 000)	40 000 (20 000–60 000)	30 000 (17 500–200 000)	5000 (0–28 000)	0
Expected no. of MSC for days in culture	728 064	7 864 320	999 424	2 621 440	33 554 432
Recovered/expected (%)	6	0.003	3	0.2	0
Sensitivity: successful experiments (%)	100	100	100	50	0
Purity: % male cells isolated	100	100	100	100	0

Expected number of MSC is calculated for a mean doubling time in culture of 30 h. Values expressed as median (range).

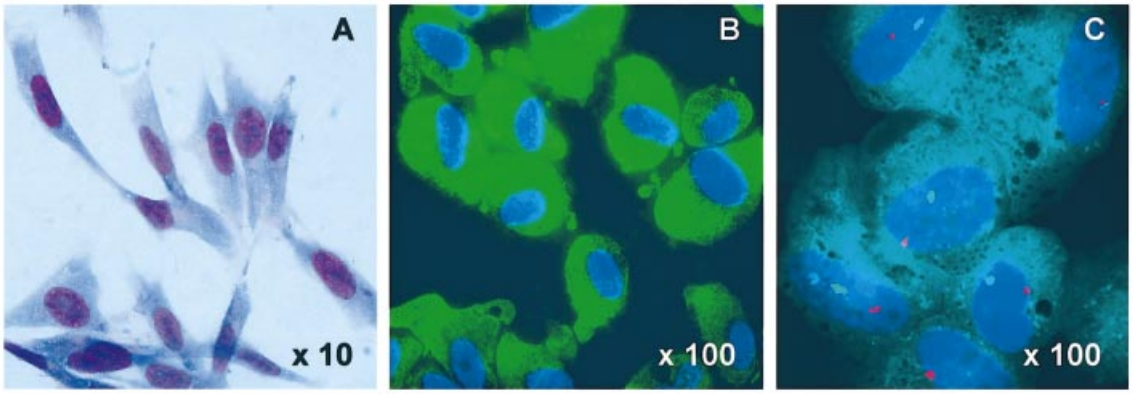

Figure 1. Mesenchymal stem cells isolated from male fetal in adult female dilutions show mesenchymal morphology (**A**; Wright's stain), Vimentin positive immunostaining (**B**) and are 100% fetal in origin (**C**; XY fluorescence in-situ hybridization).

concentration, and, for greater accuracy, cell numbers of ⩽50 were obtained by micromanipulation under light microscopy. All blood samples were processed using the standard enrichment protocol described above and enriched cells were maintained in culture for 2–4 weeks (Table I).

Colonies of male MSC were enriched in all experiments of fetal MSC cultured at the 1 in 10^{-5}, 10^{-6} and 10^{-7} dilutions with female cells (Table I). MSC were identified by their characteristic morphology and immunophenotype and were 100% XY on FISH (Figure 1). In contrast, once the dilution of male fetal MSC in female blood was increased, MSC were more difficult to detect, being identified in half of the experiments (i.e. 4/8; Table I) at a dilution of one MSC in 2.5×10^7 female cells. When the dilution was increased to 1 in 10^8, we were unable to identify any male fetal MSC in female blood. No adherent female cells were expanded at the 10^{-5}, 10^{-6}, 10^{-7} or 10^{-8} dilutions. As blood from pregnant women was used in the model mixtures, a series of control samples ($n = 12$) from an equivalent group of women at similar gestations was not spiked with male fetal MSC but enriched through the same protocol. In no case were adherent MSC cultured.

Thus, the sensitivity of fetal MSC enrichment using our standard protocol lies between 1 in 10^7 and 1 in 2.5×10^7, possibly as this dilution was just on the threshold level of detection. Although this degree of sensitivity is better than most reports of enrichment of other candidate cell types, we tried various strategies to improve the system further, including medium supplemented with MSC supernatant and co-culture systems, where small numbers of cells proliferate over an established MSC feeder layer. Addition of filtered supernatant from cultured MSC ($n = 5$) and co-culture of fetal MSC/maternal mixtures with MSC feeder layers ($n = 5$) had no effect on the detection of small numbers of fetal MSC in maternal blood.

Enrichment of fetal MSC from first trimester maternal blood: in-vivo model

Since our enrichment protocol gave a predicted sensitivity of >1 in 10^7 cells and would be predicted to be clinically useful, we went on to test this approach by attempting to isolate fetal MSC from maternal peripheral blood collected shortly after first trimester termination of pregnancy. Peripheral blood samples ($n = 20$; median volume 38 ml, range 30–68) were obtained from 20 women between 7^{+6} and 13^{+5} weeks of pregnancy ($n = 20$; median 10 weeks) 5–15 min after surgical termination of pregnancy. In all samples, fetal gender was confirmed as male after XY FISH on fetal blood or trophoblast.

Following the standard enrichment protocol described above, the CD45– GPA– cell fraction was maintained in culture in 10% FBS for 2–4 weeks (median 27 days, range 11–33). Fetal MSC were successfully enriched from one (5%) of the 20 post-termination maternal blood samples tested: 80 000 cells were trypsinized from culture on day 22, confirmed as male by XY FISH, identified as mesenchymal by their immunophenotype and demonstrated to be stem cells by successful differentiation into bone and fat (Figure 2).

Discussion

To overcome the problems of poor sensitivity and lack of purity that have beset prenatal diagnosis using fetal cells in maternal blood, we

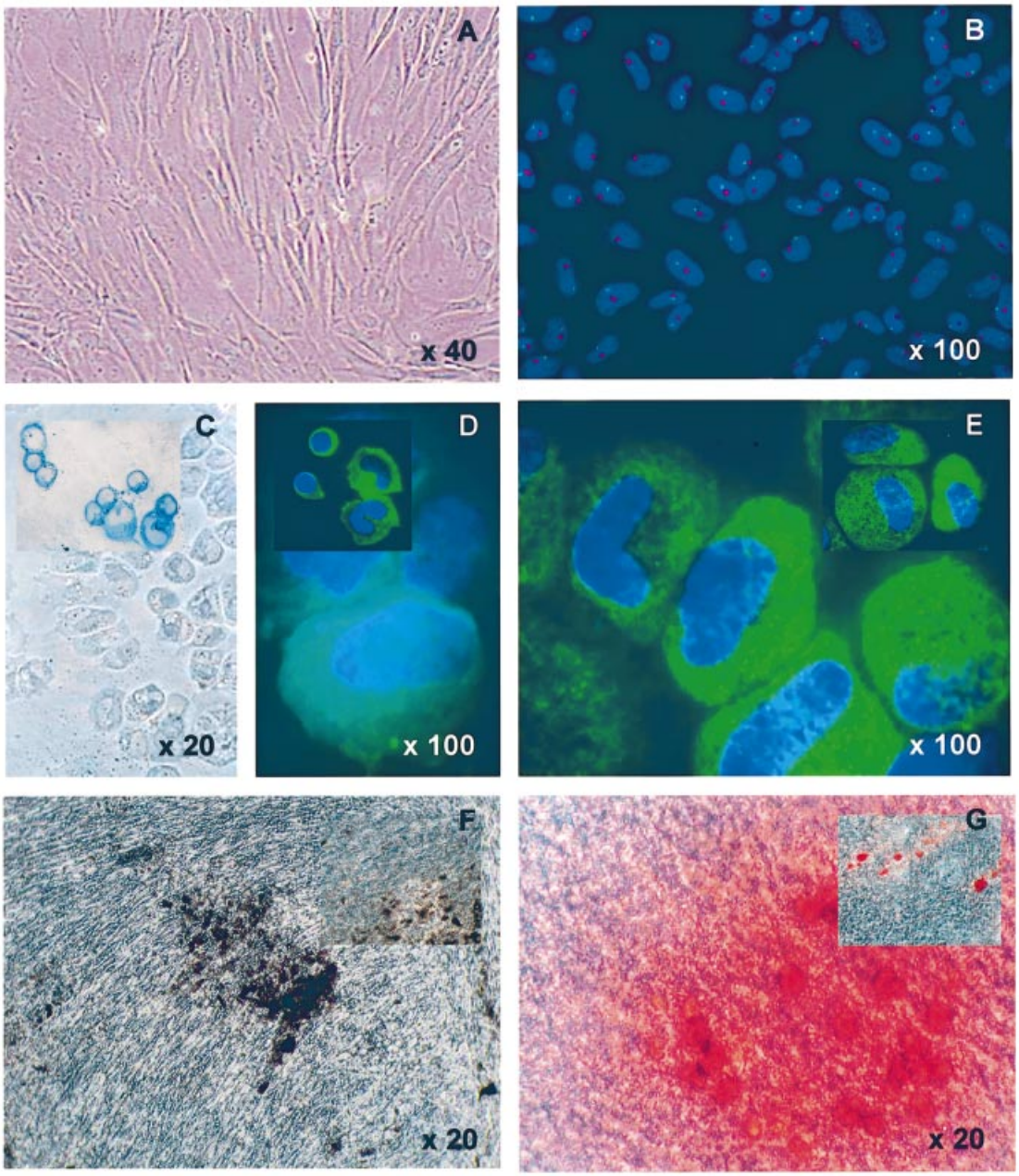

Figure 2. Mesenchymal stem cells (MSC) enriched from post-termination maternal blood at 10 weeks gestation show a mesenchymal growth pattern in culture (**A**), are 100% male on XY FISH (**B**), have a mesenchymal immunophenotype, CD45– (**C**, **D**) and vimentin+ (**E**) and, when grown in selective media, differentiate into bone and fat as demonstrated by Von-Kossa staining (**F**) and Oil Red O staining (**G**). Insets show controls: CD45+ mononuclear cells from adult peripheral blood (**C**, **D**), Vimentin+ MSC from fetal blood (**E**), and osteogenic differentiation (**F**) and adipogenic differentiation (**G**) of MSC in fetal blood.

focused our investigations on fetal MSC. On theoretical grounds, they should be ideal target cells, both because they are unique to fetal rather than maternal blood, and because they can be clonally expanded into a pure source of fetal cells.

Using a simple enrichment protocol, we developed a method for isolating small numbers of fetal MSC from 30–40 ml adult blood, and in dilution experiments with adult nucleated cells we were able to detect one fetal MSC in 10^7 maternal cells and to derive a 100% pure population of fetal cells. As the reported frequencies of fetal cells in maternal blood range from 1 in 5000 (Hamada *et al*., 1993) to 1 in 10 000 000 (Price *et al*., 1991; Bianchi *et al*., 1997), an enrichment method with a sensitivity of 1 in 10^7 should be readily applicable to first trimester non-invasive prenatal diagnosis. Indeed, our sensitivity is significantly better than the 1 in 10^1–10^6 reported in other model systems developed to test enrichment procedures (Bianchi, 1994; Troeger *et al*., 1999; Voullaire *et al*., 2001) for nucleated red blood cells (Troeger *et al*., 1999; Choolani *et al*., 2003), fetal liver cells (Bianchi *et al*., 1996a) and expanded erythroid cells from cord blood (Jansen *et al*., 1999).

Feto-maternal haemorrhage after first trimester termination of pregnancy (Bianchi *et al*., 2001) results in an 80-fold increase in fetal cell numbers in maternal blood, and is a useful biological model with which to evaluate fetal cell enrichment and expansion strategies under development (Choolani *et al*., 2003). We therefore applied our

enrichment protocol to the isolation of MSC from first trimester post-termination maternal blood. Although it was possible to isolate fetal MSC in maternal blood, fetal cells were identified in only one of 20 patients tested. This suggests that fetal MSC are likely to circulate at a very low frequency in maternal blood, since our enrichment protocol in model mixtures was sensitive enough to detect one MSC among 2.5×10^7 adult nucleated cells or one cell in 3.3 ml whole blood.

There are several possible reasons for the low frequency of fetal MSC detected in maternal blood. Firstly, fetal MSC may only be present in the circulation in extremely low numbers at or below the level of sensitivity of the culture assay we used. Non-haemopoietic cells account for only 0.4% of nucleated cells (Campagnoli *et al*., 2001) and the fetal cell frequency in first trimester fetal blood has been estimated by quantitative PCR to be 1.2 fetal cells per ml of maternal blood (Bianchi *et al*., 1997). If one cell in 250 fetal nucleated cells is an MSC, it is possible that >250 ml of blood would have to be processed to obtain the needed cell number, which is neither practical nor clinically appropriate. However, precise estimation of fetal cell frequency is hampered by many variables such as gestational age and sensitivity of the PCR methods used to quantify fetal cell DNA equivalents in whole blood (Bianchi *et al*., 1997). More recent data suggest that there is a much higher normal fetal cell load in maternal blood, with as many as two to six fetal cells per ml of maternal blood between 18 and 22 weeks of pregnancy (Krabchi *et al*., 2001) and also in the first trimester (R.Drouin, personal communication). This implies that 40–125 ml of blood could be processed to enrich fetal MSC, and significantly less would be needed after feto-maternal haemorrhage, e.g. after termination of pregnancy. In reality, the true prevalence of all types of fetal cells in maternal blood remains unknown.

Second, the detection method may not be optimal. To improve this, we are currently investigating alternative enrichment strategies, using positive selection for fetal MSC cell surface antigens such as SH2 [also referred to as CD105 (Haynesworth *et al*., 1992; Barry *et al*., 1999)] and negative selection using Rosette Sep™ (Stem Cell Technologies), a depletion antibody cocktail designed to enrich MSC from adult bone marrow. While a fluorescence-activated cell sorting (FACS)-based strategy is another possibility, the low concentration of fetal cells in maternal blood makes this technically challenging; indeed the NIFTY study (Bianchi *et al*., 2002) showed FACS to be considerably inferior to MACS for fetal cell enrichment.

Finally, although fetal MSC are likely to cross the placenta based on both theoretical considerations and from our findings that fetal MSC are detectable in at least a proportion of maternal blood samples, the subsequent destination of fetal MSC in the mother is not clear and has never been studied. While they may persist in an undifferentiated state in blood in undetectable amounts, it seems more likely that MSC engraft in maternal tissues, particularly bone marrow. Fetal MSC may persist indefinitely in maternal tissues, as described for other fetal cell types, such as CD34+ haemopoietic progenitors and CD3, CD14 or CD19+ white blood cells ((Bianchi *et al*., 1996b; Artlett *et al*., 1998; Evans *et al*., 1999). The expression of a variety of adhesion molecules, including VCAM-1, CD44, the integrins CD29, CD49b and CD49e (de la Fuente *et al*., 2002), along with adherent culture properties of MSC *in vivo*, suggests that these cells may disperse widely, implant and persist in connective tissues. In addition, adult bone marrow-derived MSC readily engraft in most organs in large animal models, but preferentially home to bone marrow after infusion (Liechty *et al*., 2000; Devine *et al*., 2001; Mackenzie and Flake, 2001). We therefore speculate that fetal cell microchimerism in maternal organs, which may contribute to the aetiology of autoimmune disease (Bianchi, 2000; Nelson, 2002), might result from feto-maternal haemorrhage of MSC in early pregnancy.

In conclusion, we have isolated fetal MSC from maternal blood for the first time, but acknowledge that the rarity of fetal MSC circulating in maternal blood appears to preclude clinical application in non-invasive prenatal testing. Notwithstanding this, the discovery of a unique fetal stem cell circulating in maternal blood with the potential to persist in tissues years after pregnancy provides information about feto-maternal trafficking in early pregnancy and emphasizes increasing awareness that cellular trafficking may have far-reaching biological consequences.

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