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Recent advances in non-invasive prenatal DNA diagnosis through analysis of maternal blood

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Abstract

Prenatal diagnosis of aneuploidy and single-gene disorders is usually performed by collecting fetal samples through amniocentesis or chorionic villus sampling. However, these invasive procedures are associated with some degree of risk to the fetus and/or mother. Therefore, in recent years, considerable effort has been made to develop non-invasive prenatal diagnostic procedures. One potential non-invasive approach involves analysis of cell-free fetal DNA in maternal plasma or serum. Another approach utilizes fetal cells within the maternal circulation as a source of fetal DNA. At the present time, fetal gender and fetal RhD blood type within RhD-negative pregnant women can be reliably determined through analysis of maternal plasma. Furthermore, genetic alterations can be diagnosed in the maternal plasma when the mother does not have the alterations. However, the diagnosis of maternally inherited genetic disease and aneuploidy is limited using this approach. Non-invasive prenatal diagnosis through examination of intact fetal cells circulating within maternal blood can be used to diagnose a full range of genetic disorders. Since only a limited number of fetal cells circulate within maternal blood, procedures to enrich the cells and enable single cell analysis with high sensitivity are required. Recently, separation methods, including a lectin-based method and autoimage analyzing, have been developed, which have improved the sensitivity of genetic analysis. This progress has supported the possibility of non-invasive prenatal diagnosis of genetic disorders. In the present article, we discuss recent advances in the field of non-invasive prenatal diagnosis.

Key words: cell-free DNA, fetal cell, maternal blood, non-invasive prenatal diagnosis, nucleated erythrocyte.

Introduction

Prenatal diagnosis is an established part of modern obstetrics. In the absence of prenatal diagnosis, 1 in 50 babies are born with serious physical or mental handicaps, and as many as 1 in 30 have some form of congenital malformation.[1] This may be the result of structural or chromosomal abnormalities, or single-gene disorders. In the 1950s, genetic counseling was the only modality available. For prenatal diagnosis, at that time, couples could be provided with a recurrence risk in the few recognized Mendelian conditions, but otherwise no diagnosis could be made, and no intervention was possible. In 1968, this situation changed when amniocentesis started being performed for diagnostic purposes. In 1984, first-trimester prenatal diagnosis via chorionic villus sampling (CVS) was shown to be a feasible alternative and as safe as amniocentesis.[2] In fact, over the past 25 years a number of methods for prenatal diagnosis of genetic disorders have become available and are used in laboratory research and clinical genetics. Prenatal diagnosis of aneuploidy and single-gene disorders is usually performed by collecting fetal samples through

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amniocentesis or CVS. However, both of these procedures are associated with some degree of risk to the fetus and/or mother. For example, abortion owing to hemorrhage or infection occurs in 0.2–0.4% of pregnancies in which amniocentesis is performed. Furthermore, CVS carries a potential risk of fetal limb malformation in 0.01–0.03% of cases.[3] Prenatal diagnosis is thus generally considered when the perceived risk of an abnormal pregnancy as estimated by maternal age, maternal serum biochemistry and/or fetal ultrasonography, outweighs the procedure-related miscarriage risk. Therefore, recent efforts have been directed toward less invasive approaches for prenatal diagnosis. Currently, the most utilized strategy for detecting aneuploidy is mid-trimester maternal serum screening using human chorionic gonadotrophin (hCG), estriol (E3), and alpha-fetoprotein (AFP), followed by amniocentesis in screen-positive cases. The serum tests detect approximately 60% of cases of trisomy 21 with a calculated false positive rate of 5%.[4] Although addition of another marker, such as diametric inhibin A, increases the sensitivity to 75%,[5] serum testing still does not provide a definitive diagnosis; rather, it estimates a woman's adjusted (or posterior) risk of various chromosomal aneuploidies. Another method by which to screen for fetal trisomy 21 is sonography to measure the thickness of a fluid-filled space behind the fetal neck, otherwise known as the nuchal translucency (NT) measurement. In a study of 96 127 singleton pregnancies, combining the risk of maternal age with the NT measurement enabled detection of 77% of fetuses with trisomy 21 with a 5% false positive rate.[6] Furthermore, ultrasound detected an absence of the nasal bone in approximately 65% of fetuses with trisomy 21 and in 1% of fetuses with a normal karyotype.[7] Combining absence of the nasal bone with first-trimester NT and serum screening scores resulted in detection of 90% of fetuses with Down syndrome with a false-positive rate of 5%.[8] However, while these approaches are useful in screening for Down syndrome, they have limited application in diagnosing fetal aneuploidy.

In recent years, considerable effort has been directed toward the development of non-invasive prenatal diagnostic methods. One potential non-invasive approach involves analysis of cell-free (extracellular) fetal DNA within maternal plasma or serum. The other approach utilizes fetal cells within the maternal circulation as a source of fetal DNA. In this article, we discuss recent advances in non-invasive fetal DNA diagnosis.

Fetal DNA in plasma and its use in prenatal DNA diagnosis

Human plasma has been regarded as an unlikely source of genetic material for a long time, except in the field of virology. In 1948, Mandel and Metais[9] reported the presence of nucleic acid within the plasma of both healthy and sick individuals. In the serum of patients with systemic lupus erythematosus,[10] rheumatoid arthritis,[11] and cancer,[12] high levels of circulating DNA have been reported. In 1996, Nawroz *et al*.[13] and Chen *et al*.[14] simultaneously reported DNA molecules with tumor-specific characteristics within the plasma and serum of patients with cancer, giving rise to the possibility of serum as a source of DNA. A new area of research followed the discovery of large amounts of circulating cell-free fetal DNA within maternal plasma and serum. In 1997, Lo *et al*. demonstrated that cell-free fetal DNA circulates within the plasma and serum of pregnant women.[15] Real-time polymerase chain reaction (PCR) was used to quantify the amount of fetal DNA by targeting SRY, a single-copy Y-chromosome-specific sequence, in pregnant women carrying male fetuses.[16] Surprisingly, high mean concentrations of fetal DNA (3.4–6.2%) in total maternal plasma DNA were demonstrated. A mean of 25.4 copies of fetal DNA in 1 mL of maternal plasma was detected in early gestation, a much higher concentration than that of fetal cells in maternal blood.[16] We also quantified fetal DNA in the plasma of 156 pregnant women at 7–16 weeks. A median of 36.5 genome-equivalents (GE) of fetal DNA was detected per milliliter of plasma.[17] Since the half-life of fetal DNA in maternal plasma is reportedly 16.3 min,[18] fetal DNA is a suitable target for prenatal DNA diagnosis without being affected by any previous pregnancies.

Fetal gender determination

Fetal DNA was initially used for determination of fetal gender[16,17] and fetal rhesus D (RhD) blood type.[19] The detection of Y-chromosome-specific sequences indicated a male fetus. Their absence indicated female sex. Tang *et al*. further positively identified female fetuses through detection of paternally inherited short tandem repeats (STR) on the X chromosome within maternal plasma.[20] Y-chromosome-specific sequences, such as SRY,[16] DYS14,[15,17] and DAZ[21] loci are commonly used to detect fetal gender. In 2001, we assessed fetal gender using maternal blood samples from 302 pregnant women at 7–16 weeks gestation, the largest sample size to date. The Y-chromosome-specific DYS14 sequence

was amplified using real-time quantitative PCR, based on which fetal gender was determined.[17] Fetal gender was determined in 298 of 302 cases with an overall sensitivity of 97.2%, specificity of 100%, and positive and negative predictive values of 100% and 97.5%, respectively, for identification of a male fetus.[17] Since the DYS14 sequence was then detected on re-analysis of plasma samples from all four false negative cases, fetal DNA is likely present within the plasma of all pregnant women, providing an accurate and reliable method for determination of fetal gender at ≥7 weeks gestation.[17] Identification of fetal gender via this non-invasive technique can reduce the number of invasive procedures required to examine X-linked genetic disorders. To limit invasive prenatal testing for identification of X-linked genetic diseases or ambiguous development of the external genitalia, non-invasive prenatal diagnosis of fetal gender using maternal plasma has been performed in pregnancies at risk for congenital adrenal hyperplasia and X-linked recessive disorders, such as Duchenne muscular dystrophy and hemophilia, and the usefulness and accuracy of this method thus demonstrated.[22–24]

Fetal rhesus D genotyping

RhD blood group incompatibility between a pregnant woman and her fetus is a significant problem given the potential for maternal alloimmunization and subsequent hemolytic disease of the newborn. The Rh^- blood group is found among 15% of Caucasians, 3–5% of black Africans, and is rare among Asian populations. As such, management of Rh^- pregnant women is an important part of prenatal practice. Fetal DNA within maternal plasma has been used to determine fetal RhD status in Rh^- pregnant women.[19] Unlike more conventional methods, such as amniocentesis and CVS, the risk of feto-maternal hemorrhage and further sensitization is removed using this method. Thus, it provides a basis for administration of anti-D immunoglobulin as prophylaxis only in pregnancies with a confirmed Rh^+ fetus, thereby preventing unnecessary administration of anti-D immunoglobulin.[25] Lo *et al*. accurately diagnosed fetal RhD blood type in 45 of 57 cases using plasma samples from Rh^- pregnant women obtained during the second trimester or later. They concluded that non-invasive fetal RhD genotyping can be performed rapidly and reliably using maternal plasma beginning in the second trimester of pregnancy.[19] As part of routine prenatal care, non-invasive diagnosis of fetal RhD genotype is already being performed in the UK, France, and the Netherlands.[26] Several studies regarding the accuracy of non-invasive prenatal RhD genotyping have thus surfaced in recent years using relatively large series of patients.[27–29] In one such study, a total 2838 Rh^- pregnant women were tested and the diagnosis was concordant in 97.4% of cases.[29] Thus, non-invasive prenatal diagnosis of RhD blood type can be accurately performed; however, there remain a small percentage of false-positive or false-negative cases.[26] Recently, we have learned more about the RhD locus and its variants. Although most Rh^- Caucasians have a deletion of the RhD gene, only 18% of Rh^- black Africans have the deletion, with 82% of Rh^- black Africans having one of two RhD variants, the RhD pseudogene (67%) or the RhD-Cde^S hybrid gene (15%).[30] These non-functional variants produce non-specific amplification of the RhD gene. False-negative cases are either due to a lack of fetal DNA in plasma during early gestation, or a lack of sensitivity in detecting low amounts of fetal DNA. At the present time, a large-scale clinical trial is being performed in the European Union to identify ways to reduce false-positive and false-negative results. Development of a newer method for determination of fetal RhD genotype, which takes into account racial differences, is expected. Prenatal determination of fetal RhD blood type might prove a clinically useful means by which to limit unnecessary testing or therapeutic intervention, including administration of antenatal anti-D immunoprophylaxis, by identifying Rh^- fetuses.

Diagnosis of fetal single-gene disorders

Fetal cell-free DNA in maternal plasma has also been used for prenatal diagnosis of single-gene disorders. In 2000, we achieved non-invasive prenatal DNA diagnosis of achondroplasia (ACH).[31] This was first report in which a fetal single-gene disorder was diagnosed using maternal plasma. ACH is the most common genetic form of dwarfism and is inherited as an autosomal dominant gene, although most cases are sporadic.[32] Shiang *et al*. demonstrated that >90% of ACH patients display a missense mutation in the same locus of the transmembrane domain of a fibroblast growth factor-receptor 3 (FGF-R3) gene, comprising a G–A or G–C transition at nucleotide (nt) 1138.[32] We investigated prenatal DNA diagnosis of ACH using plasma from a woman at 30 weeks of gestation whose fetus displayed a suspected short-limb disorder on ultrasonography. Maternal plasma DNA was extracted and the FGF-R3 sequence with nt1138 for ACH was amplified by PCR using specific primers and conditions.[32] The PCR products were then restricted using *Sfc*I to detect a G–A transition at nt1138. The ACH mutation creates a *Sfc*I

site, resulting in two extra bands of 111 and 55 bp upon digestion. Since this revealed the mutant allele within DNA from plasma of the pregnant woman, we established that the fetus of this pregnant woman had the G–A transition at nt1138 and was therefore affected by ACH.[31] Another group has reported prenatal diagnosis of ACH using cell-free DNA in maternal plasma.[33] Recently, Amicucci *et al*. were able to establish a prenatal diagnosis of myotonic dystrophy using maternal plasma.[34] Furthermore, prenatal exclusion of cystic fibrosis,[35,36] beta-thalassemia[37–39] and congenital adrenal hyperplasia,[40] has been described in at-risk pregnancies through analysis of fetal DNA in maternal blood.

Tsui *et al*. have developed a method for quantitative mass spectrometric analysis of fetal single-nucleotide differences, which may permit the diagnosis of single-gene disorders. This method may be a potential sensitive approach. Poon *et al*.[41] have further developed an epigenetic approach to identify a single nucleotide polymorphic site within a region at the IGF2-HI9 locus which is methylated when the allele is maternally inherited and unmethylated when the allele is paternally inherited.

A fetus-derived mutant gene could thus be detected in maternal plasma for prenatal diagnosis of single-gene disorders caused by paternally inherited genes or mutations that are distinguishable from maternally inherited counterparts. However, this plasma DNA approach only allows identification of disorders in which the gene of interest is present in the fetal genome and absent from the maternal genome. Thus, the use of fetal DNA for the diagnosis of single-gene disorders using this method is limited.

Altered fetal DNA concentrations in some complications of pregnancy

Fetal DNA concentrations are affected by various conditions of pregnancy. Some researchers have evaluated alterations in fetal DNA concentrations to see if they might be predictive of complications of pregnancy or fetal aneuploidies.

Pre-eclampsia

Lo *et al*. have reported elevated fetal DNA concentrations in the plasma of pregnant women with pre-eclampsia, and have concluded that circulating DNA levels might be useful as a marker for diagnosing and/or monitoring this condition.[42] Zhong *et al*. have also reported elevated levels of cell-free fetal and maternal DNA in the plasma of pregnant women with pre-eclampsia.[43] Likewise, we observed increased fetal DNA levels in the plasma of pregnant women with pre-eclampsia, but not in the plasma of pregnant women with fetal growth restriction (FGR) without pre-eclampsia.[44] However, increased fetal DNA levels have been reported in a select population of pregnant women in which abnormal uterine arteries were identified on Doppler ultrasonography.[45]

The pathogenesis of pre-eclampsia is poorly understood, but it is likely associated with failure of the uterine vasculature to undergo adequate physiological remodeling by extravillous trophoblasts.[46] Insufficient invasion of extravillous trophoblasts into the uterine vasculature results in a failure of placental vascular resistance to decrease, causing inadequate oxygenation of blood within the placental intervillous spaces bordering the villous trophoblasts. This may damage the villous trophoblasts, resulting in the release of DNA due to cell damage or apoptosis into the intervillous spaces, and from there into the maternal circulation.[47] Moreover, Lau *et al*.[48] have demonstrated impaired clearance of fetal DNA from maternal plasma in pre-eclampsia. However, the exact mechanism by which a quantitative increase in plasma DNA occurs requires further investigation.

Since fetal DNA in maternal plasma might be a marker of placental damage, we have previously examined whether fetal DNA levels in maternal plasma might be associated with the severity of pre-eclampsia. Estimated fetal DNA concentrations were 2.25- and 5.06-fold greater among patients with mild and severe pre-eclampsia, respectively, compared to controls.[49] We have also examined the relationship between proteinuria and hypertension and fetal DNA levels. We found that both proteinuria and hypertension were independently and strongly associated with increased fetal DNA concentrations in maternal plasma, and that proteinuria was associated with greater increases in fetal DNA levels than hypertension.[49] These findings support previous reports indicating that proteinuria is more closely associated with FGR or placental dysfunction than hypertension in pre-eclampsia. Total DNA concentrations in maternal plasma were further assessed by analyzing the beta-globin gene, revealing that beta-globin levels in the plasma of pregnant women with pre-eclampsia were fourfold higher than in controls.[50] Among women with pre-eclampsia, the values of cases with FGR were almost double those of cases without FGR.[50] These findings indicate that beta-globin values in maternal plasma are associated with the severity of pre-eclampsia.

Leung *et al*. have reported elevated fetal DNA concentrations in the plasma of pregnant women prior to the development of pre-eclampsia.[51] We have also reported fetal DNA levels approximately 2.39-fold greater among pregnancies that went on to develop pre-eclampsia, compared to matched controls (at about 20 weeks of gestation).[52] Furthermore, beta-globin levels in the plasma of pregnant women around 20 weeks prior to the development of clinical symptoms of pre-eclampsia were approximately double those of controls.[53] When fetal DNA, total DNA, or both values were used to predict the development of pre-eclampsia, detection rates of 33%, 46% and 62% were achieved, respectively, with an overall false positive rate of 5%.[53] Plasma DNA concentrations can thus potentially be used for early prediction of pre-eclampsia.

Hyperemesis gravidarum

We have previously investigated whether increased fetal DNA levels might be detected in the maternal plasma in cases of hyperemesis gravidarum (HG), since hyperactivation of the maternal immune system is thought to destroy trophoblast cells. We therefore examined the relationship between fetal cell-free DNA concentrations and clinical severity of HG among women with varying severities of HG: mild HG (nausea and vomiting, but no need for hospitalization); moderate HG (admission required for intravenous hydration but absence of any of the criteria used to define severe HG); and severe HG (admission for HG with ketonuria $>3^{+}$ based on urine dipstick test and weight loss >3 kg). Blood samples were obtained from 45 patients with HG for quantification of fetal DNA concentrations. Fetal DNA levels in mild, moderate and severe HG were found to be 1.26-, 1.62-, and 2.41-fold greater, respectively, than controls.[54,55]

Although the pathogenesis of HG remains obscure, Minagawa *et al*. have reported that functional activation of natural killer and cytotoxic T cells is more prominent in the blood and uterine decidua of women with HG than in women with uncomplicated pregnancies.[56] Hyperactivation of the maternal immune system may be responsible for the onset of HG, probably while maternal immune tolerance to the semiallograft is being established. As the primary source of fetal DNA is thought to be placental trophoblasts, this theory is concordant with the finding that the severity of HG is related to fetal DNA levels.[55]

Invasive placenta (abnormal adherence of the placenta)

We have also speculated that invasion of trophoblasts into the uterine musculature of patients with invasive placenta might result in increased concentrations of cell-free fetal DNA within maternal plasma, as trophoblasts might be destroyed by the maternal immune system upon invasion of the myometrium. Fetal DNA levels were thus assessed in two patients with invasive placenta. Concentrations of fetal DNA in both cases were greater than in gestational age-matched controls. This finding suggests that antenatal detection of invasive placenta might be achievable through analysis of fetal DNA concentrations in maternal plasma.[57] Furthermore, we have also reported on a case of placenta increta, in which a small part of the placenta remained adherent despite manual removal of the placenta at the time of delivery. The patient was followed up by monitoring plasma concentrations of beta-human chorionic gonadotrophin (β-hCG) and fetal DNA levels after delivery.[58] In this patient, fetal DNA was detected until 10 weeks after delivery, whereas plasma β-hCG could not be detected after 11 days postpartum. Intermittent vaginal bleeding continued until fetal DNA was no longer detected in plasma. This finding suggests that concentrations of fetal DNA in plasma might provide a useful marker by which to follow patients with retained placental tissue after delivery.[58] These findings also support the placenta as an important source of cell-free DNA in maternal plasma.

Preterm delivery

Leung *et al*. have reported that fetal DNA is present in greater concentrations within the plasma of pregnant women who undergo preterm labor, compared to those who deliver at term.[51] This finding has recently been confirmed in a high-risk population by Farina *et al.*, with early preterm delivery (<30 weeks) more likely to occur (with a cumulative probability of 45%) among women with increased fetal DNA concentrations, compared to only 14% for women with lower plasma fetal DNA values.[59]

Fetal aneuploidy

Lo *et al*. have reported elevated concentrations of cell-free DNA in the plasma and serum of pregnant women with a trisomy 21 fetus.[60] Zhong *et al*. have also reported significantly elevated fetal DNA levels in pregnancies with trisomy 21, but not in those with trisomy 18.[61] In a matched case–control study, Farina *et al*. have confirmed increased fetal DNA levels in

maternal serum from the second trimester in cases of Down syndrome with a detection rate of about 20% and a false positive rate of 5%.[62] However, Ohashi *et al*. and Hromadnikova *et al*. have reported no differences in fetal DNA levels within maternal serum among pregnancies with fetuses of normal karyotype and those with trisomy 18 or 21.[63,64] Spencer *et al*. have observed elevated total (maternal and fetal) DNA concentrations, but not specifically fetal DNA concentrations, among the plasma of women carrying fetuses with abnormal karyotypes.[65] The reported link between elevated fetal DNA levels in maternal plasma or serum and aneuploidy thus remains controversial. If in fact fetal DNA levels in maternal plasma are significantly elevated in pregnant women with aneuploid fetuses, quantitative analysis of these levels may provide additional information in the detection of some fetal chromosomal abnormalities.

Origin of fetal DNA in maternal plasma or serum

The origin of fetal DNA remains controversial. Possible sources of fetal DNA in maternal plasma include: (i) destruction of fetal cells in maternal blood; (ii) transplacental transfer of fetal cell-free DNA; or (iii) destruction of villous trophoblasts bordering the intervillous spaces.

We have previously observed apoptotic changes in 43% of fetal nucleated red blood cells (NRBC) circulating within maternal blood, demonstrating that the increased oxygen concentration of maternal blood induces apoptotic changes in fetal NRBC transferred to maternal blood.[66,67] We thus speculate that apoptosis might be the mechanism by which fetal cells are cleared from the maternal circulation in the absence of a maternal immune response during pregnancy, and that fetal cells in maternal blood represent an important source of fetal DNA in maternal plasma. It has been reported that tumor cell death is associated with the release of tumor-derived circulating DNA.[68,69] Plasma DNA concentrations have been found to correlate with levels of circulating nucleosomes, which are characteristic by-products of apoptosis.[70] However, significantly reduced concentrations of fetal NRBC have been observed in maternal blood, compared to fetal DNA concentrations in maternal plasma.[16] The concentration of fetal DNA in maternal plasma has not been found to increase in plasma samples extracted from blood after 24 h[71] Furthermore, while elevated fetal DNA concentrations have been detected in women that undergo preterm labor,[51] elevated numbers of fetal NRBC in maternal blood are not observed.[72] These findings indicate that there is not a direct relationship between increased fetal cell numbers in maternal blood and elevated fetal DNA levels in maternal plasma. The majority of fetal DNA thus does not appear to originate from fetal cells transferred to maternal blood.

To assess the possibility of transplacental transfer of fetal cell-free DNA, we have also evaluated the bidirectional transfer of plasma DNA between the maternal and fetal circulations.[47] Cell-free fetal DNA concentrations in maternal plasma were found to far exceed maternal DNA concentrations in umbilical plasma, and cell-free maternal DNA concentrations within fetal blood were unaffected by pre-eclampsia, which is associated with trophoblastic damage and elevated fetal DNA concentrations within the plasma of pregnant women.[42,44,47,49] These findings suggest an unequal transfer of plasma cell-free DNA among the fetal and maternal circulations. We have thus concluded only a limited amount of cell-free fetal DNA is transferred from the fetal circulation to maternal plasma.

Another possibility is that trophoblasts are the primary source of fetal DNA. In the placenta, villous trophoblasts border the intervillous spaces, which are filled with maternal blood. In pre-eclampsia, insufficient invasion by extravillous trophoblasts is thought to result in hypoxic damage to villous trophoblasts, inducing apoptosis of villous trophoblasts.[73] Apoptosis is frequently detected by TUNEL staining of syncytiotrophoblasts within the placentas of women with pree-clampsia.[74] Cell-free and fragmented DNA is likely released into the intervillous spaces. Apoptosis of villous trophoblasts has been observed even in normal pregnancies and villous trophoblasts are very important for maintenance of placental function. Functional and structural characteristics of the placenta are likely responsible for the high concentrations of fetal DNA found in maternal plasma. Furthermore, as mentioned above, we have previously identified elevated concentrations of fetal DNA within the maternal plasma of women with placenta previa, particularly in those with invasive placenta.[57,58] In these cases, trophoblasts invading the myometrium are attacked and destroyed by the maternal immune system, resulting in increased levels of fetal DNA in maternal plasma. Furthermore, fetal DNA concentrations in maternal plasma increase as pregnancies advance,[16,75] and placental apoptosis increases with gestational age.[76] Circulating fetal DNA has also been found to correlate with maternal human chorionic gonadotrophin concentra-

tions,[54,77] which is produced by syncytiotrophoblasts. mRNA of placental origin has also been detected in maternal plasma, suggesting that fetal nucleic acids, at least in the form of RNA molecules, are released from the placenta.[78,79] Although the concentration of fetal cell-free DNA in maternal plasma has not been observed to correlate with first-trimester placental volume, as estimated by 3-D ultrasonography,[80] it remains likely that the majority of cell-free fetal DNA in the plasma of pregnant women originates from damaged trophoblasts.

Fetal Cells in the Maternal Circulation

Fetal gender and fetal RhD blood type in Rh^- pregnant women can be reliably determined by analyzing maternal plasma. Furthermore, genetic diseases where the mother does not have the genetic alteration can be diagnosed by analyzing maternal plasma. However, plasma analysis cannot be used for prenatal diagnosis of maternally inherited genetic diseases and aneuploidies. Another method of non-invasive prenatal diagnosis that does not have these limitations is analysis of intact fetal cells circulating in maternal blood. Since only a limited number of fetal cells circulate in maternal blood, procedures to enrich the cells and enable single cell analysis with high sensitivity are required. We outline below the progress made to date on examining fetal cells from maternal blood.

Fetal cell type

Trophoblasts

It has long been recognized that trophoblasts circulate in maternal blood. At the turn of the 19th century, German pathologist Schmorl demonstrated trophoblasts in the lungs of women who had died from eclampsia.[81] In 1959, Douglas *et al*. detected trophoblasts in maternal blood using light microscopy.[82] However, the limited number of trophoblasts normally present in maternal blood led to difficulty reproducing their findings. It is difficult to isolate trophoblasts since they are large multinucleated cells which become trapped in the lungs and are rapidly cleared from the maternal circulation,[83] even among women with hypertension in pregnancy. In these women, trophoblasts have been recovered from the uterine vein, inferior vena cava[84] and peripheral circulation.[85] Also, trophoblast cell trafficking does not commonly occur in pregnancy.[86] Furthermore, enrichment of trophoblasts is difficult due to a lack of specific antibodies.[87,88] Moreover, the karyotype of 1% of placental cells differs from that of the fetus due to confined placental mosaicism.[89,90] This also limits the use of trophoblasts in maternal blood as a tool for prenatal genetic diagnosis. However, despite these obstacles, some diagnostic success has been achieved through detection of Y chromosome sequences by PCR amplification and fluorescence *in situ* hybridization (FISH) techniques using trophoblasts from maternal blood.[91–93] Hawes *et al*. have also accurately detected a fetal beta-globin mutation in trophoblasts from maternal blood.[92]

Leukocytes

Through successful enrichment of leukocytes from the maternal circulation, it has been demonstrated that fetal leukocytes traverse the placenta. This has made non-invasive prenatal diagnosis possible. Although Schmorl discovered the presence of trophoblasts in the maternal circulation almost a century ago, the use of fetal cells for prenatal diagnosis was not considered feasible until 1969 when Walknoska *et al*. observed cells with a 46,XY karyotype in cultured lymphocytes from 21 pregnant women, 19 of whom subsequently delivered male infants.[94] This finding was later confirmed by other investigators in the early 1970s, who also demonstrated Y-chromatin within cells in the maternal circulation of women carrying male fetuses.[95–101] However, hundreds of maternal cells needed to be analyzed in order to document the presence of just a few fetal cells in the maternal circulation, a labor-intensive process. Thus, these studies required methods to enrich fetal cells for detection, including use of a nylon wool column and selective cell culturing.[102,103]

Herzenberg *et al*. were the first to use fluorescence-activated cell sorting (FACS) to successfully enrich fetal leukocytes from the maternal circulation.[104,105] This group showed a significant correlation between male gender of the fetus at birth and human leukocyte antigen HLA-A2 with detection of a quinacrine-positive Y body in flow-sorted cells. This method, which requires HLA-typing of the father, was validated in a subsequent study using PCR amplification of Y-chromosome-specific sequences after flow-sorting based on paternal HLA polymorphisms.[86] However, other investigators have had limited success using this method, even following selection based on several HLA differences using monoclonal antibodies. One study found only 18 HLA-informative couples out of 78 screened.[106]

Moreover, all observations to date suggest a persistence of fetal cells in maternal blood after delivery since

male DNA has been detected with high frequency among women with male offspring. Early interest in fetal leukocytes for prenatal diagnosis was based on their ability to proliferate *in vitro*, suggesting that they might also proliferate *in vivo* within maternal organs. However, this approach could produce incorrect diagnoses in cases of multigravida. An early study by Schroder *et al*.[107] determined that fetal leukocytes can still be detected in the maternal circulation up to 1 year after birth. This was determined by mitogen-stimulation of leukocytes in order to detect Y chromatin in a woman who had previously borne a son. This has been confirmed by others, in some instances, up to 5 years postpartum.[108–110] Moreover, Bianchi *et al*. have used FACS-enrichment of T and B lymphocytes to identify the presence of fetal progenitor cells (CD34+, 38+) in the maternal circulation 27 years postpartum.[111] In another study, T lymphocytes (CD3+, 4+, 5+) were found to persist, for as many as 6 years in one instance, after birth. These studies indicate that cell types with a short-life span should be used for prenatal diagnosis when examining fetal cells in maternal blood.

Fetal erythrocytes/nucleated red blood cells

In 1976, Kleihauer *et al*. demonstrated the presence of immature erythrocytes circulating in maternal blood using a new staining method.[112] In 1964, Clayton *et al*. observed NRBC more frequently under certain circumstances, such as rhesus incompatibility, or following amniocentesis and termination.[113] Since NRBC are one of the first hemopoietic cell lines produced during fetal development and are abundant in the fetal circulation during early pregnancy,[114] they are detectable early in pregnancy. When blood pools at the interface between fetal and maternal tissue, transfer of erythrocytes, including NRBC, into the maternal circulation predominates over other cell types, including leukocytes and trophoblasts. NRBC are mononuclear and relatively well differentiated. They also have a short lifespan compared to fetal lymphocytes[115] given their limited proliferative capacity, making them unlikely to persist throughout pregnancy. These characteristics make NRBC particularly suitable for non-invasive prenatal diagnostic testing.

In 1990, Bianchi *et al*. discovered a way to enrich NRBC containing fetal DNA for FACS using a monoclonal antibody against the transferrin receptor (CD71), which is highly expressed on erythroblasts.[116] The ability to do so has since been confirmed by other investigators using a variety of monoclonal antibodies and cell enrichment techniques.[117–120] One such method is magnetic cell sorting (MACS) using an antibody to CD71.[121] Ganshirt-Ahlert *et al*. have used MACS following NRBC enrichment to correctly identify fetal aneuploidy.[84,122] These successful attempts at NRBC enrichment and fetal determination demonstrate the potential of using NRBC for non-invasive prenatal diagnosis.

However, isolation of fetal cells from the maternal circulation presents considerable challenges, given their limited numbers. Fetal cells are estimated to range from 1 in 10^5 to 1 in 10^9 in maternal blood.[120,123] Hamada *et al*. used FISH on mononuclear cells isolated by density gradient separation from maternal blood to find Y-chromosome-bearing cells.[124] They needed to screen as many as 144 000 nuclei to find a single fetal cell containing DNA that hybridized to the Y chromosome. An increased frequency of fetal cell isolation with gestational age was observed, from less than 1 in 10^5 during the first trimester to 1 in 10^4 at term. Bianchi *et al*. then examined the number of fetal-cell DNA equivalents present in maternal blood by PCR amplification of a Y-chromosome-specific sequence and found approximately one fetal cell per 1 mL of maternal blood.[125] Thus, although the presence of fetal NRBC in maternal blood is well established and they are considered the best target for non-invasive prenatal diagnosis at the present time, their detection remains problematic.

Undoubtedly, some NRBC, even after fetal cell enrichment, are of maternal origin.[114,126–128] De Graff *et al*. have used fetal hemoglobin to differentiate maternal from fetal NRBC; however, 20% of all HbF-positive NRBC are still of maternal origin.[129] Recently, Troeger *et al*. used single cell PCR analysis on single micromanipulated NRBC identified by May–Grunwald Giemsa (MGG) staining to demonstrate that almost half of NRBC in maternal blood are of fetal origin.[130] These findings suggest that the origin of each cell must be confirmed for reliable clinical use when performing non-invasive prenatal diagnosis through analysis of cells recovered from maternal blood.

Another approach is to culture NRBC. If selective induction of NRBC proliferation occurs *in vitro,* fetal genetic material can be amplified for non-invasive prenatal diagnosis. Lo *et al*.[131] were the first to culture mononuclear cells and to isolate fetal erythroid progenitors from the peripheral blood of pregnant women. Subsequently, other investigators have successfully cultured colony-forming units, as well as erythroid and mature burst-forming units and erythroid colonies, from fetal hemopoietic progenitors enriched from maternal blood.[132] However, these results have not been repli-

cated by other investigators, and thus far selective amplification of fetal over maternal hemopoietic progenitors has not been successful.[133,134]

Physiological variation of the maternal circulation

Data indicating the timing of fetal cell trafficking into the maternal circulation exists. In mice, fetal cell migration is a rare event.[135] In general, it is thought that the proportion of NRBC to non-nucleated erythrocytes diminishes in fetal blood as gestation progresses in humans. In accordance with placental growth, the interface between fetal and maternal tissue expands, such that more fetal cells may traverse the placental barrier in the early stages of gestation. In a study of two pregnant women bearing male fetuses following *in vitro* fertilization, Y-chromosome-specific DNA was detected as early as 33 and 40 days gestation.[136,137] PCR amplification of a Y-chromosome-specific sequence has been achieved in maternal blood between 6 and 12 weeks in two separate studies.[124,138] Other studies using flow-sorted NRBC have demonstrated reliable detection of fetal DNA at less than 16 weeks gestation.[139]

It has been reported that more fetal cells are recovered when the fetus is aneuploid.[125,140] This might be associated with the ultrastructure of the placenta in pregnancies affected by aneuploidy.[141,142] It might also be associated with erythrocyte size, which differs from that in cytogenetically normal individuals of the same gestational age.[143] Aneuploid fetal cells express CD71[144] or FB3-2 and H3-3[145] antigens. Simpson and Elias demonstrated, using FISH analysis with chromosome-specific probes, that, on average, 19.6% of enriched cells from maternal samples were trisomic fetal cells (ranging from 0 to 74%).[146] Using the same method, Ganshirt-Ahlert *et al*. found that 10% of the final population of enriched cells were trisomic fetal cells.[122]

Increased proportions of fetal cells have been detected in women with pre-eclampsia, which follows historical observations by Schmorl[81] and Clayton *et al*.[113] Holzgreve *et al*. have also noted a large increase in the number of NRBC (38 *vs* 7) in male-bearing pregnancies with pre-eclampsia.[147] Other factors that likely influence the degree of transfer of fetal cells into the maternal circulation are multiple gestation, feto-maternal blood incompatibilities, and other maternal complications, such as diabetes or bleeding. Another issue is the possible influence of autoimmune diseases, such as scleroderma.[148]

Various methods to enrich NRBC from maternal blood

Nucleated red blood cells are thought to hold promise for non-invasive diagnosis using fetal cells from the maternal circulation; however, fetal NRBC are rarely detected in the maternal circulation. Thus, enrichment of NRBC is essential. Methods for enrichment of NRBC include FACS, MACS, density gradient centrifugation, charged flow separation, selective erythrocyte lysis, and the lectin base method. Efficient selection of NRBC is essential for analysis of fetal genetic abnormalities. Although a number of reports describe successful enrichment of NRBC, a preferred method has yet to be established.

FACS and MACS

Fluorescence-activated cell sorting and MACS were preferred methods used by researchers in the 1990s for fetal cell enrichment. Both techniques rely on antigen–antibody recognition using NRBC-specific monoclonal antibodies. In order to perform FACS, the antibodies are first labeled with a fluorescent dye, while they are labeled with magnetic beads for MACS. As previously mentioned, Bianchi *et al*. first used a monoclonal antibody against CD71 to enrich NRBC in 1990.[116] In doing so, they sorted CD71-positive cells from the blood of pregnant women at 12–17 weeks gestation, after which they performed PCR amplification of a Y-specific sequence in the sorted cells. They detected the Y-specific sequence in 75% of women bearing male fetuses. However, after selection using the CD71 antibody, the purity of NRBC remained low. Subsequent to this, their group established a new NRBC marker, a monoclonal antibody against gamma-hemoglobin. This marker markedly improved the purity of isolated NRBC.[149,150] Ganshirt-Ahlert *et al*. employed MACS separation using magnetic beads combined with antibody against CD71[122,151] to successfully identify fetal cells with chromosomal abnormalities using FISH in blood samples from 15 pregnant women bearing fetuses with chromosomal abnormalities. Some investigators have also used glycophorin A for enrichment of NRBC.[152] Purity can be further enhanced by MACS depletion of maternal cells and fetal lymphocytes with anti-CD45 prior to positive selection for CD71-positive cells. Using this method, approximately 20 fetal cells are obtained from a 20 mL maternal blood sample.[153] However, even following double MACS separation, the purity of NRBC remains low, and a prolonged interval is required for NRBC detection. The

advantages of using MACS include the short time required for the procedure itself and its relatively low cost. A disadvantage is that selection is based on only one criterion, thus explaining the low purity of NRBC obtained.

In order to improve the recovery of NRBC, Bianchi *et al*. primarily examined samples collected after termination procedures, which are thought to increase the number of NRBC in maternal blood. They recovered fetal NRBC from maternal blood in all post-termination samples.[154–158] We further observed an improvement in the recovery of NRBC by MACS negative selection of CD45 prior to FACS separation using gamma-hemoglobin antibody.[150]

National Institute of Child Health and Human Development Fetal Cell Isolation Study

Based on the success of fetal NRBC detection using FACS-FISH and/or MACS-FISH analysis, NRBC were thought to have the potential for non-invasive fetal diagnosis. In the USA, to examine this possibility, a large-scale multicenter study funded by the National Institute of Health, known as the National Institute of Child Health and Human Development Fetal Cell Isolation Study, otherwise known as NIFTY, was performed between 1995 and 1999.[159] The purpose of the study was to assess the reliability of non-invasive prenatal diagnosis of fetal aneuploidy using NRBC from the maternal circulation. Subjects thought to be at risk of carrying an aneuploid fetus (>35 years of age), or with serum screening or sonographic results suggestive of aneuploidy, about to undergo an invasive diagnostic procedure were selected. The results were compared with the karyotype obtained following the invasive procedure as the gold standard. A very low sensitivity of fetal cell detection was obtained in this study, in which over 2700 maternal blood samples were examined. The sensitivity ranged from 13% using FACS to 44% using MACS. Thus, MACS separation yielded a better recovery of fetal NRBC than FACS separation.[159] Although the purity of NRBC was high following FACS separation using gamma-hemoglobin antibody, fewer NRBC were recovered. In contrast, the greater number of cells separated using MACS led to improved recovery of NRBC, despite an overall decrease in the proportion of NRBC to total cells collected. Thus, recovery of NRBC using MACS was slightly better than FACS. However, fetal NRBC were difficult to detect with both methods. This is likely due to the extremely low number and proportion of fetal NRBC in maternal blood. Consequently, this study concluded that separation of NRBC based on interactions between NRBC-specific antigens and their corresponding antibodies is limited.

Density gradient centrifugation and other methods

To separate NRBC, removal of mature erythrocytes is a very important initial step. Methods to do so include bulk separation, lysis, and various forms of selective centrifugation. Since efficient separation of NRBC using the cell-lysis method has not been achieved, more recent protocols start with some form of density gradient centrifugation intended to enrich a population of mononuclear cells. In the early 1990s, a 1.077 g/mL density gradient was primarily used. However, in 1993, Bhat *et al*. showed a 25-fold enhancement of NRBC isolation using a triple density gradient.[160] Using this protocol, fetal NRBC were successfully isolated from the maternal blood of aneuploid pregnancies in the second and third trimesters.[122]

In 1995, Takabayashi *et al*.[161] used discontinuous gradients to enhance enrichment. They placed 2-mL maternal venous blood samples on double density gradients of 1.075 and 1.085 g/mL Percoll solution. Following centrifugation, cells with the targeted densities were placed on slides and stained with May–Giemsa for morphological NRBC identification. Takabayashi *et al*. identified a large proportion of NRBC among the nucleated cells obtained using this approach. An average of 4.1 NRBC (ranging from 1 to 22) were identified per sample analyzed, from which fetal sex could be accurately determined in 10 out of 11 samples.[161] NRBC were detected as early as 8 weeks gestation. Later, we evaluated the effects of various density gradients on recovery of NRBC. We used FACS separation based on gamma-hemoglobin and subsequent FISH analysis.[150] The number of NRBC recovered using a gradient of 1.090 g/mL was threefold greater than with a gradient of 1.085 g/mL. Furthermore, we recovered twice as many NRBC using 1.119 g/mL, compared to 1.090 g/mL[158] Moreover, Voullarie *et al*. examined the density of NRBC using a continuous Percoll density gradient and revealed that the majority of NRBC are denser than white blood cells.[162] Some investigators prefer to use Ficoll 1119.[163,164] Given that NRBC have greater density than white blood cells, it is necessary to use increased density gradients to recover a high yield of NRBC.

Other methods used for enrichment of fetal cells include avidin–biotin columns, magnetic ferro-fluids, and cell culturing to increase yields. To enhance enrich-

ment, some investigators have sought more specific antibodies or biochemical markers, while some have relied on chemical assays for analysis, such as 2,3-biophosphoglycerate (BPG),[118] carbonic anhydrase (CA)[129,165] and thymidine kinase (TK).[166] BPG is thought to identify fetal hemoglobin by exposing the fetal haem-iron to oxidization in a sequential peroxidase reaction, thereby forming a colored substrate-associated complex. Using a fluorescent TK thymidine analog, fetal cells can be differentiated from adult cells based on TK enzyme activity, since enzyme activity is virtually absent in adult cells. Fetal NRBC are also less susceptible to ammonium chloride lysis than adult NRBC, since CA activity is fivefold less, and acetozolamide permeability approximately 10-fold greater. Other specific antibodies include HAE9[167] and those developed by Genzyme (FB3-2, 2–6B/6 and H3-3).[145] The possibility of using an erythropoietin assay has also been explored.[168] However, at the present time, most researchers still either use anti-CD71 or anti-gamma globin antibodies for enrichment of NRBC.

Other investigators have isolated very high numbers of NRBC by charge flow separation (CFS),[169,170] which permits sorting of cells according to their characteristic surface charge densities. Using this method, several thousand NRBC (average: 6910) were enriched from a 20 mL maternal venous blood sample in one study, from which both fetal sex and ploidy could be accurately examined. However, this result has not been repeated.

Lectin-based method

A more recent study has used a galactose-specific lectin for isolation of fetal NRBC from maternal blood.[171] This method is based on the observation that erythroid precursor cells express large numbers of galactose molecules on their cell surface, associated with development and maturation of the cell. In this study, they used soybean agglutinin (SBA) as a galactose-specific lectin to enrich high level galactose-expressing erythroid cells, from which they recovered one to several hundred NRBC (mean ± SD: 7.8 ± 8.5) in 2.3 mL of peripheral blood from 96% of pregnant women between 6 and 27 weeks. The isolated NRBC were then analyzed using a Y-chromosome-specific FISH probe in eight cases carrying male fetuses, for which Y-signals were detected in all eight cases and more than half of all NRBC collected were of fetal origin. Subsequently, Babochkina *et al*. compared the lectin method with MACS/CD71 separation, and revealed an eightfold increase in NRBC recovery using this method.[172] Thus, the lectin method is the most promising method for NRBC separation to date. Using this method, we evaluated how many NRBC could be separated from the blood of normal pregnant women at early gestation.[173] We detected NRBC in all 55 samples examined (1–82 cells/sample). The median number of NRBC detected in normal pregnancy was 12.5 cells per 6 mL of maternal blood. This finding confirms that NRBC circulate in the blood of pregnant women. In order to identify the NRBC by microscopy, we manually screened two slides per case, which was very labor intensive. However, following enhancement using the lectin-based method, the majority of contaminant cells were non-nucleated erythrocytes. The burden of NRBC identification could be considerably reduced by this method. Again, the lectin-based method seems superior to other methods, such as FACS and MACS.

Autoimage analyzer

Since enrichment of NRBC produces a low purity sample, the requirement for subsequent screening is labor intensive. This has created interest in automated detection systems, using laser-mediated scanning or a charge-coupled device (CCD) with a video computer-aided capture and dot counting analysis system. Zheng *et al*.[174] and Ferguson *et al*.[175] have used an autodetection system in the screening of NRBC. In their study, they stained NRBC with fluorescence-conjugated anti-fetal hemoglobin antibodies. Following detection of NRBC, FISH analysis can then be performed to diagnose fetal aneuploidies. However, this system is still plagued by difficulties, since morphological identification of NRBC is still required and the optimal staining conditions have yet to be determined.

Recently, a new system has been developed. Following lectin-based separation, the enriched cells are stained and the entire slide examined under microscopy and the images loaded into a computer using a CCD camera, from which NRBC are identified based on their morphology. Takabayashi *et al*. have developed a similar system. This computer system identifies nucleated cells in images from the CCD camera and evaluates the morphology of each cell. After selecting candidate NRBC, cells on the slideglass are highlighted by the computer and manual determination of the cell type is performed. Using the system, we are currently comparing the rate of this system for identification of NRBC with manual detection. At present, we use the lectin-based method to separate NRBC. Using samples obtained by lectin separation, high efficiency of the autoimage analyzer is expected given that the majority

of contaminant cells are non-nucleated erythrocytes. It is likely that the efficiency and reliability of the image analyzer system is sufficient for use in routine NRBC detection. Further development of this system will save time in the detection of NRBC and allow processing of samples with strong reliability. This approach is becoming more feasible as the image-processing capacity of computers improves.

DNA analysis

In the late 1980s, FISH and PCR methods became available. Because these methods are sensitive enough to analyze single cells, non-invasive prenatal diagnosis using maternal blood became all the more possible. Two methods are now used to analyze fetal cells. One is the FISH method, which can analyze fetal gender and aneuploidies. Another is the PCR method, which permits analysis of mutations in single-gene disorders, as well as fetal gender and RhD blood type.

FISH analysis

Since Price *et al*. first reported diagnosis of fetal trisomy 21 by isolation of NRBC from maternal blood,[120] the FISH method has been used for non-invasive prenatal diagnosis of fetal aneuploidies, as well as fetal gender. In our own experiments, analysis of maternal blood samples by FISH following termination procedures resulted in detection of NRBC by positive staining for gamma-hemoglobin, all of which were fetal in origin.[176] However, when blood samples from normal pregnant women were examined, the majority of NRBC were not suitable for FISH. We then learned that 43% of the fetal NRBC collected from maternal blood were apoptotic.[156] When NRBC migrate into maternal blood, they circulate under relatively high oxygen tension. As a result, they are more subject to apoptosis[67] and the size of their nuclei is diminished.[177] This might be why FISH signals are not detected within nuclei. This problem warrants further investigation. Recently, we have developed a modified method of FISH in NRBC with highly condensed nuclei. At any rate, the ability to perform FISH on NRBC recovered from maternal blood makes non-invasive prenatal diagnosis of aneuploidies more feasible.

PCR analysis

Through PCR amplification, we can evaluate fetal DNA in NRBC-enriched samples. In fact, Bianchi *et al*. have reported the presence of a Y-chromosome specific sequence in 75% of male-bearing maternal blood samples.[116] However, this approach is not considered sufficiently accurate for use in clinical practice. For diagnostic purposes, individual NRBC of fetal origin must be examined, since half of NRBC are maternal in origin. Unfortunately, thus far, micromanipulation remains the only method by which to isolate individual NRBC for analysis.

In 1995, Takabayashi *et al*. pioneered a micromanipulation method by which to isolate NRBC based on morphology. They accurately determined fetal sex in 10 out of 11 cases.[161] We subsequently used this NRBC separation method to retrieve individual NRBC and diagnose fetal single-gene disorders, such as Duchenne type muscular dystrophy and ornithine transcarbamylase deficiency.[178,179] We illustrated that PCR can be performed on genetic material obtained from a single cell, a finding made possible by whole genome random primer extension preamplification (PEP). As a result, we were then able to determine whether a NRBC was of fetal origin using differences in ZFX/ZFY loci (to diagnose the gender of the cell), and also to examine particular genes of interest. Although we were the first to diagnose a fetal single-gene disorder, over 100 cycles of PCR amplification, including PEP and subsequent PCR of the target genes, were required to diagnose the origin of the cell, as well as the DNA alterations leading to one or more single-gene disorders. Thus, there are definite limitations to use of this technology for clinical applications. Cheung *et al*. have also prenatally diagnosed hemoglobinopathies using multiple singly manipulated fetal NRBC identified by antifetal hemoglobin staining.[180] In order to perform PCR, several cells need to be retrieved in order to circumvent the problem of allele dropout, a phenomenon that frequently occurs when using single or low template copies for PCR.

Thus, while PCR methods are highly sensitive, PCR amplification errors involving a single base and allele dropouts may still occur.

Future prospects

In Japan, several groups have been engaged in research using fetal NRBC from maternal blood for non-invasive prenatal diagnosis as mentioned above. As such, I organized a research consortium (The Study Group for Fetal DNA Diagnosis from Maternal Blood) with Dr Kitagawa and Dr Takabayashi in 2006. We examined the success of NRBC separation among each group and attempted to design an optimal protocol. As a result of this collaboration, we have recently developed a new method for NRBC separation for subsequent genetic analysis by FISH. To assess the efficiency of NRBC

separation using the galactose-specific lectin method, we evaluated the recovery and enrichment of NRBC from 55 maternal blood samples from pregnant women, which was successful in every case. The mean number of NRBC recovered was 12.5 NRBC per 6 mL of blood from normal pregnant women.[173] Furthermore, we optimized our FISH method and performed fetal gender determination in 20 normal pregnant women (median: 15; range: 10–18 weeks). Fetal gender analysis using FISH in 20 pregnant women was successful by comparison with fetal gender determination by analysis of maternal plasma, thus confirming the presence of fetal NRBC in maternal blood. In male-bearing pregnancies, XY cells were detectable in 45% of NRBC. Thus, we speculate that almost half of all NRBC circulating in maternal blood are of fetal origin.[181]

We have recently begun a multicenter study, named the FeDiM study, to evaluate the accuracy of non-invasive prenatal diagnosis of fetal gender, trisomy-21, 18, and 13. If the results of this study are encouraging and the reliability of this method can be confirmed, our method might be used for clinical applications. We therefore believe that non-invasive prenatal diagnosis will be used for routine prenatal testing in the near future.

Conclusion

Non-invasive prenatal diagnosis of fetal aneuploidies or genetic disorders has become a realistic goal for routine prenatal care. Fetal DNA can be detected in maternal plasma after 7 weeks. Fetal DNA within plasma can be used for accurate fetal gender determination and fetal RhD blood typing in Rh^- pregnant women. Furthermore, it can be applied to the identification of paternally inherited diseases and sporadic genetic disorders. However, fetal DNA from maternal plasma cannot be used to diagnose maternally inherited diseases and fetal aneuploidies.

Thus, analysis of NRBC in maternal blood has some advantages. If sufficient NRBC can be recovered from the blood of pregnant women, fetal cells can be used for the prenatal diagnosis of every kind of genetic disorder. Recent progress with regard to lectin separation, autoimage analyzing, and FISH technology, makes the possibility of non-invasive prenatal diagnosis of aneuploidy more likely. The development of techniques for non-invasive prenatal diagnosis using cell-free DNA and fetal cells in maternal blood will contribute greatly to the field of perinatal medicine and result in safer antenatal care.

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References

1. Harper P. *Practical Genetic Counseling*, 5th edn. Oxford: Butterworth-Heinemann, 1998.
2. Kuliev AM, Modell B, Jackson L *et al.* Risk evaluation of CVS. *Prenat Diagn* 1993; **13**: 197–209.
3. Firth HV, Boyd PA, Chamberlain PF, MacKenzie IZ, Morriss-Kay GM, Huson SM. Analysis of limb reduction defects in babies exposed to chorionic villus sampling [see comments]. *Lancet* 1994; **343**: 1069–1071.
4. Wald NJ, Cuckle HS, Densem JW *et al.* Maternal serum screening for Down's syndrome in early pregnancy. *BMJ* 1988; **297**: 883–887.
5. Wald NJ, Densem JW, George L, Muttukrishna S, Knight PG. Prenatal screening for Down's syndrome using inhibin-A as a serum marker. (Published erratum appears in *Prenat Diagn* 1997; 17: 285–290.) *Prenat Diagn* 1996; **16**: 143–153.
6. Snijders RJ, Noble P, Sebire N, Souka A, Nicolaides KH. UK multicentre project on assessment of risk of trisomy 21 by maternal age and fetal nuchal-translucency thickness at 10–14 weeks of gestation. Fetal Medicine Foundation First Trimester Screening Group [see comments]. *Lancet* 1998; **352**: 343–346.
7. Cicero S, Curcio P, Papageorghiou A, Sonek J, Nicolaides K. Absence of nasal bone in fetuses with trisomy 21 at 11–14 weeks of gestation: an observational study. *Lancet* 2001; **358**: 1665–1667.
8. Cicero S, Avgidou K, Rembouskos G, Kagan KO, Nicolaides KH. Nasal bone in first-trimester screening for trisomy 21. *Am J Obstet Gynecol* 2006; **195**: 109–114.
9. Mandel P, Metais P. Les acides nucleiques du plasma sanguin chez l'homme. *CR Acad Sci Paris* 1948; **142**: 241–243.
10. Tan EM, Schur PH, Carr RI, Kunkel HG. Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J Clin Invest* 1966; **45**: 1732–1740.
11. Leon SA, Ehrlich GE, Shapiro B, Labbate VA. Free DNA in the serum of rheumatoid arthritis patients. *J Rheumatol* 1977; **4**: 139–143.
12. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 1977; **37**: 646–650.
13. Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* 1996; **2**: 1035–1037.
14. Chen XQ, Stroun M, Magnenat JL *et al.* Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med* 1996; **2**: 1033–1035.
15. Lo YM, Corbetta N, Chamberlain PF *et al.* Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997; **350**: 485–487.

16. Lo YM, Tein MS, Lau TK *et al.* Quantitative analysis of fetal DNA in maternal plasma and serum: implications for non-invasive prenatal diagnosis. *Am J Hum Genet* 1998; **62**: 768–775.
17. Sekizawa A, Kondo T, Iwasaki M *et al.* Accuracy of fetal gender determination by analysis of DNA in maternal plasma. *Clin Chem* 2001; **47**: 1856–1858.
18. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 1999; **64**: 218–224.
19. Lo YM, Hjelm NM, Fidler C *et al.* Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998; **339**: 1734–1738.
20. Tang NL, Leung TN, Zhang J, Lau TK, Lo YM. Detection of fetal-derived paternally inherited X-chromosome polymorphisms in maternal plasma. *Clin Chem* 1999; **45**: 2033–2035.
21. Stanghellini I, Bertorelli R, Capone L *et al.* Quantitation of fetal DNA in maternal serum during the first trimester of pregnancy by the use of a DAZ repetitive probe. *Mol Hum Reprod* 2006; **12**: 587–591.
22. Hromadnikova I, Houbova B, Hridelova D *et al.* Replicate real-time PCR testing of DNA in maternal plasma increases the sensitivity of non-invasive fetal sex determination. *Prenat Diagn* 2003; **23**: 235–238.
23. Hyett JA, Gardener G, Stojilkovic-Mikic T *et al.* Reduction in diagnostic and therapeutic interventions by non-invasive determination of fetal sex in early pregnancy. *Prenat Diagn* 2005; **25**: 1111–1116.
24. Chi C, Hyett JA, Finning KM, Lee CA, Kadir RA. Non-invasive first trimester determination of fetal gender: a new approach for prenatal diagnosis of haemophilia. *BJOG* 2006; **113**: 239–242.
25. Budka H. Prions and transfusion medicine. *Vox Sang* 2000; **78** (Suppl 2): 231–238.
26. Bianchi DW, Avent ND, Costa JM, van der Schoot CE. Non-invasive prenatal diagnosis of fetal Rhesus D: ready for Prime(r) Time. *Obstet Gynecol* 2005; **106**: 841–844.
27. Brojer E, Zupanska B, Guz K, Orzinska A, Kalinska A. Non-invasive determination of fetal RHD status by examination of cell-free DNA in maternal plasma. *Transfusion* 2005; **45**: 1473–1480.
28. Hengstschlager M, Holzl G, Ulm B, Bernaschek G. Raising the sensitivity of fetal RhD typing and sex determination from maternal blood. *J Med Genet* 1997; **34**: 350–351.
29. Van der Schoot CE, Soussan AA, Koelewijn J, Bonsel G, Paget-Christiaens LG, de Haas M. Non-invasive antenatal RHD typing. *Transfus Clin Biol* 2006; **13**: 53–57.
30. Tax MG, van der Schoot CE, van Doorn R, Douglas-Berger L, van Rhenen DJ, Maaskant-vanWijk PA. RHC and RHc genotyping in different ethnic groups. *Transfusion* 2002; **42**: 634–644.
31. Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. *Lancet* 2000; **356**: 1170.
32. Shiang R, Thompson L, Zhu Y *et al.* Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell* 1994; **78**: 335–342.
33. Li Y, Holzgreve W, Page-Christiaens GC, Gille JJ, Hahn S. Improved prenatal detection of a fetal point mutation for achondroplasia by the use of size-fractionated circulatory DNA in maternal plasma – case report. *Prenat Diagn* 2004; **24**: 896–898.
34. Amicucci P, Gennarelli M, Novelli G, Dallapiccola B. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. *Clin Chem* 2000; **46**: 301–302.
35. Nasis O, Thompson S, Hong T *et al.* Improvement in sensitivity of allele-specific PCR facilitates reliable noninvasive prenatal detection of cystic fibrosis. *Clin Chem* 2004; **50**: 694–701.
36. Gonzalez-Gonzalez MC, Garcia-Hoyos M, Trujillo MJ *et al.* Prenatal detection of a cystic fibrosis mutation in fetal DNA from maternal plasma. *Prenat Diagn* 2002; **22**: 946–948.
37. Vrettou C, Traeger-Synodinos J, Tzetis M, Malamis G, Kanavakis E. Rapid screening of multiple beta-globin gene mutations by real-time PCR on the LightCycler: application to carrier screening and prenatal diagnosis of thalassemia syndromes. *Clin Chem* 2003; **49**: 769–776.
38. Li Y, Di Naro E, Vitucci A, Zimmermann B, Holzgreve W, Hahn S. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. *JAMA* 2005; **293**: 843–849.
39. Chiu RW, Lau TK, Leung TN, Chow KC, Chui DH, Lo YM. Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. *Lancet* 2002; **360**: 998–1000.
40. Chiu RW, Lau TK, Cheung PT, Gong ZQ, Leung TN, Lo YM. Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study. *Clin Chem* 2002; **48**: 778–780.
41. Poon LL, Leung TN, Lau TK, Chow KC, Lo YM. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. *Clin Chem* 2002; **48**: 35–41.
42. Lo YM, Leung TN, Tein MS *et al.* Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem* 1999; **45**: 184–188.
43. Zhong XY, Holzgreve W, Hahn S. Circulatory fetal and maternal DNA in pregnancies at risk and those affected by preeclampsia. *Ann NY Acad Sci* 2001; **945**: 138–140.
44. Sekizawa A, Jimbo M, Saito H *et al.* Cell-free fetal DNA in the plasma of pregnant women with severe fetal growth restriction. *Am J Obstet Gynecol* 2003; **188**: 480–484.
45. Caramelli E, Rizzo N, Concu M *et al.* Cell-free fetal DNA concentration in plasma of patients with abnormal uterine artery Doppler waveform and intrauterine growth restriction – a pilot study. *Prenat Diagn* 2003; **23**: 367–371.
46. Roberts JM, Cooper DW. Pathogenesis and genetics of pre-eclampsia. *Lancet* 2001; **357**: 53–56.
47. Sekizawa A, Yokokawa K, Sugito Y *et al.* Evaluation of bidirectional transfer of plasma DNA through placenta. *Hum Genet* 2003; **113**: 307–310.
48. Lau TW, Leung TN, Chan LY *et al.* Fetal DNA clearance from maternal plasma is impaired in preeclampsia. *Clin Chem* 2002; **48**: 2141–2146.
49. Sekizawa A, Farina A, Sugito Y *et al.* Proteinuria and hypertension are independent factors affecting fetal DNA values: a retrospective analysis of affected and unaffected patients. *Clin Chem* 2004; **50**: 221–224.
50. Sekizawa A, Farina A, Koide K *et al.* Beta-globin DNA in maternal plasma as a molecular marker of preeclampsia. *Prenat Diagn* 2004; **24**: 697–700.

51. Leung TN, Zhang J, Lau TK, Hjelm NM, Lo YM. Maternal plasma fetal DNA as a marker for preterm labour. *Lancet* 1998; **352**: 1904–1905.
52. Farina A, Sekizawa A, Sugito Y *et al.* Fetal DNA in maternal plasma as a screening variable for preeclampsia. A preliminary nonparametric analysis of detection rate in low-risk nonsymptomatic patients. *Prenat Diagn* 2004; **24**: 83–86.
53. Farina A, Sekizawa A, Iwasaki M, Matsuoka R, Ichizuka K, Okai T. Total cell-free DNA (beta-globin gene) distribution in maternal plasma at the second trimester: a new prospective for preeclampsia screening. *Prenat Diagn* 2004; **24**: 722–726.
54. Sekizawa A, Sugito Y, Iwasaki M *et al.* Cell-free fetal DNA is increased in plasma of women with hyperemesis gravidarum. *Clin Chem* 2001; **47**: 2164–2165.
55. Sugito Y, Sekizawa A, Farina A *et al.* Relationship between severity of hyperemesis gravidarum and fetal DNA concentration in maternal plasma. *Clin Chem* 2003; **49**: 1667–1669.
56. Minagawa M, Narita J, Tada T *et al.* Mechanisms underlying immunologic states during pregnancy: possible association of the sympathetic nervous system. *Cell Immunol* 1999; **196**: 1–13.
57. Sekizawa A, Jimbo M, Saito H *et al.* Increased cell-free fetal DNA in plasma of two women with invasive placenta. *Clin Chem* 2002; **48**: 353–354.
58. Jimbo M, Sekizawa A, Sugito Y *et al.* Placenta increta: postpartum monitoring of plasma cell-free fetal DNA. *Clin Chem* 2003; **49**: 1540–1541.
59. Farina A, LeShane E, Romero R *et al.* High levels of fetal cell-free DNA in maternal serum: a Risk factor for spontaneous preterm delivery. *Am J Obstet Gynecol* 2005; **193**: 421–425.
60. Lo YM, Lau TK, Zhang J *et al.* Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* 1999; **45**: 1747–1751.
61. Zhong XY, Burk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. *Prenat Diagn* 2000; **20**: 795–798.
62. Farina A, LeShane ES, Lambert-Messerlian GM *et al.* Evaluation of cell-free fetal DNA as a second-trimester maternal serum marker of Down syndrome pregnancy. *Clin Chem* 2003; **49**: 239–242.
63. Ohashi Y, Miharu N, Honda H, Samura O, Ohama K. Quantitation of fetal DNA in maternal serum in normal and aneuploid pregnancies. *Hum Genet* 2001; **108**: 123–127.
64. Hromadnikova I, Houbova B, Hridelova D *et al.* Quantitative analysis of DNA levels in maternal plasma in normal and Down syndrome pregnancies. *BMC Pregnancy Childbirth* 2002; **2**: 4.
65. Spencer K, de Kok JB, Swinkels DW. Increased total cell-free DNA in the serum of pregnant women carrying a fetus affected by trisomy 21. *Prenat Diagn* 2003; **23**: 580–583.
66. Sekizawa A, Samura O, Zhen D, Falco V, Farina A, Bianchi DW. Apoptosis in fetal nucleated erythrocytes circulating in maternal blood. *Prenat Diagn* 2000; **20**: 886–889.
67. Kondo T, Sekizawa A, Saito H, Jimbo M, Sugito Y, Okai T. Fate of fetal nucleated erythrocytes circulating in maternal blood: apoptosis is induced by maternal oxygen concentration. *Clin Chem* 2002; **48**: 1618–1620.
68. Fournie GJ, Courtin JP, Laval F *et al.* Plasma DNA as a marker of cancerous cell death. Investigations in patients suffering from lung cancer and in nude mice bearing human tumours. *Cancer Lett* 1995; **91**: 221–227.
69. Giacona MB, Ruben GC, Iczkowski KA, Roos TB, Porter DM, Sorenson GD. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. *Pancreas* 1998; **17**: 89–97.
70. Holdenrieder S, Stieber P, Chan LY *et al.* Cell-free DNA in serum and plasma: comparison of ELISA and quantitative PCR. *Clin Chem* 2005; **51**: 1544–1546.
71. Angert RM, LeShane ES, Lo YM, Chan LY, Delli-Bovi LC, Bianchi DW. Fetal cell-free plasma DNA concentrations in maternal blood are stable 24 hours after collection: analysis of first- and third-trimester samples. *Clin Chem* 2003; **49**: 195–198.
72. Hoesli I, Danek M, Lin D, Li Y, Hahn S, Holzgreve W. Circulating erythroblasts in maternal blood are not elevated before onset of preterm labor. *Obstet Gynecol* 2002; **100**: 992–996.
73. Loke YW, King A. Immunology of human placental implantation: clinical implications of our current understanding. *Mol Med Today* 1997; **3**: 153–159.
74. Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez JB, Samoto T, Maruo T. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. *Am J Obstet Gynecol* 2002; **186**: 158–166.
75. Ariga H, Ohto H, Busch MP *et al.* Kinetics of fetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. *Transfusion* 2001; **41**: 1524–1530.
76. Smith SC, Baker PN. Placental apoptosis is increased in post-term pregnancies. *Br J Obstet Gynaecol* 1999; **106**: 861–862.
77. Ohashi Y, Miharu N, Honda H, Samura O, Ohama K. Correlation of fetal DNA and human chorionic gonadotropin concentrations in second-trimester maternal serum. *Clin Chem* 2002; **48**: 386–388.
78. Ng EK, Tsui NB, Lau TK *et al.* mRNA of placental origin is readily detectable in maternal plasma. *Proc Natl Acad Sci USA* 2003; **100**: 4748–4753.
79. Ng EK, Leung TN, Tsui NB *et al.* The concentration of circulating corticotropin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. *Clin Chem* 2003; **49**: 727–731.
80. Wataganara T, Metzenbauer M, Peter I, Johnson KL, Bianchi DW. Placental volume, as measured by 3-dimensional sonography and levels of maternal plasma cell-free fetal DNA. *Am J Obstet Gynecol* 2005; **193**: 496–500.
81. Schmorl G. *Pathogisch-anatomische untersuchungen uber puerperaleklampsie*. Leipzig: Vogel, 1893.
82. Douglas GW, Thomas L, Carr M, Cullen NM, Morris R. Trophoblast in the circulating blood during pregnancy. *Am J Obstet Gynecol* 1959; **78**: 960–973.
83. Benirschke K. Anatomical relationship between fetus and mother. *Ann NY Acad Sci* 1994; **731**: 9–20.
84. Ganshirt-Ahlert D, Basak N, Aidynli K, Holzgreve W. Fetal DNA in uterine vein blood. *Obstet Gynecol* 1992; **80**: 601–603.
85. Covone AE, Mutton D, Johnson PM, Adinolfi M. Trophoblast cells in peripheral blood from pregnant women. *Lancet* 1984; **2**: 841–843.

86. Sargent IL, Choo YS, Redman CW. Isolating and analyzing fetal leukocytes in maternal blood. *Ann NY Acad Sci* 1994; **731**: 147–153.
87. Bertero MT, Camaschella C, Serra A, Bergui L, Caligaris-Cappio F. Circulating 'trophoblast' cells in pregnancy have maternal genetic markers. *Prenat Diagn* 1988; **8**: 585–590.
88. Covone AE, Kozma R, Johnson PM, Latt SA, Adinolfi M. Analysis of peripheral maternal blood samples for the presence of placenta-derived cells using Y-specific probes and McAb H315. *Prenat Diagn* 1988; **8**: 591–607.
89. Henderson KG, Shaw TE, Barrett IJ, Telenius AH, Wilson RD, Kalousek DK. Distribution of mosaicism in human placentae. *Hum Genet* 1996; **97**: 650–654.
90. Goldberg JD, Wohlferd MM. Incidence and outcome of chromosomal mosaicism found at the time of chorionic villus sampling. *Am J Obstet Gynecol* 1997; **176**: 1349–1352; discussion 1352–1353.
91. Mueller UW, Hawes CS, Wright AE *et al.* Isolation of fetal trophoblast cells from peripheral blood of pregnant women. *Lancet* 1990; **336**: 197–200.
92. Hawes CS, Suskin HA, Kalionis B *et al.* Detection of paternally inherited mutations for beta-thalassemia in trophoblast isolated from peripheral maternal blood. *Ann NY Acad Sci* 1994; **731**: 181–185.
93. Tse DB, Anderson P, Goldbard S, Gown AM, Hawes CS, Donnenfeld A. Characterization of trophoblast-reactive monoclonal antibodies by flow cytometry and their application for fetal cell isolation. *Ann NY Acad Sci* 1994; **731**: 162–169.
94. Walknowska J, Conte FA, Grumbach MM. Practical and theoretical implications of fetal-maternal lymphocyte transfer. *Lancet* 1969; **1**: 1119–1122.
95. Grosset L, Barrelet V, Odartchenko N. Antenatal fetal sex determination from maternal blood during early pregnancy. *Am J Obstet Gynecol* 1974; **120**: 60–63.
96. Schroder J, De la Chapelle A. Fetal lymphocytes in the maternal blood. *Blood* 1972; **39**: 153–162.
97. Siebers JW, Knauf I, Hillemanns HG. Antenatal sex determination in blood from pregnant women. *Humangenetik* 1975; **28**: 273–280.
98. Parks DR, Herzenberg LA. Fetal cells from maternal blood: their selection and prospects for use in prenatal diagnosis. *Methods Cell Biol* 1982; **26**: 277–295.
99. Schindler AM, Martin-du-Pan R. Prenatal diagnosis of fetal lymphocytes in the maternal blood. *Obstet Gynecol* 1972; **40**: 340–346.
100. Takahara H, Kadotani T, Kusumi I, Makino S. Some critical aspects on prenatal diagnosis of sex in leucocyte cultures from pregnant women. *Proc Jpn Acad* 1972; **48**: 603–607.
101. Whang-Peng J, Leikin S, Harris C, Lee E, Sites J. The transplacental passage of fetal leukocytes into the maternal blood. *Proc Soc Exp Biol Med* 1973; **142**: 50–53.
102. Schroder J. Are fetal cells in maternal blood mainly B lymphocytes? *Scand J Immunol* 1975; **4**: 279–285.
103. Schroder J, Schroder E, Cann HM. Fetal cells in the maternal blood. Lack of response of fetal cells in maternal blood to mitogens and mixed leukocyte culture. *Hum Genet* 1977; **38**: 91–97.
104. Herzenberg LA, Bianchi DW, Schroder J, Cann HM, Iverson GM. Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. *Proc Natl Acad Sci USA* 1979; **76**: 1453–1455.
105. Iverson GM, Bianchi DW, Cann HM, Herzenberg LA. Detection and isolation of fetal cells from maternal blood using the flourescence-activated cell sorter (FACS). *Prenat Diagn* 1981; **1**: 61–73.
106. Tharapel AT, Jaswaney VL, Dockter ME *et al.* Inability to detect fetal metaphases in flow-sorted lymphocyte cultures based on maternal-fetal HLA differences. *Fetal Diagn Ther* 1993; **8**: 95–101.
107. Schroder J, Tiilikainen A, De la Chapelle A. Fetal leukocytes in the maternal circulation after delivery. I. Cytological aspects. *Transplantation* 1974; **17**: 346–354.
108. Hsieh TT, Pao CC, Hor JJ, Kao SM. Presence of fetal cells in maternal circulation after delivery. *Hum Genet* 1993; **92**: 204–205.
109. Ciaranfi A, Curchod A, Odartchenko N. [Post-partum survival of fetal lymphocytes in the maternal blood]. *Schweiz Med Wochenschr* 1977; **107**: 134–138. (In French.)
110. Liou JD, Hsieh TT, Pao CC. Presence of cells of fetal origin in maternal circulation of pregnant women. *Ann NY Acad Sci* 1994; **731**: 237–241.
111. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci USA* 1996; **93**: 705–708.
112. Kleihauer E, Braun H, Betke K. [Demonstration of fetal hemoglobin in erythrocytes of a blood smear.] *Klin Wochenschr* 1957; **35**: 637–638. (In German.)
113. Clayton EM Jr, Feldhaus WD, Whitacre FE. Fetal erythrocytes in the maternal circulation of pregnant women. *Obstet Gynecol* 1964; **23**: 915–919.
114. Ganshirt D, Garritsen H, Miny P, Holzgreve W. Fetal cells in maternal circulation throughout gestation. *Lancet* 1994; **343**: 1038–1039.
115. Pearson HA. Life-span of the fetal red blood cell. *J Pediatr* 1967; **70**: 166–171.
116. Bianchi DW, Flint AF, Pizzimenti MF, Knoll JH, Latt SA. Isolation of fetal DNA from nucleated erythrocytes in maternal blood. *Proc Natl Acad Sci USA* 1990; **87**: 3279–3283.
117. Holzgreve W, Garritsen HS, Ganshirt-Ahlert D. Fetal cells in the maternal circulation. *J Reprod Med* 1992; **37**: 410–418.
118. Von Koskull H, Gahmberg N. Fetal erythroblasts from maternal blood identified with 2,3-bisphosphoglycerate (BPG) and in situ hybridization (ISH) using Y-specific probes. *Prenat Diagn* 1995; **15**: 149–154.
119. Wachtel S, Elias S, Price J *et al.* Fetal cells in the maternal circulation: isolation by multiparameter flow cytometry and confirmation by polymerase chain reaction. *Hum Reprod* 1991; **6**: 1466–1469.
120. Price JO, Elias S, Wachtel SS *et al.* Prenatal diagnosis with fetal cells isolated from maternal blood by multiparameter flow cytometry. *Am J Obstet Gynecol* 1991; **165**: 1731–1737.
121. Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. *Cytometry* 1990; **11**: 231–238.
122. Ganshirt-Ahlert D, Borjesson-Stoll R, Burschyk M *et al.* Detection of fetal trisomies 21 and 18 from maternal blood using triple gradient and magnetic cell sorting. *Am J Reprod Immunol* 1993; **30**: 194–201.
123. Ganshirt-Ahlert D, Pohlschmidt M, Gal A, Miny P, Horst J, Holzgreve W. Ratio of fetal to maternal DNA is less than 1 in

5000 at different gestational ages in maternal blood. *Clin Genet* 1990; **38**: 38–43.
124. Hamada H, Arinami T, Kubo T, Hamaguchi H, Iwasaki H. Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. *Hum Genet* 1993; **91**: 427–432.
125. Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW, Shuber AP. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. *Am J Hum Genet* 1997; **61**: 822–829.
126. Hamada H, Arinami T, Sohda S, Hamaguchi H, Kubo T. Mid-trimester fetal sex determination from maternal peripheral blood by fluorescence in situ hybridization without enrichment of fetal cells. *Prenat Diagn* 1995; **15**: 78–81.
127. Slunga-Tallberg A, el-Rifai W, Keinanen M *et al.* Maternal origin of nucleated erythrocytes in peripheral venous blood of pregnant women. *Hum Genet* 1995; **96**: 53–57.
128. Slunga-Tallberg A, el-Rifai W, Keinanen M *et al.* Maternal origin of transferrin receptor positive cells in venous blood of pregnant women. *Clin Genet* 1996; **49**: 196–199.
129. de Graaf IM, Jakobs ME, Leschot NJ, Ravkin I, Goldbard S, Hoovers JM. Enrichment, identification and analysis of fetal cells from maternal blood: evaluation of a prenatal diagnosis system. *Prenat Diagn* 1999; **19**: 648–652.
130. Troeger C, Zhong XY, Burgemeister R *et al.* Approximately half of the erythroblasts in maternal blood are of fetal origin. *Mol Hum Reprod* 1999; **5**: 1162–1165.
131. Lo YM, Morey AL, Wainscoat JS, Fleming KA. Culture of fetal erythroid cells from maternal peripheral blood. *Lancet* 1994; **344**: 264–265.
132. Valerio D, Aiello R, Altieri V, Malato AP, Fortunato A, Canazio A. Culture of fetal erythroid progenitor cells from maternal blood for non-invasive prenatal genetic diagnosis. *Prenat Diagn* 1996; **16**: 1073–1082.
133. Bohmer RM, Johnson KL, Bianchi DW. Fetal and maternal progenitor cells in co-culture respond equally to erythropoietin. *Prenat Diagn* 2001; **21**: 818–823.
134. Campagnoli C, Roberts IA, Kumar S *et al.* Expandability of haemopoietic progenitors in first trimester fetal and maternal blood: implications for non-invasive prenatal diagnosis. *Prenat Diagn* 2002; **22**: 463–469.
135. Bonney EA, Matzinger P. The maternal immune system's interaction with circulating fetal cells. *J Immunol* 1997; **158**: 40–47.
136. Thomas MR, Williamson R, Craft I, Rodeck CH. The time of appearance, and quantitation, of fetal DNA in the maternal circulation. *Ann NY Acad Sci* 1994; **731**: 217–225.
137. Thomas MR, Williamson R, Craft I, Yazdani N, Rodeck CH. Y chromosome sequence DNA amplified from peripheral blood of women in early pregnancy. *Lancet* 1994; **343**: 413–414.
138. Lo YM, Patel P, Baigent CN *et al.* Prenatal sex determination from maternal peripheral blood using the polymerase chain reaction. *Hum Genet* 1993; **90**: 483–488.
139. Bianchi DW, Stewart JE, Garber MF, Lucotte G, Flint AF. Possible effect of gestational age on the detection of fetal nucleated erythrocytes in maternal blood. *Prenat Diagn* 1991; **11**: 523–528.
140. Ganshirt D, Borjesson-Stoll R, Burschyk M *et al.* Successful prenatal diagnosis from maternal blood with magnetic-activated cell sorting. *Ann NY Acad Sci* 1994; **731**: 103–114.
141. Rochelson B, Kaplan C, Guzman E, Arato M, Hansen K, Trunca C. A quantitative analysis of placental vasculature in the third-trimester fetus with autosomal trisomy. *Obstet Gynecol* 1990; **75**: 59–63.
142. Kuhlmann RS, Werner AL, Abramowicz J, Warsof SL, Arrington J, Levy DL. Placental histology in fetuses between 18 and 23 weeks' gestation with abnormal karyotype. *Am J Obstet Gynecol* 1990; **163**: 1264–1270.
143. Sipes SL, Weiner CP, Wenstrom KD, Williamson RA, Grant SS. The association between fetal karyotype and mean corpuscular volume. *Am J Obstet Gynecol* 1991; **165**: 1371–1376.
144. Thilaganathan B, Meher-Homji NJ, Nicolaides KH. Blood transferrin receptor expression in chromosomally abnormal fetuses. *Prenat Diagn* 1995; **15**: 282–284.
145. Zheng YL, Zhen DK, DeMaria MA *et al.* Search for the optimal fetal cell antibody: results of immunophenotyping studies using flow cytometry. *Hum Genet* 1997; **100**: 35–42.
146. Simpson JL, Elias S. Isolating fetal cells from maternal blood. Advances in prenatal diagnosis through molecular technology. *JAMA* 1993; **270**: 2357–2361.
147. Holzgreve W, Ghezzi F, Di Naro E, Ganshirt D, Maymon E, Hahn S. Disturbed feto-maternal cell traffic in preeclampsia. *Obstet Gynecol* 1998; **91**: 669–672.
148. Nelson JL, Furst DE, Maloney S *et al.* Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. *Lancet* 1998; **351**: 559–562.
149. DeMaria MA, Zheng YL, Zhen D, Weinschenk NM, Vadnais TJ, Bianchi DW. Improved fetal nucleated erythrocyte sorting purity using intracellular antifetal hemoglobin and Hoechst 33342. *Cytometry* 1996; **25**: 37–45.
150. Sekizawa A, Farina A, Zhen DK *et al.* Improvement of fetal cell recovery from maternal blood: suitable density gradient for FACS separation. *Fetal Diagn Ther* 1999; **14**: 229–233.
151. Ganshirt-Ahlert D, Burschyk M, Garritsen HS *et al.* Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood. *Am J Obstet Gynecol* 1992; **166**: 1350–1355.
152. Bigbee WL, Grant SG. Use of allele-specific glycophorin A antibodies to enumerate fetal erythroid cells in maternal circulation. *Ann NY Acad Sci* 1994; **731**: 128–132.
153. Busch J, Huber P, Holtz J, Pfluger E, Radbruch A. Simple and fast 'double-MACS' sorting of fetal erythroblasts from maternal blood for PCR-based paternity analysis. *Ann NY Acad Sci* 1994; **731**: 144–146.
154. Zhen DK, Wang JY, Falco VM *et al.* Poly-FISH: a technique of repeated hybridizations that improves cytogenetic analysis of fetal cells in maternal blood. *Prenat Diagn* 1998; **18**: 1181–1185.
155. Wang JY, Zhen DK, Falco VM *et al.* Fetal nucleated erythrocyte recovery: fluorescence activated cell sorting-based positive selection using anti-gamma globin versus magnetic activated cell sorting using anti-CD45 depletion and anti-gamma globin positive selection. *Cytometry* 2000; **39**: 224–230.
156. Sekizawa A, Samura O, Zhen DK, Falco V, Farina A, Bianchi DW. Apoptosis in fetal nucleated erythrocytes circulating in maternal blood. *Prenat Diagn* 2000; **20**: 886–889.
157. Sekizawa A, Samura O, Zhen DK, Falco V, Bianchi DW. Fetal cell recycling: diagnosis of gender and RhD genotype in the same fetal cell retrieved from maternal blood. *Am J Obstet Gynecol* 1999; **181**: 1237–1242.

158. Samura O, Sekizawa A, Zhen DK, Falco VM, Bianchi DW. Comparison of fetal cell recovery from maternal blood using a high density gradient for the initial separation step: 1.090 versus 1.119 g/mL. *Prenat Diagn* 2000; **20**: 281–286.
159. Bianchi DW, Simpson JL, Jackson LG *et al.* Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. *Prenat Diagn* 2002; **22**: 609–615.
160. Bhat NM, Bieber MM, Teng NN. One-step enrichment of nucleated red blood cells. A potential application in perinatal diagnosis. *J Immunol Meth* 1993; **158**: 277–280.
161. Takabayashi H, Kuwabara S, Ukita T, Ikawa K, Yamafuji K, Igarashi T. Development of non-invasive fetal DNA diagnosis from maternal blood. *Prenat Diagn* 1995; **15**: 74–77.
162. Voullaire L, Ioannou P, Nouri S, Williamson R. Fetal nucleated red blood cells from CVS washings: an aid to development of first trimester non-invasive prenatal diagnosis. *Prenat Diagn* 2001; **21**: 827–834.
163. Troeger C, Holzgreve W, Hahn S. A comparison of different density gradients and antibodies for enrichment of fetal erythroblasts by MACS. *Prenat Diagn* 1999; **19**: 521–526.
164. Prieto B, Alonso R, Paz A *et al.* Optimization of nucleated red blood cell (NRBC) recovery from maternal blood collected using both layers of a double density gradient. *Prenat Diagn* 2001; **21**: 187–193.
165. Boyer SH, Noyes AN, Boyer ML. Enrichment of erythrocytes of fetal origin from adult-fetal blood mixtures via selective hemolysis of adult blood cells: an aid to antenatal diagnosis of hemoglobinopathies. *Blood* 1976; **47**: 883–897.
166. Hengstschlager M, Bernaschek G. Fetal cells in the peripheral blood of pregnant women express thymidine kinase: a new marker for detection. *FEBS Lett* 1997; **404**: 299–302.
167. Savion S, Carp H, Shepshelovich J *et al.* Use of antibodies against the human antigen of erythroblasts for the detection of nucleated erythrocytes in the maternal circulation. *Biol Neonate* 1997; **71**: 126–130.
168. Valerio D, Aiello R, Altieri V. Isolation of fetal erythroid cells from maternal blood based on expression of erythropoietin receptors. *Mol Hum Reprod* 1997; **3**: 451–455.
169. Wachtel SS, Sammons D, Manley M *et al.* Fetal cells in maternal blood: recovery by charge flow separation. *Hum Genet* 1996; **98**: 162–166.
170. Wachtel SS, Sammons D, Twitty G *et al.* Charge flow separation: quantification of nucleated red blood cells in maternal blood during pregnancy. *Prenat Diagn* 1998; **18**: 455–463.
171. Kitagawa M, Sugiura K, Omi H *et al.* New technique using galactose-specific lectin for isolation of fetal cells from maternal blood. *Prenat Diagn* 2002; **22**: 17–21.
172. Babochkina T, Mergenthaler S, Lapaire O *et al.* Evaluation of a soybean lectin-based method for the enrichment of erythroblasts. *J Histochem Cytochem* 2005; **53**: 329–330.
173. Purwosunu Y, Sekizawa A, Farina A *et al.* Enrichment of NRBC in maternal blood: a more feasible method for noninvasive prenatal diagnosis. *Prenat Diagn* 2006; **26**: 545–547.
174. Zheng YL, Carter NP, Price CM *et al.* Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting. *J Med Genet* 1993; **30**: 1051–1056.
175. Ferguson-Smith MA, Zheng YL, Carter NP. Simultaneous immunophenotyping and FISH on fetal cells from maternal blood. *Ann NY Acad Sci* 1994; **731**: 73–79.
176. Samura O, Pertl B, Sohda S *et al.* Female fetal cells in maternal blood: use of DNA polymorphisms to prove origin. *Hum Genet* 2000; **107**: 28–32.
177. Babochkina T, Mergenthaler S, De Napoli G *et al.* Numerous erythroblasts in maternal blood are impervious to fluorescent in situ hybridization analysis, a feature related to a dense compact nucleus with apoptotic character. *Haematologica* 2005; **90**: 740–745.
178. Sekizawa A, Kimura T, Sasaki M, Nakamura S, Kobayashi R, Sato T. Prenatal diagnosis of Duchenne muscular dystrophy using a single fetal nucleated erythrocyte in maternal blood. *Neurology* 1996; **46**: 1350–1353.
179. Watanabe A, Sekizawa A, Taguchi A *et al.* Prenatal diagnosis of ornithine transcarbamylase deficiency by using a single nucleated erythrocyte from maternal blood. *Hum Genet* 1998; **102**: 611–615.
180. Cheung MC, Goldberg JD, Kan YW. Prenatal diagnosis of sickle cell anaemia and thalassaemia by analysis of fetal cells in maternal blood. *Nat Genet* 1996; **14**: 264–268.
181. Sekizawa A, Purwosunu Y, Farina A *et al.* Development of noninvasive fetal DNA diagnosis from nucleated erythrocytes circulating in maternal blood. *Prenat Diagn* 2007; **27**: 846–848.