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Novel Approaches to Manipulating Foetal Cells in the Maternal Circulation for Non-Invasive Prenatal Diagnosis of the Unborn Child

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ABSTRACT

Due to the risks to the foetus with invasive prenatal diagnosis, non-invasive prenatal diagnosis (NIPD) is gaining tremendous interest but no reliable method that can be widely used has been developed to date. Manipulation of foetal cells and foetal cell-free genetic material in the maternal blood are two promising approaches being researched. The manipulation of foetal cells in the maternal circulation is more popular as it can provide complete genetic information of the foetus particularly the diagnosis of aneuploidies. However, the foetal cell numbers in the maternal circulation are small and their enrichment and ex vivo culture remain two major challenges for NIPD. Primitive foetal erythroblasts (pFEs) have been considered as a good potential candidate for early first trimester NIPD but their nature, properties and manipulation to provide adequate cell numbers remain a challenging task and several approaches need to be meticulously evaluated. In this review we describe the current status of NIPD and suggest some novel approaches in manipulating pFEs for future clinical application of NIPD. These novel approaches include (1) understanding the pFE enucleation process, (2) enriching pFE numbers by individual pick-up of pFEs from maternal blood using micromanipulation and microdroplet culture, (3) expansion of pFEs using mitogens and (4) decondensation of the pFE nucleus with histone deacetylase (HDAC) inhibitors followed by reprogramming using gene delivery protocols with/without small reprogramming molecules to improve reprogrammed pFE proliferation rates for successful NIPD. J. Cell. Biochem. 112: 1475–1485, 2011.

KEY WORDS: FOETAL CELLS; MATERNAL BLOOD; NON-INVASIVE PRENATAL DIAGNOSIS; PRIMITIVE FOETAL ERYTHROBLAST

Prenatal diagnosis has become an established routine tool for detecting birth defects in the foetus thus relieving the anxiety of parents seeking such evaluation of a suspected genetic defect in their unborn child. Although the history of prenatal diagnosis can be traced back to almost hundred years ago when the first diagnosis using X-ray was reported in 1916, it was not until 1966 that the first reliable genetic diagnosis using aspirated amniotic fluid (amniocentesis) was performed by Steele and Breg [1966]. Later, first trimester chorionic villus sampling (CVS) was shown to be a safe and reliable approach for earlier prenatal diagnosis [Kuliev et al., 1993]. Both these two invasive techniques have remained today as the gold standards used in modern day prenatal diagnosis even at the expense of potential risks of miscarriage to the foetus. Because of these risks, non-invasive prenatal diagnosis (NIPD) approaches have been aggressively pursued in the past two decades for diagnosis of chromosomal anomalies and single gene defects of the foetus [Hahn et al., 2008]. Examples of such approaches being pursued include the genomic analysis of cell-free DNA, mRNA and foetal cells in the maternal circulation. However, there are several limitations associated with these approaches such as the fact that cell-free DNA is insufficient to provide complete chromosomal information such as aneuploidies for diagnosis. The utilisation of foetal cells circulating in the maternal blood has been considered a very promising strategy for detecting aneuploidies which also offers more complete detailed genetic information of the foetus. Unfortunately, the occurrence of foetal cells in maternal blood is small and difficulty in separating them from maternal cells has existed for a long time. The separation and expansion of these scarce foetal cells ex vivo remains a major challenge to obtaining adequate material for carrying out genetic diagnosis. In this review we discuss existing invasive and NIPD methods and provide some novel ideas that need to be researched as potential reliable approaches for future NIPD using foetal cells present in maternal blood.

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INVASIVE PRENATAL DIAGNOSIS

AMNIOCENTESIS

Chromosome analysis on aspirated amniotic fluid in the mid trimester of pregnancy (amniocentesis) was first carried out more than four decades ago [Kubli and Hindermann, 1966]. Today, it is the conventionally used method at 16–18 weeks of pregnancy causing low risk to the foetus after withdrawal of about 10–20 ml of fluid. Cells in the amniotic fluid are separated after the aspiration procedure and are grown in vitro in specially designed culture media to amplify cell numbers for later diagnosis. Approximately, $2–3 \times 10^5$ cells [Weise et al., 1984] can be harvested from 10 ml of amniotic fluid and the origins of these cells are from the inner lining of the amniotic membrane, skin, respiratory and urogenital tract of the foetus resulting from sloughing due to swallowing, urination and physical movements of the foetus within the amniotic sac. Amniocentesis has been routinely used for detecting chromosomal anomalies such as Down's, Edward's and Patau's syndromes, neural tube defects and other genetic disorders. Although amniocentesis is a routine tool for prenatal diagnosis in many hospitals, there are risks of miscarriage associated with its invasiveness. The rate of amniocentesis-related miscarriages has been reported to be about 1% [Tabor et al., 2009].

CHORIONIC VILLUS SAMPLING (CVS)

CVS as a tool for prenatal diagnosis was first developed in 1984 [Kuliev et al., 1993]. In this procedure, samples of chorionic villi are aspirated transcervically or transabdominally and the foetal cells within the tissue are used for diagnosis. The advantage of CVS over amniocentesis is that it can be performed at an earlier stage of pregnancy (usually before 14 weeks of gestation) providing early diagnosis and thus relieving much anxiety to the parents. CVS has been used for the detection of similar chromosomal anomalies as in amniocentesis. In the CVS method also there are risks of miscarriage in the region of about 2% [Tabor et al., 2009].

FOETAL BLOOD SAMPLING

Foetal blood sampling, also known as percutaneous umbilical cord blood sampling is a procedure to remove a small amount of blood from the foetal umbilical cord. The foetal blood sample can be used for detecting chromosomal abnormalities, blood and metabolic disorders and infections and is useful when information cannot be obtained through amniocentesis and CVS. Foetal blood sampling is usually performed in late gestation after 17 weeks and has a high miscarriage rate of 2–3% [Buscaglia et al., 1996; Antsaklis et al., 1998].

NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

ULTRASONOGRAPHY

Ultrasonography is a common non-invasive procedure done routinely for prenatal care. It helps visualising the foetus in the uterus and provides information on its growth status. Important developmental abnormalities such as Down's syndrome can be detected using ultrasonography by evaluating the thickness of the nuchal fold and its translucency, and foetal organs. Although ultrasonography is very useful in monitoring foetal growth and detecting the phenotypes of developmental abnormalities, its false-positive rates are high. For example, in the nuchal translucency thickness test, about 5% of positive foetuses do not actually have Down's syndrome [Muller et al., 2003]. Most foetal defects are caused by abnormal genetic make-up (chromosome number and morphology, gene defects) and as such many of them cannot be detected via ultrasound scan.

MATERNAL SERUM SCREENING ('TRIPLE TEST')

Maternal serum screening, also called the 'triple test' is a blood test which helps determine the risk of certain chromosomal abnormalities such as Down's syndrome and neural tube defects. It is usually done in the first or second trimester of pregnancy by measuring a combination of serum levels of alpha-fetoprotein, oestriol and human chorionic gonadotropin. A positive test indicates a high risk of the particular disorder but the diagnosis is not definitive. If the result is positive, then the diagnosis has to be further confirmed with other invasive procedures such as amniocentesis or CVS.

PREIMPLANTATION GENETIC DIAGNOSIS (PGD) IN IN VITRO FERTILISATION (IVF)

Preimplantation genetic diagnosis (PGD) is a special prenatal diagnosis test for patients going through in vitro fertilisation (IVF). In older IVF patients (>40 years) with potential aneuploidies due to age-related egg defects and those IVF patients with habitual miscarriages after embryo transfer due to possible imbalanced translocations, PGD on embryo biopsies has become a routine diagnostic tool. PGD is also available for many monogenic disorders such as cystic fibrosis and beta-thalassemia and some chromosomal abnormalities. The main advantage of this method is that it can avoid selective termination of pregnancy because only genetically normal embryos are transferred but its disadvantage is that it is reserved only for IVF patients.

CELL-FREE FOETAL DNA AND mRNA IN MATERNAL BLOOD

Large amounts of cell-free foetal DNA circulating in maternal plasma and serum was first reported by Lo et al. [1997] and 3 years later cell-free foetal mRNA was also observed in the maternal plasma [Poon et al., 2000]. Cell-free foetal DNA carries valuable genetic information of the foetus and was shown to be suitable for diagnosing foetal sex, foetal rhesus D (RhD) blood type, and some single-gene disorders such as beta-thalassemia and achondroplasia (ACH) [Saito et al., 2000; Chiu et al., 2002; Vrettou et al., 2003; Li et al., 2005]. However, because of the small amounts of cell-free foetal DNA in the total free DNA in the maternal plasma, the quantification of specific foetal loci from the cell-free foetal material is difficult and inefficient. This problem was overcome by the use of foetal mRNA which is specific to placental tissue minimising maternal background contamination. However, in using cell-free foetal DNA or mRNA, diagnosis of foetal aneuploidy remains the major difficulty for NIPD. Shotgun sequencing of cell-free foetal DNA was shown to give an accurate diagnosis of foetal aneuploidy [Fan et al., 2008]. In a more recent study, the whole genetic profile of the foetus could be revealed by sequencing foetal cell free-DNA in the maternal blood [Lo et al., 2010]. The same group

conducted a larger scale study on maternal plasma samples using multiplexed DNA sequencing analysis and showed that it was able to rule out foetal trisomy 21 among high-risk pregnancies thus avoiding invasive diagnostic procedures [Chiu et al., 2011]. However, before these newly developed techniques can be used for routine clinical application, trials on large sample numbers need be carried out to optimise the protocols and their cost effectiveness needs to be considered.

FOETAL CELLS IN MATERNAL BLOOD

The presence of foetal cells in the maternal circulation was first documented by in 1893 when multinucleated syncytial trophoblasts were found in the lung tissue of pregnant women who died from eclampsia [Schmorl, 1893]. This important observation laid the foundation for the use of foetal cells in maternal blood for NIPD. Intact foetal cells, compared to cell-free foetal DNA and mRNA, have the advantage of retaining complete foetal genetic information in the nucleus and cytoplasm that can provide information in complex genetic diagnosis. There are a variety of foetal cell types present in maternal blood, viz., trophoblasts, lymphocytes, erythroblasts and haematopoietic stem cells (HMCs). Trophoblast cell trafficking does not occur commonly in pregnancy and their enrichment from the maternal blood is difficult due to lack of specific makers. Additionally, due to placental mosaicism, the karyotype of about 1% of placental cells is different from the actual karyotype of the foetus [Henderson et al., 1996; Goldberg and Wohlferd, 1997] leading to false-positive and false-negative genetic diagnosis. Therefore, the use of trophoblasts in maternal blood for NIPD is limited. Foetal lymphocytes present in maternal blood were previously shown to be useful for foetal sex determination [Walknowska et al., 1969; Schroder and De la Chapelle, 1972; Grosset et al., 1974] because of their ability to proliferate in vitro. However, their disadvantage is that it was shown that they could persist in maternal blood for a very long period of time even after 27 years postpartum [Bianchi et al., 1996] leading to misdiagnosis of the current pregnancy. Thus, a cell type with short life span is preferred and as such foetal erythroblasts (FEs) or nucleated red blood cells are good candidates for NIPD because they can be detected early in pregnancy, have short life spans, and specific antibodies can be developed for their enrichment from maternal blood. However, major challenges exist in the use of FEs because the number of FEs in maternal blood is small and estimated at 1 in 10^5 to 1 in 10^9 of mononuclear cells [Ganshirt-Ahlert et al., 1990; Price et al., 1991]. On average approximately 20 FEs are obtained after enrichment of 20 ml of maternal blood [Busch et al., 1994]. The final purity of FEs is low even when specific antibodies are used to sort them out from the maternal blood. Some workers have cultured foetal erythroid progenitors from the maternal blood which were proliferative with the hope that increased numbers of foetal cells could be obtained for prenatal diagnosis. However, because of the contamination of maternal cells in the culture, selective amplification of foetal over maternal progenitors was not successful in these studies [Lo et al., 1994; Bohmer et al., 2001; Campagnoli et al., 2002]. Similar problems occurred when culturing CD34+ HMCs from maternal blood for prenatal diagnosis. Both the foetal and maternal CD34+ cells proliferated in culture, making it difficult to separate the two populations and perform a diagnosis on the foetal cells. CD34+ foetal stem cells have also been found to remain post-delivery in maternal blood. Besides the problems of cell numbers and enrichment and in vitro expansion of foetal cells in maternal blood, the PCR analysis of these cells is often associated with a high level of allele dropout as a result of the non-amplification of one of the two alleles. This would require the analysis of at least five to six foetal cells in order to offset the dropout rate [Sinuhe et al., 2009].

Methods of enrichment of FEs from maternal blood include fluorescence-activated cell sorting (FACS), magnetic activated cell sorting (MACS), density gradient centrifugation, charged flow separation, selective erythrocyte lysis and lectin-based methods. FACS and MACS are based on antigen-antibody recognition using specific antibodies for the target foetal cells. The first monoclonal antibody used to enrich FE was CD71 [Bianchi et al., 1990] but the purity of FEs after sorting remained low. Purity was later improved by using MACS depletion of anti-CD45 prior to positive selection of CD71 positive cells [Busch et al., 1994]. To assess the potential of FEs for NIPD, a large-scale multicentric study was carried out, known as NIFTY, between 1995 and 1999 [Bianchi et al., 2002]. Results from this study showed that MACS separation yielded better recovery of FEs compared to FACS separation. However, in both methods, FEs were difficult to detect, most likely due to the scarce number of these cells in maternal blood. This study concluded that the separation method based on interactions between the cell-specific antigen and the corresponding antibodies has its limitations. To remove mature erythrocytes from maternal blood, selective density gradient systems were developed to enrich mononuclear cells. Various density gradients using Percoll and Ficoll were tried on separating FEs from the rest of the cells in the maternal blood but the recovery rates were still very low [Troeger et al., 1999; Samura et al., 2000; Prieto et al., 2001; Voullaire et al., 2001]. The ideal enrichment protocol for FEs from maternal blood with high recovery rates and purity still needs to be developed.

NOVEL APPROACHES OF MANIPULATING PRIMITIVE FOETAL ERYTHROBLASTS FOR NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

Despite the limitations of using foetal cells in maternal blood for NIPD, studies on their enrichment, culture and PCR efficiency still continue. The first trimester pFE is still considered a promising candidate for early NIPD because it appears early in gestation within the first trimester, has a short half-life and can be distinguished from maternal nucleated erythrocytes using unique embryonic haemoglobin markers [Choolani et al., 2003]. However, like other foetal cells, pFEs are very rare in the maternal blood. The cell number problem still poses difficulties of using them for a reliable prenatal diagnosis even if they can be enriched with specific markers. What is more, in the human, this type of erythroblast has not been extensively studied probably due to its transient presence in the foetus and limited accessibility. In the following sections, recent information on the nature and properties of pFEs will be described and some novel approaches will be suggested and discussed on how

this foetal cell type may be manipulated to increase cell numbers for NIPD.

UNDERSTANDING PRIMITIVE FOETAL ERYTHROBLASTS (pFEs)

Primitive erythropoiesis. Two distinct types of erythropoiesis have been reported during mammalian embryo development (1) primitive erythropoiesis in the yolk sac and (2) definitive erythropoiesis mainly in the foetal liver and bone marrow. While definitive erythropoiesis is well studied in the adult, primitive erythropoiesis is less understood due to its transient presence in the early foetus. It was recognised very early that primitive erythroid cells emerged in the yolk sac blood islands [Maximow, 1909] and primitive erythropoiesis was the first developmental process producing erythrocytes to supply oxygen for the survival and growth of the foetus. One study showed that human primitive erythroblasts were the only circulating red cells present within the foetus and its yolk sac until week 6 [Pereda and Niimi, 2008]. Some studies have indicated, however, that primitive erythropoiesis arises from haemangioblast precursors in the mesoderm layer of the yolk sac wall [Choi et al., 1998; Ema et al., 2006; Lugus et al., 2009].

Primitive red cells, also called primitive erythroblasts are distinguishable from definitive red blood cells in that they start circulating in the blood stream as nucleated cells and contain an embryonic type of haemoglobin viz., epsilon-globin. The dogma existed for a long time that primitive erythroblasts share many features with their non-mammalian counterparts such as being nucleated throughout the whole life span. Whether primitive erythroblasts enucleate has been a puzzle ever since they were discovered and extensively studied in murine models in recent years. In the mouse foetus, there was evidence showing that primitive erythroblasts undergo enucleation in the foetal liver [Kingsley et al., 2004; Fraser et al., 2007]. After erythroblast enucleation there were two populations of cells generated, namely reticulocytes and pyrenocytes which are excluded nuclei surrounded by a thin rim of cytoplasm. Pyrenocytes are rapidly removed by macrophages by phagocytosis [Yoshida et al., 2005; McGrath et al., 2008]. In the human, due to ethical sensitivities and practical reasons, not many studies have been done on primitive erythropoiesis. Van Handel et al. [2010] recently suggested that the human placenta was the anatomical site for primitive erythroblast enucleation and that enucleation probably required interaction with macrophages in the chorionic villi.

Morphology of the primitive foetal erythroblast (pFE). The human primitive erythroblast has a high cytoplasmic–nuclear ratio and is a larger size compared to definitive erythroblasts and it shares some common properties with definitive erythroblasts. Interestingly, high nuclear–cytoplasmic ratio is a feature of human embryonic stem cells (hESCs) [Bongso et al., 1994]. Primitive erythroblasts undergo morphological changes consistent with definitive erythroblast maturation. As primitive erythroblasts mature, increasing amounts of haemoglobin are accumulated in their cytoplasm [De la Chapelle et al., 1969], they become smaller in size [Henery and Kaufman, 1992], their nuclei condense progressively [Sasaki and Kendall, 1985] and they lose intermediate filaments with the nucleus being free to move about in the cytoplasm [Sangiorgi et al., 1990]. One distinct feature of primitive erythroblasts is that they circulate in the blood in a nucleated state unlike definitive erythroblasts which enucleate before they enter the circulation. Primitive erythroblasts isolated from the maternal blood of pregnant women have slightly different morphology compared to those in the foetus. They usually possess pyknotic nuclei which are very condensed. This may be caused by the difference in oxygen concentration between the maternal and foetal blood [Babochkina et al., 2005].

Manipulating the human primitive foetal erythroblast (pFE) for non-invasive prenatal diagnosis (NIPD). The primitive erythroblast is a good target cell for first trimester NIPD. Most of our knowledge on the nature and properties of primitive erythroblasts is gained from murine models and it is therefore difficult to make conclusions on human primitive erythroblast maturation, enucleation and other properties. If human primitive erythroblasts do enucleate in vivo, it may be worth studying this phenomenon in vitro for the application of NIPD. The nucleus should be retained within the cytoplasm before a genetic diagnosis can be performed and therefore ways of delaying the enucleation process may be needed if enucleation does occur in vitro. Recent studies on mouse definitive erythroblasts showed that histone deacetylation induced by histone deacetylase (HDAC) played an important role in definitive erythroblast nuclear condensation and enucleation [Popova et al., 2009; Ji et al., 2010]. When HDAC inhibitors such as trichostatin A was added to mouse definitive erythroblasts, nuclear condensation and enucleation were inhibited. Therefore one might ask will HDAC inhibitors delay the enucleation of primitive erythroblasts as well. Our preliminary studies showed that HDAC inhibitors decondense the nucleus of the human primitive erythroblast and may play an important role in enucleation. However, the mechanisms behind primitive erythroblast enucleation may be different from that of the definitive erythroblast.

EX VIVO CULTURE OF PRIMITIVE FOETAL ERYTHROBLASTS (pFE)

For many years the ex vivo expansion in culture of foetal cells from maternal blood has been attempted by many groups to provide adequate cell numbers for reliable NIPD (Table I). Foetal erythroid precursor cells, HMCs, mesenchymal stem cells and endothelial precursor cells were used for such expansion culture because they had the potential to proliferate ex vivo. However, two major challenges exist in such culture expansion, (1) the enrichment of the few target foetal cells from the large number of maternal blood cells and (2) developing the optimal culture system that will favour the growth of foetal cells over maternal cells. When the sex of the cultured cells was examined using fluorescent in situ hybridisation (FISH), PCR and foetal haemoglobin staining techniques, most of the expanded cells turned out to be of maternal origin despite the various culture systems used [Chen et al., 1998; Han et al., 1999; Bohmer et al., 2002]. This was most likely due to the scarcity of foetal cells in the maternal blood. A specific marker for distinguishing the foetal cells from maternal cells is therefore critical in the enrichment step. An optimal combination of cytokines allowing foetal over maternal cell growth needs to be developed.

Thus far no study has been done on the ex vivo culture of first trimester human primitive erythroblasts from maternal blood. The reasons for this may be that human primitive erythroblasts are more

TABLE I. Ex Vivo Culture of Foetal Cells From Maternal Blood for Non-Invasive Prenatal Diagnosis (NIPD)

Refs.	Cell type	Gestation in weeks	No. of samples	Assays
Lo et al. [1994]	Erythroid cells	11–20	5	FISH
Valerio et al. [1996]	BFU-E, CFU-E	14–16	8	PCR, FISH, HbF
Little et al. [1997]	CD34+ cells	10–13	42	FISH
Valerio et al. [1997]	BFU-E, CFU-E, CFU-GEMM	17–22	7[a]	FISH
Chen et al. [1998]	BFU-E, CFU-E	9–17	16	PCR, FISH, HbF
Han et al. [1999]	CD71/GPA+ cells	10	1	PCR, HbF
Bohmer et al. [1999]	Nucleated red cells	18	1	FISH
Jansen et al. [2000]	CD34+ cells	7–16	65	FISH
Valerio et al. [2000]	Erythroid cells	19	1[a]	FISH
Huber et al. [2000]	Erythroid cells	12, 17	26	PCR, FISH, HPLC
Tutschek et al. [2000]	BFU-E, CFU-GM	14–20	14	PCR
Coata et al. [2001]	CD34+ cells	11–16	31	PCR, FISH
Han et al. [2001]	Erythroid cells	8–14	10	PCR, HbF
Campagnoli et al. [2002]	CD34+ cells	10–40	49	FISH
Zimmermann et al. [2002]	Erythroid cells	13–41	16	PCR
Manotaya et al. [2002]	CD34+ cells	5–21	17	PCR, FISH
Gussin et al. [2002]	Endothelial precursor cells	13–26	13	FISH
Bohmer et al. [2002]	Nucleated red cells	11–25	25	PCR, FISH
Donoghue et al. [2003]	Foetal MSC	7–13	20	FISH

[a]Trisomy.

predominant in early gestation [Choolani et al., 2001, 2003], and the knowledge generated for this cell type is limited. How the human primitive erythroblast behaves ex vivo is not known but from what is already known in murine primitive erythropoiesis, the primitive erythroblast at an early differentiation stage is able to proliferate for a few cycles in order to give rise to more mature daughter cells [Palis et al., 2010]. Thus, the cell cycle profile of primitive erythroblasts at different weeks of gestation and the percentage of cells at various mitotic phases that can be analysed need to be studied. With such information, it is possible to know at what gestational age primitive erythroblasts have more proliferation potential for ex vivo culture for NIPD.

Use of mitogens. Mitogens are chemical substances that encourage a cell to commence cell division. Usually they trigger signalling pathways such as the mitogen-activated protein kinase (MAPK) pathway which leads to mitosis. Concanavalin A, phytohaematoglutinnin and pokeweed mitogen are common mitogens routinely used to stimulate lymphocyte proliferation in clinical laboratory medicine. Upon exposure to these mitogens, lymphocytes are activated and able to divide again and produce immune responses. Since pFEs are also of a blood lineage like lymphocytes it would be interesting to examine the effects of these mitogens on FEs to stimulate mitosis and increase cell numbers for NIPD. Some signalling pathways may be triggered and as a result these mitogens may improve the proliferative potential of the human pFE. Our preliminary studies showed that pokeweed mitogen could extend the viability of FEs significantly in vitro.

Micromanipulation and microdroplet culture of primitive foetal erythroblasts (pFEs). Besides separation methods using density gradients and flow sorting to enhance enrichment and purity of FEs from maternal blood, the micromanipulation methods used in IVF programs to pick-up single spermatozoa with micropipettes under the microscope for intracytoplasmic injection into oocytes (ICSI) would be a novel method of separating pFEs from maternal cells. We successfully developed an efficient system using a Zeiss–Narashige micromanipulation system and 20 μm bore micropipettes to pick up individual pFEs. Additionally, we used 10 μl microdroplets of culture medium under sterile mineral oil (Sigma Chemical Co, MO) to house and observe single pFE enucleation and monitor their cell behaviour and growth in vitro. The microdroplets of culture medium contained pure populations of pFEs (Fig. 1). Their growth behaviour and number can be accurately tracked over time because there is no contamination of other cell types which are commonly present in the blood. With this microdroplet culture system, the effects of different gas environments, medium composition, mitogens and growth factors on FEs can be studied so as to develop the optimal culture environment for the expansion of primitive FEs.

DOES THE PRIMITIVE FOETAL ERYTHROBLAST (pFE) HAVE STEMNESS PROPERTIES?

In the adult bone marrow, HSCs are able to self-renew and differentiate into all the blood lineages. When the specific differentiation pathway is decided for HSCs towards an erythroid lineage, the downstream progenitor cells are also capable of self-renewal, but in a more restricted way as they continue to further differentiate. In adult definitive erythropoiesis, immature erythroid-restricted progenitors called erythroid burst-forming units (BFU-E) have a better proliferative ability than late-stage erythroid progenitors [erythroid colony-forming units (CFU-E)]. CFU-E subsequently differentiate into a cascade of erythroid precursors which undergo three to four cell divisions as they progress from proerythroblast to basophilic, polychromatophilic and orthochromatic erythroblast stages. Orthochromatic erythroblasts finally enucleate to produce reticulocytes which enter the blood stream and soon become erythrocytes. Interestingly, a type of definitive erythroid precursor cell derived from early mouse embryos (yolk sac and foetal liver) was recently found to have extensive proliferation potential ex vivo, much better than their adult counterparts [England et al., 2010]. In the same study, erythroid precursors from primitive erythropoiesis were also cultured but found not to be proliferative.

Although primitive erythropoiesis was suggested to originate from the haemangioblast, the maturation process of primitive

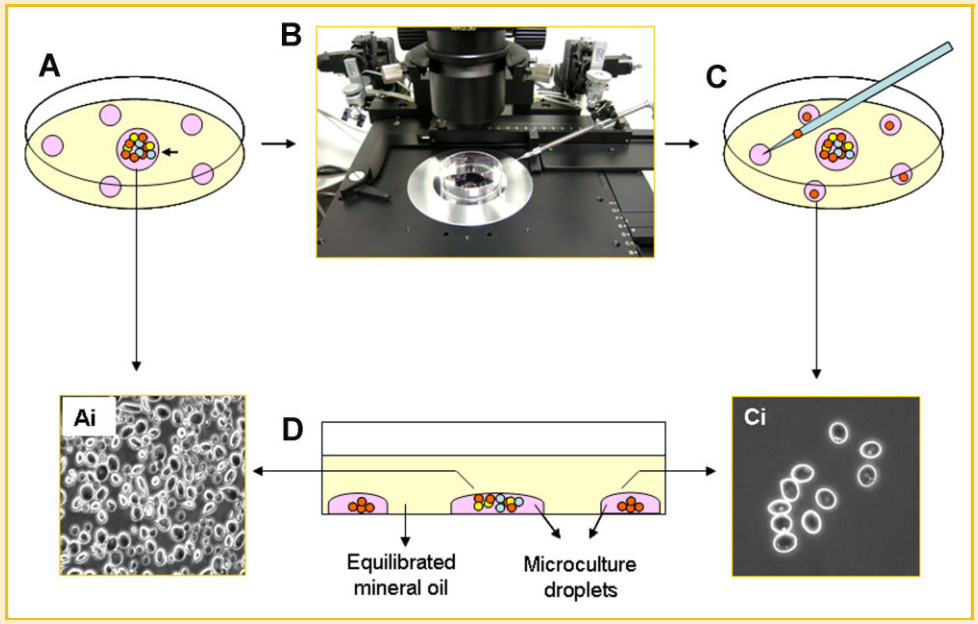

Fig. 1. Micromanipulation–microdroplet culture methodology developed for isolation of primitive fetal erythroblasts (pFEs) from a chorionic villus sample. A: Heterogeneous population of cells in a CVS lying in the centre large droplet (arrow) which was previously subjected to separation with a Percoll gradient. Ai: Magnified phase contrast image of the heterogeneous cell population before micromanipulation. B, C: pFEs from the CVS in the large centre droplet are individually picked up using a 20 μm bore micropipette and Zeiss–Narashige micromanipulator under the microscope, and transferred to 10 μl peripheral microculture droplets under sterile mineral oil to obtain homogenous populations of pFEs. Ci: Magnified phase contrast image of pFEs in the peripheral microculture droplets that can be individually monitored. D: Cross-sectional view showing the microculture droplets of medium in a large Petri dish (60 mm) covered by a thin layer of equilibrated mineral oil. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

erythroid cells is similar to that of definitive erythropoiesis as shown in the murine model [Palis et al., 2010]. Human primitive erythropoiesis is not well studied but may share many common features with the mouse model. Nevertheless, it is not clear yet whether human primitive erythroblasts undergo the same differentiation process as mouse primitive erythroblasts which eventually enucleate to form reticulocytes. The self-renewal potential of human primitive erythroblasts has also not been explored probably due to ethical concerns and difficulty of accessing such cells. Given the fact that morphologically, FEs have varied nuclear and cytoplasmic sizes like hESCs and the fact that they are blast cells, it would be important to investigate whether they possess stemness properties. It thus may be useful to investigate the presence of stemness-related surface marker antigens and CD marker profiles on the human primitive erythroblasts. There is a whole battery of characteristic markers for identifying stem cells with long-term self-renewal property. Since primitive erythroblasts originate from the mesoderm of the early embryonic yolk sac membrane, markers of hESCs, mesenchymal stem cells and HMCs should be evaluated on this cell type. hESCs have typical surface marker antigens such as SSEA3, SSEA4, Tra-1-60 and Tra-1-81 and nuclear antigens such as OCT4, SOX2 and NANOG. HMCs are positive for CD34, CD45 and CD117 while mesenchymal stem cells markers vary from one cell type to another but generally they are positive for CD73, CD90, CD105, and negative for CD45, CD34 and CD14. In terms of transcription profile, hESCs have high expressions of OCT4, SOX2, NANOG, LIN28, c-MYC and KLF4, all of which help maintain self-renewal capacity and pluripotency. Increased alkaline phosphatase and telomerase levels are also two markers indicative of stemness in embryonic cells. After checking all these stemness markers on human primitive erythroblasts it may be possible to identify which differentiation stage it belongs to in the primitive haematopoiesis process and whether it is a progenitor cell that can self-renew and differentiate further or whether it is already a terminally differentiated cell.

NUCLEAR REPROGRAMMING OF PRIMITIVE FOETAL ERYTHROBLASTS (pFEs)

Viral and non-viral reprogramming. A recent breakthrough in the field of stem cell biology has been the generation of induced pluripotent stem cells (iPSC) from somatic cells by ectopic expression of a battery of four genes viz., KLF4, OCT4, SOX2 and c-MYC (KOSM factors) [Takahashi et al., 2007]. It is now clear that differentiated somatic cells can be reverted back to the embryonic state by using nuclear reprogramming techniques including somatic cell nuclear transfer (SCNT), cell fusion, cell-free extracts and the iPSC approach. In nuclear reprogramming, the cell DNA content will not be lost during cell differentiation, but the transcription status of all the genes change dynamically and constantly by epigenetic modifications in order to suit the functional role the cell plays at a particular developmental stage and in a particular location in the body. After the report of Takahashi et al. [2007], another group later showed that by using LIN28 instead of KLF4 in the KOSM factors the same iPSCs could be generated from somatic cells [Yu et al., 2007]. These six genes used for production of iPSCs play essential roles in maintaining the pluripotency and indefinite self-renewal potential in embryonic stem cells. In the differentiated cells, these genes may

have been shut down or expressed at very low levels to avoid unnecessary cell potencies. By introducing these active genes back into the cell, they will be transcribed and translated to active transcription factors that act on the cell genome and activate certain gene expressions, thus changing the transcription profile of the cell and reverting it back to an embryonic state. Initially, retroviral and lentiviral vectors carrying the reprogramming factors were used to generate iPSCs because of their higher gene delivery efficiency compared to non-viral transfection methods. However, viral integration in the host cell genome may lead to insertional mutagenesis which poses a big concern for clinical application. Soon after the success of producing iPSC from several types of somatic cells, non-viral approaches were rapidly developed to avoid the use of viruses for iPSC generation. In the recently devised piggyback transposon/transposase system, transgenes flanked by piggyBac terminal repeats were inserted into the host genome and removed after the pluripotency was established [Woltjen et al., 2009]. Here, there was no residual genomic integration in the host cell and thus the host cell genome was not changed but activated to the embryonic state. Transient repeated transfection of cells with only plasmids has also resulted in iPSC generation although the efficiency was extremely low [Okita et al., 2010]. Other than transgene-based methods, direct delivery of proteins [Kim et al., 2009; Zhou et al., 2009] and messenger RNA encoding the transcription factors [Yakobov et al., 2010] were also able to generate iPSCs.

Use of small molecules for reprogramming. Interestingly, many small molecules have been shown to be able to enhance the efficiency of iPSC generation significantly [Li and Ding, 2010]. For example, using the small molecules SB431542 and PD0325901 to inhibit TGF and MAPK/ERK pathways, human fibroblasts could be reprogrammed with the KOSM factors with 200-fold enhanced efficiency [Lin et al., 2009]. Some of the small molecules can even replace one or more of the reprogramming factors. For example, human fibroblasts were reprogrammed to iPSCs with only OCT4, SOX2 and addition of valproic acid (VPA) [Huangfu et al., 2008]. VPA, a HDAC inhibitor, can increase histone acetylation thus activating the transcription of certain genes whose promoters were previously repressed due to histone deacetylation. In a recent study, neonatal human keratinocytes were reprogrammed to iPSCs with only Oct4 and a cocktail of chemical compounds that modulate epigenetic status, cellular metabolism and signalling pathways [Zhu et al., 2010]. Interestingly, this study indicated that a metabolic switch to anaerobic glycolysis which is mainly used by pluripotent cells is important for reprogramming somatic cells to the pluripotent state. Small molecules that modulate different aspects of cellular activities may eventually lead to the goal of reprogramming cells with only chemical molecules without transgenes.

Efficiency of reprogramming. Besides safety issues, reprogramming efficiency of iPSC technology is another big concern when considering its clinical applications. Viral-mediated reprogramming methods have higher efficiency than non-viral methods, but yet it can only reach up to 1% efficiency with the help of small molecules. Non-viral approaches are more ideal but overall have very low efficiency ranging from 0.001% to 0.01% [Kiskinis and Eggan, 2010]. The efficiency of reprogramming also depends on the target cell type. Less differentiated cells such as progenitor cells and stem cells can be reprogrammed at a much higher efficiency compared to terminally differentiated cell types such as adult skin fibroblasts and lymphocytes from peripheral blood. This is probably due to the fact that in a fully differentiated cell, more features that are required for differentiation need to be changed before the cell can go back to a non-differentiated state. Based on current knowledge, iPSC reprogramming itself is a stochastic process, but amenable to acceleration [Hanna et al., 2009]. The use of small molecules may greatly enhance reprogramming efficiency by pre-setting the target cells to a more reprogrammable status which allows the immediate effects of the reprogramming factors on the cells.

Cell reprogramming for non-invasive prenatal diagnosis (NIPD). As iPSC technology advances, safer reprogramming strategies targeting a broader range of cell types with higher efficiencies will be developed in the near future. It will be beneficial in the field of foetal medicine if the iPSC technique can be applied to foetal cells. Foetal cells from amniotic fluid and chorionic villi have been reprogrammed to iPSCs successfully [Ye et al., 2010] which in turn can be differentiated into desirable tissues for intra-uterine foetal therapy. Additionally, foetal cells may have a higher reprogramming efficiency to iPSCs or require a lesser number of reprogramming factors than adult cells due to minimal somatic mutations and higher endogenous expression levels of the reprogramming factors. iPSC generation from human blood has gained much interest recently because blood is readily available and the collection procedure is not so invasive. iPSCs have been successfully generated from HMCs, T lymphocytes and myeloid cells [Staerk et al., 2010] but not from erythroid cells (Table II). In the setting of NIPD using foetal cells from maternal blood, if the small number of enriched foetal cells could be reprogrammed to iPSCs (Fig. 2), cell numbers would no longer be a problem for performing a reliable diagnosis as the ensuing iPSCs will be proliferative and at the same time can be differentiated for cell-based therapies. Since the pFE is a good target cell for NIPD, it may be useful to apply

TABLE II. Generation of Induced Pluripotent Stem Cells (iPSCs) From Blood Cells Using Viral Reprogramming Methods

Cell type	Reprogramming factors	Method of delivery	Efficiency (%)	Refs.
CD34+ cells from mobilised human peripheral blood	Oct4, Sox2, c-Myc, Klf4	Retroviral vectors	0.01–0.02	Loh et al. [2009]
Human cord blood-derived endothelial cells	Oct4, Sox2, Nanog, Lin28	Lentiviral vectors	0.01–0.03	Haase et al. [2009]
Human cord blood-derived CD133+ cells	Oct4, Sox2, c-Myc, Klf4	Retroviral vectors	0.002–0.007	Giorgetti et al. [2009]
Mouse mature B lymphocytes	Oct4, Sox2, c-Myc, Klf4, c/EBPa	Doxycycline-inducible lentiviral vectors	0.01–0.1	Hanna et al. [2008]
Human T lymphocytes from peripheral blood	Oct4, Sox2, c-Myc, Klf4	Doxycycline-inducible polycistronic lentiviral vectors	0.0002–0.001	Staerk et al. [2010]

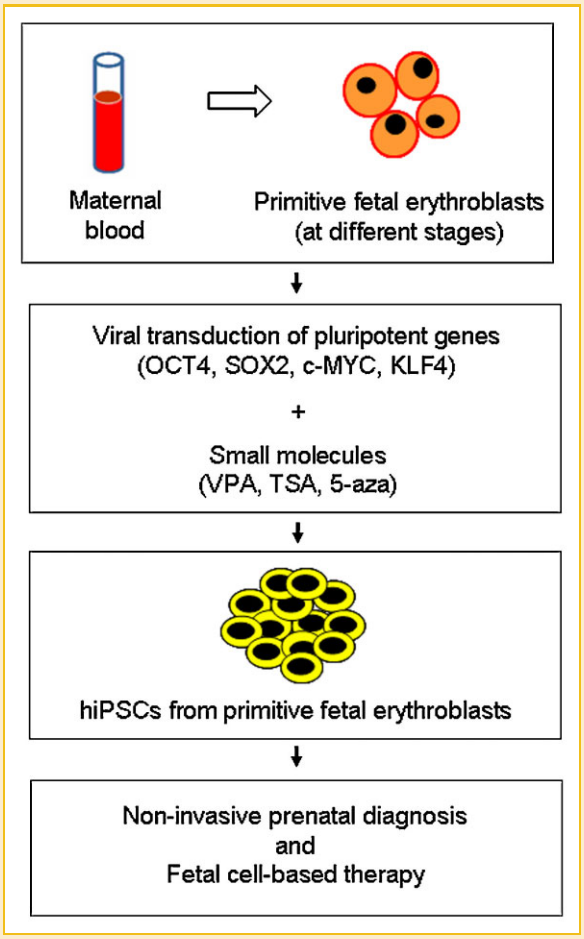

Fig. 2. Schematic representation of human induced pluripotent stem cells (hiPSCs) generated from primitive fetal erythroblasts (pFEs) using pluripotent genes and small molecules. VPA: Valproic acid; TSA: Trichostatin A; 5-aza: 5-azacytidine. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

various iPSC methods on it to see whether it could be reprogrammed and its numbers could be amplified via the generation of actively dividing hESC-like colonies. There would be technical challenges in producing iPSCs from small numbers of pFEs given the overall low reprogramming efficiency. As reprogramming technology becomes more and more efficient, refined and established, manipulating foetal cells in the maternal blood for both NIPD and foetal cell-based therapy may become a reality in the future.

CONCLUSIONS

NIPD of foetal genetic disorders and aneuploidies has become an important goal for routine prenatal care in the near future. Both cell-free foetal DNA and foetal cells present in maternal blood are being explored extensively for its application for NIPD in the clinic. Although foetal DNA has been used for foetal sex determination, RhD blood typing and diagnosing some single gene disorders, it cannot provide accurate results for foetal aneuploidies and maternally inherited diseases. Foetal cells in the maternal blood will still be the ideal target cell for NIPD despite the many difficulties of enriching and identifying them. The novel approaches suggested for manipulating first trimester foetal primitive erythroblasts in this article may provide additional exploratory strategies for use of the same cell type for NIPD. Studying the nature and properties of human primitive erythropoiesis will enrich our knowledge on this special phenomenon. A better understanding of the FE behaviour ex vivo will give us clues to expand and make them feasible for NIPD. By using some of the recent stem cell technologies such as iPSC generation, foetal cells in maternal blood may be reprogrammed to become pluripotent stem cells with proliferative potential that will hold great promise not only for NIPD but also for other clinical applications such as intra- and extra-uterine foetal therapies.

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