SEARCH FOR UNIQUE MEMBRANE PROTEIN OF FIRST TRIMESTER PRIMITIVE ERYTHROBLAST

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Summary

Current methods for obtaining fetal cells for prenatal diagnosis are invasive and carry a small but definitive risk of fetal loss. Recovery of first trimester fetal erythroblasts (NRBCs) in maternal blood represents an attractive and promising alternative for early non-invasive prenatal diagnosis. However, these cells are rare and it is technically challenging in recovering them from maternal blood due to the lack of a fetal specific surface marker that could be used to isolate fetal NRBCs from adult RBCs.

This thesis investigated the membrane protein profiles of first trimester primitive fetal NRBCs and adult RBCs using proteomic approaches and immunocytochemical screening with an aim to identify unique surface marker(s). To enhance the recovery of membrane proteome from a limited amount of fetal NRBC sample, an efficient proteomic strategy was developed for membrane proteome analysis, that is, sequential use of organic solvents methanol (MeOH) and 2,2,2-trifluoroethanol (TFE) to recover both hydrophilic and hydrophobic peptides and identification of proteins using two-dimensional liquid chromatography coupled with matrix-assisted laser desorption/ionisation-time of flight/time of flight tandem mass spectrometry (2-D LC-MALDI-TOF/TOF-MS). The use of this strategy to analyse fetal NRBC membrane enabled us to present its first relatively comprehensive membrane proteome, and to identify twenty-three unique fetal NRBC membrane proteins when compared with adult RBC membrane proteome. In addition, three differentially/uniquely expressed surface antigens were identified using immunocytochemical screening.

Of the twenty-six potentially useful markers, surface antigen CD147 was tested and demonstrated to be a very useful target for the separation of fetal NRBCs

from adult RBCs in model mixture, by either immunomagnetic cell sorting or fluorescence-activate cell sorting. I envisage that CD147, and/or other potential targets after further investigation, would be useful for the development of an efficient protocol to isolate fetal NRBCs from maternal blood in the first trimester of pregnancy, for early non-invasive prenatal diagnosis.

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List of Abbreviations

All units are standard SI (international system) units and standard statistical abbreviations are used

1-DE	One-dimensional gel electrophoresis
2-DE	Two-dimensional gel electrophoresis
ACN	Acetonitrile
AU	Arbitrary units
BSA	Bovine serum albumin
CFU	Colony forming unit
CHAPS	3 [(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHCA	α–cyano-4-hydroxycinnamic acid
CID	Collision-induced dissociation
CVS	Chorion villus sampling
DNA	Deoxyribonucleic acid
DIGE	Difference gel electrophoresis
DTT	Dithiothreitol
EC	Endothelial cells
EDTA	Ethylene-diamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Erythropoietin
ESI	Electrospray ionisation
FACS	Fluorescence-activated cell sorting
FBS	Fetal blood sampling
FISH	Fluorescence *in situ* hybridisation
g	Grams
g	Centrifugal *g* force

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GO	Gene Ontology
GPA	Glycophorin A
GRAVY	Grand average of hydropathicity
Hb	Haemoglobin
HbA	Haemoglobin A
HbF	Haemoglobin F
HBSS	Hank's balanced salt solution
HEPES	(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IgA	Immunoglobin A
IgG	Immunoglobin G
IMAC	Immobilised metal affinity chromatography
LC	Liquid chromatography
M	Molarity (number of moles of a given substance per litre of solution)
MAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MALDI	Matrix-assisted laser desorption/ionisation
Min	Minute(s)
MS	Mass spectrometry
mRNA	Message ribonucleic acid
NaCl	Sodium chloride
NHG	National Health Group
NRBC	Nucleated red blood cell
NP40	Nonidet P-40
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
p*I*	Isoelectric point
PMF	Peptide mass fingerprinting
ppm	Parts per million
PUBS	Percutaneous umbilical cord sampling
Rh	Rhesus
RBC	Red blood cell
RNA	Ribonucleic acid
RP LC	Reversed phase liquid chromatography
RT-PCR	Reverse transcriptase-Polymerase chain reaction
rpm	Revolutions per minute
SBA	Soyabean agglutinin
SCX	Strong cation exchange
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate- polyacrylamide-gel electrophoresis
Sec	Second(s)
SELDI	Surface-enhanced laser desorption/ionisation
SRY	Sex-determining region Y
TCEP	Tris-carboxyethyl phosphine hydrochloride
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TMD	Transmembrane domain
TOF	Time of flight
TOP	Termination of pregnancy
UV	Ultraviolet
WBC	White blood cell

Chapter 1 Introduction

1.1 Overview

Current prenatal diagnosis of genetic birth defects such as aneuploidies and monogenic disorders requires invasive diagnosis by amniocentesis, chorionic villus sampling (CVS) and fetal blood sampling (FBS). These procedures carry a 1-4% risk of fetal loss (Buscaglia et al., 1996; Lippman et al., 1992; Rhoads et al., 1989; Tabor et al., 1986; Wald et al., 1998) and 1 in 5 at-risk women (>35 years old) would decline the invasive tests (Cuckle 1996; Kocun et al., 2000). Thus the importance of developing non-invasive prenatal diagnosis is significant.

Detection of cell-free fetal DNA in maternal plasma and serum (Lo et al., 1997) and enrichment of fetal cells from maternal blood (Bianchi 1997, 1999; Choolani et al., 2003) offer an alternative source of fetal DNA for non-invasive prenatal diagnosis. The relatively high concentration of cell-free fetal DNA (Lo et al., 1997; Lo et al., 1998b) and its rapid clearance after delivery (Lo et al., 1999a) are very useful to determine fetal gender in pregnancies as early as 5 gestational weeks (Guibert et al., 2003; Ho et al., 2003; Honda et al., 2002) and single gene disorders such as RhD status (Lo et al., 1998c; Zhong et al., 2000a) and β-thalassaemia (Chiu et al., 2002b). The determination of RhD status using cell-free fetal DNA in maternal plasma is routine clinical application by the International Blood Group Reference Laboratory (IBGRL, UK). However, maternal DNA masks maternally-inherited fetal DNA and thus only paternally-inherited fetal-specific alleles can be examined from cell-free fetal DNA in maternal circulation.

On the other hand, fetal cells in the maternal blood contain the full complement of fetal genes. Theoretically, all fetal aneuploidies and single gene disorders could be detected by analysing these cells. Since trophoblasts were first found in the maternal circulation (Schmorl 1893), other types of fetal cells such as leukocytes (Walknowska et al., 1969), progenitor and stem cells (Campagnoli et al., 2001b; Little et al., 1997; O'Donoghue et al., 2003) and fetal nucleated red blood cells (NRBCs) have been demonstrated to be present in the maternal circulation. Of these cells, fetal NRBCs are considered as an ideal candidate for non-invasive detection of aneuploidies and monogenic disorders (Ho et al., 2003), as they are short-lived, morphologically distinct from maternal blood cells and have a highly specific fetal cell marker useful for their identification (Choolani et al., 2001).

The potential value of fetal NRBCs for non-invasive prenatal diagnosis has led to many attempts to enrich these cells from maternal blood (Bianchi et al., 1990; Bianchi 1997, 1999; Ganshirt et al., 1994a; Hahn et al., 1998; Holzgreve et al., 1992; Wachtel et al., 1991). Attempts using either fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) represent the commonest and most successful approaches for the enrichment of fetal NRBCs from maternal blood. Both of them usually use density gradient centrifugation to remove the bulk of maternal red blood cells (RBCs) followed by an antibody-based cell separation. The use of enriched fetal NRBCs could give a 100% accuracy in the determination of fetal gender (Bianchi et al., 1993) and aneuploidies (Ganshirt-Ahlert et al., 1993). These initial promising results led to a large-scale clinical study called National Institutes of Health Fetal Cell Study (NIFTY) (Bianchi et al., 1999). In this study, either FACS with anti-haemoglobin F (HbF) or MACS with anti-CD71 was used to enrich fetal NRBCs from maternal blood samples by four participating centres. The overall sensitivity and false-positive rate for detection of aneuploidies from the analysis of enriched fetal

NRBCs were 74.4% and 0.6-4.1% respectively, which indicated that it could be used as a screening tool but not yet for clinical diagnostic application. Other efforts were made to use a more optimal density gradient centrifugation (Samura et al., 2000; Sekizawa et al., 1999) or to couple with laser capture micromanipulation to isolate fetal cells (Di Naro et al., 2000). However, the complexity of enrichment step would not only cause a significant loss of target cells (Huber et al., 1996) but also increase the burden of procedure, rendering them to be very time-consuming and cost-ineffective, and thus of little clinical value.

The difficulty in current enrichment of fetal NRBCs is due to the rarity of fetal cells in maternal blood (Bianchi et al., 1997; Krabchi et al., 2001) and the lack of surface markers that could be used to separate fetal NRBCs from adult RBCs. The targeting intracellular antigen HbF makes the purification steps subject to more cell loss as the fragile fetal NRBCs need to be permeabilised (Huie et al., 2001); and the use of anti-CD71, which targets surface antigen but is not highly specific to fetal NRBCs, does not yield satisfactory results as shown in the NIFTY trial. Moreover, only about 68% of primitive fetal NRBCs express CD71 and the expression is weak compared to that of definitive fetal NRBCs (Choolani et al., 2003), rendering much difficulty in enrichment of primitive fetal NRBCs from maternal blood of first trimester pregnancy. In this thesis, I aimed to identify the differential expression of membrane proteins between primitive fetal NRBCs and adult RBCs, which can be potentially useful for the development of an efficient protocol to enrich primitive fetal NRBCs from maternal blood.

1.2 Current methods of prenatal diagnosis

Currently, there are four prenatal diagnostic methods used to diagnose fetal structural, chromosomal and genetic abnormalities. Ultrasonography is the only non-invasive method, and can assess and evaluate gestational age, fetal position, growth, development, and many structural birth defects. When performed by highly experienced operators, ultrasonography can detect fetal structural abnormalities with up to 96% accuracy (Andrews et al., 1994; Carrera et al., 1995; Markov et al., 2006). However, the use of ultrasonography for the detection of chromosomal and genetic abnormalities has relatively low sensitivity and specificity (DeVore 2001; Queisser-Luft et al., 1998). For the major chromosomal abnormality of Down syndrome, ultrasonography can only detect about 53% of the affected pregnancies (Smith-Bindman et al., 2007). The other three methods (amniocentesis, CVS and FBS) are often used to detect fetal chromosomal and genetic abnormalities. They are highly accurate and reliable for diagnosis of fetal chromosomal and genetic abnormalities, but carry a 1-4% risk of fetal loss.

Amniocentesis. Amniotic fluid contains hormones, enzymes and amniocytes. The procedure to obtain amniotic fluid is often performed in the second trimester of pregnancy (15-18 gestational weeks) for at-risk pregnant women. In this procedure, a thin needle is inserted through abdomen and uterus into amniotic sac with ultrasonic guidance and 10-20 ml of amniotic fluid is withdrawn. The levels of hormones (e.g. α-fetoprotein) in the supernatant of amniotic fluid can be measured directly for screening various abnormalities such as anencephaly, spina bifida and omphalocele (Szabo et al., 1990). The enzymes and some metabolites can be used to detect fetal abnormality as well, but this has been largely replaced by molecular testing to achieve higher accuracy. The cells in

amniotic fluid can be cultured and used for analysis of chromosomal abnormalities and genetic disorders. The test results will be obtained within 2-3 weeks.

Recent use of quantitative fluorescent polymerase chain reaction (QF-PCR) assays (Adinolfi et al., 1997; Bili et al., 2002; Cirigliano et al., 2001) and fluorescence *in situ* hybridisation (FISH) (Eiben et al., 1998) to analyse uncultured amniocytes have allowed not only fast, but accurate detection of chromosomal aneuploidies and genetic disorders. The results can be obtained within hours. Use of real-time PCR technique to analyse uncultured amniocytes has also been demonstrated to be fast, reliable and specific for the diagnosis of aneuploidies (Hu et al., 2004).

Chorionic villus sampling. CVS is performed earlier in pregnancy (10-12 gestational weeks) as compared with amniocentesis. Placental villi which protect the fetus may be obtained through transcervical or transabdominal access to the placenta with ultrasonic guidance. Large amounts of fetal DNA can be isolated from the villi and genetic analysis can be done within 24-48 hours. This quick results and its use at earlier gestational age provide more time for counselling and decision-making, and if termination of pregnancy is elected, it can be performed much safer at this early stage (Weatherall 1991). There is no difference in fetal loss rates after transcervical or transabdominal CVS (Jackson et al., 1992). However, the risk of miscarriage and other complications after CVS is slightly higher than the risk after midtrimester amniocentesis (Caughey et al., 2006; Lippman et al., 1992; Mujezinovic et al., 2007).

Transabdominal fetal blood sampling. This method is also called percutaneous umbilical blood sampling (PUBS) and is usually performed after 20 gestational

weeks. A needle is inserted into the umbilical vein and fetal blood is drawn. The procedure presents the highest risk of miscarriage and is usually recommended only when diagnostic information can not be gathered via other tests or the results of those tests are inconclusive. For example, it may be useful to further evaluate chromosomal mosaicism discovered after CVS or amniocentesis is performed (ACOG 2007).

1.3 Disadvantages of current prenatal diagnostic tests

These relate to reliability, safety, accuracy of the procedures, and timing of availability of results.

Ultrasonography. The ultrasonography scanning is the only non-invasive diagnostic method and preferred by many women. However, whether the specificity and sensitivity are high enough as a diagnostic method largely depends on expertises of operators and the nature of the abnormalities. In addition, although significant progress has been made in the ability to detect fetal anomalies by ultrasound, some fetal anomalies cannot be detected during early pregnancy (Achiron et al., 1991). Furthermore, this method is mainly limited to the detection of structural defects, and used often in conjunction with maternal serum α-fetoprotein screening for prenatal screening.

Amniocentesis. The second trimester amniocentesis is considered as the "gold standard" for prenatal diagnosis, but it involves 0.5-1.0% risk of miscarriage (Wilson 2000), fetal injury, and maternal complications such as Rh sensitisation and chorioamnionitis. In addition, the procedure is performed at relatively late gestational age and the results may not be available until 18 weeks. Thus, termination of pregnancy, when indicated, may not be as safe as that in the first

trimester. The concept of the procedure (e.g., a needle in the abdomen) can be very distressing to some women with needle anxiety. Early amniocentesis, which is performed at 10-12 gestational weeks, would provide results earlier. However, higher risks are involved, that include increased risk of fetal loss, a higher rate of amniotic fluid leakage, a higher incidence of cell culture failure as well as orthopaedic and respiratory problems among children (Himes 1999). It generally takes longer to receive test results because fewer cells are present to initiate the cell culture for early amniocentesis. The Canadian Early and Mid-trimester Amniocentesis Trial (CEMAT) Group (Winsor et al., 1999) found the risk of miscarriage to be 2.6% for early amniocentesis as compared to 0.8% for the second trimester amniocentesis, and the risk of limb-defects after early amniocentesis was higher as well (1.3% vs. 0.1%).

Chorion Villi Sampling. Potential risks associated with this test include miscarriage and pregnancy complications. The risk of fetal loss is slightly higher for CVS than amniocentesis in the second trimester and less than early amniocentesis (Alfirevic et al., 2003). Although there were several reports of an associations between CVS and limb reduction defects (e.g. missing fingers and toes) (Firth et al., 1991; Mastroiacovo et al., 1993), the risk for these abnormalities may be only increased when CVS is preformed before 9 weeks of gestation (Botto et al., 1996) and this risk relates to the specific device used for sampling and the size of the sample. Other complications after CVS include vaginal spotting or bleeding, which may occur in up to 32.2% of patients after transcervical CVS is performed. The incidence of vaginal bleeding after transabdominal CVS is performed is less than that in transcervical CVS (Brambati et al., 2004). The incidence of culture failure, amniotic fluid leakage, or infection after CVS procedure is less than 0.5% (Brambati et al., 2004).

In addition, CVS has very limited use in the detection of neural tube defects (Cunniff 2004). Some cytogenetic laboratories reported that chromosomes from CVS were usually too short to identify micro-deletions or subtle chromosomal abnormalities (Nicolaides et al., 1996). Certain metabolic disorders are not expressed in villus cells, preventing prenatal diagnosis by CVS (Delisle et al., 1999).

Fetal blood sampling. This procedure is associated with a high risk of miscarriage (~2%) (Buscaglia et al., 1996). Thus, it is usually performed for those pregnancies in which the information required about the fetus (e.g. fetal blood type, fetal anaemia and infection) cannot be obtained accurately, completely, and/or in sufficient time to benefit pregnancy management by other prenatal diagnostic procedures. The main causes of fetal loss are rupture of membranes, chorioamnionitis, and puncture of the umbilical artery, bleeding from the puncture site and prolonged bradycardia (Antsaklis et al., 1998).

The disadvantages of current prenatal tests related to timing, accuracy, low but definite risk of fetal loss, fetal and maternal complications worry many women who undergo the procedures. Because of this and testing costs, only at-risk pregnant women determined by screening tests (e.g. serum screening, nuchal translucency assessment) are currently offered the invasive prenatal diagnostic tests, but 1 in 5 women would decline the invasive tests (Cuckle 1996) and this rate is at an increasing trend (Kocun et al., 2000). Maternal serum analyte screening and ultrasound are non-invasive methods, and can identify individuals at risk of fetal aneuploidy. However, their roles in the diagnosis of genetic disorders are limited due to the relatively low sensitivity and high false positive rate. Thus, the ideal test for aneuploidies and monogenic diseases should be

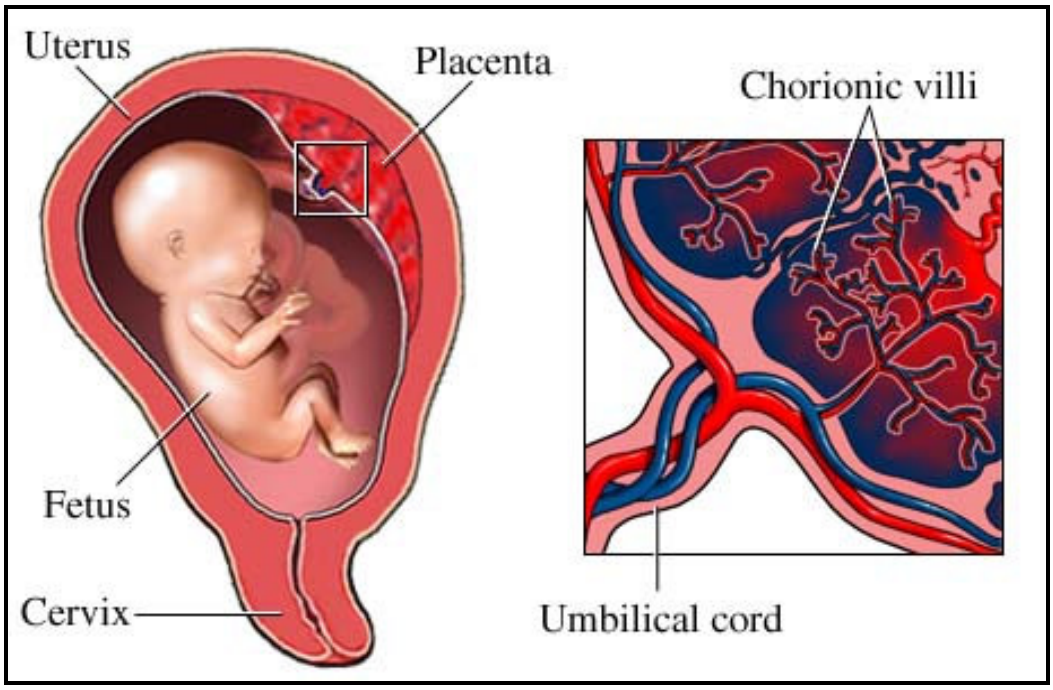
non-invasive, reliable and early prenatal diagnosis, which would be preferred and could be offered to all pregnant women.

1.4 Developmental biology relevant to non-invasive prenatal diagnosis

1.4.1 Placental development: the fetal-maternal interface

One week after fertilisation of an egg, it is developing into blastocyst which includes three structures: the trophoblast, which is the layer of cells that surrounds the blastocyst; the blastocoele, which is the hollow cavity inside the blastocyst; and the inner cell mass, which is a group of cells at one end of the blastocoele. The inner cell mass develops into the fetus while the trophoectoderm implants and eventually become part of the placenta (Cross et al., 1994). In the fully developed placenta Figure 1-1), fetal-derived tissue forms “finger-like growths” (villi) that enter and intermingle with the surface layer (endometrium) of the uterus. The fetal circulation extends down to the umbilical cord and branches into capillaries inside these villi. The villi are surrounded by a network of intervillous spaces, and the mother's endometrial arteries fill these spaces with blood. Endometrial veins remove the blood from these spaces. As a result, maternal blood continuously flows around the villi. The villi are the sites for exchanging materials between the fetal and maternal circulatory systems. The mother’s body supplies the fetus with oxygen, nutrients, and antibodies, removes the fetus’s carbon dioxide and other metabolic waste materials, and helps regulate fetal growth and physiology by circulating hormones (Gude et al., 2004). The fetus produces hormones that help to bring about changes in the mother's body to maintain the pregnancy.

Figure 1-1 Placenta and chorionic villi



Picture from http://www.nucleusinc.com

1.4.2 Fetal-maternal trafficking

The fetal and maternal circulations are separated by the placental membranes. However, this barrier is incomplete to both nucleic acid and cellular trafficking between fetus and its mother.

Nucleic acid trafficking. The demonstration of DNA trafficking came in 1997 with the application of sensitive modern molecular methods (Lo et al., 1997). In the pioneering study, cell-free fetal DNA was found to be present in both maternal plasma and serum. More recently, the presence of stable fetal RNA in maternal plasma was demonstrated (Ng et al., 2003a; Poon et al., 2000). As the fetal nucleic acid has relatively high concentration in maternal circulation (Lo et al., 1998b) and is quickly cleared out after delivery (Lo et al., 1999a), the use of this fetal nucleic acid for the development of non-invasive prenatal diagnosis has generated much interest (Lo 2005).

Shortly after the finding of fetal DNA in maternal circulation, the presence of maternal DNA was described in fetal circulation. Using real-time PCR amplification of maternal specific gene, Lo et al. (2000) found that maternal DNA were present in 30% of cord plasma samples (n=50), which is higher than maternal cellular fraction in cord blood (24%) (n=50). In another study, Bauer et al. (2002) used QF-PCR amplification of nine short tandem repeat markers and found that maternal DNA was present in 43 of 57 (75%) umbilical cord plasma. In contrast to fetal DNA in maternal circulation, maternal DNA has much lower concentration in fetal circulation, and is not affected by complications of pregnancy such as preeclampsia, suggestive of unequally transferred mechanism between fetus and mother (Sekizawa et al., 2003).

Cellular trafficking. Fetal cells were found in maternal circulation in as early as 1893, when trophoblasts were described in maternal pulmonary vasculature (Schmorl 1893). Since then, various types of fetal cells, such as fetal lymphocytes (Herzenberg et al., 1979; Walknowska et al., 1969), fetal erythroblastic cells (Bianchi et al., 1990) and their progenitors (Lo et al., 1994; Valerio et al., 1996), and fetal mesenchymal stem cells (O'Donoghue et al., 2003), have been detected and/or isolated from maternal blood. Not only can fetal cells cross into maternal circulation, but also some of them can persist in maternal circulation for years after delivery (Bianchi et al., 1996).

The observation of maternal cells trafficking into fetus was reported since 1960s, when routine karyotyping of newborn male infants showed the presence of sex chromosome mosaicism (el-Alfi et al., 1969; Turner et al., 1966). The presence of circulating maternal lymphocytes is common in peripheral blood of infants with severe combined immunodeficiency syndrome (Pollack et al., 1982). More recently, maternal cells were found in umbilical cord blood (Lo et al., 1996; Petit

et al., 1995), third trimester fetal blood (Petit et al., 1997) and even fetal blood at 13 gestational weeks (Lo et al., 1998a). Maternal cells can also circulate in fetal blood and/or deposit in all kinds of fetal tissues (Srivatsa et al., 2003).

The bi-directional fetal-maternal trafficking of nucleic acids and cells via placenta during pregnancy is well established (Hahn et al., 2005). The focus is now to understand the possible biological significance of fetal cells/DNA in maternal circulation and maternal cells/DNA in fetal circulation, and to isolate fetal cells/DNA from maternal circulation for non-invasive prenatal diagnosis.

1.4.3 Ontological development of erythropoiesis

During early human fetal development, two waves of erythropoiesis occur: primitive and definitive erythropoiesis. Primitive erythropoiesis begins in the yolk sac as early as day 18 of gestational age (Moore et al., 1970), which produces large (~25 μm), nucleated primitive erythroblasts expressing embryonic haemoglobin (ε, ζ) (Palis et al., 1998) and some primitive macrophages. These large primitive erythroblasts are predominant cell type in the fetal circulation during the first trimester pregnancy (Choolani et al., 2003) and likely cross into maternal blood. The primitive erythroblasts are probably able to enucleate during the circulation (Kingsley et al., 2004).

Definitive erythropoiesis originates in the aorto-gonado-mesonephros region, and migrates to the fetal liver at about 6 gestational weeks, then spleen and bone marrow (Galloway et al., 2003). It generates definitive erythroblasts that are smaller, expressing fetal globins (γ), and these cells terminally differentiate into anucleate erythrocytes (Peschle et al., 1985). Fetal globin is widely used as a fetal-specific marker for the enrichment and identification of fetal erythroblasts,

but it is not nearly specific enough for accurate fetal erythroblast identification because of increased maternal fetal globin production in pregnancy (Pembrey et al., 1973) and in β-thalassaemia patient (Weatherall 2000). In contrast, embryonic globins are now favoured because of higher specificity.

Besides their difference in sites of their production, timing, globin expression and morphology, primitive and definitive haematopoiesis require different transcriptional control and growth regulation. Definitive haematopoiesis requires both transcription factors myb and acute myeloid leukemia-1 whereas primitive erythropoiesis does not (Muller et al., 1994; Okuda et al., 1996; Wang et al., 1996). Similarly, c-kit receptor tyrosine kinase and its ligand are essential for progression into the definitive program, but are not required for the establishment of primitive erythropoiesis (Nocka et al., 1989). In addition, erythropoietin (EPO) was found to be essential for maturation of definitive erythroid lineage, but not for primitive erythropoiesis (Lee et al., 2001). These distinct features raise the possibility that they could have distinct cell surface antigen profiles as well.

1.5 Non-invasive prenatal diagnosis for chromosomal and single gene disorders

In the last decade, considerable efforts were made on development of non-invasive or minimally invasive techniques for prenatal diagnosis. These include approaches based on the analysis of fetal nucleated cells in maternal blood, the analysis of cell-free fetal DNA and RNA present in maternal plasma and serum, and the identification and isolation of fetal trophoblastic cellular elements shed into the uterine cavity and the endocervical canal.

1.5.1 Transcervical samples

In 1971, Shettles et al. (1971) observed that fetal cells (trophoblastic cellular elements) could shed into the uterine cavity and then into the endocervical canal. This opened the possibility of performing minimally invasive prenatal diagnostic tests by recovering fetal cells from transcervical cell samples. The collection of transcervical cell samples can be started as early as 5 gestational weeks (up to 15 gestational weeks). The recovery rate of fetal cells depends on the expertise of operators and the methods used for the collection (Rodeck et al., 1995). High rates of success (70-97%) have been reported using aspiration, lavage, or a cytobrush (Adinolfi et al., 2001).

Investigations have demonstrated the usefulness of transcervical samples for gender determination (Falcinelli et al., 1998), detection of aneuploidy (Sherlock et al., 1997) and RhD phenotype (Adinolfi et al., 1995). However, poor recovery of fetal cells (Overton et al., 1996), contamination by foreign genetic material (Antsaklis et al., 1998), and considerable variation in the composition and quality of recovered material (Miller et al., 1999) limit its applicability for non-invasive prenatal diagnosis.

1.5.2 Fetal DNA in maternal blood

In 1997, Lo et al. (1997) first demonstrated that cell-free fetal DNA was present in maternal plasma and serum using quantitative real-time PCR amplification of fetal sex-determining region Y (SRY). Subsequently, they found that fetal DNA has relatively high concentration in both maternal plasma and serum in early and late pregnancy (Lo et al., 1998b), and that fetal DNA is quickly cleared from maternal circulation after delivery (Lo et al., 1999a). These characteristics allow the diagnostic test by analysing cell-free fetal DNA to be more sensitive, and less susceptible to false positive results from previous pregnancy.

Two common applications of fetal DNA in maternal circulation are determinations of fetal gender and fetal RhD genotype. A 100% sensitivity and specificity could be achieved in fetal gender determination by amplification of fetal SRY gene in maternal plasma and serum at as early as 5 gestational weeks (Guibert et al., 2003; Ho et al., 2003; Honda et al., 2002). This highly accurate, non-invasive determination of fetal gender has important clinical implication in at-risk pregnant women bearing a fetus with an X-linked disorder. RhD genotype determination during pregnancies has been validated (Zhong et al., 2000a), and a 100% accuracy could be achieved with the use of maternal plasma from second trimester of pregnancy onwards (Lo et al., 1998c). Similarly, a 95-100% sensitivity and specificity were achieved in detection of RhD and E allele of RhCE using fetal DNA in maternal plasma (Legler et al., 2002).

Use of cell-free DNA to detect many other single gene disorders has been explored recently. These include the detection of achondroplasia (Saito et al., 2000), myotonic dystrophy (Amicucci et al., 2000), cystic fibrosis and Huntington disease (Gonzalez-Gonzalez et al., 2003a; Gonzalez-Gonzalez et al., 2003b), congenital adrenal hyperplasia (Chiu et al., 2002a), and β-thalassaemia (Li et al., 2005b).

The origin of cell-free fetal DNA in maternal circulation remains unclear, but accumulating data suggest that placenta is the major source (Bianchi 2004). An elevated level of fetal DNA was observed in complications of pregnancy, such as preeclampsia initiated by a placental lesion (Bianchi 2004), or fetal cytogenetic abnormalities (Bianchi 2004; Zhong et al., 2000b). Thus, the concentration of fetal DNA alone may serve as a marker for certain fetal chromosomal abnormalities, in particular trisomies 21 and 13. Indeed, a quantitative study has shown that a 2-fold increase in fetal DNA levels for pregnancy with trisomy 21

compared to normal cases (Lo et al., 1999b). However, development and standardisation of protocols for quantitation and amplification of fetal DNA would be required to make the test of fetal DNA in maternal plasma a clinically relevant analytical method.

To date, developments have been achieved in the use of fetal DNA in maternal plasma to detect some single gene disorders. However, the inability to distinguish maternally inherited fetal DNA from native maternal DNA is clearly a diagnostic impediment. Moreover, the use of fetal DNA in maternal circulation is thus far inapplicable to accurately diagnose fetal chromosomal disorders.

1.5.3 Fetal RNA in maternal blood

Presence of fetal RNA in maternal plasma was described shortly after the detection of cell-free fetal DNA in maternal circulation (Poon et al., 2000). Fetal RNA in maternal plasma is likely present in the form of apoptotic bodies, hence rendering them more stable as compared to 'free RNA'. Both placenta and haematopoietic cells probably contribute to the stable RNA pool in the maternal plasma as evidenced by the detection of placenta specific genes (Ng et al., 2003b) and erythroid specific epsilon/gamma gene (Xu et al., 2003) respectively, but probably much of them originates from the placenta (Wong et al., 2005). Similar to fetal DNA in maternal circulation, fetal RNA is rapidly cleared following delivery (Chiu et al., 2006).

Fetal RNA in maternal plasma is detectable from 4 gestational weeks, and increases with the advance of gestational age (Chiu et al., 2006). Concentration of fetal RNA is higher in the carriers of the female fetus than in the carriers of the male fetus in the first trimester (Ge et al., 2005). Elevation of fetal RNA was shown in maternal plasma with certain pregnancy disorders such as

preeclampsia (Ng et al., 2003b). Collectively, these may allow profiling of fetal RNA in maternal plasma for non-invasive prenatal diagnosis. In addition, circulating fetal RNA can be applied to all pregnancies without the limitations of fetal gender. However, there is lack of fetal- and disease-specific plasma RNA marker currently and thus more investigation is needed.

1.5.4 Fetal cells in maternal blood

As early as in 1893, fetal cells were thought to be present in maternal circulation (Schmorl 1893). However, definitive proof of fetal cells in maternal blood did not come until 1960s when leukocytes bearing chromosome-Y-specific DNA sequences were detected in maternal blood (Walknowska et al., 1969). Successful detection and enrichment of fetal leukocytes using FACS was reported in the late 1970s (Herzenberg et al., 1979) and early 1980s (Iverson et al., 1981). More convincing evidence of the existence of fetal cells in maternal blood came in the 1990s with the application of modern molecular techniques such as PCR and FISH to detect unique fetal DNA sequences from cellular components of maternal blood (Bianchi et al., 1990; Bianchi et al., 1992; Camaschella et al., 1990; Ganshirt-Ahlert et al., 1993).

Fetal erythroblastic cells have been recovered and identified from maternal blood by the combined use of FACS cell sorting and PCR or FISH detection technique (Bianchi et al., 1990; Holzgreve et al., 1992). Trophoblasts have also been successfully isolated and identified from an abnormal pregnancy (XXY fetus) using FACS and FISH (Cacheux et al., 1992). Similar results were obtained when MACS was used for cell sorting of fetal NRBCs (Ganshirt et al., 1994b; Ganshirt-Ahlert et al., 1993) and trophoblasts (Hawes et al., 1994; Mueller et al., 1990).

However, despite considerable progress in detection and isolation of fetal cells from maternal blood, the reproducibility and reliability remain poor, probably because of the rarity and variability of fetal cells among pregnancies. Thus, several groups attempted to quantify fetal cells in maternal circulation using either unsorted (Bianchi et al., 1997; Hamada et al., 1993; Krabchi et al., 2001) or sorted samples (Price et al., 1991). Bianchi et al. (1997) reported an estimated 1 fetal cell per ml of maternal blood using quantitative PCR to amplify Y chromosome-specific sequence of unsorted maternal blood samples. However, the number may be overestimated in their study in that there were possible false positive detections as 25.7% of 109 female pregnancy samples appeared to have Y-cell equivalents, and that they included all kinds of fetal cells (e.g. progenitors, leukocytes and lymphocytes), some of which tend to persist postpartum and thus could be from a previous pregnancy (Bianchi et al., 1996; Ciaranfi et al., 1977; Schroder et al., 1974). Hamada et al. (1993) examined the frequency of fetal cells in maternal blood across three trimesters in unsorted samples using FISH technique. They reported an even higher frequency of fetal cells in maternal blood and showed that the frequency of fetal nucleated cells was positively correlated with gestational age: fetal cells changed from 1 in 100,000 maternal nucleated cells (~10 fetal cells per ml maternal blood) in the first trimester to 1 in 10,000 at term. However, using similar method, Krabchi et al. (2001) found only 2-6 fetal nucleated cells per ml of second trimester maternal blood.

Specifically, Price et al. (1991) quantified fetal NRBCs using sorted blood sample. They enriched fetal NRBCs from first and second trimester blood samples before invasive procedure using Ficoll density gradient centrifugation and FACS with anti-CD71 and anti-Glycophorin A (GPA) antibodies. PCR amplification of Y chromosome-specific sequence gave a ratio of 1 fetal NRBC: 10^8 maternal nucleated cells (equivalent to 1 fetal NRBC in 10 ml of maternal blood).

However, this study also has limitations. First, while CD71 was strongly positive on 100% of fetal definitive erythroblasts and on 96% of maternal NRBCs, it was only expressed on ~68% fetal NRBCs in the first trimester (Choolani et al., 2003). As such, first trimester fetal NRBCs could be lost during FACS sorting. Second, the density of fetal NRBCs varies in a wide range and a significant loss was reported even with optimised density gradient centrifugation (Choolani et al., 2003).

Despite the differences in the observed frequencies of fetal cells in maternal blood, there is consensus of elevated fetomaternal cellular trafficking in some abnormal pregnancies. The elevated number of fetal NRBCs in the maternal circulation in pregnancies affected by preeclampsia and before the onset of preeclampsia symptoms were first shown in 1969 (Jones et al., 1969) and confirmed by several recent studies (Al-Mufti et al., 2000; Ganshirt et al., 1998; Holzgreve et al., 1998; Simchen et al., 2001). The increase of fetomaternal trafficking in pregnancies with abnormal Doppler of the uterine artery without symptoms of preeclampsia (Al-Mufti et al., 2000), fetal growth restriction and polyhydramnios (Zhong et al., 2000c) was also observed. Moreover, it was shown of a 6-fold elevation of fetal cells in the maternal blood in pregnancies bearing trisomy 21 fetus (Bianchi et al., 1997; Parano et al., 2001), and 2-5 times higher in other aneuploid pregnancies including trisomy 18, 13, triploidy (69,XXX), aneuploidy (47,XXX, 47,XXY, 47,XYY, 47,XY,r(22),+r(22)) (Krabchi et al., 2006).

The usefulness of fetal cells in maternal blood for non-invasive prenatal diagnosis has been demonstrated in gender determination (Bianchi et al., 1993; Mavrou et al., 1998), diagnosis of fetal chromosomal aneuploidies (Lin et al., 2002; Wang et al., 2000a), and single gene disorders such as Mendelian disorders (Camaschella et al., 1990; Cheung et al., 1996), human leukocyte antigen (HLA)

polymorphisms, and fetal RhD genotype (Toth et al., 1998). By examining fetal cells isolated from maternal blood, the NIFTY clinical trial demonstrated a 74.4% detection rate in the determination of common aneuploidies (13, 18, 21, X and Y), with a false positive rate as low as 0.6%, which is slightly better than current non-invasive methods such as serum screening or ultrasound (Bianchi et al., 1999; Bianchi et al., 2002).

1.5.5 Candidate of fetal cells for non-invasive prenatal diagnosis

Trophoblasts. As the first fetal cell type found to have crossed into maternal circulation (Schmorl 1893), trophoblasts appeared as an attractive cell type for non-invasive prenatal diagnosis. They have distinct morphology which permits microscopic identification and are commonly deported into maternal circulation extensively during the first trimester pregnancy. The early separation of this cell type came in 1980s. Goodfellow et al. (1982) reported that trophoblasts were detected in 6 out of 10 pregnant women by immunofluorescence stain of sorted maternal blood sample. Since then, many studies have demonstrated successful isolation of trophoblasts from maternal blood using other antibodies. Mueller et al. (1990) screened 6,800 antibodies and found five that were specific to trophoblasts. When two of them (FDO161G and FDO66Q) were applied to enrich trophoblasts from maternal blood for gender determination, they correctly determined fetal sex in 11 out of 12 pregnancies but with 1 false positive result. Bruch et al. (1991) isolated trophoblasts from peripheral blood of women bearing male fetuses using three monoclonal antibodies (GB17, GB21, and GB25) against trophoblasts. They were able to detect PCR amplification of Y-chromosome specific sequence in two of three samples. Van Wijk et al. (1996) isolated trophoblasts by depletion of maternal white blood cells (WBCs) using anti-α-CDw50 from the enriched mononuclear cells by Percoll density gradient

centrifugation, and obtained a 91.7% success rate in fetal gender determination (n=36).

However, some recent studies reported the inability to identify trophoblasts in maternal blood. Hviid et al. (1999) could not recover trophoblasts from maternal blood using anti-LK26, despite the successful use of this antibody for the separation of trophoblasts in spiked sample. Kuhnert et al. (2000) used anti-EGF-receptor, which was shown to be specific to trophoblasts (Durrant et al., 1994), to isolate fetal cells from maternal blood and yielded no fetal cells. Similarly, Schueler et al. (2001) used both trophoblast-specific antibodies and separation strategies that were successfully used by others (Durrant et al., 1996; Mueller et al., 1990) to isolate fetal cells from 30 ml maternal blood in the first trimester. No positive identification was possible with either immunoreactive proteins or mRNAs for specific expressed genes for trophoblasts.

These conflict data indicate that although trophoblast deportation is a common biological phenomenon, trophoblasts may not be consistently detectable for all pregnancies. Indeed, it is known that trophoblasts are often cleared rapidly by the pulmonary circulation in a normal pregnancy (Attwood et al., 1961), and that there is apparent paucity of highly specific antibodies for their enrichment. In addition, trophoblasts are part of the placenta, which carries a 1% incidence of chromosomal mosaicism (Bianchi 1999). As such, the isolated trophoblasts may not be fully representative of fetal genotype. Moreover, syncytiotrophoblasts are multinucleate and not suitable for FISH analysis of aneuploidy. Thus, trophoblasts do not appear to be a viable candidate for fetal screening using maternal blood as the source.

Leukocytes. Walknowska et al. (1969) first described a Y chromosome in mitogen-stimulated lymphocytes in blood cells from pregnant women bearing male fetuses. This report provided practical and theoretical implications of fetomaternal lymphocyte transfer. Ten years later, fetal leukocytes were recovered from maternal blood using FACS with antibodies against paternally-derived HLA antigens (Herzenberg et al., 1979) and subsequently, fetal gender and HLA type were predicted using the isolated lymphocytes (Iverson et al., 1981). However, the clinical use of this cell type is considered somewhat impractical in that it requires to perform HLA tests of both parents prior to cell sorting and that some apparent fetal lymphocytes in the maternal blood could persist years after delivery (Schroder et al., 1974). Moreover, fetal leukocytes were not consistently isolated from maternal blood (Adinolfi 1995).

Stem cells and progenitors. The problem of the rarity of fetal cells in maternal blood could be overcome if the enriched fetal cells can be expanded *in vitro*. This would allow the amplification of the limited number of cells enriched and thus more genetic materials for diagnostic tests. In this regards, fetal stem cells and progenitors would be ideal cell types.

Early, investigators demonstrated some success of *in vitro* expansion of fetal erythroid progenitors isolated from maternal blood (Lo et al., 1994; Valerio et al., 1996). However, in a similar experiment, Chen et al. (1998) were not able to reproduce these data. Other groups focused on CD34+ fetal cells because of their higher proliferative potential. Little et al. (1997) found a 0-7 fetal CD34+ cells in 20 ml maternal blood. Coata et al. (2001) reported similar number (0-11 cells) of fetal cells in 20 ml maternal blood after culturing CD34+ cells. The small number and the inability to recover CD34+ fetal cells from all maternal samples limit their potential for clinical tests. In addition, these cells are able to persist in

the maternal circulation for as long as 27 years (Bianchi et al., 1996), thereby complicating diagnosis in subsequnt pregnancies.

Campagnoli et al. (2001b) recently identified mesenchymal stem cells in fetal blood. These cells are present in fetal blood from first trimester pregnancy onwards and account for 0.4% of fetal nucleated cells. The frequency of these cells declines with gestational age. Their presence in maternal blood was demonstrated in 1 out of 20 post-termination maternal blood by the culture of CD45-/GPA- cell fraction (O'Donoghue et al., 2003). Clearly, this low frequency and possible persistence after delivery render this cell type inapplicable for non-invasive prenatal diagnosis in clinical setting.

Fetal erythroblasts. The ideal cell type for non-invasive prenatal diagnosis should be short-lived within the maternal circulation, morphologically distinct from maternal cells, and possess cell surface markers to ease enrichment in all pregnancies. These requirements led to the choice of fetal NRBCs as target cells. Moreover, fetal NRBCs are consistently found in maternal blood during pregnancy (Parano et al., 2001), bear a fully representative complement of fetal genes, are the predominant fetal cell type in the first trimester gestation fetal blood (Thomas et al., 1962), and harbour developmentally-specific markers (e.g. embryonic globin).

The transfer of this cell type between mother and fetus is an early documented phenomenon (Schroder 1975), but considerable effort to isolate them from maternal blood came in 1990s. Bianchi et al. (1990) successfully enriched fetal NRBCs using anti-CD71 and FACS in 75% maternal blood samples prior to amniocentesis. Wachtel et al. (1991) enriched fetal NRBCs using multiparameter FACS and correctly determined fetal gender in 17 of 18 pregnancies by PCR

amplification of Y-chromosome specific sequences. Subsequently, Bianchi et al. (1993) demonstrated that a 100% gender determination could be achieved using enriched fetal NRBCs from either anti-GPA alone or in combination with anti-CD71 or anti-CD36 enrichment.

One of the earliest use of enriched fetal NRBCs for prenatal diagnosis was reported in 1991 (Price et al., 1991). Many chromosomal abnormalities have since been detected using fetal NRBCs enriched by either FACS or MACS (Bianchi et al., 1992; Ganshirt-Ahlert et al., 1992; Ganshirt-Ahlert et al., 1993). These promising results had led to a large scale clinical trial (NIFTY) to use fetal NRBCs in maternal blood for the determination of fetal gender and the detection of fetal aneuploidies (Bianchi et al., 2002).

1.5.6 Fetal NRBCs for non-invasive prenatal diagnosis: current state of the art

1.5.6.1 Current enrichment of fetal NRBCs from maternal blood

Due to the rarity of fetal cells in maternal blood, a rapid, simple, and consistent procedure for their isolation has to be developed before their clinical applications for non-invasive prenatal diagnosis. During the last decade, many approaches were developed to recover fetal NRBCs from maternal blood, that include density gradient centrifugation, filtration, selective RBC lysis, charge flow separation, lectin-affinity separation and antibody-based separation (Bianchi 1999, 2000; Wachtel et al., 2001).

Density. The buoyant densities of RBCs, WBCs and fetal NRBCs in maternal blood are different. Most of fetal NRBCs have a density close to that of mononuclear cells and slightly lower than RBCs. Thus, density gradient

centrifugation can be used as an initial enrichment step to deplete the bulk of maternal RBCs. For this purpose, single, double or triple density gradients have been developed as a routine first step to isolate fetal NRBCs from maternal blood using either Percoll or Ficoll density solutions (Al-Mufti et al., 1999; Prieto et al., 2001; Samura et al., 2000).

In 1993, Bhat et al. (1993) described a discontinuous triple density gradient and obtained a 25-fold enrichment of fetal NRBCs in their spiking experiment. Ganshirt-Ahlert et al. (1993) used a triple density gradient (Ficoll 1077, 1110 and 1119) that enabled them to recover 100 to 1000 NRBCs from 40 ml of maternal blood. Takabayashi et al. (1995) developed a discontinuous Percoll density gradient with which they enriched a mean of 4.1 fetal NRBCs from 2 ml of maternal blood (n=11). Other investigators used continuous Ficoll gradients with different densities varying from 1077 to 1119 (Lewis et al., 1996; Oosterwijk et al., 1998b; Troeger et al., 1999). In 1999, Sekizawa et al. (1999) introduced an appropriate density gradients: Percoll density gradient 1.083 and 1.090, with which they recovered a mean of 13.3 and 11 fetal NRBCs from 10 ml maternal blood respectively.

The denser gradients allow more fetal NRBCs to be recovered from the gradient interface and less fetal cells to settle in the cell pellet (Samura et al., 2000; Troeger et al., 1999). Over time, single denser gradients are preferred and most investigators prefer Ficoll 1119. This density gradient has been shown to be useful in the separation of fetal NRBCs from maternal blood (Prieto et al., 2001; Samura et al., 2000; Troeger et al., 1999). In 2003, Choolani et al. (2003) showed, in the separation of first trimester primitive fetal NRBCs, that although Ficoll 1119 gave the most promising recovery (32%) in all Ficoll solutions, Percoll 1118 yielded much better recovery rate (64.1%) of fetal NRBCs.

More recently, Sitar et al. (2005) described a novel method to enrich fetal NRBCs by modifying their densities using non-physiological conditions. In their study, whole maternal blood was suspended into a medium of low pH and slightly high osmolarity. Under such conditions, cell volume perturbations occurred either by osmolarity and/or pH changes and the composition of extracellular fluids which eventually enlarged the density difference between fetal NRBCs and maternal cells. Fetal NRBCs were then separated from maternal cells (RBCs and WBCs) using one-step density gradient centrifugation. In the study, they could detect fetal NRBCs and correctly diagnosed fetal aneuploidies by FISH in all 105 maternal blood samples.

Enrichment of fetal NRBCs by density gradient centrifugation is currently the most commonly used method. However, this method is associated with an inevitably high loss of fetal NRBCs, usually between 30% and 70%. Meanwhile, the resulting fetal NRBCs are still dispersed in a large amount of maternal WBCs. As such, further enrichment would be required, usually by an antibody dependent procedure such as FACS or MACS.

Filtration. Fetal NRBCs are larger than adult RBCs (5-8 μm). Based on this property, fetal NRBCs can be enriched using a molecular filter (non-woven cloth) in place of commonly used density gradient centrifugation by filtering through of the bulk of maternal RBCs. This strategy enabled Wachi et al. (2004) to recover as many as 19.5 (± 12.8) NRBCs from 3.5 ml maternal blood samples (n=12), and 63.6% of them were fetal origin. The recovery rate of fetal NRBCs were 4.2 (± 5.0) times higher than commonly used Ficoll density separation. However, this approach has not been used by other groups, probably due to the need to make in-house optimal molecular filters.

Charge flow separation. Negative charge on most blood cells is conferred by sialic acid molecules and the negative surface charge density on RBCs is dependent on the sialic acid content (Suzuki et al., 1998). As most blood cells have their surface charge densities characterised, they can be sorted by charge flow separation. It was demonstrated that fetal NRBCs could be separated from maternal blood cells under optimal charge conditions (Shulman et al., 1998; Wachtel et al., 1996) but the efficiency is lower compared to that of surface antigen based separation.

Lectin-binding method. The sugar moiety resulted from protein glycosylation on the surface of fetal NRBCs and RBCs was suggested to be different (Wachi et al., 2004). Such a difference could be used for cell separation by differential lectin-binding selection. One of the lectins, Soyabean agglutinin (SBA), recognises N-acetylgalactosamine and galactose and can simultaneously bind to blood cells and polymers coated on plates (Reisner et al., 1981). The usefulness of SBA for the enrichment of fetal NRBCs was recently demonstrated by Kitagawa et al. (2002). In this study, one to several hundreds NRBCs were recovered from only 2.3 ml of maternal blood in 96% of 131 pregnant women between 6 and 27 gestational weeks. In a similar study, Shinya et al. (2004) identified fetal gene in all seven cases using SBA isolation, micromanipulation and PCR analysis.

Selective RBC lysis. RBC lysis in isotonic solutions containing ammonium chloride (NH_4Cl), ammonium bicarbonate (NH_4HCO_3), and a carbonic anhydrase enzyme inhibitor (acetazolamide) is a function of RBC enzyme activity and permeability of cells to the inhibitor. Carbonic anhydrase is at least 5-fold greater and acetazolamide permeability about 10-fold lower in RBCs compared to fetal NRBCs (Boyer et al., 1976). As a result, fetal NRBCs are much more resistant to NH_4Cl lysis than RBCs. This characteristic could be used for the development of

a protocol to enrich fetal NRBCs from maternal blood by selective lysis of RBCs (de Graaf et al., 1999). However, recent data suggested that the lysis procedure may affect the fetal NRBCs by modifying the membrane properties, which causes significant clumping and poor cell morphology, and hence has a limited role in fetal NRBC enrichment (Choolani et al., 2003).

Intracytoplasmic marker. Fetal globin (e.g. γ-globin) is expressed predominantly in fetal NRBCs but rarely in adult RBCs, and thus can be useful marker for enrichment and identification of fetal NRBCs. DeMaria et al. (1996) developed an intracellular staining protocol that combined anti-γ-globin monoclonal antibody and Hoechst 33342 to enrich fetal NRBCs using FACS. In their sorting of spiking sample with male umbilical cord cells and female adult, non-pregnant peripheral blood mononuclear cells, the yield of NRBCs was 39-100% with a purity of 59-73%. Subsequent FISH analysis demonstrated that 83-100% (mean: 98%) of γ-globin enriched cells were male, whereas 82-100% (mean: 92.5%) of γ-globin negative cells were female. However, targeting intracellular antigens makes the purification steps more subject to cell loss, as the fragile erythroid cells need to be permeabilised to make globin chain accessible to these antibodies.

Surface antigen. At present, no surface antigen highly specific to fetal NRBCs has yet been found. A combination of antibodies is commonly used for separating fetal NRBCs from maternal blood. Possible targeting surface antigens include CD45, GPA, CD71, CD36, CD35, CD47 and erythropoietin receptor (EPO-r). GPA is present on all erythrocytes while CD45 is absent. CD71 is a transferrin receptor that is expressed on all cells that incorporate iron such as activated lymphocytes, trophoblasts and erythroid cells from the burst forming units-erythroid (BFU-e) to the reticulocyte stage (Loken et al., 1987) and definitive

fetal NRBCs in maternal blood (Cheung et al., 1996). CD71, CD36 and EPO-r expression are lost beyond the reticulocyte stage, while CD35 is only expressed on mature erythrocytes (Telen 2000).

Thus, strategies for enrichment of fetal NRBCs could be developed using EPO-r (Valerio et al., 1997), CD71 (Price et al., 1991; Zhong et al., 2000c), CD36 or their combinations (Bianchi et al., 1993) after density gradient centrifugation. Valerio et al. (1997) showed that the enrichments by anti-EPO-r and anti-CD71 displayed similar detection sensitivity for fetal gender (55% EPO-r, 61% CD71). This relatively low sensitivity was mainly due to the lack of EPO-r and CD71 expression specificity. A higher sensitivity could be obtained by enhancing the purity of fetal NRBCs using dual MACS sorting, in which depletion of WBCs (Ganshirt et al., 1998) was followed by positive selection of erythroid cells. It has been demonstrated that fetal NRBCs could be found in about 70-90% pregnancies in a combined use of CD45 with CD14 (Busch et al., 1994; Reading et al., 1995) or with CD32 (Andrews et al., 1995; Zheng et al., 1993) to deplete maternal nucleated cells and CD71 to select fetal NRBCs.

Bianchi et al. (1993) compared the effectiveness of CD71, CD36 and GPA alone or their combinations for the separation of fetal NRBCs from maternal blood by flow sorting. The use of CD71 and CD36 alone gave 57% and 88% gender prediction respectively; whereas the use of GPA alone or in combination with CD71 or CD36 gave 100% gender prediction. This suggested that CD71 alone might not be efficient for fetal NRBC enrichment. Indeed, only some of fetal NRBCs express CD71 in healthy pregnancies throughout the first trimester and the expression on positive cells was weak compared to that of definitive fetal NRBCs (Choolani et al., 2003).

Thus, instead of using CD71, some investigators used CD45 and/or GPA for selection of fetal NRBCs followed by selective lysis of maternal RBCs (de Graaf et al., 1999). However, the use of anti-GPA for selection is also problematic because GPA separation often causes agglutination of non-NRBCs, thereby changing both light scatter and fluorescence properties of cells by flow cytometry (Lewis et al., 1996) and complicating FISH analysis. Thus far, there is no specific surface marker that can efficiently separate fetal NRBCs from maternal RBCs.

Device for cell sorting. Systems suitable for cell sorting include Dynal system, ferrofluid suspensions, micromanipulation, FACS and MACS. Of these, FACS and MACS are the two most commonly used systems for antibody labelled cell sorting.

FACS was first used for isolation of fetal cells by Herzenberg et al. (1979). FACS is able to enrich cells with high purity so that slides with sorted cells can be readily scanned manually. Furthermore, it also allows multiparameter sorting: simultaneous analysis of several criteria on a single cell and can be adapted for use with intracytoplasmic antigens. Its limitations are the high cost, high maintenance and the need for specially trained laboratory staff. Reported studies and clinical trials suggested that MACS was more suitable than FACS for the enrichment of fetal cells from maternal blood, as the target cell recovery and fetal cell detection were better with MACS than FACS (Bianchi et al., 2002).

The use of MACS for non-invasive prenatal diagnosis was first recommended by Ganshirt-Ahlert et al. (1992). MACS is a fast and economical bench top technique that is able to process higher cell numbers. This technique can be performed without the requirement for equipped personnel. Positive selection or depletion by MACS can be carried out by exploiting the antigenic differences

between cells. The disadvantages include that cell separation can only be accomplished in order as cell selection is based on one antigen at a go, and that purity is poorer as compared with FACS (Wang et al., 2000b).

1.5.6.2 Identification of fetal origin of enriched NRBCs from maternal blood

The accuracy of prenatal diagnosis using fetal cells enriched from maternal blood depends on the specificity of their identification. Methods to identify fetal origin of enriched cells include the use of morphological criteria, immuno-labelling with embryonic (ε, ζ) and fetal (γ) globin, and molecular techniques PCR and FISH to detect Y-chromosome specific sequence.

Fetal NRBCs include primitive NRBCs and definitive NRBCs. Definitive fetal NRBCs, which generated from fetal liver, spleen and bone marrow, are usually isolated from maternal blood of late first trimester and second trimester pregnancy. These cells contain fetal globin (γ). It was early suggested that fetal origin of NRBCs could be confirmed by labelling intracytoplasmic, developmentally-specific fetal globin (Zheng et al., 1993). Since then, most investigators used γ-globin for fetal cell identification (de Graaf et al., 1999; DeMaria et al., 1996; Parano et al., 2001). More recently, it has been suggested that the γ-globin mRNA specific probes could be used to detect and isolate fetal NRBCs from maternal blood (Larsen et al., 2003). However, the use of γ-globin has its limitation as some of maternal NRBCs also express γ-globin (Pembrey et al., 1973). This “leaky” expression of γ-globin in adults led Cheung et al. (1996) to suggest the use of the embryonic ζ-globin instead. They have demonstrated this principle in the case of sickle cell anaemia and also in the case of β-thalassaemia on micromanipulated, ζ-globin positive NRBCs enriched from maternal blood between 10-12 gestational weeks. However, although ζ-globin is

less likely to be present in adult blood cells, its expression exists in adults with the α-thalassaemia trait (Chung et al., 1984). Moreover, its expression is not completely switched off after the embryonic period and ζ-globin is present in 53% of definitive erythrocytes between 15-22 gestational weeks and 34% at term (Luo et al., 1999).

Primitive fetal NRBCs generated from yolk sac are large and contain embryonic globins and fetal globin. Thus, it is possible that primitive NRBCs, enriched from first trimester pregnancy, can be identified morphologically, but more conclusive evidence has been usually achieved by immuno-labelling with embryonic globin. The use of ζ-globin for fetal NRBC identification has its limitation as discussed above. The ε-globin was earlier demonstrated to be present in male fetal NRBCs from post-CVS maternal blood samples (Mesker et al., 1998). Subsequently, Mavrou et al. (1999) described the use of ε-globin for the identification of fetal NRBCs from supernatant fluid during CVS procedure and suggested that it was more reliable and specific than ζ-globin for the identification of fetal NRBCs in maternal blood, which was confirmed by other studies (Choolani et al., 2003; Christensen et al., 2003).

The use of fetal specific protein expression for identification of fetal NRBCs has an important role currently. However, modern molecular techniques FISH and PCR for the detection of fetal specific gene (Y-sequence in male pregnancy, or paternally inherited fetal HLA-DQa gene sequences (Geifman-Holtzman et al., 1995)) are considered more reliable, and usually used for the confirmation of fetal identity.

1.5.6.3 Diagnosis of chromosomal and monogenic disorders by analysing fetal NRBCs from maternal blood

The three most important molecular techniques that have allowed genetic analysis of enriched fetal cells are PCR, reverse transcription-polymerase chain reaction (RT-PCR) and chromosomal FISH.

PCR. The ability of PCR to amplify minute quantities of DNA over a billion-fold, in particular, in combination with micromanipulation to reduce PCR noise, has been exploited by several investigators to demonstrate the possibility of prenatal diagnosis of monogenic disorders using fetal cells enriched from maternal blood. Sekizawa et al. (1996) demonstrated that it was possible to select a single fetal NRBC by micromanipulation and to potentially diagnose genetic conditions such as Duchenne muscular dystrophy by PCR. One limitation of that study was that NRBCs identified by morphology alone were presumed to be fetal. Cheung et al. (1996) used micromanipulation to pick ζ-globin positive NRBCs and correctly diagnosed fetal haemoglobinopathy in two pregnancies between 10-12 gestational weeks. The usefulness of micromanipulation prior to PCR has since been confirmed by Watanabe et al. (1998) who demonstrated the prenatal diagnosis of the X-linked ornithine transcarbamylase deficiency syndrome and by Samura et al. (2001) who demonstrated the adjunctive use of PCR and FISH to correctly diagnose trisomy 21. Other investigators have used fluorescent PCR to detect single gene defects and/or aneuploidy (Griffin et al., 1999; Osborne et al., 2005). The main advantage of single-cell PCR based analysis is the relative high efficiency and specificity. The disadvantage is that the collection of single cells needs a lot of experience with regard to micromanipulation and transferring the cells to reaction tubes and is associated with a significant loss of the rare fetal cells.

RT-PCR. In cells expressing a particular gene, there are many more copies of the RNA message compared with only one or two alleles within the genome. Thus, RT-PCR for the analysis of fetal mRNA could be more sensitive than PCR amplification of genomic DNA. Indeed, a double sensitivity for the non-invasive prenatal diagnosis of fetal RhD status using RT-PCR was observed (Al-Mufti et al., 1998). However, a later study showed that PCR detection of fetal DNA in maternal plasma was more sensitive than RT-PCR detection of fetal RNA, as fetal RNA is more susceptible to enzymatic degradation in maternal blood than fetal DNA (Poon et al., 2000). These conflict data suggested that the value of RT-PCR in non-invasive prenatal diagnosis remains controversial.

Chromosomal FISH. The place of FISH in non-invasive prenatal diagnosis has been firmly established. Price et al. (1991) demonstrated the use of this techniques in the prenatal diagnosis of trisomies 18 and 21. Elias et al. (1992) extended these observations by diagnosing fetal trisomy 21 in the maternal blood of a 42-year-old woman taken before CVS. The first series was reported by Ganshirt-Ahlert et al. (1993) who confirmed five cases of trisomy 18 and ten cases of trisomy 21 using NRBCs enriched from maternal blood. Subsequently, Trisomy 13 was also reported to be non-invasively diagnosed by FISH analysis at 11 gestational weeks pregnancy (Oosterwijk et al., 1998b). Ever since, many studies have reported to non-invasively detect these commonest trisomies using fetal NRBCs from maternal blood by FISH (Hromadnikova et al., 2002; Yang et al., 2003).

Meanwhile, non-invasive diagnosis of other chromosomal abnormalities was also reported by FISH analysis of enriched fetal cells. Cacheux et al. (1992) showed the reliable detection of sex chromosome aneuploidy and Bischoff et al. (1995) could detect sex chromosome mosaicism. Similarly, Pezzolo et al. (1997)

described to correctly diagnose fetal triploidy by FISH on enriched fetal cells. Some investigators attempted to increase the number of chromosomes that could be analysed by interphase FISH by performing simultaneous, dual-colour (Al-Mufti et al., 1999) and multicolour FISH (Bischoff et al., 1998) or by sequential hybridisation of chromosome pairs (Zhen et al., 1998). The main advantage of FISH is that the counting of fetal cells could be realised automatically in the future. With such automatic analysis it could be possible to examine a large number of cells in short time; it could also be more sensitive than manual evaluation (Oosterwijk et al., 1998a). The disadvantage of FISH for fetal cells is low efficiency as compared to PCR.

1.6 Challenges to the use of fetal NRBCs in maternal blood for non-invasive prenatal diagnosis

The initial promising results using fetal cells in the maternal blood to diagnose or screen fetal chromosome abnormalities, either by PCR, RT-PCR or FISH had led to a large scale clinical trial (NIFTY) (Bianchi et al., 1999). However, the relatively low accuracy to detect fetal aneuploidies indicates that currently fetal cells derived from maternal blood could only be used alone or (more likely) in combination with other modalities (e.g. biochemical tests) as a screening method, but not yet as a diagnostic tool. This is mainly due to the difficulty in recovering enough fetal NRBCs from maternal blood. Thus, while some researchers are working to improve the efficiency of enrichment techniques, other groups attempted to expand fetal erythroblasts *in vitro* before or after the enrichment.

1.6.1 Inability to expand fetal cells from maternal blood *in vitro*

Fetal erythroid cells are more sensitive to EPO than their adult counterparts (Weinberg et al., 1992). Thus it is possible to preferentially expand fetal erythroid

cells over adult cells under an EPO-enriched culture medium (Lo et al., 1994). Indeed, the formation of colony forming unit (CFU)-erythroid containing fetal cells by culturing anti-EPO selected cells was demonstrated (Valerio et al., 1996) but these results were not reproduced by independent group (Chen et al., 1998). More recently, Han et al. (1999) developed an optimal two-phase liquid culture protocol to expand fetal erythroid cells: mononuclear cells from maternal blood were firstly cultured for 4-5 days and non-adherent cells were re-cultured with EPO for another 3-4 days. They found that fetal erythroid cells were up to about 5.7% of the total erythroid cells displayed by fetal globin staining, and that male DNA was detectable by PCR in 7 out of 10 cases. However, 4 out of the 7 SRY positive pregnancies and one of SRY negative pregnancy were actually bearing male fetuses. This suggests the low sensitivity and specificity of the culture protocol. Other efforts were made to optimise fetal erythroid cell selection methods and/or culture conditions using model experiment where a known number of fetal cells were spiked with adult blood (Bohmer et al., 2002; Huber et al., 2000). It was often shown that an optimised culture condition for fetal cell preferential growth could be developed, but when similar culture condition was applied to maternal blood, no fetal cell could be detected by FISH or PCR analysis. Similar scenario occurred in the attempts to culture fetal progenitor cells. Jansen et al. (2000) showed that fetal cord blood CD34+ cells were expanded in a 1500-fold over their maternal counterpart in spiking experiment, but no preferential growth of fetal cells was observed in maternal blood samples. Similarly, CD34+ fetal haematopoietic progenitors were shown to selectively expand *in vitro* in model mixture (Campagnoli et al., 2002) but not in maternal blood samples (Manotaya et al., 2002).

Some attempts were also made to culture the whole mononuclear cells from maternal blood. This idea is attractive due to its simple step and thus minimal cell

loss. With this strategy, Tutschek et al. (2000) reported a successful culture and isolation of single clones of fetal progenitor cells. If this could be reproduced or confirmed by other groups, it would ease the recovery of fetal cells from maternal blood and non-invasive prenatal diagnosis would be possible in the near future. However, the results were questioned by several independent groups (Campagnoli et al., 2001a; Zimmermann et al., 2002).

Thus, although many efforts for optimal fetal cell culture conditions that favour the proliferation of fetal over adult cells have been made, the culture of fetal haematopoietic cells from maternal blood samples has not yet been achieved reproducibly. The inability to expand fetal erythroid progenitors *in vitro* from maternal circulation might be due to the fact that the frequency of expandable fetal haematopoietic progenitor cells in maternal blood are very rare or that they require some as yet unidentified culture conditions (Elicha Gussin et al., 2002). To overcome all the difficulties, our group chose to *in vitro* expand fetal primitive erythroblasts using a novel nuclear reprogramming strategy and achieved encouraging results (Choolani and Zhang, unpublished data).

1.6.2 Lack of cell surface markers specific to fetal NRBCs

As discussed in section 1.5.6.1, majority of studies have used density gradient centrifugation followed by anti-CD71 in combination with MACS or FACS to enrich fetal NRBCs from maternal blood (Al-Mufti et al., 2003; Bianchi et al., 1990; Ganshirt et al., 1998; Hennerbichler et al., 2003). However, the recovery of less dense fetal NRBCs during density gradient centrifugation is accompanied by a significant cell loss and a high contamination by maternal cells, mainly WBCs, which may complicate subsequent purification and identification of fetal NRBCs. Moreover, CD71 is not highly specific to fetal NRBC marker (Larrick et al., 1979).

These may explain the low efficiency of current protocols for the isolation of fetal NRBC from maternal blood.

Several attempts were made to search for a more specific marker for fetal NRBCs. Zheng et al. (1997) found FB3-2 and H3-3 to be more specific to fetal cells compared to CD71 and were suitable for fetal cell enrichment. However, there is no further application of these antigens. Troeger et al. (1999) compared the expression of five antigens-CD71, GPA, HAE9, CD36 and i type antigen and found that i blood type antigen was most fetal cell specific, but subsequent attempts to enrich fetal NRBCs from maternal blood samples using anti-i antibody yielded no fetal cell.

With an aim to produce more specific antibodies against fetal NRBCs, Alvarez et al. (1999) used hybridoma technology to generate fetal NRBC specific antibodies. One of the generated antibodies was shown to be useful in the enrichment of fetal NRBCs from maternal blood. However, subsequent characterisation showed that this antibody was targeting a known surface antigen (CD71). In another study, Huie et al. (2001) used a phage library screening approach to generate fetal liver NRBCs specific antibodies and yielded a panel of antibodies which may also target known antigens: CD71, CD36, I/i etc. Although some of these generated antibodies were suggested to have a higher specificity compared to commercial anti-CD71 (Fernandez et al., 2005), data demonstrating their successful application for the enrichment of fetal NRBCs from maternal blood are very limited. Moreover, these antibodies might have very limited role in the isolation of fetal cells from first trimester pregnancy as liver fetal NRBCs were used for antibody production.

Many attempts to identify antibodies more specific to fetal NRBCs have been made as mentioned above, but all of them are far from satisfactory. The recent advance of mass spectrometry (MS) based "proteomics" is a highly sensitive and high throughput method for the protein identification, and thus offers an opportunity for a comprehensive analysis of surface antigens. Proteomic approach was explored in my study to investigate the surface antigens expressed on the membranes of primitive fetal NRBCs and adult RBCs.

1.7 Proteomics for protein identification and biomarker discovery

The term "proteomics" was coined in 1995 as global analysis of the proteins expressed in a cell and/or tissue (Wasinger et al., 1995). MS is currently most important tool for proteomic study. In the last decade, the advance of two key elements, MS technology and various genome projects, has allowed detection and identification of relatively low abundance proteins. Concurrently, considerable effort has been focused on and thus improved sample preparation and separation to reduce sample complexity prior to MS analysis, which further enhances the yield of low abundance proteins. The integration of good sample preparation, sensitive MS and powerful data analysis has now enabled efficient protein identification from a complex sample and become a major tool for biomarker discovery.

1.7.1 Sample preparation for proteomic study

Despite the use of high performance technology for protein separation (e.g. HPLC) and identification (e.g. sensitive MS), study of proteome presents considerable challenge because of their extraordinary complexity and vast dynamic range of proteins in cells, tissues, body fluids and organisms (Banks et al., 2000). In human cells, for example, actin could have a concentration up to

10^8 molecules per cell (Corthals et al., 2000), whereas some cellular receptors are present at only 10^2-10^3 molecules per cell. Such a dynamic range (10^5-10^6) limits the simultaneous identification of both high abundance and low abundance proteins. This situation is even worse in some tissues or body fluids (e.g. serum and plasma), where dynamic range is up to 10^9. Thus, it is often required to prepare partial proteomes of a given sample in order to maximise the coverage of the proteome and to increase the chance to identify low abundance proteins. At present, two approaches are commonly used for this purpose, that is, conventional sample fractionation based on chemical and physical properties (e.g. density, size, solubility) and affinity separation based on biological properties of a sample. More details of these methods are reviewed below.

1.7.1.1 Conventional sample fractionation

Differential centrifugation. The process of differential centrifugation is based on the fact that organelles have difference in size, shape and density. As a result, the effect of gravity on each is different, which make it possible to isolate them by centrifugation. Often, cells and tissues are first homogenised and then applied to differential centrifugation that leads to several distinct fractions: (1) nuclei, heavy mitochondria, cytoskeletal networks and plasma membrane, (2) light mitochondria, lysosomes and peroxisomes, (3) Golgi, endosomes and microsomes, and (4) cytosol fraction (Huber et al., 2003). Differential centrifugation can also be used in combination with density gradients such as sucrose, Percoll, or Nycodenz gradients to isolate highly pure organelles. Bell et al. (2001) used sucrose density gradient to purify Golgi and subsequently identified a large number of Golgi membrane proteins including a novel Golgi-associated protein using one-dimensional gel electrophoresis (1-DE) and MS. Similar approach has also been applied to the analysis of mitochondria (Hanson et al., 2001) and nucleus (Andersen et al., 2002), where a large set of proteins

including a number of novel proteins were identified respectively. Murayama et al. (2001) described a novel approach that used Nycodenz solution for fractionation of organelles from rat liver homogenate and two-dimensional gel electrophoresis (2-DE) for subsequent analysis. They showed that this protocol allowed separating endoplasmic reticuli (ER), lysosomes, mitochondria, and peroxisomes in a single step. The application of selective purification of cellular organelles for proteomic study was recently reviewed (Jung et al., 2000) and has meanwhile led to numerous efforts concerning organelle proteomics (Huber et al., 2003; Li et al., 2005a).

In addition to isolation of organelles based on their native densities, several groups demonstrated successful fractionation of a specific cell compartment by modifying its density. De Duve group purified lysosomes from rat liver after using Triton WR-1333 to modify its density (Leighton et al., 1968). Gupta and Beaumelle group used colloidal gold to coat endocytic organelles and plasma membranes for their purifications respectively (Beaumelle et al., 1990; Gupta et al., 1989). The usefulness of this strategy for proteomic study has been recently demonstrated. Rahbar et al. (2004) reported a large number of plasma membrane proteins from cancer cell membranes purified by cationic colloidal silica coating and differential centrifugation. Similarly, Derr et al. (2004) isolated plasma membranes from luminal endothelial cells (ECs) of rat lungs and from cultured rat lung microvascular ECs using a modified silica-coating methodology. They selectively coated the luminal EC surface by perfusing lung vasculature via pulmonary artery *in situ*, and coated cultured rat lung microvascular ECs by overlaying confluent monolayers of cell culture. Both silica-coating methods enabled them to isolate highly pure plasma membranes for subsequent protein identification.

The advantage of differential centrifugation is that a high purity target compartment can be obtained and thus allowing to recover a more comprehensive proteome. However, this method requires a relatively long process time, and the yield is very low and hence a large amount of starting material is required (Lehner et al., 2003).

Sequential extraction. In this method, cells or tissue samples are solubilised in different solvents sequentially to extract proteins with differing properties. It can be used alone or in combination with differential centrifugation to reduce protein cross-contamination from different organelles. Detergent Triton X-100, Triton X-114, Tween-40 and sodium dodecylsulfate (SDS) are often used in this method. Nonionic detergent Triton X-100 is very useful for the separation of Triton-soluble membrane proteins from cytoskeleton proteins (Patton et al., 1989; Patton et al., 1990). Triton X-114 can be used to fractionate cells or tissue sample into cytoskeleton, membrane and cytosol fraction (Bordier 1981). The combined use of Triton X-100 with EDTA and Tween-40 allows a more dedicate fractionation that can separate cell lysate into four distinct fractions: cytosol, membrane/organelle, nuclear and cytoskeletal fractions (Ramsby et al., 1994). The steroidal compound digitonin, which enhances the permeability of cell membrane, is widely used to extract cytosolic proteins and/or purification of membrane proteins for MS analysis (Foucher et al., 2006; Heinemeyer et al., 2004; Hoffmann et al., 2001; Lehner et al., 2003; Nielsen et al., 2005).

Use of Triton X-114 to extract a specific class of proteins provides a significant advantage for the identification of low abundance proteins. Taylor et al. (2000) presented a first relatively comprehensive rat liver Golgi protein map by the combined use of Triton X-114, 2-DE and MS. Bell et al. (2001) applied similar approach to analyse hepatic Golgi and identified 81 proteins including a novel

Golgi-associated protein of 34 kDa. Sinha et al (2005) used this method to analyse membranes of *Mycobacterium tuberculosis* and successfully identified 105 membrane proteins including 9 new proteins.

Triton X-100 is extensively used in the study of lipid raft proteins. Lipid rafts are glycolipid- and cholesterol-enriched membrane microdomains containing many proteins that are important for the molecule transporting and cell signalling, and they are insoluble or resistant to Triton X-100. The purification of lipid rafts can thus be achieved by separating lipid rafts from Triton X-100 soluble fraction. Indeed, this approach has been used by many researchers to mine lipid raft proteins. Blonder et al. (2004b) analysed lipid rafts of Vero cells from Triton X-100 insoluble fraction using liquid chromatography (LC) coupled with MS/MS, and identified 380 proteins including a large number of integral membrane proteins (24%). In a similar approach, Li et al. (2004) identified 71 proteins including 45 novel proteins from monocyte lipid rafts. Borner et al. (2005) analysed proteins from Triton X-100 insoluble extracts of plant cells (*Arabidopsis callus*) and identified many "lipid raft proteins", suggesting that lipid domain phenomena was also present in plant cell membranes.

Use of organic solvents for protein fractionation, in particular membrane protein fractionation, was recently reported. The propensity of hydrophobic proteins to partition in organic chloroform/methanol ($CHCl_3$/MeOH) phase rather than water allowed the successful enrichment of membrane proteins from chloroplast cells (Ferro et al., 2000) and rat liver cells (Zhang et al., 2006). In these studies, not only a high number of peripheral membrane proteins but also integral membrane proteins were identified. In another phase partition mixture of chloroform/ trifluoroethanol ($CHCl_3$/TFE), TFE phase was able to satisfactorily extract membrane proteins with a wide range of properties (Deshusses et al., 2003).

Similar use of $CHCl_3$/TFE mixture for membrane protein extraction enhanced the resolution of spots, and allowed double the number of spots to be visualised in 2-DE when compared with membrane extraction using 1% SDS buffer (Zuobi-Hasona et al., 2005). These observations indicate a higher efficiency for membrane protein extraction using the organic solvent TFE compared with strong detergents such as SDS.

1.7.1.2 Affinity enrichment

Affinity separation is one of the most powerful sample preparation techniques. It utilises the interactions between target proteins and ligands (e.g. biotin, lectin, antibodies) which are either immobilised with a solid support surface or by inclusion in the pores of a porous solid matrix. The proteins captured by the ligands can be released under certain conditions (e.g. high pH, high salt) and thus lead to the enrichment of target proteins. Commonly used affinity separation methods include biotin affinity, lectin affinity, metal binding, hydrophobic binding (reversed phase) and immunoaffinity.

Biotinylation. Biotin affinity separation is utilising the interaction between primary amino groups of proteins and biotinylation reagent. Hydrophilic biotinylation reagent S-NHS-LC-biotin has been shown to be effectively label almost all detectable proteins but unable to access hydrophobic membranes (Bradburne et al., 1993), which makes it possible to selectively purify cell surface proteins by the biotinylation. Indeed, Sabarth et al. (2002) used this strategy to enrich surface proteins of *Helicobacter pylori*. Subsequent analysis of the enriched membrane using 2-DE and MS enabled them to identify 18 surface proteins which include 9 novel proteins. Shin et al. (2003) applied a similar approach to analyse surface membrane proteins of cancer cells, and discovered that many chaperone proteins

(e.g. GRP94, GRP78, GRP75, HSP90A/B, HSP70, HSP60, HSP54) were present at the surface membrane of cancer cells.

Besides direct biotin affinity purification, proteins can be purified by indirect biotin labelling. For example, many phosphorylated proteins (e.g. phosphoserine), which often bind avidin, can be labelled with avidin beads, and separated through an immobilised biotin column (avidin-biotin interaction) (Thaler et al., 2003). However, this technique does not work for phosphotyrosine and has limited use for phosphothreonine due to poor reactivity. An alternative was to perform site-specific modification of phosphoseryl/phosphothreonyl residues, based on the fact that the phosphate moiety on these residues is labile and can be chemically replaced by biotinylated moieties (Oda et al., 2001). The target proteins can then be purified by the affinity handles using immobilised avidin column through the interaction with the added biotinylated moiety.

Lectin affinity. Lectins are proteins that can specifically bind carbohydrate components (Rudiger et al., 2001). This specificity forms the basis for various lectin-based affinity separations of glycoproteins and glycopeptides. Lectin affinity separation is fast and targets specific molecular species or classes of glycoproteins (Geng et al., 2001), and can be used to analyse membrane glycoproteins (Ghosh et al., 2004). Combined use of different lectins (e.g. Wheat Germ Agglutinin, Concanavalin A, Elderberry lectin, Maackia amurensis lectin) yields more types of glycoproteins and is useful for global study of glycoproteins in cells or body fluid: Zhao et al. (2006) demonstrated the usefulness of this strategy for the identification of serum biomarkers of pancreatic cancer patients. In their study, after depletion of the 12 high abundance proteins by an IgY-12 antibody column, they enriched sialated glycoproteins from normal and cancer serum using three different lectin columns. The comparative proteomic analysis

of the enriched samples enabled them to identify a pancreatic cancer marker (sialylated plasma protease C1 inhibitor).

Metal binding. It has been estimated that more than 30% of the proteins in a given mammalian cell are phosphorylated (Larsen et al., 2005). These proteins are usually low abundant but play an essential role in many cellular processes (e.g. proliferation, differentiation, and apoptosis). To efficiently study these proteins, it is often required to perform some enrichment procedures prior to MS analysis. Immobilised metal affinity chromatography (IMAC) (e.g. Fe^{3+} or Ga^{3+} columns) allows selectively bind negatively charged phosphate groups from mixture (Larsen et al., 2001) and thus is very useful for the enrichment of phosphorylated proteins for MS analysis (Schmidt et al., 2007). The use of multiple IMAC columns (Cu^{2+}, Ni^{2+} and Zn^{2+}) would enhance the overall recovery of various phosphoproteins (Sun et al., 2005).

Reversed phase liquid chromatography (RP LC). RP LC separations are based on hydrophobic interactions between the immobilised RP material and target proteins. The captured proteins by RP LC column can be eluted out by increasing the proportion of an organic solvent in the mobile phase, thereby gradually eluting more hydrophobic proteins. This approach allows not only efficient separation but also concentration of low abundance proteins, which helps to visualise and identify low abundance proteins. Indeed, Van den Berth et al. (2003) compared 2-DE patterns of tissue lysates with and without RP LC fractionation and found that significant more protein spots were observed in RP LC fractionated sample as compared to unfractionated tissue lysate. Similar approach and results have been shown in the analysis of human breast epithelial cell lysates (Reid et al., 1998).

Immunoaffinity separation. When an immune protein is used as the ligand to target a specific protein, the affinity separation can be termed as immunoaffinity separation. Its application involves enrichment or depletion of a specific protein or a class of proteins. One of its common applications is to deplete abundance proteins in serum and plasma using antibody immobilised on a matrix (e.g. sepharose). Brzeski et al. (2003) used this strategy to remove an abundance protein (albumin) from plasma and showed an improved spot detection for glycoprotein analysis in 2-DE. Pieper et al. (2003) developed a more efficient method by using a multi-component immunoaffinity chromatography column. Ten high abundance proteins (albumin, IgG, IgA, transferrin, α-1-antitrypsin, haptoglobin, α-2-macroglobulin, hemopexin, α-1-acid glycoprotein, and α-2-HS glycoprotein) were selectively removed in this method and as a result, 350 low abundance proteins were identified from the depleted plasma sample.

In summary, various sample preparation or fractionation methods are available to reduce sample complexity and to enrich low abundance proteins. Clearly, there is no simple universal strategy that is suitable for all proteomic analyses. Each specialised method has its advantages and disadvantages. The values of these methods must be weighed in relation to the specific samples and specific application.

1.7.2 Protein separation techniques

Prior to analysis by MS, a mixture of proteins or peptides, even after dedicate sample fractionation, must be further separated to reduce sample complexity. In this regard, three approaches have been commonly used: (1) protein separation using 1-DE, (2) protein separation using 2-DE, and (3) protein and peptide separation using LC. These techniques help for comparative analyses, lower the

dynamic range, simplify the identification of the proteins and allow more accurate results.

1.7.2.1 One-dimensional gel electrophoresis (1-DE)

In 1-DE (also referred as SDS-PAGE), the denaturing detergent SDS is included in the sample solution and buffer. This inclusion enhances protein solubilisation and adds negative charges to the proteins. SDS is considered to bind proteins fairly specifically in a mass ratio of 1.4: 1. Thus, the negative charge to mass ratio of any protein and detergent complex is approximately constant, and the separation is determined solely by the apparent molecular weight of the proteins passing through the sieving gel. One lane in the gel is usually run along with proteins of known molecular weights, to which the unknown proteins are characterised. This method can provide rough molecular weight estimations with an accuracy of 5-10%. 1-DE is simple to perform and has good reproducibility. Moreover, it has good protein solubility that is capable to separate hydrophobic proteins and resolve proteins with wide range of molecular masses (10 to 300 kDa). The limitation of this approach is that the single dimensional separation constrains its capability to resolve complex proteome samples. Thus, when a relatively complex protein mixture (e.g. whole cell lysate) is encountered, 2-DE is usually recommended.

1.7.2.2 Two-dimensional gel electrophoresis (2-DE)

2-DE technique was originally described by O'Farrell (1975), where the proteins are separated according to their isoelectric points (p*I*s) in the first dimension and their apparent molecular mass in the second. This orthogonal approach allows the separation of more than 1000 proteins simultaneously in a single gel. After the separation, the protein spots can be visualised with various staining methods (e.g. silver stain, Coomassie blue stain and fluorescent dye stain), and excised

from the gel for enzymatic digestion followed by MS identification. The appearance or disappearance of protein spots can provide information about differential protein expression, while the intensity of those spots provides quantitative information about protein expression levels.

These features allow 2-DE to be very useful for the identification of all proteins in a sample as well as the comparison of differential expression for two or more samples. Indeed, most of the relatively comprehensive proteomes of subcellular organelles were initially obtained by the combined use of 2-DE and MS, for example, mitochondria (Hanson et al., 2001), lysosome (Chataway et al., 1998), Golgi (Fialka et al., 1997), ER (Vlasuk et al., 1980) and other endovacuolar organelles (Scianimanico et al., 1997). The comparison of differential expression using 2-DE allows the identification of biomarkers for normal or diseased states, and the assessment of the effects of some exposure (e.g. drugs or stimuli) on cells. Ueno et al. (2000) used 2-DE and MS to analyse plasma proteins in patients with Alzheimer's disease and identified three disease-specific protein markers. In a similar use, Skaar et al. (1998) identified 7 protein markers that were associated with estrogen-induced proliferation in MCF-7 breast cancer cells. In addition, 2-DE can distinguish protein isomeric forms. Thus, it can play an important role in the study of protein post-translational modification such as phosphorylation (Kovarova et al., 2003) and glycosylation (Packer et al., 1998).

The limitations of conventional 2-DE include poor reproducibility, poor sensitivity and limited dynamic range, which constrain 2-DE to identify only relatively high abundance proteins (Gygi et al., 2000). The use of narrow-range IPG strips could minimise these problems by expanding the resolving power, thereby increasing 2-DE resolution and enhancing the recovery of low abundance proteins. The introduction of difference gel electrophoresis (DIGE) (Unlu et al., 1997), on the

other hand, has dramatically improved the reproducibility and applicability. In the DIGE, control and treated protein samples are covalently bound with structurally similar but spectrally distinct fluorophores, e.g. Cy 3 or Cy 5. The dye-tagged proteins are mixed and separated on the same 2-DE. The same form of a given protein from each sample will migrate to the same position on 2-DE. The differential expression could then be identified by scanning the gel using excitation and emission wavelengths that are unique to each dye.

These technical advances improved the reproducibility and enhanced the yield of low abundance proteins but a number of problems remain. It is still a labour-intensive and time-consuming process. A typical 2-DE experiment can take two days, and a maximum two samples can only be analysed per gel. It is clear that the proteins of high molecular weight and extreme acidity or basicity (proteins with p*I*s below pH 3 and above pH 10) are intractable, and that its usefulness is limited when hydrophobic proteins (e.g. membrane proteins) are studied (Nouwens et al., 2000). Under-representation of hydrophobic proteins is due to the inability of detergents to effectively solubilise these proteins in the aqueous solution used for the isoelectric focusing (Molloy 2000), and the use of chaotrope mixtures (Rabilloud 1998), novel detergents (Luche et al., 2003) and organic solvents (Molloy et al., 1999) demonstrated little improvement over detergent based protocols. Until now, hydrophobic proteins whose grand average of hydropathicity (GRAVY) scores are larger than 0.1, 0.15, and 0.3 in *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *E. coli*, respectively, have not been detected by 2-DE (Kashino 2003).

The limitations of 2-DE lead to the development of an alternative chromatography strategy for the effective separation of protein mixtures. Indeed, the gel-free separation technique allows automated, sensitive, reproducible, and quantitative

analysis of complex samples through a single chromatography unit or the combination of various chromatography methods, which are gaining incremental popularity.

1.7.2.3 Liquid chromatography

Liquid chromatography (LC) is one of the most powerful protein separation methods that are based on the specific interaction between an immobilised ligand and a target protein or peptide. Depending on the absorbents used, LC can include ion-exchange, size exclusion, reversed phase. Accordingly, the bounded proteins or peptides in the column can be eluted off by changing the properties of the buffer (mobile phase). Different types of LC can be used as a single operating unit or as a combination under the two- or multi-dimensional LC to achieve higher resolving power.

LC has many applications. It can be used for protein sample preparation prior to gel electrophoresis as discussed in section 1.7.1.2. It is often used for the purification and enrichment of proteins or peptides after gel electrophoresis. For example, lectin affinity LC is widely used to isolate a specific class of glycoproteins and followed by trypsin digestion and MS analysis (Madera et al., 2007). The commonest application of LC in proteomic study, however, is to separate enzymatic peptides into simple components for direct MS analysis. This approach is also referred to shotgun proteomics (Link et al., 1999), where proteins are first enzymatically digested in solution and the resulting peptides are fractionated using various types of LC, in most cases, strong cation exchange (SCX) LC and RP LC. In SCX LC a peptide sample is passed through a charged column. The peptides are captured in the column based on the interaction between charged groups of a peptide and oppositely charged groups on the ion exchange support. Changing the pH of the mobile phase buffer allows the elution

of captured peptides. RP LC is a separation technique which is based on hydrophobic interactions between the peptides and the immobilised ligands on a support material fixed. Captured peptides are eluted by gradually increasing the proportion of an organic in mobile phase. The eluted peptides can be analysed by an on-line or off-line coupled MS. This approach, first described by John Yates group (Link et al., 1999), has now become a major tool for large scale protein identification (Wu et al., 2002), biomarker discovery (McDonald et al., 2002) and analysis of post translational modification (Cantin et al., 2004).

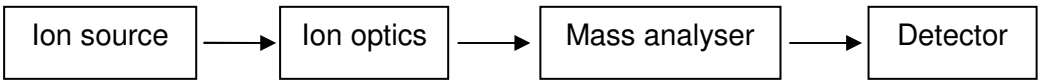
Similar to 2-DE in DIGE, the LC method allows for quantitative protein analysis by selective labelling with isotope-coded affinity tag (ICAT) (Gygi et al., 1999) or isobaric tags (iTRAQ) (Ross et al., 2004). In both approaches, the peptides from different samples (e.g. normal and disease) are labelled with distinct affinity mass tags, mixed together and then analysed using LC-MS/MS. The peak intensity in MS or MS/MS spectra provides quantitative information of protein expressions and is therefore useful for biomarker discovery. The ICAT is used for quantitative analysis of two cell states, whereas iTRAQ reagent strategy contains four isotopically distinct tags, enabling quantitative analysis of four cell states simultaneously. Although iTRAQ was introduced only recently (Ross et al., 2004), it has gained enormous interests for biomarker discovery (Chong et al., 2007; DeSouza et al., 2005; Liu et al., 2006).

In summary, the LC-based approaches are automated, sensitive and reproducible. It can be on-line or off-line coupled with MS to allow rapid protein characterisation and quantitative analysis of complex samples, which are far more efficient than gel-based approaches.

1.7.3 Mass spectrometry

Mass spectrometry (MS) continues to play a central and essential role in proteomic study. MS consists of three basic components: an ion source, a mass analyser and an ion detector (Figure 1-2). Protein and peptide must be converted into gas phase ions prior to MS analysis. A mass analyser sorts these ions according to their mass-to-charge (m/z) ratios. The ions motion is then recorded based on the number of events or electrical currents created.

Figure 1-2 Simplified diagram of mass spectrometer
Transfer of ions from liquid or solid phase to gas phase, and separation of ions according to their *m/z* ratio.



1.7.3.1 Ionisation techniques

The electrospray ionisation (ESI) (Whitehouse et al., 1985) and matrix-assisted laser desorption/ionisation (MALDI) (Karas et al., 1988) are two most commonly used ionisation techniques. They are belonging to “soft” ionisation techniques, which can ionise large biomolecules such as proteins and peptides without significant change of their integrity.

ESI operates at atmospheric pressure and generates a continuous stream of charged droplets when a high electric potential is set between a capillary and the inlet to a mass spectrometer. As the droplets evaporate in a drying gas or heat atmosphere, the charged droplets shrink and eventually desolvated ions are formed (Fenn et al., 1989). The introduction of microelectrospray and nanoeclectrospray (Emmett et al., 1994; Wilm et al., 1996), coupled with LC (McCormack et al., 1997), allows analysis of a very small amount of sample

mixture. Additionally, ESI produces multiply charged ions which lowers the m/z values for higher molecular weight compounds and thus allows measurement of m/z values in MS with limited m/z ranges. Multiple protonation of peptides and proteins could further dissociate under activation. These features of ESI have led to its widespread application in proteomic study.

In MAIDI instead, sample is incorporated into matrix to form co-crystals, ionisation occurs with ultraviolet (UV) absorption from laser energy. The gas phase analytes are ionised through proton transfer from the matrix, cation adduct formation, electron transfer or electron capture. Single charged ions resulting from proton transfer from sample to matrix are observed in the gas phase. Usually, small UV absorbing molecules such as 2, 5-dihydroxybenzoic acid and alpha-cyano-4-hydrozycinnamic (CHCA) are used as matrix. In contrast to ESI, MALDI has a relatively good tolerance to salts which reduces complications for sample preparation, in particular, integral membrane proteins and hydrophobic peptides. Furthermore, ions generated are in bunches coincident with a pulsed laser beam rather than a continuous mist, which allows customised MS/MS analysis. Recently, the coupling of MALDI-TOF/TOF with LC to generate an off-line MS platform (Zhen et al., 2004) has improved the applicability for analysing protein and peptide complexes.

1.7.3.2 Mass analysers

As ions exit the ion source, they pass into a mass analyser. The mass analyser is responsible for separating ions by their mass-to-charge ratios. Mass analysers use electric and/or magnetic fields to manipulate ions in a mass-dependent manner. There are four basic types of mass analysers used in proteomic research: Ion trap, Time-of-flight (TOF), Quadrupole and Fourier transform ion cyclotron analysers. These analysers can be stand alone or, in some cases, put

together in tandem to take advantage of the strengths of each. Currently, TOF/TOF and linear Ion trap are the most commonly used tandem mass analysers for protein analysis by peptide fingerprinting and peptide sequence analyses.

TOF/TOF analysers are usually coupled to MALDI ionisation method, whereas linear Ion traps have mostly been coupled to ESI. In the MALDI-TOF/TOF, ions of a particular *m*/*z* are selected in a first TOF, fragmented in a collision-induced dissociation (CID) cell and the fragment ion masses are detected by a second TOF analyser. The separation of ions is based on the fact that the MALDI generated ions contain the same amount of energy but have different masses, the lighter ions travel faster and reach the detector ahead of the heavier ions. These instruments have high sensitivity, resolution and excellent mass accuracy. The resulting fragment ion spectra are often more extensive and informative than those generated in Ion trap instruments.

In the ESI linear Ion traps, the ions are first captured or 'trapped' for a certain time interval and are then subjected to fragmentation in CID. It has a propensity to generate multiply charged analyte ions, thus enabling multiple stages of MS. Linear Ion traps are robust, sensitive and relatively inexpensive. A disadvantage of linear Ion traps is their relatively low mass accuracy. The comparison of linear Ion trap and TOF/TOF-MS is shown in Table 1-1.

Table 1-1 Comparison of performance characteristics for two common tandem MS

Instrument	**Resolution**	**Mass accuracy**	**MS scan rate**	**MS/MS scan rate**
Linear Ion traps	2–15,000	100–300 ppm	Moderate to fast	Moderate to fast
TOF/TOF	20-25,000	10-20 ppm	Fast	Slow

(ppm: parts per million)

1.7.4 Database searching and bioinformatic analysis

Database searching through appropriate algorithms is used for protein identification by correlating MS or MS/MS spectrum with theoretical spectrum. It can include *de novo* peptide sequence, uninterpreted MS/MS database searching, peptide mass fingerprinting (PMF) and peptide sequence. PMF and peptide sequence are two most commonly used methods for protein identification. Bioinformatic tools, on the other hand, are integral tools providing functional information and correlating this information to some specific biological pathways. For example, gene ontology tool Gofigure can help to functionally annotate identified proteins.

Peptide mass fingerprinting (PMF) database searching. In this method, the mass spectrum of an enzymatic peptide of an unknown protein is compared to spectrum in database of known proteins. The high number of peptide matches and better matches allow for protein identification with higher confidence. PMF is simple, which does not require sophisticated MS/MS machine. The disadvantage is the ambiguity in protein identification. This is because many different peptides may have same mass, which causes a single MS spectrum matched with more than one theoretical spectrum in a database. In practice, it is often required a large number of peptide matches for a confident protein identification. Also, higher confidence of protein identification can be achieved by correlating identified protein with apparent molecular weight obtained from 1-DE or 2-DE separation. Other disadvantages include that the use of PMF is limited to completely sequenced and well annotated genome, and that PMF does not work well with protein mixtures (Cottrell 1994).

Peptide sequence database searching. This method is the most specific for protein identification and is often used in MS/MS analysis. The ionised peptides

selected by mass in the first MS are subjected to the subsequent fragmentation during collision with a gas (in CID). The resultant smaller fragments are used for determination of amino acid sequence, which is done by comparing experimental fragmentation with a theoretical fragmentation of the peptide in database. Once the amino acid sequence of a peptide is determined, it can be used to search databases to find the protein from which it is derived. Peptide sequence has higher mass accuracy as compared with PMF and is suitable for analysing protein mixtures.

Searching programmes or algorithms. A number of novel algorithms for database searching are available through the internet and listed in Table 1-2. Algorithms are developed to determine peptides and/or their amino acid sequences *de novo* from MS or MS/MS data. The simplest algorithm for protein search in database matches experimentally obtained and theoretical mass spectra and ranks database proteins according to number of matching peptides. Such algorithm often overestimates large proteins, for which a greater number of calculated peptides is possible. This is the case for the algorithm used for PMF. More effective algorithms (e.g. MASCOT, SEQUEST) use additional information such as protein p*I*, Mw, and amino acid composition. These tools allow for the identification of proteins with higher sensitivity and specificity than algorithms used for PMF.

Table 1-2 Various tools for MS-based protein identification

SPONSOR	URL
Eidgenossische Technische Hochschule	http://cbrg.inf.ethz.ch
European Molecular Biology Laboratory (EMBL)	http://www.mann.emblheidelberg.de
Swiss Institute of Bioinformatics (ExPASy)	http://www.expasy.ch/tools
Matrix Science (Mascot)	http://www.matrixscience.com
Rockefeller University (PepFrag, ProFound)	http://prowl.rockefeller.edu
Human Genome Research Centre (MOWSE)	http://www.seqnet.dl.ac.uk
University of California (MS-Tag, MS-Fit, MS-Seq)	http://prospector.ucsf.edu
Institute for Systems Biology (COMET)	http://www.systemsbiology.org
University of Washington (SEQUEST)	http://thompson.mbt.washington.edu/sequest

Bioinformatic tools. In proteomic study, bioinformatic tools may include all methods that are involved in data analysis, storage, management, search and retrieval. More specifically, they can be categorised into two groups. One group is referred to tools for mass spectra analysis for protein identification and characterisation as reviewed by Palagi et al. (2006). The raw output of a MS study consists most of a list of peaks from MS spectra. Bioinformatic tools (e.g. MASCOT) that correlate the peak information with database (e.g. Swiss-Prot, IPI) are required to identify proteins. The other is referred to tools related to "post-identification" annotations which often elucidate biological significance of the identified proteins (Lisacek et al., 2006). In this context, the annotations may describe functional properties of identified proteins and refer to pathway data, and protein-protein interactions. Such information is commonly integrated and stored in on-line public databases, such as an integral protein sequence database with high-quality annotation (Swiss-Prot: http://au.expasy.org/sprot/), protein family databases defined on sequence similarity (e.g., Pfam: http://www.sanger.ac.uk/Software/Pfam/; InterPro: http://www.ebi.ac.uk/interpro/),

structural databases defined on structure similarity (e.g., SCOP; http://scop.mrc-lmb.cam.ac.uk/scop/). In each database, there may be different criteria for grouping proteins together. Various GO tools based on gene ontology (e.g. Gofigure: http://udgenome.ags.udel.edu/gofigure/) can usually be meaningful links between independent classifications of proteins described in a collection of databases. Moreover, these GO tools allow inferring biological process, molecular function and cellular component for unknown or uncharacterised proteins. In addition, there are some tools to calculate protein physical and chemical parameters. For example, the programme ProtParam (http://au.expasy.org/tools/protparam.html) can be used to computer protein molecular weight, theoretical p*I* and grand average value of hydropathicity (GRAVY); TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) can be used to predict transmembrane domain(s) of proteins. These bioinformatic tools are integral components of proteomic analysis and essential in the discovery of sensitive and specific biomarkers.

The availability of complete sequence databases, optimised sample preparation and separation protocols, the advance of MS, and the powerful bioinformatic tools, together, converged into a mature, robust, sensitive, and rapid technology that allows a large scale of protein identification and biomarker discovery.

1.7.5 Proteomic strategies for membrane protein analysis

Cell membranes are essential for cells to maintain the integrity of their structure, and segregate processes within intracellular compartments. Study of membrane proteome is important not only for understanding of the biology of diseases and for biomarker discovery, but also to enhance the drug discovery process as more than two-thirds of the known protein targets for drugs are membrane proteins (Hopkins et al., 2002). Commonly used proteomic strategies for membrane

protein analysis include: (1) gel-based approach where proteins are separated by 2-DE or 1-DE and identified by MS, and (2) shotgun approach where proteins are digested in solution, separated by LC and identified by MS. The features of these methods were discussed in more detail in the following paragraphs.

1.7.5.1 Gel-based approach

2-DE coupled with MS. As the first technique enabling large scale, high resolution of protein separation, 2-DE coupled with MS has a key role in early proteomic analysis of membrane proteins. Shortly after the introduction of 2-DE (O'Farrell 1975), this method was used to separate membrane proteins (Ames et al., 1976). Proteins were fairly separated and many spots were visualised. However, protein identification was difficult due to lack of resources then, and thus the spots in 2-DE may not represent true membrane proteins. Indeed, a later paper found that an abundance integral membrane protein Band 3 did not enter 2-DE gel when using nonionic detergent mixtures for membrane protein analysis (Rubin et al., 1978), which was confirmed by Klein et al. (2004).

As conventional 2-DE using nonionic detergents is not effective to separate membrane proteins (Santoni et al., 2000), other detergents were used to improve 2-DE separation of membrane proteins. The zwitterionic detergent (3 [(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (CHAPS) has superior membrane protein solubilising capability as compared to nonionic detergents. Its use enabled effective separation of crude microsomal proteins (Perdew et al., 1983). Another detergent aminosulphobetaine 14 (ASB-14) was shown to be more effective than CHAPS in solubilising membrane proteins and its use allowed the separation of a number of integral membrane proteins that were not previously detected using 2-DE (Nouwens et al., 2000; Taylor et al., 2003a). Several studies suggested that the surfactant C80 was an even more potent

solvent for integral membrane proteins over ASB14 and CHAPS (Babu et al., 2004; Henningsen et al., 2002).

The use of various chaotrope mixtures and novel detergents improves 2-DE separation of membrane proteins, and enables recovery of some, or a small class of membrane proteins. However, the usefulness of 2-DE for global membrane protein analysis remains very limited as the diversity, low abundance, and high hydrophobicity of membrane proteins. Galeva et al. (2002) compared 2-DE and 1-DE for the analysis of rat ER membrane proteins, and found that while twenty-two ER membrane proteins were identified from 1-DE separated proteins, only three were from 2-DE separation. Vanrobaeys et al. (2005) identified 38 proteins from myelin membrane using 2-DE method, whereas the number was doubled (93) in shotgun approach. These studies suggested that 2-DE should not be a first choice for membrane proteins analysis.

1-DE coupled with MS. In contrast to 2-DE, the protein solubility in 1-DE is seldom a problem as the strong detergent SDS is included in the sample buffer. This advantage has allowed 1-DE coupled with MS to become one of most effective strategies in various membrane protein analyses. Galeva et al. (2002) used this strategy to analyse rat ER membrane proteins, and identified 22 out of 34 known ER membrane proteins in a single experiment. In a similar approach, Bell et al. (2001) identified all known Golgi membrane proteins and a novel membrane protein GPP34. The successful application of this approach was also reported in the analyses of membrane proteins from chloroplasts (Ferro et al., 2002), *mycobacterium tuberculosis* (Sinha et al., 2005), human macrophage (Slomianny et al., 2006) and rat liver (Zhang et al., 2006).

The limitation of this approach is the relatively poor resolving power of 1-DE. It is often noted that more than one proteins are present in a fine band when studying complex protein mixture, thereby limiting the identification of low abundance proteins. This problem can be overcome by the use of LC to resolve the eluted peptides from each band prior to MS analysis (Simpson et al., 2000). Indeed, this strategy has been demonstrated as a powerful tool to study membrane proteins. Brugiere et al. (2004) used this approach to analyse mitochondrial membrane and reported a total of 114 proteins including ~40% novel proteins. In a similar approach, Bagshaw et al. (2005) analysed lysosome membranes and identified 215 proteins including 20 novel proteins; Rahbar et al. (2004) analysed plasma membranes and presented 366 proteins including 43% of plasma membrane proteins.

Despite the effectiveness, however, this approach is inherently not quantitative (Han et al., 2001) and that peptides may not efficiently recovered with tedious in-gel digestion procedure (Zhang et al., 2004). In addition, difficulty is encountered with low molecular weight proteins due to the low number of peptides available, and this situation is worse if only a small amount starting sample is available. Finally, this approach requires multiple LC separations prior to MS analysis, which is very time-consuming.

1.7.5.2 Shotgun approach

Compared to gel-based strategy, shotgun approach was introduced more recently (Link et al., 1999). In this approach, proteins are firstly digested in solution, and then resolved by one-dimensional LC or multidimensional LC prior to MS analysis. It circumvents some challenges that 2-DE faces, for example, the digestion of intact proteins into peptides creates more soluble analytes, which

eases subsequent separation. It is faster, high throughput and more efficient in peptide recovery as compared with 1-DE based method.

In shotgun approach, the major challenge is the protein solubility. A buffer, which is effectively solubilising membrane proteins and compatible with enzymatic digestion, is necessary to increase peptide recovery. For this purpose, three solubilisation buffers for extraction and analysis of membrane protein with shotgun approach have been used and demonstrated to be effective, that are, detergent, organic acid and organic solvent.

Detergent buffered solution. Strong detergent SDS has excellent solubilisation characteristics and is widely used to extract and solubilise membrane proteins. The commonly used concentrate SDS solution for membrane protein solubilisation, however, hurdles trypsin activity. In 2001, Han et al. (2001) described the use of a relatively low concentration of SDS solution (0.5%) for both pre-digestion procedures (protein extraction, reduction, and alkylation) and protein digestion prior to LC-MS analysis. More recently, a slightly modified protocol was developed (Zhang et al., 2004), where pre-digestion procedures were carried out in a concentrate SDS solution (1%), and the protein digestion was performed in a diluted solution (0.1%). This protocol is not only efficient for membrane protein solubilisation but also more compatible with trypsin digestion, and has been widely used to analyse various hydrophobic proteins such as membrane proteins (Zhang et al., 2004), lipid raft proteins (Li et al., 2004) and proteins in myelin sheath (Vanrobaeys et al., 2005).

Other detergents (e.g. urea) have also been used for shotgun analysis of membrane proteins. Urea-based protocol, similar to SDS-based protocol, often consists of two steps: use of concentrate urea solution for pre-digestion

procedures and use of a diluted urea solution for the protein digestion. Durr et al. (2004) used 8 M urea solution for pre-digestion procedures, and 1.6 M urea solution for the protein digestion. In their study, a total of 450 proteins were identified from luminal endothelial cell plasma membrane. In a similar use, Pasini et al. (2006) could present a relatively comprehensive RBC membrane proteome. Nielsen et al. (2005) used 4 M urea and 1 M urea in the two steps, which enabled them to identify a large number of membrane proteins (>500) from mouse brain cortex in a single experiment.

The detergent solution for membrane protein solubilisation and digestion in shotgun approach generally allows efficient recovery of membrane proteins, and has significant advantages over gel-based approach (Wu et al., 2006). However, the detergents can interfere with LC separations, suppress analyte ionisation, and additional manipulations to remove them could lead to the loss of significant low abundance proteins (Puchades et al., 1999), rendering its inapplicability of this approach for analysis of a small amount sample.

Organic acid solution. The usefulness of organic acid for shotgun analysis of membrane proteins has also been demonstrated. In a pioneering study (Washburn et al., 2001), yeast membranes were solubilised in 90% formic acid and proteins were chemically cleaved by cyanogen bromide into relatively large fragments. These fragments, more soluble than intact proteins, were then enzymatically digested in neutral medium (pH 7-8) and analysed by LC-MS/MS. This approach enabled them to identify 131 yeast integral membrane proteins with ≥3 TMDs. In a similar use, Xiang et al. (2004) analysed membrane proteins from two breast cancer cell lines BT474 and MCF7, and presented a total of 604 proteins from BT474 membrane and 313 proteins from MCF7 membrane.

Despite its high efficiency, this approach was less commonly used in current proteomic study, mainly because of three possible reasons: (1) cyanogen bromide is a highly combustible, toxic chemical and very difficult to handle; (2) it is a time-consuming and tedious digestion process as multiple-step digestions and pH adjustment are needed; and (3) it involves potential sample losses caused by protein precipitation (Blonder et al., 2004a).

Aqueous organic solvents. Organic solvent for shotgun proteomics was first described by Blonder et al. (2002). In their study, the membranes from Deinococcus radiodurans were solubilised into aqueous methanol (60%) with intermitting sonication. Protein mixture was denatured and digested by addition of trypsin with a 1:20 (w/w) trypsin-to-protein ratio. The subsequent analysis using LC-MS/MS enabled them to identify a total of 503 proteins that include 135 hydrophobic proteins and 53 integral membrane proteins. This organic solvent-based approach is more compatible with LC-MS/MS analysis, as sample clean-up could be avoided leading to lower sample loss and better recovery of low abundance, highly hydrophobic transmembrane peptides.

More recently, organic solvent 2,2,2-trifluoroethanol (TFE) was described for the analysis of whole cell lysate in a shotgun approach (Wang et al., 2005). In their study, mouse brain tissue or MCF7 cells were lysed in aqueous TFE (50%) and pre-digestion procedures (protein extraction, reduction and alkylation) were carried out in this solution. The protein digestion was performed in a dilute TFE solution (5%). This TFE-based approach had a comparable or slightly better efficiency than SDS-based approach in the analysis of large amount of sample, and provided much higher efficiency when analysing a small amount sample (<1 μg protein), as sample clean-up could be avoided. However, it is not yet to know if this approach is suitable for the analysis of membrane proteins.

In summary, various methods, as reviewed above, are available for membrane protein analysis and biomarker discovery. Each method has its own advantages and disadvantages and the method chosen is largely dependant on the specific sample type to be analysed. It is advisable to identify a suitable method or combination of methods using similar sample prior to the analysis of a rare sample type, e.g. fetal NRBCs.

1.8 Adult RBC membrane proteins

Human adult RBCs represent evolutionary genius. Enucleation of terminally differentiated mammalian erythrocytes is thought to be a recent evolutionary adaptation, as birds, reptiles, amphibians and fish have nucleated red blood cells (Kingsley et al., 2004). Despite not having a nucleus, internal organelles or a transcriptome, human adult RBCs are able to carry out their oxygen transport function, survive in the circulation for 120 days, and undergo programmed cell death (Bratosin et al., 2001). The size, shape and function are tightly regulated throughout their lifespan, and much of this is due to its membrane proteins and the interaction of the latter with molecular signals in the blood (Walsh et al., 2002).

Over the last 40 years, adult RBC membrane has been studied extensively via biochemical method, thus the identity, topology and function of many RBC membrane proteins have been determined. The three major groups of RBC membrane proteins include: (a) integral membrane proteins, (b) peripheral membrane proteins and (c) glycosyl-phosphatidylinositol (GPI) anchored membrane proteins.

Integral membrane proteins. Integral proteins comprise up to 80% of all RBC membrane proteins, and are embedded in the membrane matrix spanning the entire bilayer. These proteins are characterised by at least two hydrophilic domains composed mainly of polar amino acids, and by at least one hydrophobic domain composed mostly of apolar amino acids. The hydrophilic sections of the proteins are in contact with the surrounding water phases-the extracellular domain is usually glycosylated (Steck et al., 1974), whereas the cytoplasmic domain is usually phosphorylated. The important integral membrane proteins, tightly bound to the cytoskeleton architecture, include band 3, and the glycophorins. Band 3 acts as the anion transport and gas exchange channel (Bruce 2006), which consists of N-terminal cytoplasmic domain and C-terminal membrane domain. Structural models predict that the membrane domain consists of 12 to 14 transmembrane helices, and the longest, fourth loop is N-glycosylated and its single lactosamine-rich glycan chain carries over half of the red cell ABO blood group epitopes (Jarolim et al., 1998). Glycophorins are associated with the MNS blood group system (Poole 2000). About 60% of the glycophorin molecule is made up of carbohydrate chains, which provide a negative charge to the surface of RBCs (Marchesi et al., 1976). Other major integral proteins consist of glucose transporter (band 4.5), Rh polypeptides, Rh glycoproteins, and band 7 (stomatin) (Wang et al., 2000c).

Peripheral proteins. Peripheral proteins are located on the cytoplasmic surface of the membrane. They are bound loosely to the membrane, and can be readily removed in low ionic strength medium, in the presence of chelating agents, or under alkaline conditions. This group is represented by the proteins of bands 1, 2, 2.1, 2.2, 2.3, 4.1, 4.2, 4.9, 5, and 6.

Spectrin (band 1 and band 2) is the major constituent of the membrane skeleton; it accounts for 25% of the total RBC membrane proteins. The spectrin fraction consists of two fibrous proteins that can be resolved on SDS-PAGE into two closely adjacent bands, band 1 (α-chain) and band 2 (β-chain). Two α-β helices are linked end-to-end to form a single tetramer, which has binding sites for several other proteins including other spectrin molecules (Bennett et al., 1993; Branton et al., 1981; Marchesi 1985). Ankyrin (band 2.1) is a peripheral membrane protein with a molecular mass of 210 kDa. Band 2.2 and band 2.3 proteins are probably ankyrin analogues. Band 4.1 is a globular protein with a molecular mass in the range of 78 to 82 kDa. Band 4.2 (palladin) is found in RBC membrane as a homo-tetramer closely associated with band 3 protein (Jarolim et al., 1992). Band 4.9 (Dematin) is an actin-bundling protein, which contains at least two actin-binding sites, one in the headpiece domain, and another in the amino-terminal portion (Azim et al., 1995). Actin (band 5) oligomers of 10-15 units are essential components of the membrane skeleton and the molecular mass of this protein is approximately 43 kDa. Band-6 protein is a glyceraldehyde-3-phosphate dehydrogenase (G3PD) with a pivotal function in erythrocyte metabolism. Immunostaining demonstrated that G3PD is preferentially associated with the RBC plasma membrane, and approximately 10% of G3PD exists in their cytoplasmic fraction (Rogalski et al., 1989).

GPI-anchored proteins. GPI-anchored membrane proteins are a class of membrane proteins that attach to the outer surface of the cell membrane through a glycosyl-phosphatidylinositol unit. This glycolipid anchor is composed of phosphatidylinositol, phosphatidylethanolamine, one non-acetylated glucosamine and three mannose residues; the anchor unit is connected to the C-terminal amino acid of the protein via an amide bond. This complex molecule is attached

to the cell membrane by the fatty acid residues of the phosphatidylinositol moiety embedded in the outer leaflet of the bilayer (Kinoshita et al., 1995).

GPI-anchored RBC membrane proteins include complement control proteins (decay accelerating factor (CD59), and C8 binding protein), immune control proteins (LFA-3 and CD16), and various enzymes (acetylcholinesterase, alkaline phosphatase, and 5'-nucleotidase) (Rosse 1990). Furthermore, it has been shown that antigens of several different blood group systems, Cromer, Dombrock, Cartwright, and John Milton Hagen (JMH), are located on GPI-linked membrane proteins (Telen et al., 1990; Telen 2000).

Proteomic studies of RBC membrane proteins. The proteomic study of RBCs has closely followed the technical advance in the last decade. An early study of RBC membrane proteome was carried out using gel-based proteomic approach and a total of 102 proteins were identified (Low et al., 2002). Of the 102 proteins, all major RBC band proteins (bands 1-7) were identified but a few of them (<10) were transmembrane proteins. Two years later, Kakhniashvili (Kakhniashvili et al., 2004) utilised a shotgun proteomic approach to analyse RBC membrane proteome. In the study, a total of 91 proteins were identified from RBC membrane preparation and about 25% of them were transmembrane proteins. More recently, Pasini et al., (2006) used two advanced mass spectrometers (Quadropole TOF Q-STAR and linear ion trap Fourier transform LTQ-FT MS) for the analysis of RBC membrane proteins and reported a substantive increase in the number of RBC membrane proteins (341 proteins). In all these studies, many proteins (e.g. proteasomal proteins), which were thought to be absent in mature RBCs, were also identified. Some of them have been recently validated using confocal immuno-fluorescence and western blot analysis (Neelam et al., 2006).

Therefore, proteomics has now become an efficient and high-throughput tool for the analysis of RBC membrane proteins.

1.9 Experimental aims and hypotheses

The enrichment of primitive fetal NRBCs from maternal circulation would allow an early non-invasive prenatal diagnosis. Many efforts have been made to enrich this most desirable cell type from maternal blood but all of them are far from satisfactory. As discussed, the enrichment is being difficult for two reasons: small number of fetal NRBCs in maternal circulation and the lack of appropriate surface biomarker that can be used to differentiate fetal NRBCs from adult RBCs. Attempts on *in vitro* amplification of fetal cells and generation of antibodies against fetal NRBC surface antigens for enrichment protocol were also not successful. Thus an alternative would be to explore more on the surface protein/antigen profiles of fetal NRBC and adult RBC membranes, so that the enrichment procedure could be strengthened by obtaining or generating new antibodies.

Two possible means to gather surface protein information are: (1) profiling the membrane proteomes of fetal NRBCs and adult RBCs using MS-based proteomic approaches, and (2) an immunocytochemical screening of fetal NRBCs and adult RBCs using commercially available surface antibodies. My study is centred on the identification of differences between fetal NRBC and adult RBC membrane proteins. First trimester primitive fetal NRBCs obtained from placental tissue at the time of termination of pregnancy (TOP) will be used. Adult RBCs from laboratory volunteer will also be studied in parallel with an ultimate objective to find out unique surface membrane protein(s) present in either fetal NRBCs or adult RBCs.

1.9.1 Aims

1 To profile both primitive fetal NRBC and adult RBC membrane proteomes.

2 To identify surface membrane protein(s) unique to primitive fetal NRBCs by comparing the two membrane proteomes.

3 To identify surface antigen(s) differentially expressed between primitive fetal NRBCs and adult RBCs by immunocytochemical screening.

1.9.2 Hypotheses

1 The most comprehensive membrane protein profiles of fetal NRBCs and adult RBCs can be obtained by developing an efficient proteomic platform for membrane protein analysis.

2 It is possible to identify unique surface protein(s) on fetal NRBCs or adult RBCs by comparing the membrane profiling data obtained by proteomic approaches.

3 Differential expression of surface antigen(s) is (are) present in primitive fetal NRBCs or in adult RBCs, and could be identified by immunocytochemical screening using commercially available antibodies.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Human tissue and blood samples

2.1.1.1 Ethical approval for use of human tissues and blood samples

Placental tissue and adult blood collection for this research were approved by the NHG Domain Specific Review Board in compliance with national guidelines regarding the use of human sample for research purposes. All research participants gave written informed consent for the collection and use of human tissues.

2.1.1.2 First trimester placental tissues

First trimester placental tissues (7^{+0} to 11^{+4} gestational weeks) were collected immediately after surgical termination of pregnancy. The tissues were washed carefully to eliminate maternal blood contamination and kept in phosphate-buffered saline (PBS) in 50 ml polypropylene tube.

2.1.1.3 Peripheral blood from healthy male and female volunteers

Peripheral blood samples (2-10 ml) were collected via venepuncture from healthy male and female volunteers.

2.1.2 Antibodies, reagents, solutions and kits

All antibodies, reagents, solutions and kits used in the experiments are listed below together with their source.

2.1.2.1 Antibodies

Primary antibodies against following human antigens (all host in mouse unless otherwise stated): CD34, CD46, CD55, CD100, CD117 (host in rabbit), CD175s, HLA-DR (Neomarkers, LAB VISION, CA); CD29, CD31, CD35, CD36, CD44, CD45, CD45RB, CD47, CD59, CD81, CD99, CD108, CD147, CD147-FITC (fluorescein isothiocyanate), CD164, CD222, E-cadherin (BD Pharmingen, San Diego, CA); CD14, CD90, CD105, CD133, monocarboxylate transporter 1 (MCT1) and neutral amino acid transporter ASCT2 (host in rabbit) (Chemicon, Temecula, CA); CD71, HLA-ABC (DAKO, Carpinteria, CA); CD233 (Sigma-Aldrich, St Louis, MO); CLC-6 (host in rabbit) (Alpha Diagnostics, San Antonio, TX); azurocidin/CAP37 (R&D Systems, Minneapolis, MN).

Secondary antibodies: biotinylated goat anti-mouse IgG, biotinylated rabbit anti-mouse IgG (Vector Laboratories, Burlingame, CA); goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Pierce, Rockford, IL); goat anti-mouse IgG conjugated Dynabeads M-450 (Dynal, Oslo, Norway); rat anti-mouse IgG conjugated to Miltenyi-MACS microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany).

2.1.2.2 Primers

Oligo dT (18 T nucleotide) and all other primers listed in Table 2-1 were purchased from Sigma-Proligo, Singapore.

Table 2-1 Primer pairs used for the amplification for individual gene

Selected protein identifications	Gene name	Forward	Reverse	Amplified bp
Hemoglobin epsilon chain	HBE1	5'-TTTTACTGCTGAGGAGAAGGCTGC C-3'	5'-CTTGCCAAAGTGAGTAGCCAGAATAA-3'	355
Hemoglobin gamma-2 chain	HBG2	5'-ACGCCATGGGTCATTTCACAGA-3'	5'-GAGCTCAGTGGTATCTGGAGGA-3'	455
Band 3 anion transport protein	SLC4A1	5'-ACACAGCTCTTCTGTGAGCA-3'	5'-TCCGACACTCCCATCTGGTT-3'	727
BCG induced integral membrane protein BIGM103	SLC39A8	5'-GTCTGAGATGCCTGGTATATAG-3'	5'-TCTTTGGCTCCTTAAAGACTTGG -3'	314
Chloride channel protein 6	CLCN6	5'-GGGACCTTGTGCTGAGGGA-3'	5'-AGCTGCGACTGCGGCAAT-3'	246
Azurocidin precursor	AZU1	5'-GTGCTGGGTGCCTATGACCTGAGG-3'	5'-AAGAGCGCCACTCGG GTGAAGAA-3'	467
Vitamin K epoxide reductase complex subunit 1-like protein 1	VKORC1L1	5'-AGACACCTCAGGCAGCACTT-3'	5'-TATTTCACCTTTTCTGGGCG -3'	134
Protein GPR107 precursor	GPR107	5'-TCAGAACATGGTTGTTCTCCC-3'	5'-GCTTGCTCTTCCTCCACATC-3'	164
Neutral amino acid transporter B	SLC1A5	5'-TGGCTGCTGGAGTACATGTG'-3'	5'-CCCAGTGGGGGCTAGAATTC-3'	196
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	5'-AAGGACTCATGACCACAGTCCATG-3'	5'-TTGATGGTACATGACAAGGTGCGG-3'	673
Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2, isoform A	SLC3A2	5'- ATGGACCCACTACCCTTCTC -3'	5'- CATGCAGGGGTGACTTTTAT -3'	150
Solute carrier family 22 member 11, isoform 2	SLC22A11	5'- CTGGGTTCCAATCTCACCC -3'	5'- TTTTTCTGGCAGCTCTCTCA -3'	150
Antibacterial protein FALL-39 precursor	CAMP	5'- GATAACAAGAGATTTGCCCTGC -3'	5'- GGGTAGGGCACACACTAGGA -3'	146
Vesicle-associated membrane protein 2	VAMP2	5'- AGTCCCTTAACCTGCCACG -3'	5'- CTGGGATAATATGGGGGGTC -3'	165
Transferrin receptor protein 1	TFRC	5'- TAGGCAGCAGCTTTTAATACAGG -3'	5'- AAAGTAAGCGAACCACTTACAACC -3'	238
Cleft lip and palate transmembrane protein 1	CLPTM1	5'-AGGTTCCCACAGCAGCAG-3'	5'-CCTCTGCTGGCTTTGGAG-3'	155
CAAX prenyl protease 1 homolog	ZMPSTE24	5'-CCTAAG GCTAAAGAGGAGCAG-3'	5'-GCGTTGGCAATGTTTAATGT-3'	146
Steroid dehydrogenase homolog	HSD17B12	5'-TGAAATATGCAGCAAGAAGATTGG-3'	5'-AATGATGCTGATAGCAGATGGCT-3'	193

Table 2-1. Continued

Olfactory receptor 11H4	OR11H4	5’-AACAACTGAATGTCTCTTTCT-3’	5’-GGAGTCGTTACTGAATATACC-3’	483
Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1	ATP6V0A1	5’-ACCTGACCCGACCTTGTG-3’	5’-CTGAACTCTGCTTCAAACCCC-3’	96
CDNA PSEC0252 fis, clone NT2RP3003258, highly similar to Likely ortholog of mouse embryo	SLC43A3	5’-ATGTCCTAGAAGGTTTTAGG-3’	5’-CAAAGATAGTCTGTCAGAAA -3’	104
Synaptophysin-like protein	SYPL1	5’-TGCATCATAAAGGAACCTAAGTG-3’	5’-TGTAAGAATAAGAAACCTGAATCC C-3’	144
Protein C9orf5	C9orf5	5’-TAGCCCTGACCTTGCAGTCT-3’	5’-GCATTTGGAAGTAATGCTAGC C-3’	123

2.1.2.3 Reagents

Chemical reagents: methanol (MeOH) and ethanol (Fisher Scientific, Fairlawn, NJ); acetic acid, and acetone (Merck, Darmstadt, Germany); urea, tris-base, SDS and glacial acetic acid (BDH Merck, Whitehouse Station, NJ); ammonium persulphate, dithiothreitol (DTT), mineral oil, 30% bis/acrylamide, overlay agarose, rehydration buffer, protein assay dye reagent concentrate and N,N,N',N'-Tetramethylethylenediamine (TEMED) (Bio-Rad Laboratories, Hercules, CA); Tween-20 (DAKO Corporation, Carpinteria, CA); Percoll (GE Healthcare, Uppsala, Sweden); Hanks' Balanced Salt Solution (HBSS), ethylenediaminetetraacetic acid (EDTA) and (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1 M (Invitrogen, Carlsbad, CA); Nonidet P-40 (NP-40) (Roche diagnostics, Mannheim, Germany); bromophenol blue, thiourea, glycerol, N-decyl-N, N-dimethyl-3-ammonio-1-propane sulfonate (SB3-10), n-octylglucoside, tris-carboxyethyl phosphine hydrochloride (TCEP), Trition X-100, potassium ferricyanide, sodium thiosulphate, sucrose, ammonium bicarbonate (NH_4HCO_3), iodoacetamide (IAA), formic acid, trifluoroacetic acid (TFA), HPLC grade acetonitrile (ACN) and Ficoll (Histopaque) 1077 (Sigma-Aldrich, St. Louis, MO); 2, 2, 2-trifluoroethanol (TFE) (Fluka, Buchs, Switzerland); DPX mountant (RA Lamb, East Sussex, UK).

Biological reagents: bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO); complete mini® protease inhibitor cocktail (Roche diagnostics, Mannheim, Germany); fetal calf serum (Pierce, Rockford, IL); RNase-Free DNase (QIAGEN GmbH, Hilden, Germany); RNase Inhibitor (New England Biolabs, Beverly, MA); modified sequencing grade trypsin (Promega, Southampton, UK); PCR buffer, $MgCl_2$, dNTPs (PE Biosystems, Warrington, England); AmpliTaq Gold polymerase (Roche, Mannheim, Germany); precision plus protein all blue standards (Bio-Rad Laboratories, Hercules, CA).

2.1.2.4 Water and solutions

Millipure water (Mili-Q systems, 18 MΩ/cm at 25°C) (Millipore, Bedford, MA); DEPC-treated water (0.1% Diethylpyrocarbonate (DEPC) in pure water); 5× SDS-PAGE sample buffer (0.313 M tris-base, pH 6.8 at 25°C, 10% SDS, 0.05% bromophenol blue, 50% glycerol, and 0.5 M DTT); tris solution for stack gel (0.5 M tris-base, pH 6.8, 0.4% SDS); tris solution for resolving gel (1.6 M tris-base, pH 8.8, 0.4% SDS); 1× SDS/glycine electrophoresis buffer (0.25 M tris, pH 8.6, 1.9 M glycine and 1% SDS); 2-DE sample buffer (7 M urea, 2 M thiourea, 100 mM DTT, 4% CHAPS, 0.5% carrier ampholytes pH 4–7, 0.01% Bromophenol blue and 40mM Tris); equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 30% glycerol, and 2% (w/v) DTT); equilibration buffer II (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 30% glycerol and 2.5% IAA); silver stain fixative solution (40% MeOH, 10% acetic acid (v/v) in pure water); silver stain stop solution (0.5% acetic acid); trophoblast digestion buffer (0.1% trypsin and 25 mM HEPES in HBBS); protein digestion solution (12.5 ng/µl trypsin in 50 mM NH_4HCO_3 solution); HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA and 250 mM sucrose); PBST (1× PBS/0.5% Tween-20); MACS, FACS and Dynal cell sorting buffer (0.5% BSA/PBS (w/v) supplemented with 2 mM EDTA); 1.5 M NaCl solution.

2.1.2.5 Kits

Alkaline phosphatase Vector blue substrate labelling kit (Vector Laboratories, Burlingame, CA); chemiluminescence HRP substrate WEST PICO (Pierce, Rockford, IL); RC DC protein assay kit (Bio-Rad Laboratories, Hercules, CA); Sensiscript RT kit and RNeasy Mini kit with QIAshredder spin column (QIAGEN GmbH, Hilden, Germany).

2.1.3 IPG Strip, gels, membrane and film

IPG ReadyStrip/pH 3-10/7cm, RGel/4-20%Tris-HCl/IPG and CrtGel/4-20%Tris-HCl/1.0mm (Bio-Rad Laboratories, Hercules, CA); HYBOND-C EXTRA nitrocellulose membrane (GE Healthcare, Uppsala, Sweden); CL-XPOSURE film (Pierce, Rockford, IL); Chromatography paper (SARTORIUS, Goettingen, Germany).

2.1.4 Hardware

All specialised hardware used in the experiments is listed below together with their source.

2.1.4.1 Pipettes, centrifuge tubes, freezing box and filters

Positive-pressure pipettes (Gilson, Villiers-le-Bel, France); Pipette-Aid motorised (BD Diagnostics, Franklin Lakes, NJ); 1.5 ml conical-bottom polypropylene tubes, 0.2 μm filters and 70 μm cell strainers (BD Biosciences, Bedford, MA).

2.1.4.2 Blood collection tubes, needles, slides, coverslips, haemocytometer and coplin jars

Vacutainer tubes and needles (BD Vacutainer systems, Franklin Lakes, NJ); uncoated microscope slides, coverslips, coplin jars and Neubauer haemocytometer (BDH Merck, Whitehouse Station, NJ).

2.1.4.3 Immuno-cell sorting equipment

MACS multistand, miniMACS separation units and MS columns (Miltenyi Biotech, Bergisch-Gladbach, Germany); Dynal Magnetic Particle Concentrator (MPC) (Dynal, Oslo, Norway); FACStarPLUS (Becton Dickinson, San Jose, CA).

2.1.4.4 Centrifuges for polypropylene tubes, cytocentrifuge and speedvac

L-80 ultracentrifuge, bench top centrifuge for 15 ml and 50 ml polypropylene tubes (Beckman coulter, Fullerton, CA); bench top microcentrifuge for 0.5 ml and 1.5 ml polypropylene tubes (Sanyo Gallenkamp PLC, Loughborough, UK); cytocentrifuge (Shandon, Cheshire, England); Savant speedvac (Model SC110, Global Medical Instrumentation, Minnesota).

2.1.4.5 Water bath, thermo bath, thermo cycler and freeze dryer

Standard water bath (GFL, INNOV Solutions, Milan, Italy); ALB64 thermo bath (ESEL TechTra Inc, Korea); freeze dryer (ThermoSavant, Holbrook, NY); PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA).

2.1.4.6 Sonicator, electrophoresis system and supplements

Misonix sonicator (ultrasonic processor)-XL2020 (Misonix Inc., Farmingdale, NY); Electrode wicks, forceps, PowerPac Basic™, Mini-PROTEAN 3 cell, UltraRocker Rocking Platform, Tran-Blot® Semi-Dry Transfer cell, PowerPac™ 3000 power supply and GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA); M-35 X-OMAT processor (Kodak, Rochester, NY).

2.1.4.7 Peptides desalting tip and columns

ZipTip C18 tip (Millipore, Chicago, IL); Sep-Pak-plus C-18 cartridge (Waters, Milford, MA).

2.1.4.8 2-D LC, SELDI-TOF-MS and MALDI-TOF/TOF-MS

LC SCX column, LC trap column, LC RP column, Probot Micro Fraction collector; Ultimate Dual HPLC system (LC Packings/Dionex, Sunnyvale, CA); Ciphergen ProteinChip SELDI-TOF-MS system and H50 chips (Ciphergen Biosystems,

Fremont, CA); Mixing tee (Upchurch Scientific, Oak Harbor, WA); MALDI target plate and ABI 4700 Proteomics Analyser (Applied Biosystems, Framigham, MA).

2.1.4.9 Microscope and spectrophotometer

Microscope (Olympus America, Melville, NY); Beckman DU 650 spectrophotometer (Beckman Coulter, Fullerton, CA).

2.1.4.10 Computer and software

IBM R40 (IBM, Armonk, NY); Adobe Photoshop graphics software (Adobe Systems Inc., Mountain View, CA); Ciphergen software (v3.0, Ciphergen Biosystems, Fremont, CA); GPS Explorer® software (Applied Biosystems, Framigham, MA); SPSS Software (SPSS Inc., Chicago, IL); Summit software v4.3 (Dako, Fort Collins, Colorado).

2.2 Methods

2.2.1 Preparation of Percoll gradient

Commercially available Percoll has a density of 1.130 g/ml. Required Percoll gradients were prepared from this stock by mixing with 1.5 M NaCl (ρ=1.058 g/ml) (Percoll Methodology and Applicants 2nd Edition, Amersham Pharmacia Biotech, Buckinghamshire, UK). Percoll has a very low osmolality (<25 mOs/kg H_2O) and can therefore form density gradients with iso-osmotic solutions (0.15 M NaCl or 0.25 M sucrose) without significantly affecting the physiological conditions. This is important for obtaining preparations of cells with intact morphology.

To prepare 50 ml of Percoll at a specified density, the following were mixed together: 5 ml 1.5 M NaCl; and Percoll (1.130 g/ml), the volume of which was calculated as follows,

$$V_0 = V\frac{\rho - 0.1\rho' - 0.9}{\rho_0 - 1}$$, where

V_0 = volume of Percoll (1.130 g/ml) in ml

V = volume of the final working solution in ml (50 ml)

ρ = desired density of the final working solution in g/ml

ρ_0 = density of Percoll stock in g/ml (1.130 g/ml)

ρ' = density of 1.5 M NaCl (1.058 g/ml) in g/ml

Distilled water to make up to 50 ml.

2.2.2 Nucleated and red blood cell count

Up to 10 µl of a nucleated cell sample was diluted 1 in 2 with 3% glacial acetic acid and 10 µl were loaded onto Neubauer haemocytometer. Nucleated cells

were counted under the inverted microscope. At least 200 cells or four separate area were examined and the average number of cells, N, per mm^2 (0.1 µl volume) determined and the concentration of nucleated cells in the original sample calculated (N × dilution × 10^4 per ml). To count RBC concentrations manually, dilutions were of the order of 1 in 200-400 in 0.4% formalin/0.1M tri-base.

2.2.3 Cytospin preparation

Up to 5 × 10^4 nucleated cells were resuspended in 300 µl 0.5% BSA/PBS (w/v), loaded into a cytospin chamber and cytospun onto a slide at 500 rpm for 5 min inside the cytocentrifuge. Slides were air dried and processed immediately or stored in aluminium foil and parafilm at -20°C.

2.2.4 Wright's staining

Cells cytospun onto glass slides were Wright's stained by sequentially immersing in 100% MeOH containing additive for 1.5 min, methylene blue stain solution for 1 min, eosin Y stain solution for 1 min, rinsed in water. Processed slides were air dried and mounted with coverslips over DPX mountant.

2.2.5 Separation of adult RBCs from whole blood

Blood from male and female adult volunteers was diluted 1:1 in PBS and carefully layered over an equal volume of Ficoll 1077 in 15 ml propylene tubes. The samples were centrifuged at 450 × *g* at room temperature for 30 min. The supernatant containing mononuclear cell layers was discarded. The red pellet was washed with 5-10 ml PBS four times and each time, the upper 1-2 mm layer of packed cells was aspirated along with liquid phase to completely remove WBCs. The resultant RBC pellet was used for slide preparation or membrane preparation for protein analysis.

2.2.6 Recovery of fetal NRBCs from placental tissues obtained from termination of pregnancy

The placental tissues were carefully dissected off adjacent deciduas and washed twice in PBS to remove maternal blood contamination. The cleaned specimen was incubated in 45 ml of trophoblast digestion buffer at 37°C for 30 min under vigour shaking. The trypsin activity was stopped with addition of 5 ml fetal calf serum. The cell suspension was then strained through a 70 μm cell strainer to obtain single cell suspension and centrifuged at 2000 × *g* at room temperature for 10 min. The cell pellet was resuspended in 5 ml of PBS, which was layered over Percoll 1083 and then centrifuged at 2000 × *g* for 20 min. The red pellet containing fetal NRBCs was washed once using PBS and resuspended into PBS buffer. Up to 10 μl of the suspension was taken for cell count. The rest of sample was used for slide preparation, mRNA analysis or membrane protein analysis. For protein analysis, the sample was washed twice with HES buffer and then stored in 300 μl HES buffer with protease inhibitor cocktail at -80°C.

2.2.7 Adult RBC Membrane preparation

Adult RBCs were separated as in section 2.2.5. The RBC pellets were washed using PBS and pelleted down by centrifugation for 10 min. Before membrane preparation, purity of RBCs prepared was assessed by examining WBCs in cytospin slides: only 7 WBCs (average) per slide containing 5×10^4 RBCs were observed (purity >99.9%). RBC ghosts were prepared as described by Hanahan et al. (1974). Briefly, RBC pellets were resuspended in 30 mM (310 imosM) isotonic Tris-HCl buffer (pH 7.6, 4°C) to a hematocrit of 50%. Cell suspensions were diluted 7-fold with 20 imosM Tris-HCl, and incubated at 4°C for 5 min to allow hypotonic lysis. RBC ghosts were pelleted by centrifugation at 20 000 × *g* at 4°C for 45 min in an L-80 ultracentrifuge. Pellets were washed five times using

20 imosM Tris-HCl by centrifugation at 20 000 × *g* for 45 min until the membrane preparation became colourless. White pellet (ghost cells) was resuspended in 0.1 M Na_2CO_3 (pH 11), sonicated and shaken for 20 min at 4°C. Membrane fractions were pelleted down by centrifugation at 100 000 × *g* at 4°C for 45 min, and washed twice with Millipure water. Membrane pellets were lyophilised and stored at -80°C.

2.2.8 Fetal NRBC membrane preparation

Membranes from pooled fetal NRBCs (5×10^7 cells) were prepared as described by Simpson et al. (2000) with some modifications. Briefly, fetal NRBCs stored in HES buffer were lysed by thawing and sonication. Membranes were then pelleted down by centrifugation at 100 000 × *g* at 4°C for 1 hour, and washed once with high pH solution (0.1 M Na_2CO_3, pH 11) and twice with Millipure water. Membrane pellets were lyophilised and stored at -80°C.

2.2.9 Protein estimation

2.2.9.1 Bradford assay

The protein contents of samples were determined using Bio-Rad protein assay dye reagent concentrate and BSA standards (0.2, 0.6, 1 and 1.4 μg/μl). 10 μl of samples or standards were mixed with 390 μl of pure water. 100 μl of Bradford dye was added to each mixture and incubated for 5 min. Absorbance was read at 595 nm using a spectrophotometer and protein concentrations were calculated based on BSA standard curve.

2.2.9.2 RC DC protein assay

The protein contents of samples were determined using Bio-Rad RC® DC protein assay kit following the manufacturer's instructions with slight modifications. 25 μl

of BSA standards (0.2, 0.6, 1 and 1.4 μg/μl) or samples were mixed with RC® DC reagent I and II, and then centrifuged at 15 000 × *g* for 10 min. Supernatants were discarded and pellets were dissolved in 127 μl of working reagent A (20 μl of reagent S per ml reagent A). Subsequently, 1 ml of reagent B was added into the solution and incubated for 15 min. Absorbance was read at 750 nm using a spectrophotometer and protein concentrations were calculated based on BSA standard curve.

2.2.10 One-dimensional gel electrophoresis

Protein samples in SDS-PAGE sample buffer were denatured at 37°C for 30 min and centrifuged at 15 000 × *g* for 1 min. The samples were loaded into 10% self-cast polyacrylamide gels or pre-cast Criterion gel (Bio-Rad). Precision plus protein all blue standards were used as molecular weight reference. Electrophoresis on the gel was carried out at 120 V for about 90 min in PowerPac Basic™ Electrophoresis system.

2.2.11 Two-dimensional gel electrophoresis

About 100 μg protein was mixed with 2-DE sample buffer to a final volume of 200 μl and vortexed 4 times for 20 sec at 1 min intervals. This mixture was applied to ReadyStrips (7 cm, pH 4-7) and passively rehydrated at room temperature for 16 hours. Proteins were then subjected to isoelectric focusing at 50 V for 2 hours; 250 V for 1 hour; 500 V for 1 hour; 1000 V for 1 hour and rapidly ramped up to 8000 V for 3 hours and then maintained at 8000 V for a total of 40 000 V × hours. ReadyStrips from the first-dimension were equilibrated with equilibration buffer I and II, and then applied to the top of a 4-20% Tris-HCl pre-cast Criterion gel for the second dimension separation. Electrophoresis on the gel was carried out at 120 V for about 90 min in PowerPac Basic™ Electrophoresis system.

2.2.12 Protein bands and spots visualisation

After electrophoresis, gels were transferred into plastic containers. The gels were incubated in fixative solution for 30 min with gentle shaking and then washed twice in 25 ml of deionised water for 10 min each. Subsequent incubation was with 25 ml of silver staining solution. When the desired staining intensity was reached, the gels were placed in 5% acetic acid to stop the reaction and washed in deionised water for 5 min. The images of the stained gels were captured using a GS-800 calibrated densitometer.

2.2.13 Western blotting

The proteins were separated by 1-DE as described in section 2.2.10. The proteins in the gel were transferred onto nitrocellulose membrane using Tran-Blot® Semi-Dry Transfer cell at a voltage of 15 V for 60 min. The membrane was incubated with 5% non fat dry milk powder in PBST for 60 min and then incubated with primary antibody at 4°C, overnight. The membrane was washed thrice in PBST for 10 min each and subsequently incubated with secondary antibody conjugated to HRP for 60 min. The membrane was washed thrice in PBST for 10 min each. The Pierce chemiluminescence HRP substrate was added onto the membrane to develop the blotting. The membranes were exposed in the dark room onto X-ray film for appropriate time period for visualisation of the chemiluminescence signal. The X-ray film was developed with M-35 X-OMAT processor. Images of the X-ray films were captured using a GS-800 calibrated densitometer.

2.2.14 In-gel digestion of proteins for MS analysis

2.2.14.1 In-gel digestion of proteins from stained gel

Visible gel bands by silver stain were finely cut into pieces, and transferred into 600 µl PCR tubes. A small clean spatula was used to coarsely grind up the gel pieces. Destaining was performed by adding 1:1 ratio of 30 mM potassium ferricyanide and 100 mM sodium thiosulphate solution, and vortexing. The mixture was washed and dehydrated sequentially in 100 mM NH_4HCO_3, ACN/50 mM NH_4HCO_3 (50:50, v/v). The sample was dried in a speedvac centrifuge. Dried sample was reduced by incubation with a fresh solution of 10 mM DTT in 100 mM NH_4HCO_3 at 57°C for 60 min, and alkylated by incubation with 55 mM iodoacetamide solution in 100 mM NH_4HCO_3 at room temperature for 60 min. Further procedures included gel washing in 100 mM NH_4HCO_3 (thrice), dehydration in 100% ACN (twice), re-swelling in 100 mM NH_4HCO_3 (once), dehydration in 100% ACN (twice), and drying in a vacuum centrifuge (once). Dried sample was re-swelled by adding digestion solution (12.5 ng/µl trypsin in 50 mM NH_4HCO_3), and incubated at 4°C for 30 min. Excess digestion solution was removed, and 50 mM NH_4HCO_3 was added to the sample before incubation at 37°C, overnight. After incubation, the solution was centrifuged at 6000 rpm for 10 min. The pellet was incubated with 20 mM NH_4HCO_3 for 5 min and centrifuged at 6000 rpm for 10 min. The resultant pellet was finally incubated with 5% formic acid in 50% ACN in water for 5 min and centrifuged at 6000 rpm for 10 min. All three supernatants were pooled and dried in a speedvac centrifuge.

2.2.14.2 In-gel digestion of proteins from unstained gel

After 1-DE separation, a whole protein gel without staining was equally cut into 35 slices and transferred into 600 µl PCR tubes. A small clean spatula was used to

coarsely grind up the gel pieces. The destaining procedure was omitted and all the other procedures are the same as above.

2.2.15 Peptide sample clean-up

The protein digests prepared as in section 2.2.14 were dissolved in 0.1% TFA. The peptides were extracted using Zip-Tip C18 Cartridge column. Briefly, Zip-Tip column was washed with 100 µl ACN and then with pure water (1 ml) to remove ACN. 10-20 µl sample was now pipetted in the column and the sample in the column was washed (desalted) with 0.5% formic acid (500 µl). The desalted sample was first extracted using 0.5% formic acid in H_2O/ACN (1:1, v/v) mixture for 20 sec and the extract collected in a clean PCR tube. A second extraction using 100 µl of ACN for 10 sec was carried out for maximal recovery of peptides from the column. Both extracts were pooled and concentrated for MS analysis.

2.2.16 Adult RBC membrane protein extraction and in solution digestion

Fresh or lyophilised membranes were resuspended in 50 mM NH_4HCO_3 (pH 8.0) by sonication, and protein concentration was determined using the Bradford assay. Membrane fractions (containing 200 µg protein) were used for the following three extraction and digestion methods.

2.2.16.1 Protein extraction using aqueous MeOH and trypsin digestion

Membrane proteins were extracted using the protocol described by Blonder et al. (2002) with some modifications. Membrane protein extraction was carried out using MeOH/50 mM NH_4HCO_3 (60:40, v/v) mixture with intermittent sonication, and the final concentration of solubilised protein was adjusted to 2 mg/ml in MeOH/50 mM NH_4HCO_3 (60:40, v/v). Pre-digestion procedures included reduction of proteins using 2 mM DTT at 37°C for 30 min followed by alkylation at

room temperature using 8 mM iodo-acetamide for 1 hour in the dark. Alkylation was stopped by the addition of 2 mM DTT. Tryptic digestion was carried out using sequencing grade modified trypsin 1:20 trypsin-to-protein ratio (w/w) at 37°C, overnight. The tryptic digests were lyophilised and stored at -80°C.

2.2.16.2 Protein extraction using aqueous TFE and trypsin digestion

Membrane proteins were extracted as per the protocol described above, except that TFE was used as the solvent in place of methanol and a mixture of TFE/50 mM NH_4HCO_3 (50:50, v/v) was used. The protein concentration was adjusted to 2 mg/ml in TFE/50 mM NH_4HCO_3 (50:50, v/v). Protein reduction and alkylation procedures were as described above. Protein mixtures were then diluted 10 times with 50 mM NH_4HCO_3. Tryptic digestion was carried out as above. Digests were lyophilised and stored at -80°C.

2.2.16.3 Protein extraction using urea solution and trypsin digestion

Membrane proteins were extracted using 6 M urea in 50 mM NH_4HCO_3 and the protein concentration was adjusted to 2 mg/ml with solid urea and 50 mM NH_4HCO_3. Protein reduction and alkylation procedures were as described above. Protein mixtures were diluted to a final concentration of 1 M urea using 50 mM NH_4HCO_3. Tryptic digestion was carried out as above. Digests were desalted with Sep-Pak-plus C-18 cartridge, lyophilised and stored at -80°C.

2.2.17 Fetal NRBC membrane protein extraction and trypsin digestion

Fetal NRBC membranes were prepared as in section 2.2.8. MeOH and TFE were used in this membrane protein extraction and digestion procedure sequentially to maximally recover peptides for MS analysis. Proteins were extracted from the purified fetal NRBC membranes using MeOH/50 mM

NH_4HCO_3 (60:40, v/v) and protein reduction, alkylation and digestion were carried out as in section 2.2.16. After centrifugation, the pellet was washed in MeOH solution (60% MeOH in 50 mM NH_4HCO_3) twice. All supernatants, including those from wash steps, were pooled as "MeOH-derived digests". The resultant pellet was then resuspended and the protein extracted using TFE/50 mM NH_4HCO_3 (50:50, v/v). Protein mixtures were diluted 10 times with 50 mM NH_4HCO_3 for a second trypsin digestion. Supernatants recovered from this step were defined as "TFE-derived digests". The two digests from fetal NRBC membranes were lyophilised and stored at -80°C.

2.2.18 SELDI-TOF analysis of proteins and tryptic peptides

SELDI-TOF profiles for hydrophobic proteins or tryptic peptides were obtained using reversed phase protein chips H50. Protein chips were activated using 5 µl of 80% ACN with 0.1% TFA for 5 min. Five µl of proteins or their tryptic digests were spotted onto protein chips, and incubated for 3 hours in a humidity chamber. After two washes with PBS, the arrays were semi-air dried. These were then treated with saturated sinapinic acid containing 0.5% TFA and 50% ACN, and analysed using the Ciphergen protein chip® reader.

Protein mass spectra were generated using (on average) 80 laser shots per sample spot. Data acquisition of low molecular weight peptides was performed in the size range set between 0-5 kDa. Detector intensity was set at 10, and the laser intensity was set at 170 V. Mass-to-charge ratios (m/z) of each of the proteins captured on the array surface were determined according to externally calibrated standards. Mass spectra obtained from the spectrometer were processed using the Ciphergen protein chip software v3.0 for baseline correction and peak detection using standard machine settings.

2.2.19 2-D LC separation of tryptic digests

Tryptic digests were resuspended in 20 µl of solvent (98% H_2O, 2% ACN and 0.05% TFA), and suspensions were separated into insoluble fractions and supernatants by centrifugation at 13 000 × *g* for 5 min. Supernatants were separated using an Ultimate Dual HPLC system coupled with a Famos micro autosampler with UV detector and Switchos column switching device. Six µl of sample (approximately 60 µg protein equivalents) was loaded and separated first on a SCX LC column (300 µm id × 15 cm) packed with 10 µm of POROS 10S. The mobile phases A and B were 5 mM KH_2PO_4 buffer (pH 3 adjusted with H_3PO_4) + 2% ACN and 5 mM KH_2PO_4 buffer (pH 3, adjusted with H_3PO_4) + 2% ACN + 500 mM KCl, respectively. The column flow rate for SCX was 6 µl/min. Nine fractions were separated by a step gradient of mobile phase B (unbound, 0-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-50, 50-100%). Eluted fractions were captured on the PepMap trap column (300 µm id × 1 mm, packed with 5 µm C18 100 Å), and eluted by gradient elution to a RP LC column (Monolithic Capillary Column, 200 µm id × 5 cm). The mobile phases A and B used were 98% H_2O + 2% ACN + 0.05% TFA and 20% H_2O + 80% ACN + 0.04% TFA, respectively. The elution gradient was 0-60% mobile phase B in 10 min (or 60 min for fetal NRBC sample) with a flow rate of 2.7 µl/min.

The LC fractions were mixed with MALDI matrix (7 mg/ml α-cyano-4-hydroxycinnamic acid and 130 µg/ml ammonium citrate in 75 % ACN) at a flow rate of 5.4 µl/min through a 25 nl mixing tee before being spotted onto 192-well stainless steel MALDI target plates at a rate of one well per 5 sec using a Probot Micro Fraction collector.

2.2.20 MALDI-TOF/TOF analysis of tryptic peptides

Samples on the MALDI target plates were analysed using an ABI 4700 Proteomics Analyser mass spectrometer with a MALDI source and TOF/TOF™ optics. For MS analysis, typically 1000 shots were accumulated for each sample well. MS/MS analysis was performed using nitrogen, at collision energy of 1 kV and a collision gas pressure of ~3.0 × 10^{-7} Torr. A stop condition was used so that 3000 to 6000 shots were combined for each spectrum depending on the quality of the data.

2.2.21 Database searching and bioinformatics analysis

MASCOT search engine (v1.9; Matrix Science) was used to search tandem mass spectra. GPS Explorer™ software was used to create and search files with the MASCOT search engine for peptide and protein identifications. The International Protein Index (IPI) human protein database v2.28 from the European Bioinformatics Institute (EBI) was used for the search of tryptic peptides. MS/MS spectra from the LC runs were combined for the search. Cysteine carbamidomethylation, N-terminal acetylation and pyroglutamination, and methionine oxidation were selected as variable modifications. Two missing cleavages were allowed. Precursor error tolerance was set to <100 ppm and MS/MS fragment error tolerance was <0.3 Da. All the proteins identified had at least one MS/MS match with an expected value <0.05, and it must be the best match. In addition, peptides with Mascot score more than 30 were counted in the reported peptide numbers. All the MS/MS spectra were further validated manually.

Subcellular and functional categories of all identified proteins were obtained on the basis of the annotations of Gene Ontology using GoFigure (http://udgenome.ags.udel.edu/gofigure/index.html). The grand average of

hydropathicity (GRAVY), a measure of hydrophobicity of proteins and peptides, was analysed by ProtParam program (http://tw.expasy.org/tools/protparam.html). The number of trans-membrane domains of all identified proteins was predicted using TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.2.22 RT-PCR

2.2.22.1 RNA extraction and quantification

RNA was isolated using an RNeasy Mini Kit according to manufacturer's instructions. Briefly, fetal NRBCs (about 3 × 10^6 cells) were washed with PBS and then resuspended into 350 µl lysis buffer (RLT*). The suspension was further homogenised by pipetting the lysate directly onto a QIAshredder spin column and centrifuged at 15 000 × *g* for 2 min. The homogenised lysate was mixed with 350 µl of 70% ethanol and pipetted onto an RNeasy mini column, and centrifuged at 15 000 × *g* for 15 sec. The RNA trapped in the column was washed with 350 µl buffer RW1 and incubated with 10 µl of DNase in 70 µl RDD buffer at room temperature for 15 min. The RNA was washed twice with 350 µl of buffer RW1 and once with 500 µl buffer RPE# (Qiagen). The RNA was then recovered by addition of 50 µl RNase-free water onto the column and centrifugation at 15 000 × *g* for 1 min.

10 µl RNA was diluted in pure water (v/v, 1:9) and read with spectrophotometer using wavelength scan from 220 nm to 350 nm. The absorbance at 280 nm and 260 nm were read for the estimation of concentration and purity of RNA. The RNA with an A280/A260 ratio within 1.9-2.1 was used for further experiments.

2.2.22.2 RT-PCR

The cDNA template was synthesised using Sensiscript RT Kit. Briefly, five µl of RNA was mixed with appropriate amount of oligo-dT, RNase inhibitor, dNTP mix and RNase-free water, and incubated at 70°C for 5 min and chilled on ice. RT buffer and RT enzyme (in control experiment, RT enzyme was replaced with water) were added to the mixture and incubated at 25°C for 15 min, 42°C for 60 min and 72°C for 15 min. After the incubation, the mixture was cooled on ice, and used for PCR experiments.

Five µl cDNA product was mixed with $MgCl_2$, dNTP, PCR buffer, Taq Polymerase, primers and water to give final concentrations of 1× PCR buffer, 1 mM dNTP, 8 mM $MgCl_2$, 2.5 U Taq Polymerase, and 0.6 µM primers (Table 2-1). PCR was performed with a PTC-200 Peltier Thermal Cycler. Optimal annealing temperature was obtained for each primer pairs. The mixture was denatured at 94°C for 2 min and followed with a 45 cycle of amplification. A typical cycle was 94°C for 15 sec, ~60°C (depends on primer pairs) for 15 sec, 72°C for 1 min. A final extension at 72°C for 4 min was performed for each gene. The negative controls included RT control (no enzyme in RT step) and PCR control (Water blanks). After amplification, the PCR products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 g/ml) and visualised under UV light. The images were captured using a digital imager (Alpha Innotech Corp., San Leandro, CA).

2.2.23 Immunocytochemistry

The cells on glass slides were fixed in methanol/acetone (v/v, 1:1) at room temperature for 2 min. The slides were rinsed in PBS for 30 sec; air dried and framed of cell area with hydrophobic pen. The slides were rehydrated in PBST

for 1 min. They were incubated with goat serum in PBS (1:10) for 2 hours and then incubated with primary antibody for 1 hour, and were washed twice in PBST for 5 min after each incubation. Subsequent incubations were with biotinylated goat-anti-mouse antibody or goat-anti-rabbit antibody and with streptavidin alkaline phosphatase for 30 min each. The slides were washed twice in PBST for after each incubation. Vector blue substrate was added onto the glass slides and incubated in the dark for 10 min. The slides were rinsed in distilled water for 30 sec and then dehydrated in 100% ethanol for 30 sec and air dried. The slides were mounted with Vector shield mounting medium and observed under light microscope.

2.2.24 Measuring intensity of colour using Adobe Photoshop

The amount of antigen (e.g. CD147) expressed on the cell surface were compared using the luminosity histogram function on graphics software (Adobe Photoshop). Photographs of immuno-stained cells were digitised by reflective scanning at 300 dots per inch. Mean pixel intensity from up to five small cytoplasmic areas of cells was determined and luminosity (brightness) determined on 256 grey scale (arbitrary units, AU).

2.2.25 Immunomagnetic cell separation

2.2.25.1 Dynal system

Positive and negative selection using the Dynal system was performed with only minor modifications to the manufacturer's instructions. Cells to be enriched were suspended in sorting buffer (50 µl for up to 10^7 total cells) in a 1.5 ml Eppendorf tube and incubated with appropriate anti-human murine monoclonal antibody at 4°C for 30 min. After washing twice with buffer, the cell pellet was resuspended

in 500 µl sorting buffer and incubated with 5 µl pre-washed goat anti-mouse IgG conjugated Dynabeads M-450 at 4°C for 30 min with slow end-over-end rotation. The cells bound to the beads were collected by placing the tube in the MPC. The supernatant was transferred into a new tube as negative fraction. The cells bound with beads were washed twice and lastly incubated with releasing buffer (DNase I in sorting buffer) at room temperature for 15 min. 500 µl sorting buffer was added and the suspension was mixed by pipetting. The tube was placed in the MPC for 2 min and the supernatant was transferred into a new tube as positive fraction. The cells in positive and negative fraction were pelleted down and resuspended into suitable volume for cell count.

2.2.25.2 MACS sorting

Positive selection using the MACS immunomagnetic separation system was performed with only minor modifications to the manufacturer's instructions. Cells to be enriched suspended in sorting buffer (100 µl for up to 10^7 total cells) and incubated with appropriate anti-human murine monoclonal antibody at 4°C for 30 min. After washing twice with buffer, the pellet was resuspended in 80 µl of sorting buffer and incubated with 20 µl rat anti-mouse IgG conjugated to Miltenyi-MACS microbeads at 4°C for 30 min with gentle mixing every 6 min. The cell mixture was washed, and the pellet was resuspended in sorting buffer and passed through the MS columns attached to the MACS multi-stand via the miniMACS separation units. Unlabelled cells that passed through the columns were collected as the negative fraction. Cells of the positive fraction, retained within the columns, were collected by positive fraction. The cells in positive and negative fraction were pelleted down and resuspended into appropriate volume for cell count.

2.2.26 Fluorescence-activated cell sorting

Cells to be enriched suspended in sorting buffer (100 µl for 10^6 total cells) and incubated with appropriate anti-human monoclonal antibody conjugated with FITC at 4°C for 30 min. The cells were washed twice, and the pellet was resuspended in 1 ml sorting buffer and sorted by flow cytometry by using FACStarPLUS machine. Positive and negative cells were sorted into different tubes coated with 5% BSA. The cells were pelleted down and resuspended into appropriate volume for cell count.

2.2.27 Statistical Analysis

Distributions were determined by plotting the data as histograms. Statistics used include mean, sample size, standard error (SE), 95% confidence interval (CI; 1.96SE), and one way analysis of variance (ANOVA).

Chapter 3 Proteomic Analysis of RBC Membrane Proteins Using Gel-based Approach and Shotgun Method

3.1 Introduction

This thesis focuses on the identification of unique membrane protein(s) that could be used to differentiate primitive fetal NRBCs from adult anucleate RBCs. In this chapter, I aimed to analyse adult RBC membrane proteins using various proteomic strategies (including gel-based approach and shotgun method), in order to: (1) obtain a more comprehensive RBC membrane proteome for future comparison with fetal NRBC membrane proteome; and (2) determine an efficient proteomic strategy for fetal NRBC membrane protein analysis.

Adult RBCs, due to the ease in accessibility and lack of internal organelles, have been studied extensively, and thus the identity, function, and topology of many RBC membrane proteins have been determined (Kakhniashvili et al., 2004; Low et al., 2002; Steck 1974). With advance of modern MS and its associated proteomic technologies, however, a more comprehensive RBC membrane proteome can be obtained, as it was shown in the result and also in the recent report by Pasini et al. (2006).

Moreover, study of adult RBCs is necessary before the analysis of fetal NRBCs because it would help us to determine an optimal proteomic strategy for membrane protein analysis. In our study, fetal NRBCs were harvested from the trophoblastic villi of placental tissues collected at termination of pregnancy (TOP) and the cell numbers were often small (~1×10^6 cells/TOP). Thus, it was highly

desirable to determine an efficient method for membrane proteome analysis using adult RBCs, which could be then applied to the analysis of a limited amount of fetal NRBC sample.

3.2 Optimisation of RBC membrane preparation

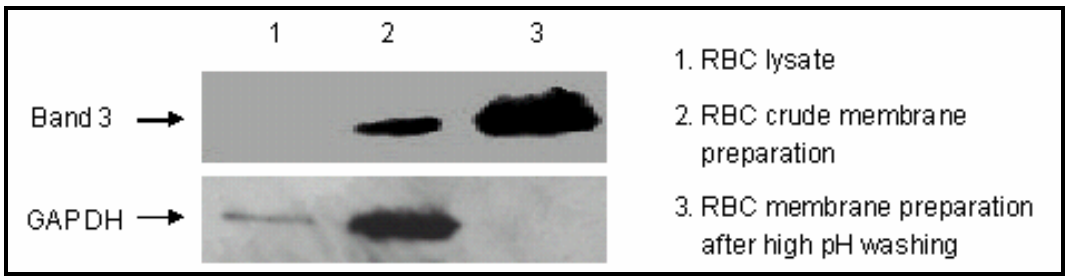
Aim. Fractionation of complex protein sample into a relatively simple components helps to increase the chance of identifying low abundance proteins and maximise the coverage of the proteome. It has been shown that high pH washing of membranes helped to enrich membrane proteins (Fujiki et al., 1982) and its use increased the identification of membrane proteins (Zhao et al., 2004). Here, I aimed to study the effect of high pH washing on our RBC membrane preparation.

Investigation. RBCs were separated from 2 ml of adult blood (~6×10^9 RBCs) using Ficoll 1077 density gradient (section 2.2.5). RBC ghosts were prepared as described by Hanahan et al. (1974) (*crude membrane preparation*). Half of the RBC ghosts were further washed in a high pH solution (0.1 M Na_2CO_3, pH 11) (section 2.2.7) (*purified membrane preparation*). The protein yields of the two preparations were estimated as described in section 2.2.9. The qualities of the two membrane preparations were assessed using western blotting by detecting two target proteins, integral membrane protein Band 3 and cytoplasmic protein Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (section 2.2.13).

Results. The protein yield was 1.42 μg per 10^6 RBCs in crude membrane preparation; whereas it was 0.55 μg per 10^6 RBCs in purified membrane preparation. The high pH washing step resulted in a lower yield of proteins but a significant enrichment of membrane proteins: there was a 3.5-fold increase in

Band 3, and a reciprocal disappearance of GAPDH to undetectable levels on western blotting (Figure 3-1).

Figure 3-1 Western blotting analysis of RBC membrane preparation with Band 3 and GAPDH monoclonal antibodies
Lane 1, RBC cell lysate; lane 2, RBC membrane preparation without high pH washing; lane 3, RBC membrane preparation with high pH washing. Protein (2 μg) was loaded in each lane. Western blotting indicates a 3.5-fold increase in Band 3 (membrane protein), and a reciprocal disappearance of GAPDH (cytoplasmic protein) to undetectable levels after high pH washing.



Interpretation. With the commonly used RBC ghost preparation protocol, the majority of cytosolic proteins (e.g. haemoglobin) could be efficiently depleted, but some of membrane-associated cytosolic proteins (e.g. GAPDH) were instead enriched together with integral membrane proteins (Figure 3-1). The use of a high pH washing helped to release membrane-associated cytosolic proteins from RBC membrane (Fujiki et al., 1982) and thus resulted in an enrichment of the membrane proteins.

The enriched membranes were then used for proteome analysis by either gel-based method (2-DE/1-DE followed by MS) (section 3.3 and 3.4) or shotgun-based approach (LC-MS/MS) (section 3.5).

3.3 2-DE separation of RBC membrane proteins

Aim. 2-DE has been used for separation of the proteins in RBC ghost preparation (Low et al., 2002), in which 102 proteins, but including only 2 integral

membrane proteins, were identified. If similar resolution (e.g. >100 spots) could be visualised in the 2-DE separation of our purified RBC membrane preparation which contained enriched membrane proteins, 2-DE and MS would be very useful for subsequent membrane protein identification. Thus, I aimed to test the suitability of 2-DE for the separation of proteins in our purified RBC membrane.

Investigation. RBC ghost preparation (crude membrane) and RBC ghost preparation followed by high pH washing (purified membrane) were carried out as described in section 2.2.7. Both crude and purified membranes were solubilised into 2-DE sample buffer for protein extraction and then separated by 2-DE in triplicate. The protein gels were silver-stained, and spot detection was performed with the PDQuest software package (v. 7.2) (section 2.2.11).

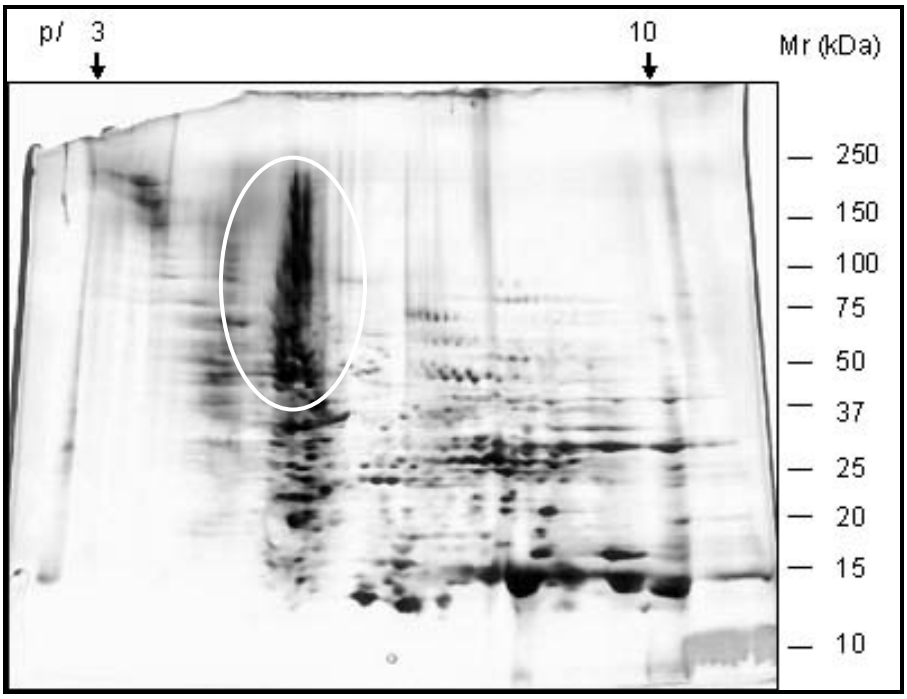
Results. 2-DE separations of proteins from crude RBC membrane and purified RBC membrane preparations were shown in Figure 3-2. While good separation of proteins from crude RBC membrane preparation was observed, only a few spots were detected from purified RBC membrane sample, suggesting that 2-DE was not suitable for separation of the proteins in our purified RBC membrane.

Interpretation. In the crude membrane preparation, many membrane-associated proteins were enriched as discussed in section 3.2. These proteins are generally relatively hydrophilic, and could be separated well using 2-DE as demonstrated by Low et al. (2002) and this study (Figure 3-2): many protein spots were observed. In contrast, hydrophilic proteins were nearly depleted in the purified membrane sample, and accordingly the protein spots were not detected. As a result, very few spots were observed in the 2-DE separation, suggesting that 2-DE was not very suitable for the separation of proteins from our purified RBC membrane preparation. Thus 2-DE was not used for further study.

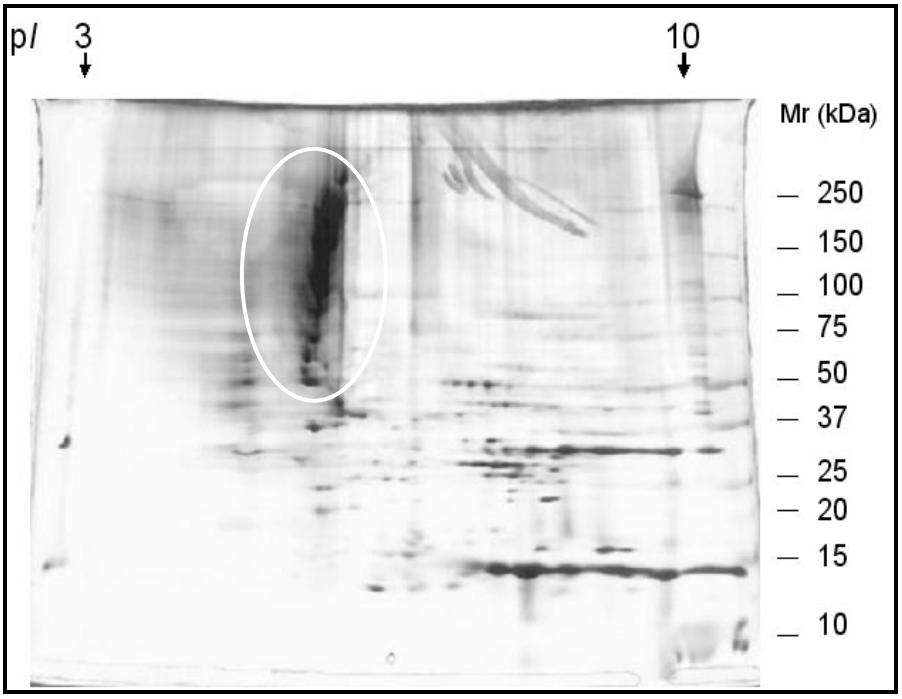
Figure 3-2 2-DE separation of RBC crude membrane and purified membrane preparations

Representative silver-stained 2-DE protein profiles of crude membrane preparation and purified membrane preparation. Protein (100 μg) was extracted and focused. The first dimension is pH 3–10 linear gradients and the second dimension is a 4–20% gradient gel. Molecular weight markers are indicated on the right and approximate isoelectric point (p*I*) is indicated across the top of the gels. The circle area corresponded to abundance protein Band 3 (with possible degradation).

(A) 2-DE of RBC crude membrane



(B) 2-DE of RBC purified membrane



3.4 1-DE separation of RBC membrane proteins and MS analysis

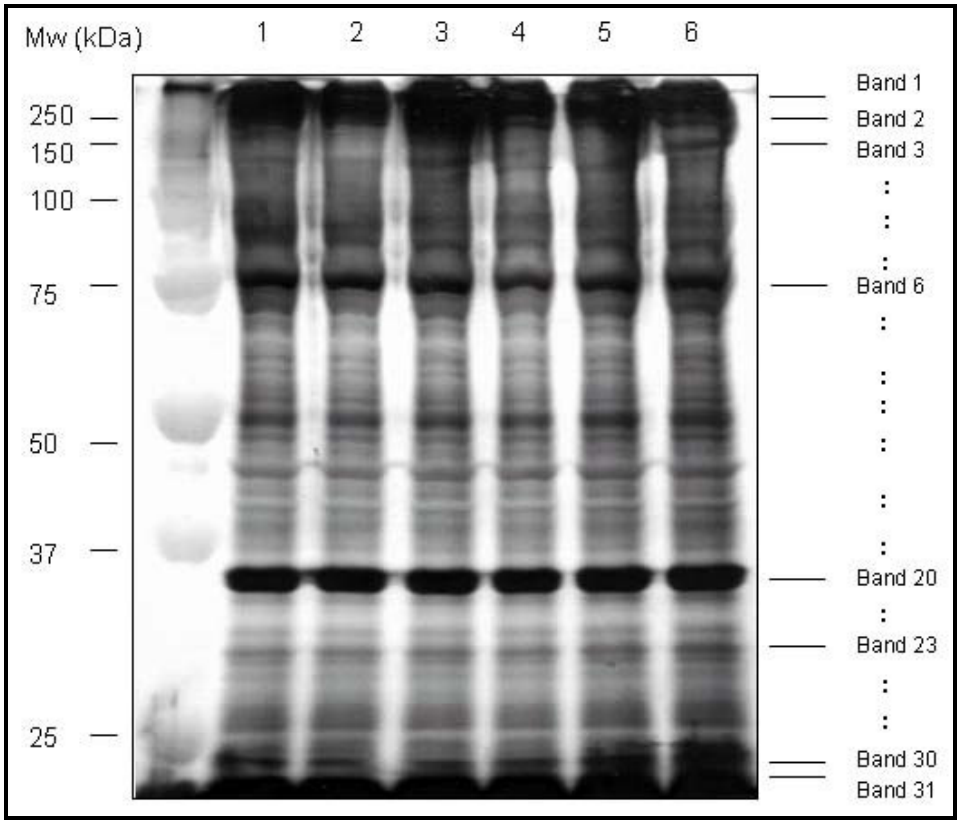
Aim. Currently, there are two common 1-DE based proteomic strategies for the analysis of membrane proteome: (1) proteins are separated using SDS-PAGE and then silver-stained. The visible bands are then cut for peptide elution and MS analysis; (2) proteins are separated by SDS-PAGE, and the whole protein gel without silver stain is equally cut into certain number of slices for peptide elution and MS analysis. Here I tested both methods for RBC membrane protein separation and identification.

3.4.1 Protein identification from silver stained 1-DE gel

Investigation. The purified RBC membrane was dissolved into SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE as described in section 2.2.10. Protein bands were visualised using Bio-Rad silver stain plus kit. The bands were cut separately (similar bands from all 6 lanes were pooled) for in-gel digestion (section 2.2.14), and the eluted peptides were desalted (section 2.2.15) and analysed using MALDI-TOF/TOF-MS.

Results. SDS-PAGE separation of RBC membrane proteins with 10 μg proteins loaded per well in an 8% polyacrylamide gel was shown in Figure 3-3. All visible bands (31) (as shown) were cut for in-gel digestion and MS analysis. With this approach, a total of 14 proteins (2 integral membrane proteins and 8 peripheral membrane proteins) were identified (Table 3-1).

Figure 3-3 SDS-PAGE separation and silver stain of RBC membrane proteins Protein (10 μg) was loaded in each lane in an 8% polyacrylamide gel. A total of 31 distinct bands were cut separately (bands from all 6 lanes were pooled), and subjected to in-gel digestion and MS analysis.



Interpretation. Membrane proteins are usually large and have high molecular weight. Thus, low percentage of polyacrylamide gel (8%) was used, as it has large-size pore and better resolution of high molecular weight proteins. In addition, similar bands from 6 lanes were pooled for in-gel digestion to elute more peptides for MS analysis. With these procedures, I was able to identify many major RBC membrane proteins (e.g. from Band 1 to 7), but unable to recover other less abundance well-known proteins, such as GPA, GPC and rhesus blood group, indicating the low efficiency of this method for RBC membrane protein analysis. Two possible reasons are: (1) the use of uniformly 8% polyacrylamide gel for protein separation does not provide good solution of all RBC membrane

proteins; and (2) silver stain of proteins interferes with their subsequent identifications as shown in a later study (Richert et al., 2004).

Table 3-1 Proteins identified from silver stained 1-DE gel

No.	Protein name	Accession Number (Gi)	Peptide count	Best ion score
1	Spectrin alpha chain, erythrocyte	1174412	4	38
2	Spectrin, beta, erythrocytic	27413156	1	32
3	Solute carrier family 4, anion exchanger, member 1	4507021	4	47
4	Ankyrin 1, erythrocyte splice form 3	1360744	5	38
5	Erythrocyte membrane protein band 4.1	42716291	1	42
6	Erythrocyte membrane protein band 4.2	112798	2	41
7	Erythrocyte membrane protein p55	4505237	1	24
8	Flotillin 2	4758394	2	35
9	Flotillin 1	5031699	6	30
10	Actin, beta	14250401	3	47
11	Tropomodulin 1	4507553	3	37
12	Aldolase A	4557305	1	28
13	Glyceraldehyde-3-phosphate dehydrogenase	31645	1	35
14	Band 7.2b stomatin	1103842	3	35

Best ion score: a MASCOT score of best match peptide

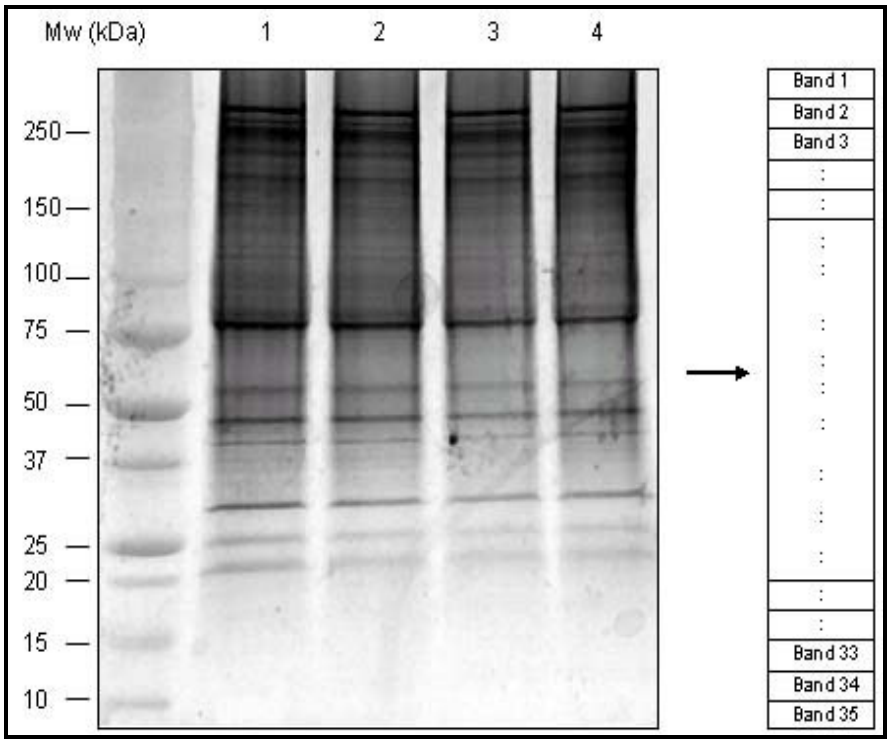
3.4.2 Protein identification from unstained 1-DE gel

Investigation. In previous section, I have shown that uniformly 8% gel for RBC membrane protein separation followed by silver stain prior to peptide elution and MS analysis yielded unsatisfactory results. In this section, the purified RBC membranes were dissolved in SDS-PAGE sample buffer. Proteins were separated using a gradient gel (Crtgel 4-20%, 1 mm, Bio-Rad). The separation of each sample was carried out in duplicate and optimal protein loading was 15 μg protein per lane for this type of gel. One gel was silver-stained as a reference for band cutting. The other without staining was equally cut into 35 slices. Each

slice was in-gel digested, and the eluted peptides were desalted and subsequently analysed using MALDI-TOF/TOF-MS.

Results. SDS-PAGE separation of RBC membrane proteins using Crtgel gel was shown in Figure 3-4. The proteins were well-separated and the bands were very fine. The unstained gel was equally cut for in-gel digestion and MS analysis as illustrated in Figure 3-4. With this approach, a total of 30 proteins (8 integral membrane proteins and 9 peripheral membrane proteins) were identified (Table 3-2).

Figure 3-4 SDS-PAGE separation of RBC membrane proteins using Crtgel gel
Protein (15 μg) was loaded in each lane. The figure shows a silver stained gel, and its duplicate gel without staining were equally cut into 35 slices for in-gel digestion (slices from all 4 lanes were pooled), and subjected to in-gel digestion and MS analysis.



Interpretation. With the gradient gel, despite more proteins loaded into each lane, the separation was better as shown that each band was very fine and clearly

separated (Figure 3-4) as compared with uniformly 8% polyacrylamide gel. The better separation reduced the possibility of less abundance proteins being masked by abundance proteins (e.g. Band 3), thereby increased the chance of identifying low abundance proteins. In addition, protein gel without silver stain was cut for in-gel digestion, which simplified in-gel digestion procedures and excluded the interference of silver with MS analysis. As a result, the number of total and integral membrane proteins identified was significantly increased as compared to that in section 3.4.1.

Using this approach, not only could major RBC membrane proteins be identified, but also many less abundant integral membrane proteins such as aquaporin-1, glycophorin C, and CD59 were identified. However, the gel-based method yielded a relatively small number of proteins when compared to a more recent shotgun proteomic analysis of RBC membrane proteins (Kakhniashvili et al., 2004), where a total of 99 proteins (including 21 integral membrane proteins) were identified from RBC membrane. Moreover, the facilities for shotgun analysis were newly available in our university. Thus, I also attempted to use shotgun approach to analyse our highly purified RBC membrane.

Table 3-2 Proteins identified from unstained 1-DE gel

No.	Protein Name	Accession Number	Peptide Count	Best Ion Score
1	Band 3 anion transport protein	IPI00022361	16	156
2	Erythrocyte band 7 integral membrane protein	IPI00219682	9	132
3	Hemoglobin beta chain	IPI00218816	6	112
4	Solute carrier family 2 (facilitated glucose transporter) member 1	IPI00220194	5	92
5	Alpha 2 globin variant	IPI00410714	2	138
6	Aquaporin-1	IPI00024689	2	137
7	Glycophorin C	IPI00218128	1	159
8	Spectrin, alpha, erythrocytic 1	IPI00220741	4	68
9	Ankyrin 1 isoform 1	IPI00216697	3	74
10	Ras-related protein RAP-2b	IPI00550907	2	63
11	Rhesus blood group, CcEe antigens	IPI00465155	2	55
12	Chromosome 9 open reading frame 19	IPI00479010	1	96
13	Erythrocyte membrane protein band 4.2	IPI00028614	2	56
14	CD59 glycoprotein precursor	IPI00011302	1	81
15	Spectrin beta isoform a	IPI00216704	2	37
16	11 kDa protein	IPI00514389	1	69
17	Similar to flotillin 2	IPI00386741	2	38
18	Stomatin-like protein 3	IPI00152125	1	62
19	Band 4.1-like protein 1	IPI00024062	1	53
20	Membrane protein C21orf4	IPI00010146	1	50
21	Actin, aortic smooth muscle	IPI00008603	1	49
22	Similar to mouse 1500009M05Rik protein	IPI00166865	1	50
23	Proteolipid protein 2	IPI00030362	1	46
24	Probable helicase senataxin	IPI00142538	1	44
25	Predicted: hypothetical protein XP_291007	IPI00216817	1	42
26	Titin protein	IPI00455173	1	41
27	Hypothetical protein FLJ42537	IPI00446235	1	41
28	Protease serine 2 isoform B	IPI00011695	1	41
29	Oxysterol binding protein-related protein 10	IPI00032971	1	41
30	N-Acetylgalactosaminyltransferase	IPI00065312	1	40

3.5 Shotgun proteomic analysis of RBC membrane proteins

SDS-PAGE coupled with MS analysis is very useful for membrane protein study, as the protein solubility is seldom a problem (section 1.7.5.1) and in-gel digestion of the denatured proteins is efficient (Lu et al., 2005). However, several limitations are associated with the gel-based method: (1) there is a significant loss of peptides during the tedious in-gel digestion procedures (Zhang et al., 2004); and (2) the limited resolving power of SDS-PAGE often results in poor separation of complex protein samples (e.g. several proteins in one small band), in which low abundance proteins are masked. This situation is even worse in the study of RBC membrane due to vast dynamic range: the most abundant membrane protein (Band 3) accounts for about 30% of the total RBC membrane proteins (1.2×10^6 molecules per RBC), whereas some receptors are only about 500 molecules per RBC (e.g. CR1). Moreover, Band 3 is easily degraded even with careful membrane preparation (Jennings et al., 1986). Thus when SDS-PAGE is used to separate RBC membrane proteins, it is often found that Band 3 is distributed in many bands as observed by us and Low et al. (2002), which could cause the failure of the identification of less abundance proteins due to being masked.

This problem could be partially overcome by using LC to separate the peptides eluted from each band prior to MS analysis, but it means that a large number of LC separations are required as there are many protein bands. Alternatively, a gel-free approach (shotgun proteomics) could be used for the RBC membrane protein analysis, where proteins are extracted and digested in solution and then analysed using LC-MS/MS. This approach is sensitive, reproducible, rapid (required only one injection for LC separation) and high throughput.

In shotgun approach, a solution, that can effectively extract membrane proteins and compatible with enzymatic digestion, is crucial for efficient peptide recovery for MS analysis. In this section, I tested three solvents (urea, MeOH and TFE) for shotgun proteomic analysis of RBC membrane proteins, and based on the results, I would determine a most efficient strategy using a solvent or their combination for the subsequent analysis of fetal NRBC membrane proteins.

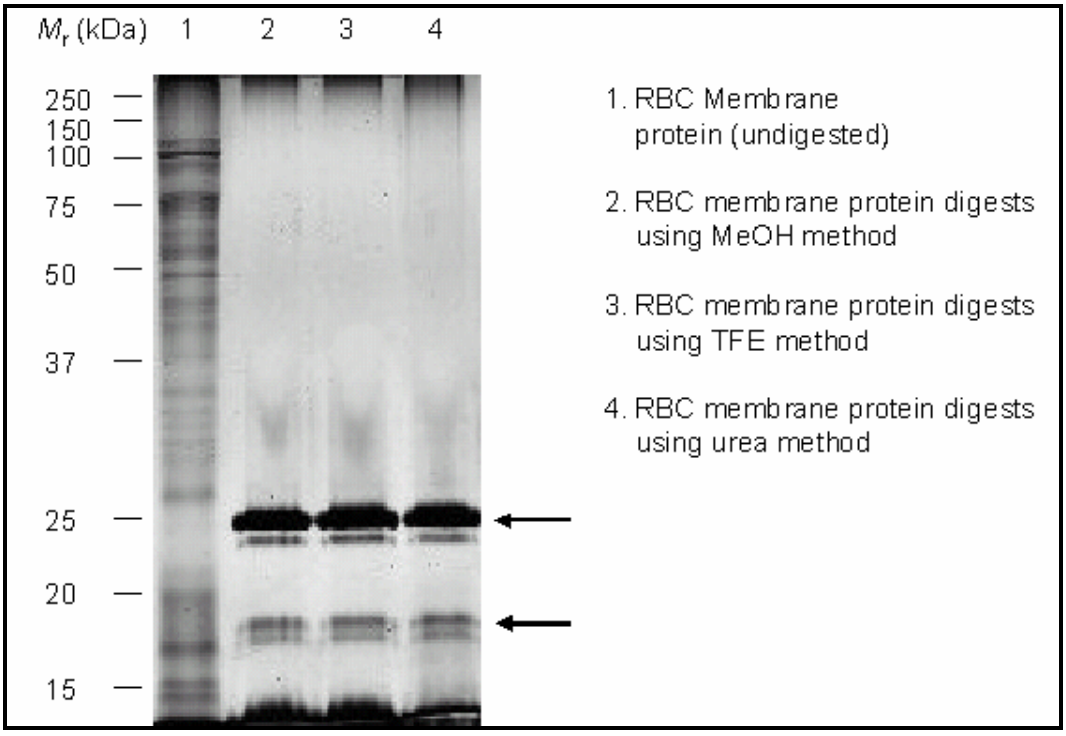
3.5.1 Membrane protein extraction and digestion

Aim. Urea solution is widely used to extract membrane proteins for 2-DE separation (Ueno et al., 2000) and shotgun proteomics (Wu et al., 2003); MeOH solution has been newly developed for membrane protein analysis in a shotgun approach (Blonder et al., 2002); and TFE is traditionally used together with $CHCl_3$ for miscible extraction of membrane proteins for 2-DE separation (Zuobi-Hasona et al., 2005) and has recently used for shotgun proteomic analysis of cell lysate (Wang et al., 2005). Here, I tested these three solvents for the extraction and digestion of proteins from RBC membrane, and the effectiveness of protein extraction and digestion processes in three solvents were assessed.

Investigation. Equal amount of purified RBC membranes (containing 200 μg proteins) was used for following three extraction and digestion procedures: (1) membrane proteins were extracted in 60% MeOH and followed by tryptic digestion in same solution; (2) membrane proteins were extracted using 50% TFE and followed by tryptic digestion in diluted solution (5% TFE); and (3) membrane proteins were extracted using 6 M urea and followed by tryptic digestion in 1 M urea (section 2.2.16). The efficiencies of protein extraction and digestion processes in three solvents were assessed and compared using SDS-PAGE and SELDI-TOF as described in section 2.2.18.

Results. SDS-PAGE comparison of the three tryptic digests, with undigested RBC membrane protein preparations as a control, revealed that extractions and digestions in MeOH, TFE and urea were effective (Figure 3-5). Only two bands, those of trypsin and its self-cleavage product (25 kDa and 17 kDa, respectively), were observed in the membrane protein digest lanes when compared with the undigested RBC membrane sample lane. These data suggested efficient extraction and adequate digestion of membrane proteins in MeOH, TFE and urea solutions.

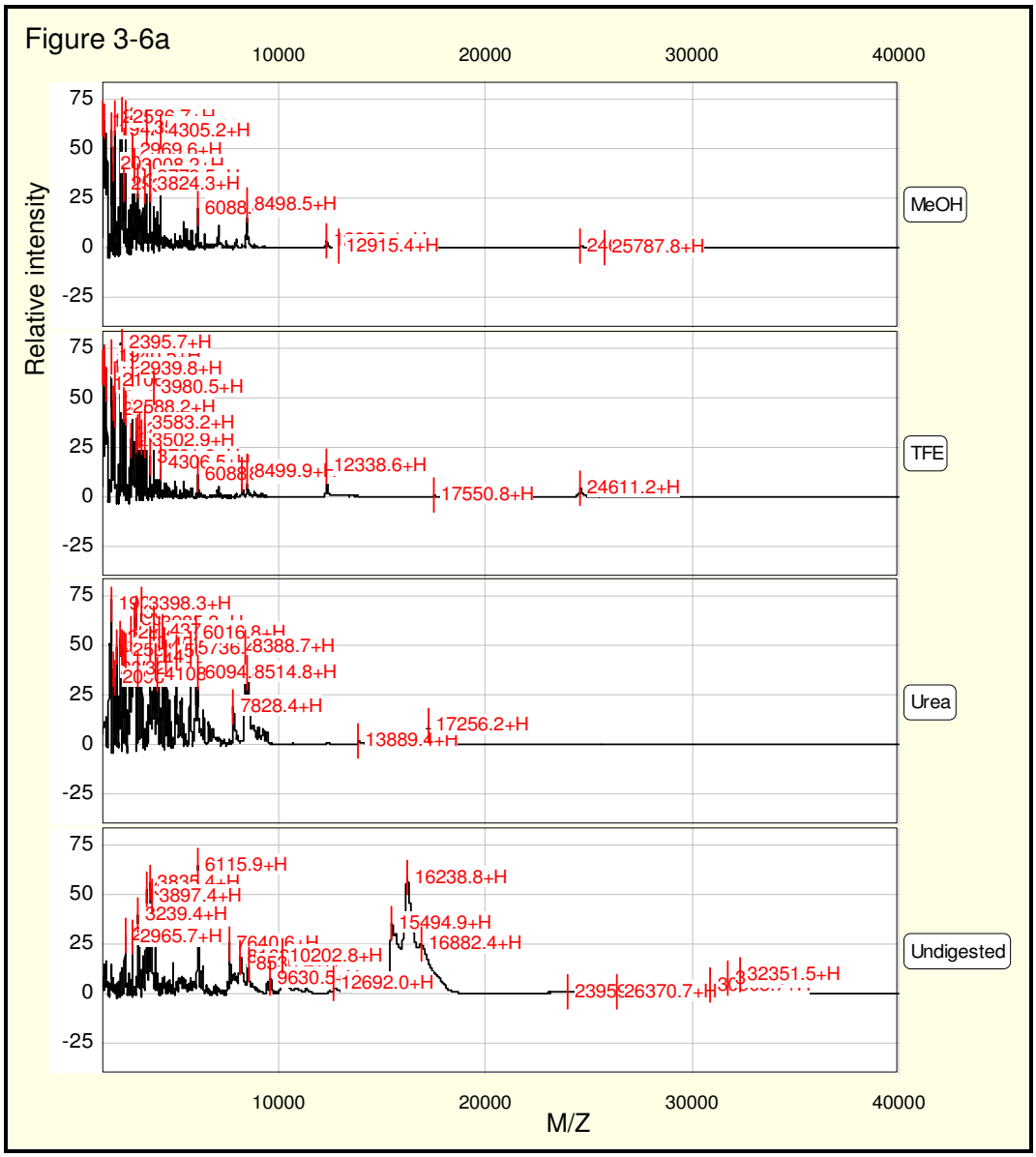
Figure 3-5 SDS-PAGE analysis of RBC membrane proteins and their tryptic digests show the effective digestion
Lane 1, RBC membrane protein (undigested); lane 2-4, RBC membrane protein digests using MeOH, TFE and urea methods respectively; 15 μg of protein or their digests was loaded in each lane. Arrows indicate bands corresponding to trypsin and its self cleavage product, 25 kDa (upper) and 17 kDa (lower) respectively.

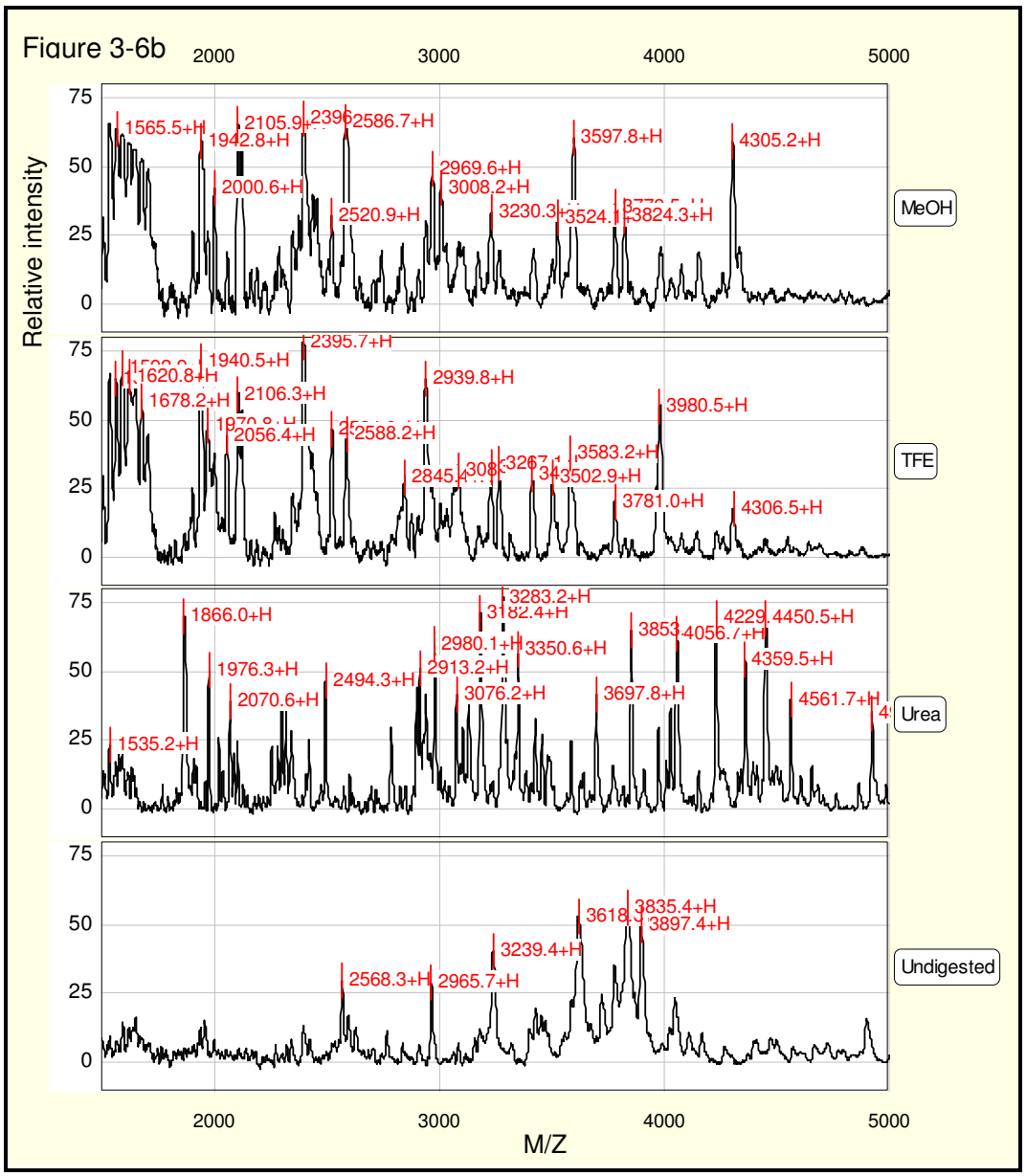


SELDI-TOF analyses of the digests using reversed phase protein chip H50, that bind mainly but not exclusively hydrophobic analytes, also confirmed efficient

digestion of the membrane proteins with trypsin (Figure 3-6). Most peaks were detected in the mass range of less than 5 kDa, and some peaks ranged between 5-10 kDa. The peaks with masses of 25 kDa and 17 kDa were similar to those observed in the SDS-PAGE separation of the digests.

Figure 3-6 SELDI-TOF analysis of RBC membrane proteins and their tryptic digests the effective digestion.
Digests analysed (0-40 kDa) is shown in Figure 3-6a. Only trypsin and self-cleaved product detected (17 kDa and 25 kDa) in high Mw range indicates the efficient digestion. Peptide peaks (<5 kDa) are shown in Figure 3-6b.





Interpretation. MeOH and urea have been previously used for membrane extraction and digestion process (Blonder et al., 2002; Wu et al., 2003). TFE is commonly used at high concentrations to solubilise, and study protein and peptide structure as it is able to stabilise protein secondary structures (especially alpha-helices) while destabilising tertiary and quaternary structure (Buck 1998). In a phase partition system with chloroform, TFE was able to satisfactorily extract membrane proteins with a wide range of properties (Deshusses et al., 2003). It is generally accepted that 2-DE is not very suitable for membrane protein

identification, but the use of TFE for isoelectric focusing has been shown to significantly increase the number and intensity of spots from membrane fractions (Deshusses et al., 2003). That these were intrinsic membrane proteins was confirmed by MS/MS. Similar use of $CHCl_3$/TFE mixtures for membrane protein extraction enhanced the resolution of spots, and allowed double the number of spots to be visualised when compared with membrane extraction using 1% SDS buffer (Zuobi-Hasona et al., 2005). These observations indicate a higher efficiency for membrane extraction using the organic solvent TFE compared with strong detergents such as SDS.

In summary, the analysis of the three digests by both SDS-PAGE and SELDI-TOF suggested efficient extraction, and adequate digestion of membrane proteins in aqueous MeOH, TFE and urea solutions. Thus all three solvents were suitable for studying membrane proteins in a shotgun approach.

3.5.2 Mass spectrometric analysis of membrane protein digests

Aim. In section 3.5.1, it was shown that the three procedures were efficient for RBC membrane protein extraction and digestion. Thus, I used these methods to prepare peptides for the subsequent 2-D LC MALDI-TOF/TOF-MS analysis in order to identify proteins from RBC membrane.

Investigation. The digests from three extraction and digestion procedures were separated using 2-D LC as described in section 2.2.19. The LC fractions were mixed with MALDI matrix and then directly spotted onto 192-well MALDI target plates using a Probot Micro Fraction collector. The samples on the plates were analysed using MALDI-TOF/TOF-MS. Database searching and bioinformatic analysis were carried out following the protocol described in section 2.2.21.

Results. Both common and unique proteins were identified from the three extraction methods. The 106 proteins identified were presented in Table 3-3. Information for each identified protein includes the protein name, protein accession number, protein molecular mass p*I*, number of identified peptides, score of best peptide match and number of transmembrane domain (TMD). Nineteen proteins were identical in all three methods, and 30, 14 and 24 unique proteins were identified using MeOH, TFE and urea, respectively (Figure 3-7). Among the 106 proteins, 47 were integral membrane proteins and contained at least 1 TMD (Figure 3-8): MeOH, 25; TFE, 22; and urea, 23, respectively. Seventeen membrane proteins were common to at least two solvents.

Figure 3-7 Schematic graphs showing the number of total proteins identified using 2-D LC-MALDI-TOF/TOF-MS from MeOH, TFE and urea recovered peptides
A total of 106 proteins were identified in this study (MeOH: 61, TFE: 46 and urea: 56; of these 57 overlapped).

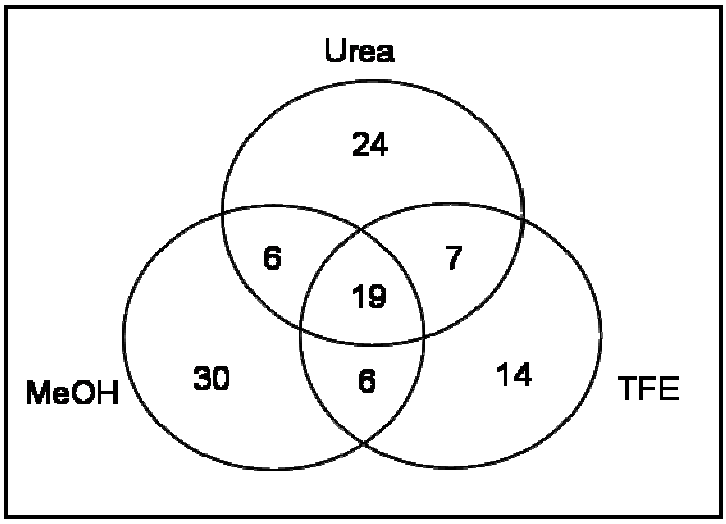


Figure 3-8 Schematic graphs showing the number of integral membrane proteins identified using 2-D LC-MALDI-TOF/TOF-MS from MeOH, TFE and urea recovered peptides
A total of 47 proteins were identified (MeOH: 25, TFE: 22 and urea: 23, of these 23 overlapped).

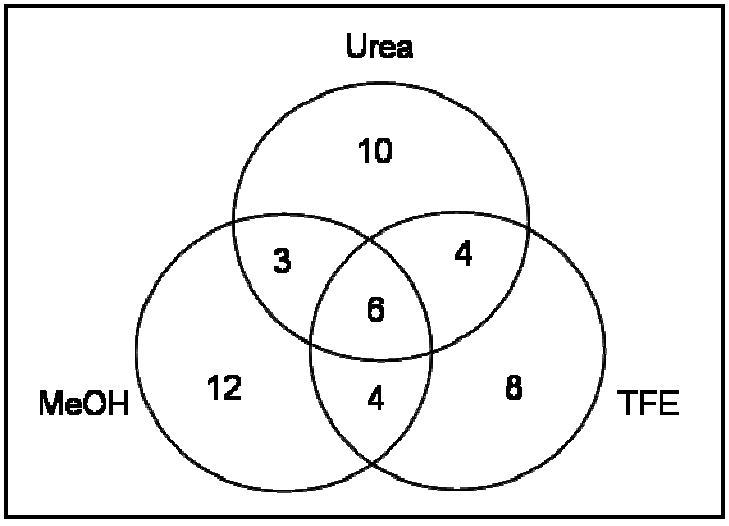


Table 3-3 The proteins identified from urea, MeOH and TFE extracted samples

No.	Protein Name	Accession Number	Protein MW	Protein p*I*	TMD	UREA		MeOH		TFE	
						Peptide Count	Best Ion Score	Peptide Count	Best Ion Score	Peptide Count	Best Ion Score
1	Ankyrin 1 isoform 4	IPI00374973	203278.55	5.72	0	56	212	78	207	42	167
2	Spectrin, alpha, erythrocytic 1 (elliptocytosis 2)	IPI00220741	280883.88	4.98	0	35	177	39	211	33	117
3	Splice Isoform 2 of Spectrin beta chain, erythrocyte	IPI00216704	267589.56	5.24	0	36	161	35	153	34	127
4	Band 3 anion transport protein	IPI00022361	101727.41	5.08	11	19	212	26	178	16	201
5	Splice Isoform 1 of Protein 4.1	IPI00003921	96957.32	5.45	0	19	154	23	183	11	110
6	Erythrocyte membrane protein band 4.2	IPI00028614	76793.61	8.27	0	17	149	15	142	13	130
7	55 kDa erythrocyte membrane protein	IPI00215610	52263.65	6.91	0	6	133	5	225	9	91
8	Erythrocyte band 7 integral membrane protein	IPI00219682	31579.70	7.9	1	11	178	6	88	7	150
9	Solute carrier family 2,facilitated glucose transporter member 1	IPI00220194	54082.52	8.93	12	10	194	12	140	7	144
10	Hemoglobin alpha-1 globin chain	IPI00410714	15247.93	8.72	0	5	184	8	171	6	103
11	Flotillin 1	IPI00027438	47325.62	7.08	0	3	114	5	134	9	116
12	Flotillin 2	IPI00029625	41659.24	5.23	0	5	74	8	130	6	112
13	Hemoglobin delta subunit	IPI00473011	15914.25	7.97	0	4	82	6	138	5	115
14	Beta-globin gene from a thalassemia patient, complete cds	IPI00382950	18918.59	6.28	0	4	136	2	57	4	121
15	Actin, cytoplasmic 2	IPI00021440	41765.79	5.31	0	5	162	2	159	4	104
16	Splice Isoform 6 of Plasma membrane calcium-transporting ATPase 4	IPI00217169	133845.70	6.04	8	1	124	6	99	3	105
17	Splice Isoform 2 of Glycophorin C	IPI00218128	11491.61	4.76	1	1	148	3	133	1	128
18	Splice Isoform Long of Dematin	IPI00292290	45486.22	8.94	0	1	76	1	73	1	61

Table 3-3. Continued

19	CD44 antigen precursor	IPI00305064	81503.40	5.13	1	1	60	1	47	1	52
20	Multidrug resistance-associated protein 4	IPI00006675	149445.70	8.48	11	2	94	1	44		
21	Ras-related protein Rab-10	IPI00016513	22526.59	8.59	0	1	44	2	44		
22	Calnexin precursor	IPI00020984	67525.85	4.47	1	3	107	1	94		
23	Aquaporin-1	IPI00024689	28377.02	7.15	6	2	195	2	59		
24	PREDICTED: similar to Zgc:66168 protein	IPI00296120	17639.35	9.3	0	2	53	3	98		
25	Splice Isoform 1of Intercellular adhesion molecule 4 precursor	IPI00396335	25914.66	10.19	0	1	47	2	52		
26	Actin, alpha cardiac	IPI00023006	41991.88	5.23	0	4	82			3	83
27	Rhesus blood group-associated glycoprotein	IPI00024094	44169.66	6.19	12	2	151			2	113
28	Kell blood group glycoprotein	IPI00220459	82770.92	8.09	1	8	114			5	96
29	Rhesus blood group CE protein	IPI00465155	45421.20	9.4	12	3	66			4	60
30	ATP-binding cassette half-transporter	IPI00465442	99649.17	9.26	9	5	126			4	49
31	Calreticulin precursor	IPI00020599	48111.82	4.29	0	1	63			1	56
32	PREDICTED: unc-13 homolog A	IPI00164441	187567.52	5.51	0	1	40			1	42
33	Cathepsin G precursor	IPI00028064	28819.07	11.19	0			1	52	1	58
34	Erythroid membrane-associated protein	IPI00044556	52571.60	8.75	1			3	68	2	96
35	Glyceraldehyde 3-phosphate dehydrogenase	IPI00219018	43990.02	9.73	0			1	46	3	78
36	Urea transporter, erythrocyte	IPI00298337	42499.76	6.79	8			2	67	1	100
37	Stomatin	IPI00377081	13466.03	7.82	1			4	72	4	113
38	Glycophorin Erik I-IV precursor	IPI00384414	9326.04	5.16	1			2	115	2	85
39	27 kDa protein	IPI00641837	27263.67	9.23	3	6	152				
40	Rhesus blood group D antigen	IPI00478119	53180.05	9.19	10	3	131				
41	Splice Isoform A of Ras-related C3 botulinum toxin substrate 1	IPI00010271	21436.24	8.77	0	2	70				
42	Semaphorin-7A precursor	IPI00025257	74776.17	7.57	0	2	49				
43	S100 calcium-binding protein A7	IPI00219806	11318.51	6.26	0	2	35				

Table 3-3. Continued

44	Probable endonuclease KIAA0830 precursor	IPI00001952	54981.26	5.55	3	1	71		
45	UPF0198 protein CGI-141	IPI00007061	15415.41	10.36	3	1	78		
46	Golgi-associated plant pathogenesis-related protein 1	IPI00007067	17076.48	9.44	0	1	99		
47	Ras-related protein Rab-21	IPI00007755	24201.21	8.16	0	1	42		
48	CD59 glycoprotein precursor	IPI00011302	14167.79	6.02	0	1	90		
49	Ras-related protein Rab-5B	IPI00017344	23691.90	8.29	0	1	67		
50	Splice Isoform 1 of Beta-adducin	IPI00019904	80803.43	5.67	0	1	76		
51	Suppressor of actin 1	IPI00022275	66908.01	6.66	2	1	43		
52	PREDICTED: similar to Reticulon protein 3	IPI00177423	25506.59	8.67	1	1	49		
53	Splice Isoform 2 of Sodium channel protein type I alpha subunit	IPI00216029	227641.81	5.65	19	1	41		
54	Similar to expressed sequence AA536743	IPI00216890	29321.88	8.57	2	1	60		
55	Triadin	IPI00220272	81374.51	9.42	1	1	54		
56	Splice Isoform 1 of Vacuolar protein sorting 13A	IPI00255301	360047.44	5.94	0	1	53		
57	Ras-related protein Rab-35	IPI00300096	23010.77	8.52	0	1	72		
58	NADH-cytochrome b5 reductase	IPI00328415	34081.68	7.31	0	1	102		
59	Multidrug resistance-associated protein 5	IPI00385383	160856.16	8.87	11	1	48		
60	22 kDa protein	IPI00478755	22062.88	5.92	0	1	41		
61	Protein C9orf32	IPI00549389	25239.80	5.31	0	1	48		
62	Splice Isoform 1 of Acid sphingomyelinase-like phosphodiesterase 3b precursor	IPI00550115	51682.49	5.5	0	1	48		
63	Hemoglobin beta chain	IPI00218816	15988.29	6.75	0			6	206
64	Similar to Calpain-like protease	IPI00550259	81699.97	8.56	0			3	84
65	Splice Isoform 2 of Guanine nucleotide-binding protein G	IPI00219835	44238.32	5.92	0			2	51
66	Splice Isoform 2 of Transforming protein p21	IPI00423570	21410.90	8.24	0			2	52

Table 3-3. Continued

67	Splice Isoform 1 of Fanconi anemia group A protein	IPI00006170	162701.38	6.13	0	1	37
68	Low affinity immunoglobulin gamma Fc region receptor III-B precursor	IPI00023858	26199.20	6.22	1	2	64
69	Syntaxin-4	IPI00029730	34158.85	5.92	1	1	39
70	Intermediate conductance calcium-activated potassium channel protein 4	IPI00032466	47664.55	9.87	5	1	61
71	Flavin reductase	IPI00219910	22579.64	7.12	0	2	61
72	Lactotransferrin precursor	IPI00298860	79802.73	8.45	0	1	42
73	RTN3-A1	IPI00398795	112541.42	4.85	3	1	40
74	Complement receptor 1	IPI00412546	272673.31	6.6	1	1	39
75	Rhesus blood group, CcEe antigens	IPI00444375	43902.44	9.85	10	2	70
76	Chromosome 9 open reading frame 19	IPI00479010	14204.11	9.55	0	1	71
77	Splice Isoform 2 of Myeloperoxidase precursor	IPI00236554	73806.61	9.3	0	1	59
78	Mesenchymal stem cell protein DSCD75	IPI00550002	23849.87	9.71	1	1	51
79	Splice Isoform 3 of Alpha adducin	IPI00220158	84250.40	5.67	0	1	41
80	Equilibrative nucleoside transporter 1	IPI00550382	60905.51	8.58	11	1	63
81	Splice Isoform 3 of Beta adducin	IPI00220241	72674.54	6.11	0	1	43
82	Zinc transporter 1	IPI00002483	55292.08	6.02	6	1	75
83	Solute carrier family 40, member 1	IPI00005547	62501.35	6.08	10	1	50
84	Splice Isoform 2 of Cell division control protein 42 homolog	IPI00016786	21245.02	6.15	0	1	57
85	Stromal cell-derived receptor-1 beta	IPI00018311	44359.50	8.11	1	1	62
86	cAMP-dependent protein kinase	IPI00021831	42954.97	5.27	0	1	54
87	Ras-related protein Rap-1A	IPI00019345	20973.71	6.38	0	1	73
88	Band 4.1-like protein 4A	IPI00030794	69332.01	9.33	0	1	52
89	Hypothetical protein FLJ10903	IPI00102165	16600.10	10.21	0	1	79

Table 3-3. Continued

90	PREDICTED: similar to KIAA0454 protein	IPI00457293	42396.36	8.87	0	1	39		
91	Galectin-3	IPI00465431	27371.69	8.89	0	1	73		
92	Novel protein	IPI00513701	21456.62	8.08	3	1	100		
93	16 kDa protein	IPI00334432	15532.11	8.76	0			3	138
94	Solute carrier family 29 (nucleoside transporters), member 1	IPI00412547	50186.36	8.61	11			2	79
95	Eosinophil cationic protein precursor	IPI00025427	18428.40	10.31	0			1	68
96	Ubiquitin and ribosomal protein L40 precursor	IPI00456429	14718.96	9.87	0			1	63
97	64 kDa protein	IPI00479914	64379.15	9.15	0			1	54
98	Membrane associated progesterone receptor component 2	IPI00005202	23803.73	4.76	2			1	49
99	Cop-coated vesicle membrane protein p24 precursor	IPI00016608	22746.38	5.08	2			1	59
100	Ras-related protein Rap-2b	IPI00018364	20491.21	4.73	0			1	45
101	Monocarboxylate transporter	IPI00024650	53922.86	8.91	11			1	78
102	28 kDa protein	IPI00069985	28213.33	8.52	1			1	52
103	Splice Isoform 2 of Reticulon 4	IPI00298289	40292.95	4.71	1			1	82
104	Lutheran blood group	IPI00328869	67362.59	5.53	1			1	53
105	Epidermal growth factor receptor pathway substrate 15	IPI00385325	83602.76	4.56	0			1	49
106	Aquaporin 1 splice variant 2	IPI00428490	14913.58	5.48	1			1	137

Interpretation. Each of the three methods enabled to identify a large set of proteins as compared with gel-based method, elucidating the higher efficiency of shotgun approach for RBC membrane protein identification.

Our approach identified a large set of proteins (106) and in particular integral membrane proteins (47) from the RBC membrane fraction using three runs of 2-D LC-MS/MS. Earlier, Low et al. (2002) identified 102 proteins including 5 integral membrane proteins using 1-D/2-DE and MALDI-TOF-MS, while a report using the Ion trap MS method reported the identification of 91 unique proteins including 21 integral membrane proteins from the erythrocyte membrane fraction (Kakhniashvili et al., 2004). Compared with these studies on RBC membrane (Kakhniashvili et al., 2004; Low et al., 2002), not only could we identify the major RBC membrane proteins such as Band 3, stomatin, rhesus blood protein, glycophorin C, Kell blood group glycoprotein (Reid et al., 2004), but we also identified relatively lower abundant membrane proteins such as urea transporter (Lucien et al., 2002) and complement receptor (CD35) (Rowe et al., 1997). Moreover, 39 of 47 integral membrane proteins were verified in a recent study (Pasini et al., 2006), confirming the effectiveness of our approach.

3.5.3 Differential recovery of hydrophobic and hydrophilic peptides

Aim. In previous section, I have shown that both unique and common proteins were identified from the three extraction methods. However, whether there is difference in the peptide recovery remains to be determined. If there is a difference, it could be very useful to sequentially use different solvents in order to maximally recover peptides from one particular sample for MS analysis and thus increase total protein identification. Thus in this section, I aimed to compare the hydropathicity of peptides identified from MeOH-, TFE- and urea-based methods.

Investigation. GRAVY values of identified peptides were obtained using the ProtParam program, and statistical significance was evaluated using the ANOVA analysis for comparison of the means of the GRAVY values of peptides recovered from MeOH-, TFE- and urea-based methods using SPSS 13.0.

Results. A GRAVY value indicates the hydrophobicity of proteins and peptides. All identified peptides with their GRAVY values were provided as supporting information in our published paper (Zhang et al., 2007). Mean GRAVY value of the peptides recovered were -0.465 (95% CI, (-0.540)-(-0.390)) in MeOH, -0.219 (95% CI, (-0.304)-(-0.135)) in urea and -0.107 (95% CI, (-0.194)-(-0.020)) in TFE. GRAVY values were significantly higher ($p<0.001$) in the peptide group recovered with urea and TFE as compared with MeOH. This suggested that more hydrophobic peptides were recovered using TFE and urea compared with MeOH in the membrane extraction and digestion process.

Interpretation. The difference between MeOH and TFE in the recovery of hydrophobic peptides could be due to differing conformations in aqueous MeOH and TFE. In MeOH, proteins tended to be denatured (Blonder et al., 2002) and thus more accessible for trypsin digestion. However the hydrophobic peptides which prefer beta-sheet formations in MeOH (Wigley et al., 1998) may be lost due to the structural changes such as intermolecular antiparallel β-sheet aggregates (Dong et al., 1998). In TFE, proteins and peptides are likely to be stabilised, possibly due to the formation of alpha-helical secondary structure (Buck 1998). Thus, this differential ability to recover hydrophilic and hydrophobic peptides from MeOH and TFE respectively indicates that these two organic solvents could be complementary for membrane protein extraction and digestion process, increase overall membrane protein identification.

3.5.4 Analysis of protein digests with longer LC elution gradient

Aim. In section 3.5.2, I have shown that the use of LC-MS/MS to identify proteins from the three digests (from urea, MeOH and TFE), where the LC elution gradients was 10 min. Recently, it has been shown that longer LC elution gradients (e.g. 60 min) has significantly higher resolution, and could greatly increase protein identification from complex digests (Vollmer et al., 2004). In this section, therefore, I attempted to find out the effects of length of LC elution gradients on identification of RBC membrane proteins.

Investigation. Membrane protein digests were prepared using MeOH as a solvent (section 2.2.16.1). The digests were separated using 2-D LC as described in section 2.2.19 except that the LC elution gradient was extended from 10 min to 60 min, and followed by MALDI-TOF/TOF-MS analysis (section 2.2.20).

Results. A total of 141 proteins (including 71 proteins with ≥1 TMD(s)) were identified (Appendix table 1) from the 60 min LC elution gradient. In contrast, only 61 proteins (including 25 proteins with ≥1 TMD(s)) were identified from 10 min elution gradient (section 3.5.2). This indicated that the extension of LC elution gradient helped to increase total identifications of proteins and membrane proteins.

Interpretation. LC plays an important role in shotgun proteomic analysis by separation of complex digests into relatively simple components prior to MS analysis. The higher LC resolution results in simpler analyte, thereby increasing overall protein identifications. The resolution of LC columns can be enhanced by

extending LC elution gradient (Vollmer et al., 2004), leading to an increasing number of protein identifications.

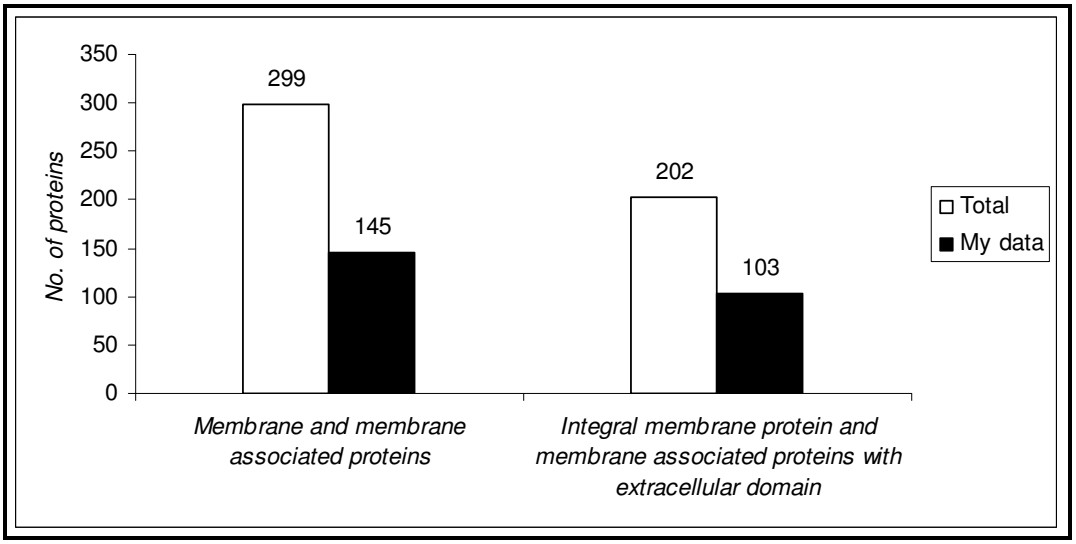
3.6 Comprehensive list of RBC membrane proteins

Adult RBCs have been extensively studied by both conventional methods (e.g. immunochemical study) and proteomic analyses. To obtain a comprehensive list of RBC membrane proteins, an in-depth literature review was made against the PubMed, and all RBC membrane proteins were retrieved and summarised. The list was further consolidated with my own proteomic dataset. In the final list, only proteins annotated as membrane protein by GoFigure were included; and redundant entries were removed regardless of proteins with different name by retrieving and comparing the sequences of all membrane proteins.

In addition, RBC membrane proteins with potential surface domain (e.g. integral membrane proteins, membrane-associated extracellular proteins) were grouped, which was used for future comparison with the counterpart of fetal NRBCs to identify unique surface membrane proteins (Chapter 5).

With these criteria, a total of 299 non-redundant membrane proteins were identified from adult RBCs (Appendix table 2). Of these, 202 proteins contain potential surface domain (Appendix table 3). In my study, nearly half of proteins in both groups were able to be identified from only four LC-MS/MS procedures (Figure 3-9), suggestive of the efficiency of organic solvents-based approaches.

Figure 3-9 Comparison of total proteins and proteins identified from my studies



3.7 Conclusion

With the proteomic studies including gel-based and shotgun proteomics, I have identified a large set of RBC proteins, which includes 145 RBC membrane proteins and 103 proteins with potential extracellular domain. In addition, by doing in-depth literature review against PubMed, a most comprehensive RBC membrane proteome to date have been obtained (Appendix table 2), which includes 299 membrane proteins and 202 proteins with potential extracellular domain.

Meanwhile, data presented in this chapter have also helped us to determine a potentially efficient proteomic strategy for subsequent fetal NRBC membrane proteins analysis. Gel-based proteomic analysis has a very limited role in studying RBC membrane proteome because of the poor solubility of membrane proteins in 2-DE separation and the limited resolving power in 1-DE separation. Shotgun approach is an efficient method as demonstrated by Kakhniashvili et al. (2004) in which they could identify 21 integral membrane proteins from RBC

membrane. More recently, Pasini et al. (2006) used a combination of 1-DE followed by multiple LC separations of eluted peptides prior to MS, and shotgun approach consisting of ≥20 LC-MS/MS experiments to in-depth analyse RBC membrane proteins, and identified 314 proteins. These studies could identify a large set of proteins from RBC membrane by using optimised sample fractionation and/or more LC-MS/MS experiments, but clearly the methods are inefficient and inapplicable in a situation when the starting sample is limited, e.g. fetal NRBCs.

In our shotgun proteomic analysis of RBC membrane proteins, firstly, I showed that two organic solvents (MeOH and TFE) had comparable efficiency in the shotgun analysis of membrane proteins as traditionally used detergent urea when a large amount of sample was used (e.g. 200 μg). In a study by Wang et al. (2005), organic solvents were shown to be much more efficient than traditionally used detergent in the analysis of limited cell lysate because they are readily evaporate, sample clean-up can be avoided and the entire process completed within a test tube, with minimal loss of sample. Together, these indicated that organic solvents (MeOH and TFE) could be more efficient in the analysis of membrane proteins of limited fetal NRBCs. Secondly, organic solvents MeOH and TFE were shown to be complementary for the recovery of both hydrophilic and hydrophobic peptides from RBC membrane proteins, indicating that they can be sequentially used for maximal recovery of peptide for MS analysis. Thirdly, the use of longer (e.g. 60 min) LC elution gradient significantly increased the number of membrane proteins identified from RBC membrane. Taken together, the sequential use of organic solvents MeOH and TFE for membrane proteins extraction and digestion procedures followed by long LC elution gradient (e.g. 60

min) separation prior to MS analysis would significantly increase the number and type of membrane proteins identified from a limited amount of fetal NRBCs.

Thus, through this chapter, I have obtained a most comprehensive RBC membrane proteome, which could allow subsequent comparison with that of fetal NRBCs in order to identify unique surface membrane proteins; I have also determined a potentially efficient proteomic strategy for fetal NRBC membrane analysis.

Chapter 4 Proteomic Analysis of Fetal NRBC Membrane Proteins Using Shotgun Approach

4.1 Introduction

Primitive fetal NRBCs are abundant in the circulation of embryo and fetus in the first trimester pregnancy, and can cross into maternal blood (Choolani et al. 2001). In recent years, extensive attempts have been made to isolate these cells from maternal blood for potentially early non-invasive prenatal diagnosis (section 1.5.6). However, little success was obtained due to their rarity in maternal blood and the lack of antibody highly specific to fetal NRBCs (section 1.6.2). The attempts to search for or to generate antibody specific to fetal NRBCs were hampered as proteins of these cells are poorly understood. To our knowledge, they were only known to contain a few proteins such as cytoskeleton protein 4.2 (Zhu et al., 1998) and spectrin (Boulanger et al., 2002), surface proteins GPA, CD47 and CD71 (Choolani et al., 2003) and cytosolic embryonic globins. Study of fetal NRBC membrane proteome is therefore essential to identify potential biomarkers that could be used to separate fetal NRBCs from adult RBCs.

This chapter is dedicated solely to the proteomic analysis of fetal NRBC membrane proteins which included isolation of these cells from placental tissues, extraction of their membrane proteins, profiling and annotation of identified proteins.

Proteomics is very sensitive and high throughput for the analysis of membrane proteins (Wu et al. 2003). In chapter 3, the efficiencies of various proteomic approaches were compared for the analysis of RBC membrane proteome. A

shotgun approach, that consists of protein extraction using two organic solvents (MeOH and TFE) and protein identification by 2-D LC-MALDI-TOF/TOF-MS, was suggested to be efficient and suitable for the analysis of membrane proteins from a limited amount of sample (section 3.7). This chapter describes the use of this shotgun approach for the analysis of fetal NRBC membrane proteome.

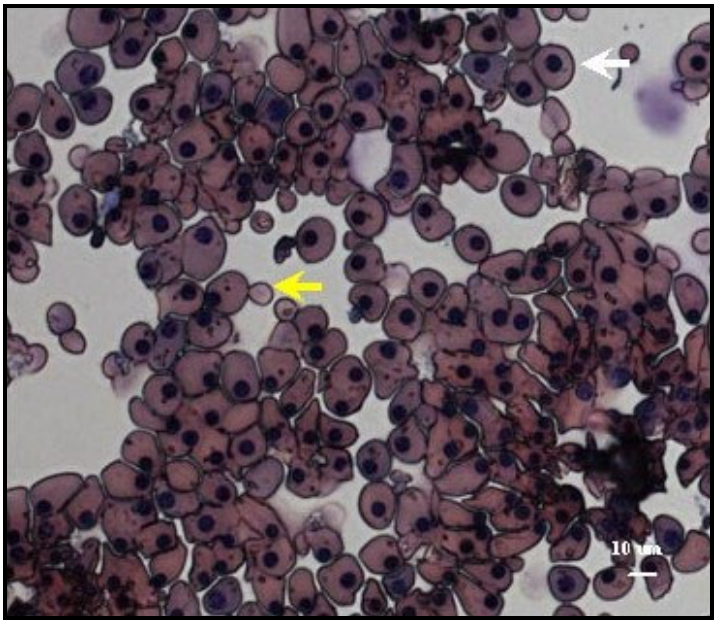
4.2 Recovery of fetal NRBCs from placental tissues

Aim. To isolate fetal NRBCs from placental tissues for the membrane proteome profiling.

Investigation. Placental tissues collected from volunteers undergoing elective termination of pregnancy were used to obtain fetal NRBCs following the protocol described in section 2.2.6. Up to 20 µl of the sample containing 5×10^4 fetal NRBCs were cytospun and Wright's stained (sections 2.2.2 to 2.2.4), and the rest was stored at -80°C for proteome analysis.

Results. Total numbers of fetal NRBCs isolated from one placental tissue sample varied from 1×10^4 to 7×10^6 depending on the gestational age and the expertise of the surgeon. Purity of the preparation varied between 1% and 98%. Samples containing at least 95% of fetal NRBCs were considered satisfactorily pure, and used for membrane protein analysis. Wright stain of a pure fetal NRBC sample is shown in Figure 4-1.

Figure 4-1 Wright stain of fetal NRBC sample from placental tissues of termination of pregnancy
Arrowed were fetal primitive NRBCs (white arrowed) and adult RBCs (yellow arrowed).



Interpretation. As the placental tissues were collected after surgical termination of pregnancy (TOP), the quantity and quality of the tissues were highly depended on the expertise of the surgeons who performed the TOP procedure. Some surgeons could provide a large quantity of placental tissues with little maternal blood contamination, while others provided a small amount of tissues with heavy contamination of maternal blood from one TOP case. As a result, the number and purity of fetal NRBC isolated from those tissues were varied.

The number and purity of target cells are crucial for an effective membrane proteome analysis which requires about 30-200 μg proteins. In our study, the protein yield was 0.55 μg per 10^6 RBCs from a highly pure membrane preparation (section 3.2). Due to sample limitation, protein quantitaion in fetal NRBC membrane preparation was not performed. Assuming that similar protein yield is to be obtained from fetal NRBCs, it is clear that fetal NRBC sample from a single

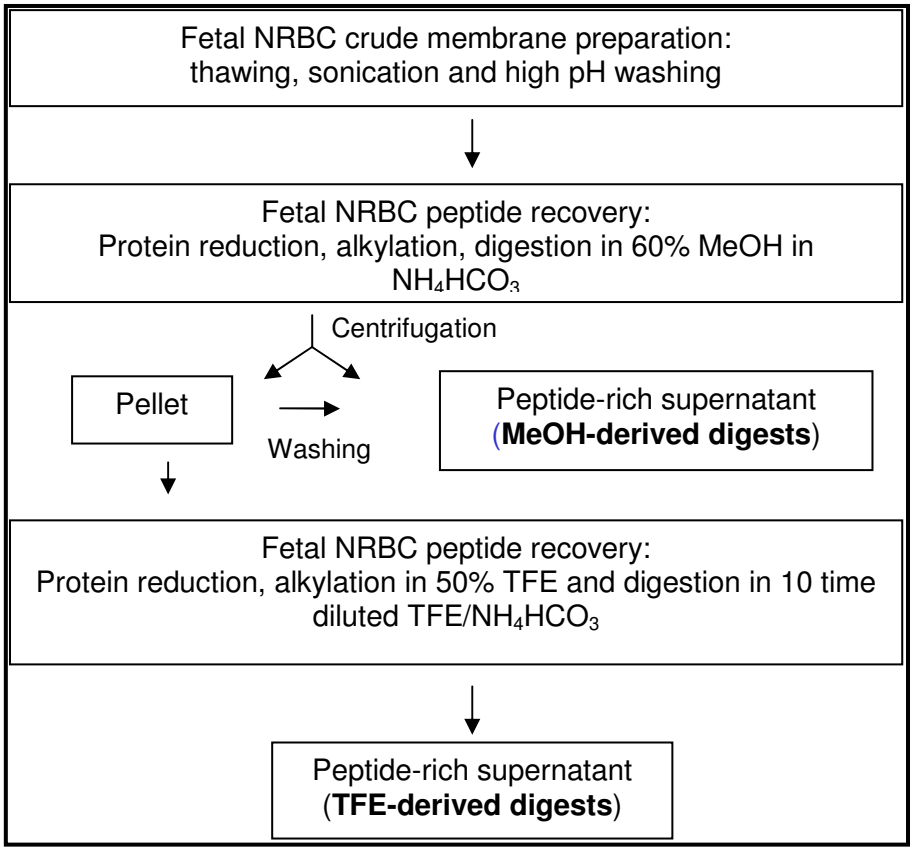
termination of pregnancy sample (usually containing ~10^6 fetal NRBCs) is technically not feasible for the study of fetal NRBC membrane proteome. Therefore, I have pooled samples with a purity >95% to make up to ~5×10^7 fetal NRBCs (estimated protein yield ~30 μg) for subsequent protein analysis.

4.3 Preparation of membrane protein digests for MS analysis

Aim. To prepare fetal NRBC membrane protein digests for 2-D LC-MALDI TOF/TOF-MS analysis.

Investigation. Stages of membrane protein digest preparation were summarised in Figure 4-2. Fetal NRBC membrane was prepared as described in section 2.2.8. MeOH and TFE were used in the membrane protein extraction and digestion procedures in a sequential manner to maximally recover peptides for MS analysis (section 2.2.17).

Figure 4-2 Flow chart showing fetal NRBC membrane protein preparation, extraction and digestion



Results. Two peptide-rich supernatants (digests) from a single fetal NRBC sample were obtained for subsequent 2-D LC-MALDI-TOF/TOF-MS analysis.

Interpretation. As only a limited amount of fetal NRBCs was available, the crucial step was to efficiently extract and recover membrane protein digests from fetal NRBCs for subsequent MS analysis. Organic solvents MeOH and TFE demonstrated an advantage for proteomic analyses: as these organic solvents readily evaporate, sample clean-up could be avoided and the entire process completed within one test tube, with minimal loss of sample during the recovery phase. In addition, these two organic solvents were shown to be complementary for the recovery of both hydrophilic and hydrophobic peptides (section 3.5.3). As such, it was anticipated that the sequential use of the two organic solvents in the

protein extraction and digestion procedures would maximise peptide recovery from the small amount of fetal NRBCs, and eventually increase total protein identifications.

4.4 Mass spectrometric and bioinformatic analyses

4.4.1 Mass spectrometric analysis of fetal NRBC protein digests

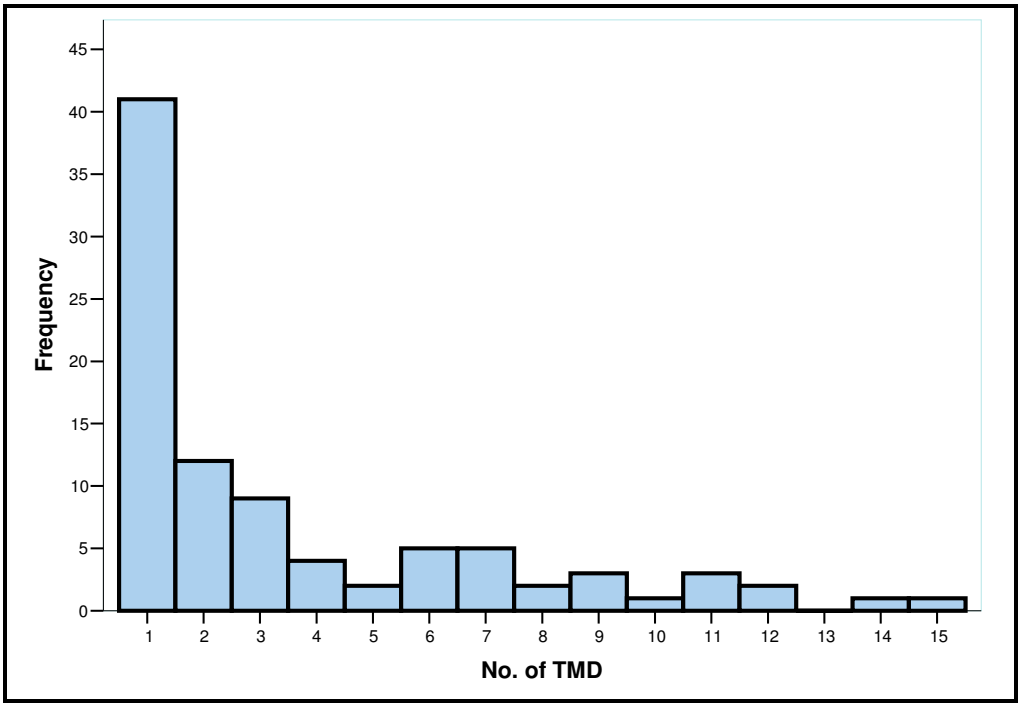
Aim. To identify proteins from fetal NRBC membrane protein digests.

Investigation. Each of the two peptide-rich supernatants (MeOH- and TFE-derived digests) from section 4.3 was lyophilised, re-solubilised into a solution (98% H_2O, 2% ACN and 0.05% TFA) and subsequently analysed using 2-D LC-MALDI-TOF/TOF-MS as described in section 2.2.19-2.2.20. MS data processing and bioinformatic analysis were performed as in section 2.2.21.

Results. A total of 315 proteins were identified (Appendix table 4). The number of proteins identified from MeOH-derived digests and TFE-derived digests were 171 and 226 respectively. Among these, 82 proteins were common for both digests. Ninety-one out of 315 proteins were considered as integral membrane proteins and contained ≥1 TMDs. Most of them contained either one or two TMDs, but as many as 38 proteins with ≥3 TMDs were also identified (Figure 4-3). In addition, 43% of identified proteins (141) were membrane proteins based on Gofigure annotation. Of these, 54 proteins were identified with potential surface domain(s) (Appendix table 5).

In addition to membrane proteins, a large number of membrane-associated proteins were also recovered. These findings could provide additional information to understand biology and/or physiology of human primitive fetal NRBCs. For example, histone H1^{o} has been reported to be expressed predominantly in terminally differentiated cells (Doenecke et al., 1997) such as mouse embryonic erythroblasts (Gjerset et al., 1982), but not in peripheral lymphocytes, granulocytes and mouse definitive erythroblasts from bone borrow (Zlatanova et al., 1994). If similar erythropoiesis occurs in human as in mouse, protein H1^{o} could serve as an important new marker for differentiating human primitive NRBCs from its definitive counterparts (e.g. maternal bone marrow NRBCs), which would be useful for the identification of primitive fetal NRBCs enriched from first trimester maternal blood.

Figure 4-3 Prediction of transmembrane domains (TMDs) of the identified proteins



Interpretation. This was the first study to present a relatively comprehensive fetal NRBC membrane proteome. The sequential use of two organic solvents enabled us to identify a large set of membrane proteins including a weakly expressed fetal NRBC surface antigen (CD71) from a small amount of fetal NRBCs (5×10^7), which clearly demonstrated the strength of our approach for maximal identification of proteins from a small amount of sample as discussed in section 3.7.

4.4.2 Subcellular and functional groups of identified proteins

Aim. To obtain global information of identified proteins in term of subcellular locations and function groups.

Investigation. Subcellular and functional group of all identified proteins were obtained by GoFigure (http://udgenome.ags.udel.edu/gofigure/index.html). Proteins, which were classified as plasma membrane proteins and had transmembrane domain(s), were identified as integral to the surface of NRBC membrane.

Results. The detailed subcellular location of all identified proteins was shown in Figure 4-4a. Forty-one proteins (13%) were classified as plasma membrane proteins, which included integral plasma membrane protein, extracellular membrane associated proteins and peripheral membrane proteins. A large number of proteins were assigned to ribosome (49), nucleus (64) and mitochondria (72), which accounted for 58% of total identifications. This was because that in our preparation, total membranes were recovered by sonication, high pH washing and ultracentrifugation, in which some of the organelles are not disrupted or resealed during the process and therefore the highly abundant proteins within the organelles, e.g. 40S, various histones, were not efficiently

depleted. The rest identified proteins included microsomal proteins (45), cytosolic proteins (21) and unclassified proteins (23).

The functional categories of identified proteins were shown inFigure 4-4b. The largest group consisted of proteins with binding function (27%). Many of them are involved in RNA and DNA binding, corresponding to abundant nuclear and mitochondrial proteins recovered. Other proteins have catalytic activity (23%), structural molecular activity (19%) and transporter activity (19%). These abundant proteins in fetal NRBCs, undoubtedly, play a vital role in the maintenance of their normal structure and function, as the erythroid cells required an active metabolism such as glycolysis, structurally extreme deformability and high transport activity for gases, water, ions and amino acids.

Interpretation. As total membranes from fetal NRBCs were prepared and analysed, the identified protein dataset represents a global recovery of membrane proteins and reveals their relative functional preponderance in the fetal NRBC membrane proteome.

Figure 4-4 Subcellular classifications (a) and functional categories (b) of fetal NRBC proteins identified from membrane preparations

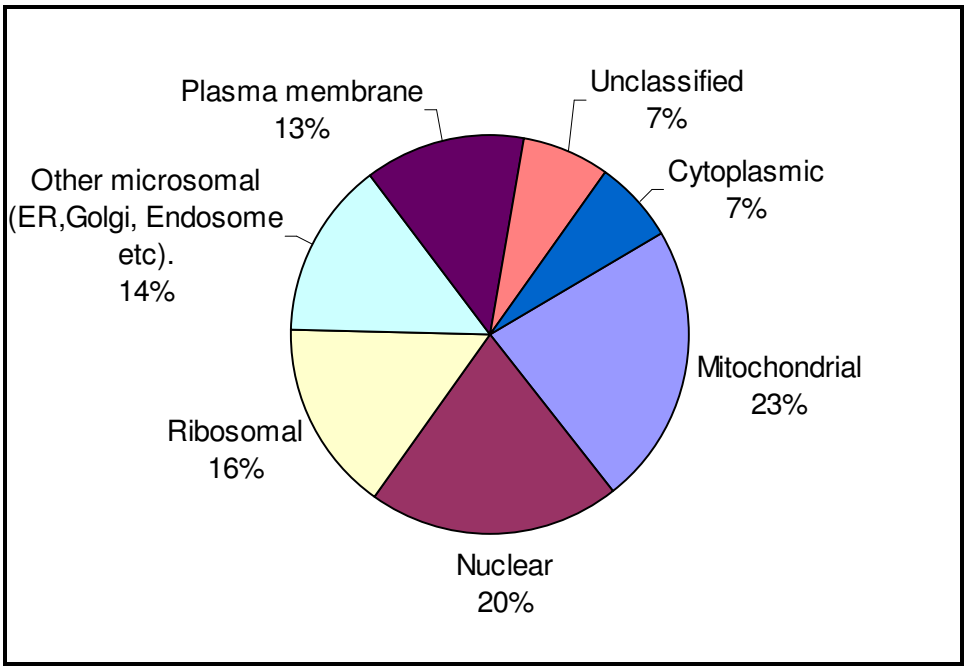


Figure 4-4a

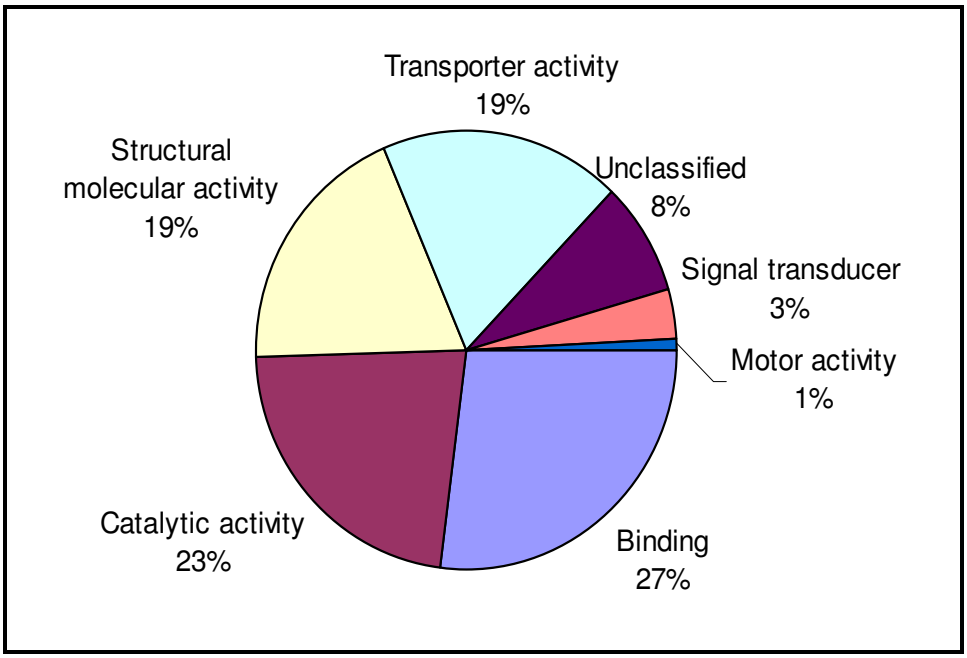


Figure 4-4b

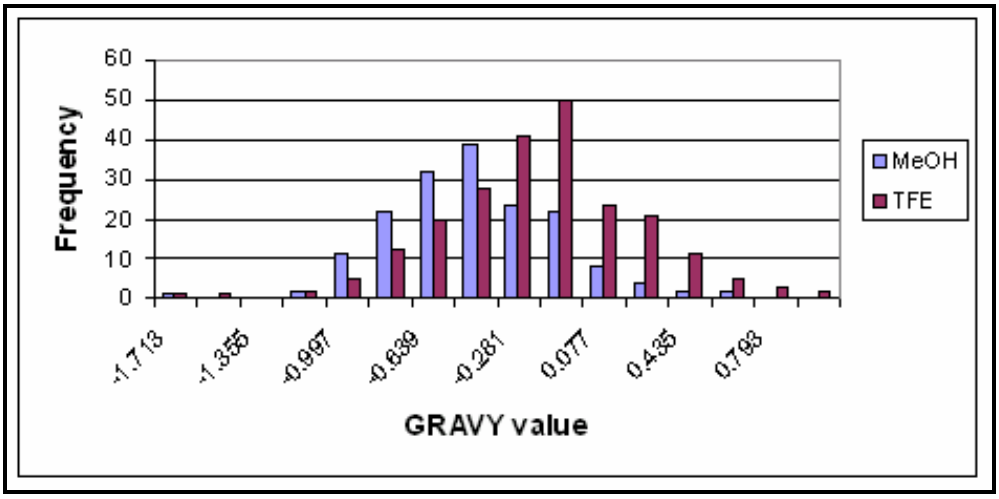
4.4.3 Hydropathy analysis of identified peptides and proteins

Aim. Fetal NRBC membrane protein digests were prepared using a sequential extraction and digestion method by two solvents (MeOH and TFE) (section 4.3) and thus two digests were obtained. Subsequent MS analyses of the two digests revealed a large number of proteins unique to MeOH- and TFE-derived digest respectively (section 4.4.1). In this section, the hydrophobicity of peptides and proteins identified from the digests were compared in order to assess the differential recovery of proteins and peptides from the two organic solvents.

Investigation. GRAVY values of identified peptides and proteins were obtained using the ProtParam program, and statistical significance was evaluated using the ANOVA analysis for comparison of the means of the GRAVY values of peptides and proteins recovered from MeOH- and TFE-based methods using SPSS 13.0.

Results. Analysis of GRAVY values of identified peptides and proteins derived from MeOH and TFE extractions of fetal NRBC membranes showed that mean GRAVY values of both, the recovered peptide groups (MeOH, mean -0.230 and 95% CI, -0.301-(-0.152); TFE, mean 0.114 and 95% CI, 0.043-0.185) and the recovered protein groups (MeOH, mean -0.467 and 95% CI, -0.532-(-0.402); TFE, mean -0.284 and 95% CI, -0.340-0.228) were significantly different ($p<0.001$) (Figure 4-5).

Figure 4-5 Hydropathy comparison of the identified proteins from MeOH and TFE sequential extraction and digestion of fetal NRBC membrane
Mean of GRAVY values of identified proteins were significant lower in MeOH-based method (-0.467) as compared to TFE-based method (-0.284) ($p<0.001$).



Interpretation. The significant difference ($p<0.001$) of mean GRAVY values in both recovered peptide and protein groups from MeOH- and TFE-based methods respectively confirmed our earlier observations: the differential recovery of hydrophobic and hydrophilic peptides of organic solvents MeOH and TFE (section 3.5.3), and as a result, the sequential use of the two solvents maximised the protein identification from a limited amount of fetal NRBCs.

4.5 Conclusion

Use of the proteomic strategy enabled us to present the first relatively comprehensive fetal NRBC membrane proteome (315 proteins), which included 141 membrane proteins, 91 integral membrane proteins, and 54 proteins with potential extracellular domain. In addition, this study also presented a large number of membrane-associated proteins, and some of which were found in erythroid cells for the first time, e.g. azurocidin. These findings would not only provide us a first relatively comprehensive fetal NRBC membrane protein list for the identification of potential biomarkers by comparing with adult RBC membrane

proteome, but also provide values to understand the biology and/or physiology of human primitive fetal NRBCs.

Chapter 5 Identification of Unique Surface Protein(s) of Primitive Fetal NRBCs by Comparing Fetal NRBC and Adult RBC Membrane Proteomes

5.1 Introduction

The primary aim of this thesis was to identify membrane proteins unique to either fetal NRBCs or adult RBCs, which could be potentially used for the immunoseparation of fetal NRBCs from adult RBCs, for non-invasive prenatal diagnosis. Earlier efforts to investigate known antigenic differences between these two cells types did not yield any differences that could be exploited (Choolani et al., 2003). In this thesis, I used a proteomic strategy to profile membrane proteomes of adult RBCs (Chapter 3) and fetal NRBCs (Chapter 4) and obtained the two most comprehensive membrane proteomes to date. I also investigated their subcellular locations. It would therefore be logical to compare the membrane proteins, specifically those with potential surface domain, in order to identify unique surface membrane proteins. Subsequently, the identified unique proteins in fetal NRBCs were validated using RT-PCR and immunocytochemistry, and functionally annotated.

5.2 Identification of unique membrane proteins

Aim. To identify unique surface proteins associated with fetal NRBCs or adult RBCs.

Investigation. Membrane proteins with potential surface domain from fetal NRBCs (Appendix table 3) and adult RBCs (Appendix table 5) were compared to identify unique surface membrane proteins.

Results. The comparison of these two groups of proteins revealed that these two cell types were highly similar in their plasma membrane proteins. The major RBC membrane proteins, which could be identified in the earler studies using MS technology with relatively low accuracy and low resolution (Kakhniashvili et al., 2004; Low et al., 2002), were also found in fetal NRBCs. These include Band 3, stomatin (Band 7), glycophorin C, rhesus blood protein, Kell blood group glycoprotein, aquaporin, calcium-transporting ATPase, sodium/potassium-transporting ATPase, solute carrier family 2, equilibrative nucleoside transporter and ATP-binding cassette half-transporter.

Moreover, the comparison resulted in the identification of 23 unique fetal NRBC membrane proteins and 187 unique RBC membrane proteins. Of the 23 fetal NRBC proteins, 10 proteins were predicted on plasma membrane, 5 were on plasma membrane/organelle and 8 proteins had unknown location but with predicted transmembrane domains (Table 5-1).

Interpretation. The number of identified membrane proteins with potential surface domain (54) in fetal NRBCs was much smaller than that of RBCs (202). This was probably because only a small amount of fetal NRBCs (5×10^7) were available for this proteomic analysis, leading to an under-represented membrane proteome. In contrast, RBCs are easily accessible and have been extensively studied, thereby leading to a much more comprehensive membrane proteome.

The common expression of major membrane proteins by fetal NRBCs and adult RBCs might elucidate that there was little success achieved in the attempt to generate antibody specific to fetal NRBCs or adult RBCs using a hybridoma technology (Alvarez et al., 1999), as only those antibodies against immunodominant epitopes tended to be generated with this approach (Groves et al., 2000).

In this study, unique fetal NRBC membrane proteins were further validated and functionally annotated in on-going paragraphs (sections 5.3 and 5.4), whereas “unique RBC membrane proteins” were used only as additional information for antibody selection in the immunocytochemical screening study (Chapter 6), as they were less reliable as “unique RBC proteins”: some of these proteins might be also expressed on fetal NRBCs but failed to be identified from the proteomic analysis due to a limited amount of sample available.

Table 5-1 Potential surface markers expressed on fetal NRBCs

No.	Accession Number	Protein Name	Subcellular location
1	IPI00022462	Transferrin receptor protein 1	Plasma membrane
2	IPI00554481	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2, isoform A	Plasma membrane
3	IPI00418660	Solute carrier family 22 member 11, isoform 2	Plasma membrane
4	IPI00034208	BCG induced integral membrane protein BIGM103	Plasma membrane
5	IPI00607576	Splice Isoform 1 of Protein C9orf5	Plasma membrane
6	IPI00168981	Olfactory receptor 11H4	Plasma membrane
7	IPI00396411	Cleft lip and palate transmembrane protein 1	Plasma membrane
8	IPI00382815	Splice Isoform 2 of Protein GPR107 precursor	Plasma membrane
9	IPI00019472	Neutral amino acid transporter B	Plasma membrane
10	IPI00305252	Splice Isoform B of Chloride channel protein 6	Plasma membrane
11	IPI00022246	Azurocidin precursor	Plasma membrane /Extracellular/Cytoplasmic
12	IPI00027180	CAAX prenyl protease 1 homolog	Plasma/ER/Golgi membrane
13	IPI00335277	Splice Isoform 2 of Synaptophysin-like protein	Plasma/Vesicle membrane
14	IPI00292532	Antibacterial protein FALL-39 precursor	Plasma membrane /Extracellular
15	IPI00553138	Vesicle-associated membrane protein 2	Plasma/Vesicle/Synapse /Mitochondrial membrane
16	IPI00166079	Vitamin K epoxide reductase complex subunit 1-like protein 1	Unclassified
17	IPI00604615	ALEX3 protein variant	Unclassified
18	IPI00031064	Hypothetical protein DKFZp586C1924	Unclassified
19	IPI00639803	8 kDa protein	Unclassified
20	IPI00646289	25 kDa protein	Unclassified
21	IPI00176708	Hypothetical protein MGC14288	Unclassified
22	IPI00394779	Splice Isoform 1 of Protein C20orf22	Unclassified
23	IPI00295621	Hypothetical protein DKFZp564K247	Unclassified

5.3 Validation of unique fetal NRBC membrane proteins

While proteomic approaches are very powerful to discover potential markers, the identified markers usually have to be validated using conventional methods before they can be considered for further use. Commonly used validation methods are antibody-based, such as enzyme-linked immunosorbent assay (ELISA), western blotting and immunocytochemistry. ELISA and western blotting

analysis require a relatively large amount of fetal NRBC sample, whereas immunocytochemistry does not. In this study, immunocytochemistry was chosen due to the sample limitation. For some proteins, especially those to which no commercial antibody was available, RT-PCR was chosen to provide additional evidence of the gene expression.

5.3.1 RT-PCR

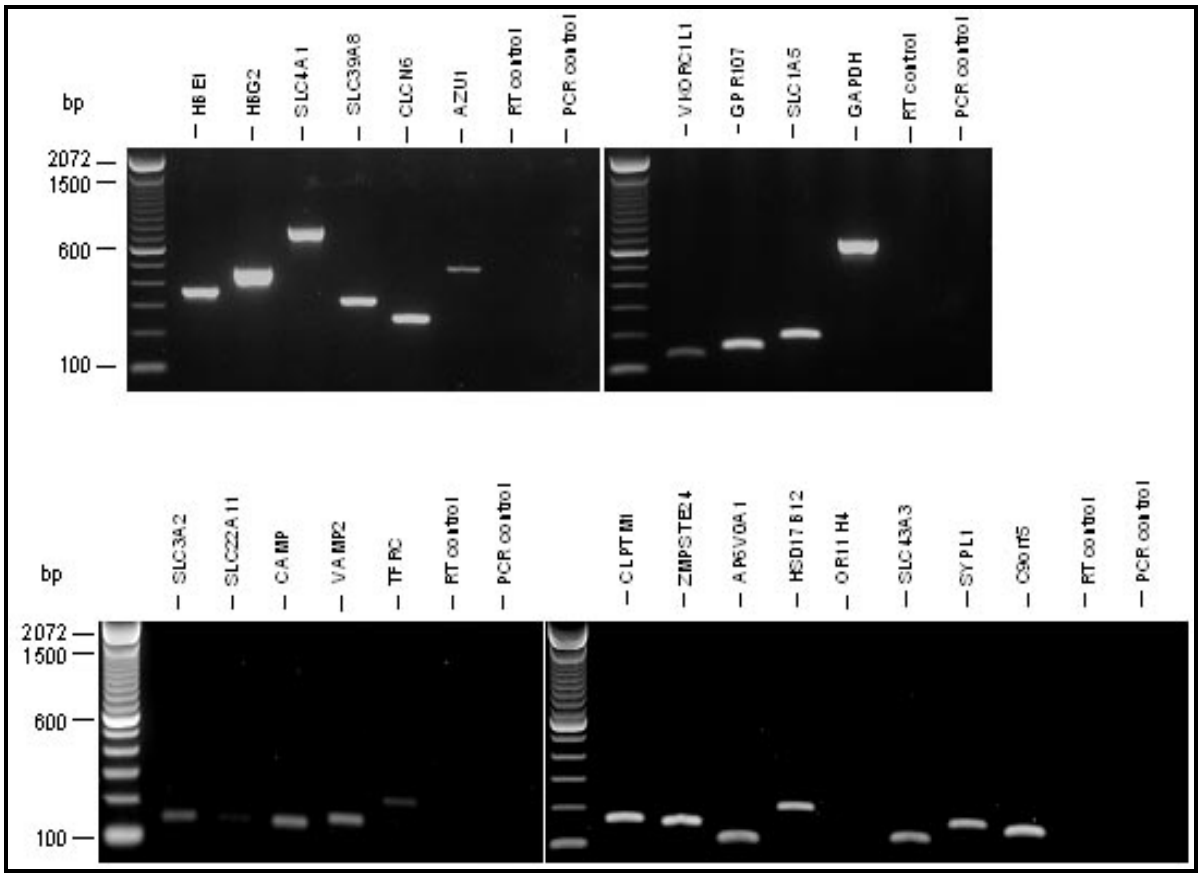
Aim. To gain additional information about the gene expression of some identified proteins including those to which no commercial antibody was available.

Investigation. The RNA was isolated from fetal NRBCs with an RNeasy Mini Kit, and cDNA template was then synthesised using Sensiscript RT Kit and Oligo dT as primers (section 2.2.22). PCR was performed with a PTC-200 Peltier Thermal Cycler. Optimal annealing temperatures were determined for each gene. Primer pairs used for the amplification for individual gene were listed in Table 2-1. The gene expressions of 23 selected proteins were tested, which includes 11 unique fetal NRBC membrane proteins to which no commercial antibody was available as yet.

Results. Of the 23 selected proteins, the mRNA expression of all targets except olfactory receptor 11H4 was detected (Figure 5-1). Of these, 4 proteins were well-known erythroid proteins (epsilon-globin, gamma-globin, Band 3, GAPDH); 15 proteins were potentially unique fetal NRBC membrane proteins (BCG induced integral membrane protein BIGM103, chloride channel protein 6, azurocidin precursor, vitamin K epoxide reductase complex subunit 1-like protein 1, protein GPR107 precursor, neutral amino acid transporter B, solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2, isoform A,

solute carrier family 22 member 11, isoform 2, antibacterial protein FALL-39 precursor, vesicle-associated membrane protein 2, transferrin receptor protein 1, cleft lip and palate transmembrane protein 1, CAAX prenyl protease 1 homolog, synaptophysin-like protein and protein C9orf5); and the other three proteins (solute carrier family 43 member 3, vacuolar proton translocating ATPase 116 kDa subunit a isoform 1, steroid dehydrogenase homolog) were common but newly identified from both fetal NRBCs and adult RBCs.

Figure 5-1 RT-PCR of the gene expression of twenty-three selected proteins
The presence of all gene expressions except olfactory receptor 11H4 was detected. The proteins were epsilon-globin, gamma-globin, Band 3, BCG induced integral membrane protein BIGM103, chloride channel protein 6, azurocidin precursor, vitamin K epoxide reductase complex subunit 1-like protein 1, protein GPR107 precursor, neutral amino acid transporter B, GAPDH, solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2, isoform A, solute carrier family 22 member 11, isoform 2, antibacterial protein FALL-39 precursor, vesicle-associated membrane protein 2, transferrin receptor protein 1, cleft lip and palate transmembrane protein 1, CAAX prenyl protease 1 homolog, vacuolar proton translocating ATPase 116 kDa subunit a isoform 1, steroid dehydrogenase homolog, olfactory receptor 11H4, solute carrier family 43 member 3, synaptophysin-like protein and protein C9orf5. RT control: no RT enzyme was added during cDNA synthesis; PCR control: water rather than cDNA was added for PCR amplification.



Interpretation. As there was no commercially available antibody to some identified proteins and the generation of a new antibody required a relatively long period, the validation of these proteins therefore presented a challenge. In this study, use of RT-PCR to detect the mRNA expression provided indirect proof of the expression of the proteins.

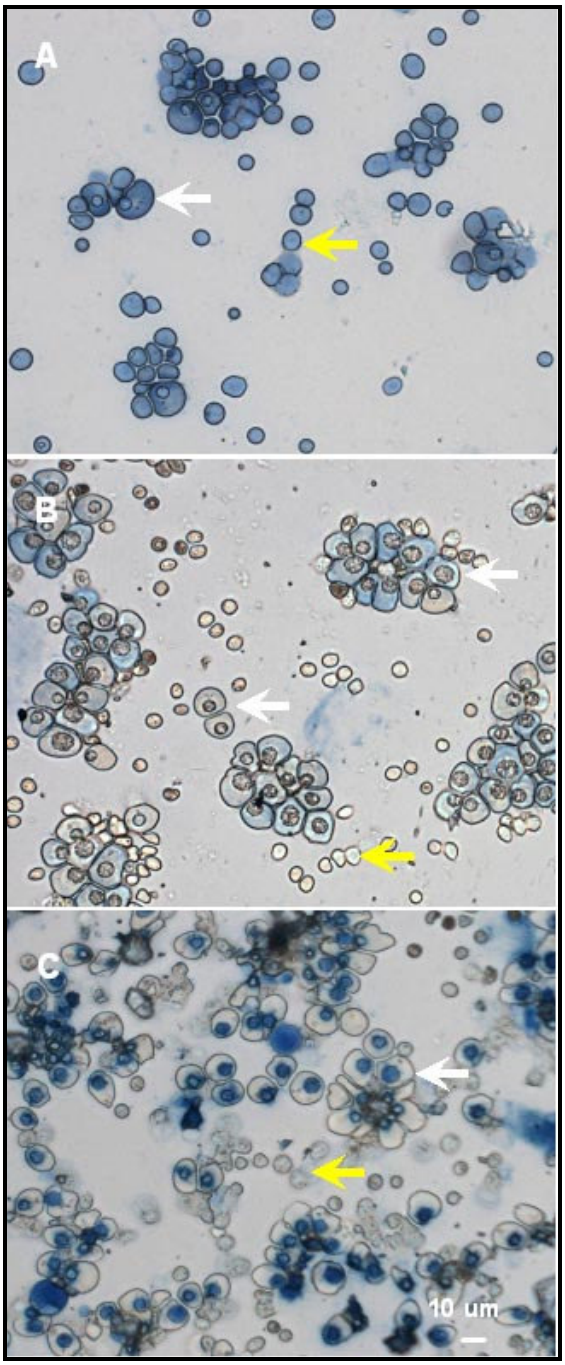
5.3.2 Immunocytochemistry

Aim. To immunocytochemically detect the protein expression of four unique fetal NRBC membrane proteins to which antibodies were commercially available.

Investigation. Primary antibodies including anti-CD71, anti-azurocidin, anti-CLC6 and anti-neutral amino acid transporter ASCT2 and anti-Band 3 were used for immunocytochemical stain of the protein expressions in fetal NRBCs and adult RBCs as described in section 2.2.23.

Results. The immunocytochemical staining on fetal NRBCs and adult RBCs were shown in Figure 5-2. CD71 expression was found to be weak/undetectable on primitive fetal NRBCs but absent on adult RBCs. While azurocidin was expressed strongly on all fetal NRBCs, it was located within nucleus and undetectable on the surface. Immunocytochemistry using anti-chloride channel protein 6 and anti-neutral amino acid transporter showed no detectable staining on both fetal NRBCs and adult RBCs, which could be due to either the low expression of targeted antigens or lack of immunoreaction due to imcompatible binding of the antibodies for the immunocytochemistry.

Figure 5-2 Immunocytochemical staining on fetal NRBCs and adult RBCs
(A) Band 3 is strongly expressed on fetal NRBCs (white arrowed) and RBC (yellow arrowed), (B) CD71 is expressed on some of fetal NRBCs but absent on RBC, (C) Azurocidin was found to be strongly expressed on nucleus of fetal NRBCs.



Interpretation. It has been reported that CD71 expression was strong in cord NRBCs, adult NRBCs and in second trimester NRBCs (Zheng et al., 1999) but weak/undetectable in first trimester fetal NRBCs (primitive fetal NRBCs)

(Choolani et al., 2003). Our data provided the first independent evidence to confirm the early observation (Choolani et al, 2003). Azurocidin, also termed heparin-binding protein or CAP37, was predominantly expressed in neutrophil granule, and with a minor part (~8%) on its plasma membrane (Tapper et al., 2002). The expression of this protein in erythroid cells has never been described. Thus, the finding of this protein in fetal NRBCs would help to understand the biological functions of this cell type. Azurocidin has a role in white blood cell apoptosis upon heparin induction (Erduran et al., 1999; Manaster et al., 1996) and a similar possibility is expected in fetal NRBCs. Indeed, it has been reported that in the enrichment of fetal NRBCs from maternal blood, the fetal NRBC detection rate was much lower when maternal blood was collected in tube with heparin anticoagulant than that collected in tube with EDTA anticoagulant (Brombacher et al., 2000), which could be presumably due to the cell loss caused by heparin induced fetal NRBC apoptosis.

5.4 Functional annotation of unique fetal NRBC membrane proteins

Many of the unique fetal NRBC proteins were annotated as molecule and ion transporters. CD71, which is responsible for cellular iron uptake, is essential for definitive erythropoiesis and completely lost during reticulocyte maturation (Knowles et al., 1997). However, it was found that the knock-out of CD71 gene only caused partially impaired primitive erythropoiesis (Levy et al., 1999). Together with our observation, it may suggest that some of primitive fetal NRBCs ($CD71^-$ NRBCs) have alternative ion uptake pathway. BCG induced integral membrane protein BIGM103 is responsible for zinc uptake. Zinc is an essential cofactor for hundreds of enzymes, and involved in protein, nucleic acid, carbohydrate, and lipid metabolism, as well as in the control of gene transcription,

growth, development, and differentiation (Taylor et al., 2003b). The identification of zinc transporter might reflect the high enzyme activity required in at least some fetal NRBCs for their continuing remodelling in the circulation such as enucleation process (Kingsley et al., 2004). Both Solute carrier family 1 and 3 (SLC1, 3) are sodium-dependent amino acid transport. SLC1A5 (Neutral amino acid transporter B) has broad substrate specificity, a preference for zwitterionic amino acids. It also acts as a cell surface receptor for feline endogenous virus RD114, baboon M7 endogenous virus and type D simian retroviruses (Rasko et al., 1999). SLC3A2 is the activator of dibasic and neutral amino acid transport and may have a role in cell differentiation (Lumadue et al., 1987). Erythroblast amino acid transporter contributes to cell viability by providing precursors for intracellular biosynthesis (Young et al., 1983) and provide an efflux route for amino acids produced during reticulocyte maturation (Tucker et al., 1980). Solute carrier family 22 member 11, also called organic anion transporter 4, mediates saturable uptake of estrone sulfate, dehydroepiandrosterone sulfate and related compounds (Cha et al., 2000). Chloride channel protein 6 is voltage-gated ion channel which has several functions including the regulation of cell volume; membrane potential stabilisation, signal transduction and trans-epithelial transport (Schwartz et al., 1997). As some of fetal NRBCs may not be a terminally differentiated cell type (Kingsley et al., 2004), thereby transcriptionally and translationally active at this stage, it was not surprised that many unique molecule and ion transporters were identified.

Two G-protein-coupled receptors (GPCR) were uniquely identified in fetal NRBCs. Olfactory receptors are expressed in sensory neurons of nasal epithelium, where they interact with odorant molecules to initiate a neuronal response that triggers the perception of a smell (Buck et al., 1991). In erythroid cells, most olfactory receptor genes were inactive (Bulger et al., 2000), but the

expression of an olfactory receptor gene at all stages of erythroid development was also described (Feingold et al., 1999). Protein GPR107 belongs to the LU7TM family (Lung seven transmembrane receptor), and has putative functions as calcium-ion binding, signal transducer, and phospholipase A2 activity.

The proteins involved in protein synthesis, modification and transportation have also been identified in fetal NRBCs. CAAX prenyl protease 1 homolog is responsible for the removal of the c-terminal three residues of farnesylated proteins. Defects in this protein are the cause of mandibuloacral dysplasia with type B lipodystrophy (MADB) (Simha et al., 2003). Synaptophysin-like protein 1 and Vesicle-associated membrane protein 2 are involved in transporting proteins or vesicle to the target locations such as plasma membrane (Johnston et al., 1989; Leube et al., 1987; Sudhof et al., 1989).

An interesting finding was that an antimicrobial protein azurocidin, together with its two neutral serine protease family members: elastase and cathepsin G, was identified in fetal NRBCs. Azurocidin binds heparin and functions to kill ingested bacteria and to regulate various physiological and pathological processes, such as inflammation (Campanelli et al., 1990; Pereira et al., 1990). The finding of azurocidin in fetal NRBCs might confer this cell type a similar role of anti-bacteria during the early fetal development.

Molecular functions of the rest 10 unique proteins are unknown. Some of them were annotated as plasma membrane proteins and others with unknown location. All these proteins contained at least one TMD and could be potential targets.

5.5 Conclusion

The comparison of the two membrane proteomes revealed both similarity and difference in the surface membrane protein expression between fetal NRBCs and adult RBCs. The major RBC surface proteins were found to be expressed in fetal NRBCs (section 5.2), and similar expression level was observed for at least the tested protein, Band 3 (section 5.3.2). Moreover, the comparison yielded 23 unique fetal NRBC surface proteins and 187 unique adult RBC surface proteins. While the unique RBC proteins were not further explored as discussed in section 5.2, the unique fetal NRBC proteins were validated using RT-PCR or immunocytochemistry, and functionally annotated. These unique fetal NRBC proteins are potential targets that could be used for the isolation of fetal NRBCs from maternal blood. In addition, these proteins have been linked to many novel functions that were not described with adult RBCs, and thus their identifications could help us to understand the biology and/or physiology of fetal NRBCs.

Chapter 6 Immunocytochemical Screening of Surface Membrane Proteins on Fetal NRBCs and Adult RBCs and Sorting of These Cells Based on Potential Candidate Identified

6.1 Introduction

Immunocytochemical screening is an alternative and complementary approach to proteomics, and can be used to systematically characterise the surface membrane protein expression. In this approach, commercially available antibodies against cell surface antigens were tested on their reactivity to fetal NRBCs and adult RBCs for the identification of potential markers. The advantages of this approach include: (1) it requires only a small amount of sample (a few cells) and is technically simple, (2) it can be used for semi-quantitative detection of differential protein expression, (3) it can detect some proteins (e.g. glycosylated proteins) that were probably missed out in proteomic analysis, and (4) the identified marker could be directly used to isolate fetal NRBCs from maternal blood by various immunoseparation techniques.

Earlier, there were some attempts to search for a more specific antigen to fetal NRBCs (section 1.6.2), but none of them systematically compared the surface antigen expressions of primitive fetal NRBCs and adult RBCs. In this study, unique antigen expression on fetal NRBCs or adult RBCs, and differential antigen expression between fetal NRBCs and adult RBCs were explored. I tested 31 commercially available antibodies that belonged to three categories: (1) antibodies that are known to target antigens on adult RBCs, (2) antibodies that potentially bind to surface antigens on NRBCs and (3) antibodies that are known

not to react with surface antigens on adult RBCs but known to react with some other blood cells and their progenitors. In particular, my emphasis was to consider well-characterised cluster of differentiation (CD) antigens, as the expression of these antigens has been linked to cellular differentiation and changes of physiological conditions.

6.2 Immunocytochemical screening of surface antigens on fetal NRBCs and adult RBCs

Aim. To identify the differential surface protein expression that could be potentially used to isolate fetal NRBCs from maternal blood.

Investigation. Antibody selection was based on the criteria described in section 6.1. Primary antibodies (all host in mouse except otherwise stated) against following human antigens were tested on fetal NRBCs and adult RBCs using immunocytochemistry (section 2.2.23): CD14, CD29, CD31, CD34, CD35, CD36, CD44, CD45, CD45RB, CD46, CD47, CD55, CD59, CD81, CD90, CD99, CD100, CD105, CD108, CD117 (host in rabbit), CD133, CD147, CD164, CD175s, CD222, CD233, CD235a, HLA-ABC, HLA-DR, E-Cadherin and Monocarboxylate transporter 1 (MCT1, host in rabbit). The image intensities of differential expression were evaluated as described in section 2.2.24.

Results. A total of 31 antibodies were tested regarding their reactivity to fetal NRBCs and adult RBCs. Of these targeting antigens, majority of them were expressed similarly on both fetal NRBCs and adult RBCs, but differential expression of three antigens were also identified (Table 6-1).

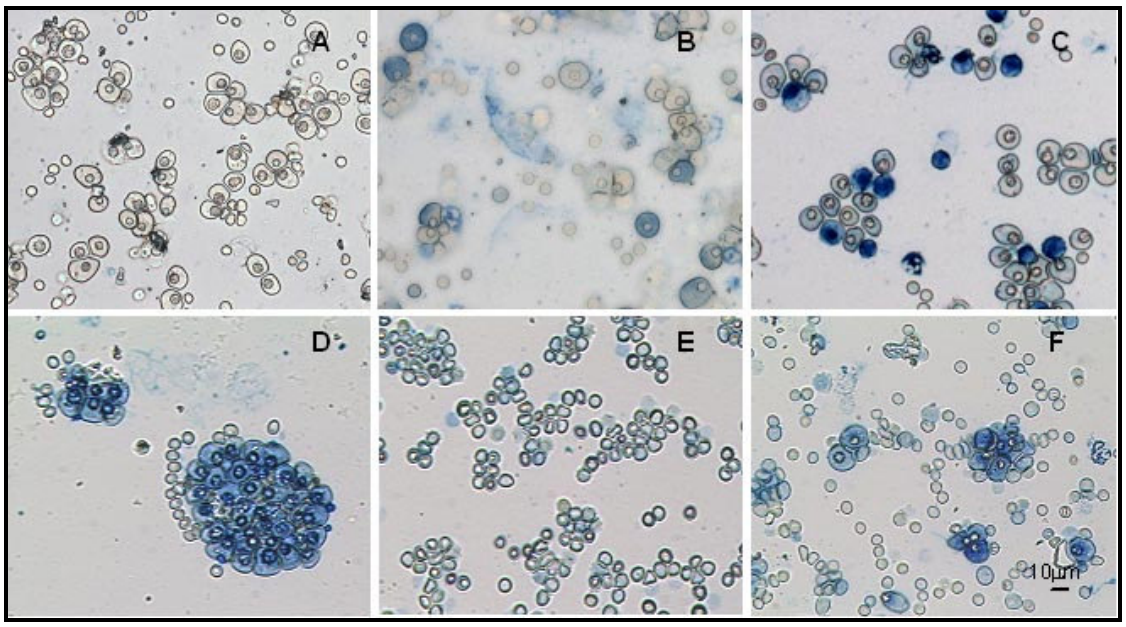
Table 6-1 Panel of antibodies tested and results for fetal NRBCs and adult RBCs

No.	Target antigen	Binding to fetal NRBCs	Binding to adult RBCs
1	CD14	-	-
2	CD29	-	-
3	CD31	-	-
4	CD34	-	-
5	CD35	-	-
6	CD36	-	-
7	CD44	+	+
8	CD45	-	-
9	CD45RB	-	-
10	CD46	-	-
11	CD47	++	++
12	CD55	+	+
13	CD59	+	+
14	CD81	-	-
15	CD90	-	-
16	CD99	-	-
17	CD100	-	-
18	CD105	-	-
19	CD108	-	-
20	CD117	-	-
21	CD133	-	-
22	**CD147**	**++**	**+**
23	**CD164**	**±**	**-**
24	CD175s	-	-
25	CD222	-	-
26	CD233	++	++
27	CD235a	++	++
28	E-cadherin	-	-
29	HLA-ABC	-	-
30	HLA-DR	-	-
31	**MCT1**	**±**	**-**

* Mean pixel intensity of negative control in immunocytochemical staining was 187.3 AU, range 185.2-189.2, n=5; strong expression (++) was defined based on the mean pixel intensity ≤110 AU.

CD164 and MCT1 were expressed on 30-50% of fetal NRBCs but completely undetectable on adult RBCs. CD147 was found to be expressed strongly on fetal NRBCs and weakly/undetectably on adult RBCs [mean pixel intensity 95.3 AU; range, 81.1-113.7; n=5; and 168.4 AU; range 156.2-176.6; n=5 respectively] (Figure 6-1), suggesting its potential use for improving current enrichment of fetal NRBCs from maternal blood.

Figure 6-1 Differential expression of surface antigens between fetal NRBCs and adult RBCs
(A) Internal control, (B) MCT1 staining: fetal NRBCs show MCT1+ or MCT1- whereas adult RBCs show MCT1-, (C) CD164 staining: fetal NRBCs show CD164+ or CD164- whereas adult RBCs show CD164-, (D-F) CD147 staining: fetal NRBCs show strong staining (CD147+) whereas adult RBCs show weak or undetectable staining.



Interpretation. This study attempted to find the differential expression of surface antigens between first trimester primitive fetal NRBCs and adult RBCs, which could potentially be explored to improve current fetal NRBC enrichment protocols. Thirty-one antibodies were tested. Most of the target antigens were expressed in similar patterns on both fetal NRBCs and adult RBCs. CD35 and CD108 were known to express on adult RBCs (Reid et al., 2004) but were not detected by our

immunocytochemistry, which could be due to the low expression of these antigens beyond the detectable limit (≤1000 molecules per cell) of light microscopy (Barclay 1998). Indeed, CD35 was expressed with only 100 to 800 molecules per RBC (Rowe et al., 1997). The copy number of CD108 (carrying JMH blood group antigen) per RBC has not been determined yet, probably due to the low copy number.

While some of erythroid progenitor antigens such as E-cadherin (Armeanu et al., 2000) and HLA-ABC (Robinson et al., 1981) were found to be absent on both of fetal NRBCs and adult RBCs, others including CD71 (Chapter 5) and CD164 (Watt et al., 2000; Zannettino et al., 1998) were found to be weakly expressed on some of fetal NRBCs but with undetectable stain on adult RBCs.

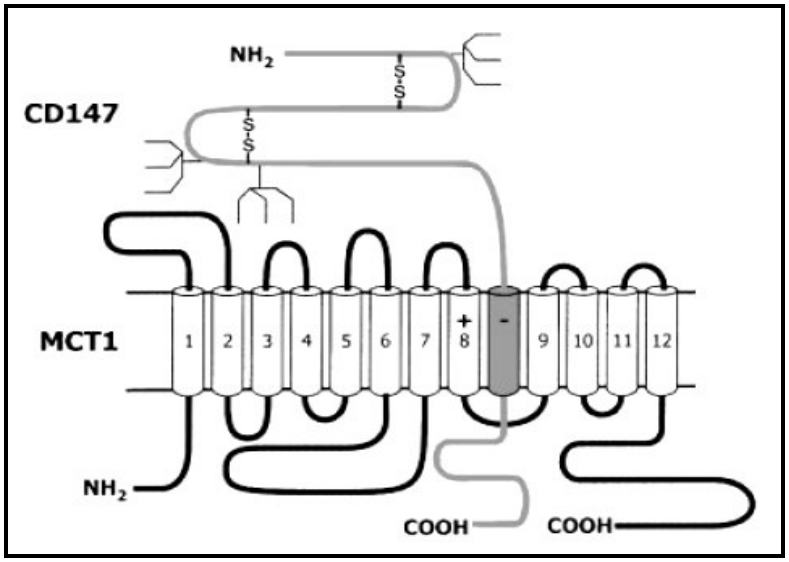
MCT1 belongs to monocarboxylate transporter family which consists of at least eight members. MCT1 is the only form in human erythrocytes and mainly facilitates the transport of lactate across cell membrane. Under physiologic conditions, the MCT1 was believed to mediate 90% of lactate transport in RBCs (Deuticke et al., 1982). The inability to detect this antigen in adult RBCs may be due to a low level of expression of MCT1. In fetal NRBCs, the MCT1 was found to be detectable in 30-50% of fetal NRBCs, indicating a possible more active glycolysis in these cells. Together, the expression of MCT1, CD71 and CD164 in some of fetal NRBCs might suggest the presence of two distinct types of primitive fetal NRBCs.

CD147 is expressed on erythrocyte lineage cells throughout erythroid development, and its presence is necessary for successful entry of RBCs into blood circulation (Coste et al., 2001). CD147 has a homophilic counterreceptor binding activity (Yoshida et al., 2000) that can function as adhesion molecules.

Abundant expression of CD147 on fetal NRBCs may facilitate the entry of these cells into maternal circulation. In addition, CD147 is a highly glycosylated protein, and its distinct expression level in fetal NRBCs and adult RBCs could serve as a potential marker that enabled the separation of fetal NRBCs from adult RBCs using lectin-affinity approach as demonstrated by Shinya et al. (2004).

CD147 was suggested to be necessary for the translocation and/or correct localisation of lactate transporters MCT1 to plasma membrane (Kirk et al., 2000). Recent studies suggested that CD147 was an accessory protein that was closely associated with MCT transporters in the plasma membrane (Table 6-2). As such, it was not surprising that both of the two antigens were found of relatively strong expression on fetal NRBCs, but with lower/undetectable expression level on adult RBCs. However, current data also showed while CD147 was strongly expressed on all fetal NRBCs, MCT1 was only expressed on less than half of fetal NRBCs. Thus, it would be worthwhile to study their co-expression in fetal NRBCs and adult RBCs. However this was not explored as this thesis focused on biomarker discovery for cell sorting.

Figure 6-2 MCT1 and CD147 topology
MCT1 is an integral membrane proteins consisting of 12 TMDs. CD147, a membrane of immunoglobulin superfamily, has three glycosylation sites, and interact with MCT1 through an arginine residue within transmembrane segment 8 of MCT1. (Picture adapted from Enerson et al. (2003)).



The unique fetal NRBC markers including CD71, CD164 and MCT1 are expressed in some of fetal NRBCs, and thus are not ideal targets for fetal NRBC enrichment due to possible loss of target cells. CD147 instead, with much higher level of expression in all fetal NRBCs as compared in adult RBCs, could be a useful marker for the separation of fetal NRBCs from adult RBCs. This possibility was subsequently explored by using common fetal cell separation methods (immunomagnetic cell sorting (section 6.3) and fluorescence-activated cell sorting (section 6.4)) to separate fetal NRBCs from the model mixtures of NRBCs/RBCs.

6.3 Immunomagnetic cell sorting using anti-CD147

Aim. The two major immunomagnetic cell sorting methods that are generally used to separate fetal NRBCs from maternal blood are immunomagnetic beads method (e.g. Dynabeads) and magnetic-activated cell sorting (MACS). In this

section, I tested the the potential of anti-CD147 for the separation of fetal NRBCs from adult RBCs by both Dynabeads and MACS techniques.

6.3.1 Dynal system

Investigation. Pure fetal NRBCs and adult RBCs were obtained as described in section 2.2.5 and. 2.2.6 respectively. Fetal NRBCs were mixed with adult RBCs in a 1:50 ratio. The mixed cells (5×10^6) were labelled with anti-CD147 by addition of 1 μg monoclonal antibody into the cell suspension (50 μl) and incubated at 4°C for 30 min. The other procedures were followed as described in section 2.2.25.1. The cells in positive and negative fractions were recovered to assess the effectiveness of CD147 for the cell separation fetal NRBCs from the mixture.

Results. The mean recovery of fetal NRBCs was 56.2% (range: 50-64.3%; n=5) and the mean purity after separation was 13.3% (range: 10.3-17.0%) which corresponded to a 92.6% (range: 88.8-94.8%) of RBC depletion (Table 6-2).

Table 6-2 Immunomagnetic cell sorting (Dynabeads) with anti-CD147

Sample No.	Recovery (%)	NRBC in negative fraction (%)	Purity (%)	Cell loss (%)
1	50.0	2.0	16.2	48.0
2	64.3	3.4	10.3	32.3
3	62.5	1.5	17.0	36.0
4	54.0	2.0	12.3	44.0
5	52.8	2.0	10.5	45.2
Mean	56.7*	2.18	13.3	41.1

* (Mean recovery 95% CI, 48.9-64.5%)

6.3.2 Magnetic-activated cell sorting

Investigation. The cell mixtures (as in section 6.3.1) were labelled with anti-CD147 by addition of 10 ng the antibody into the cell suspension (80 µl) and incubated at 4°C for 30 min. The other procedures were followed as described in section 2.2.25.2. The cells in positive and negative fractions were recovered to assess the effectiveness of CD147 for the cell separation fetal NRBCs from the mixture.

Results. The mean recovery of fetal NRBCs was 79.7% (range: 77-80.8%, n=5) and the mean purity after separation was 12.8% (range: 10.2-17.8%) which corresponded to an 89.2% (range: 85.8-92.6%) of RBC depletion (Table 6-3). Fetal NRBC loss was significantly lower ($p<0.001$) in MACS (14.0%) compared to that of Dynabeads separation (41.1%).

Table 6-3 Immunomagnetic cell sorting (MACS) with anti-CD147

Sample No.	Recovery (%)	NRBC in negative fraction (%)	Purity (%)	Cell loss (%)
1	80.0	5.0	17.8	15.0
2	80.0	0.0	13.8	20.0
3	77.0	8.0	11.5	15.0
4	80.5	12.0	10.9	7.5
5	80.8	6.8	10.2	12.4
Mean	79.7*	6.4	12.8	14.0

* (Mean recovery 95% CI, 77.7-81.5%)

Interpretation. Immunomagnetic cell sortings using anti-CD147 to separate fetal NRBCs from a mixture of fetal NRBCs and RBCs yielded relative high recovery rates (Dynabeads: 56.2%; MACS: 79.7%), whereby both methods depleted approximately 90% of RBCs. The separation principles of these two techniques

are quite different. Dynabeads have a physical size of 4.5 μm of diameter which is approximately the same diameter of RBCs. The low density of CD147 antigen and the relatively smaller size of RBCs provide limited chances of binding the IgG-conjugated Dynabeads. In contrast, such a high density of antigen and the larger size of fetal NRBCs (Figure 6-1) allowed stronger interaction with the IgG-conjugated Dynabeads. As a result, binding of NRBCs to the beads fetal NRBCs were separated from the mixtures. However, the main drawback is that binding of fetal NRBCs to these large and heavy beads (with a diameter of 4.5 μm and a density of 1.5 g/cm^3) may cause cell lysis during the separation process, one possible reason explaining the cell loss (41%). This could be circumvented with the use of a slightly smaller Dynabeads in order to reduce cell loss but it might increase binding chances of RBCs as well.

MACS cell sorting device has a high capability of recovering target cells even when there is low expression of target antigen. Initial experiments showed no enrichment due to excess antibody used during incubation with almost 100% recovery of fetal NRBC and no depletion of RBCs. Subsequent optimisation of antibody titre yielded very promising results: high recovery of fetal NRBCs and relatively high rates of RBC depletion.

Both Dynal and MACS cell sorting are often used the separation of one cell population from another based on the unique antigen(s) expressed on one of the cells. In our case, CD147 was expressed on both adult RBCs and fetal NRBCs but the expression levels were different. Thus, the recovery rate and the purity are always to be tradeoff when this antigen is targeted for the cell sorting of adult RBCs and fetal NRBCs. The higher recovery rate would be accompined with lower purity. The other technique FACS is an ideal tool for the separatioin of one

cell population from another based on differential expression and thus was used for the cell sorting in the next section.

6.4 Fluorescence-activated cell sorting with anti-CD147

Aim. FACS is one of the commonest techniques for isolation of fetal cells from maternal blood. Compared to immunomagnetic cell sorting, FACS cell soting is based on differential antigen density, and generally gives higher purity of target cells, thereby enhancing FISH efficiency and easing the identification of target cells on slide. Here, I tested the possible usefulness of CD147 for the separation of fetal NRBCs from adult RBCs by FACS sorting technique.

Investigation. Fetal NRBCs were mixed with adult RBCs in different ratios of NRBCs/RBCs: 30:70; 20:80; 10:90, 5:95 (1 set respectively), and 1:50 (5 sets). The mixed cells (10^6) were labelled with anti-CD147 conjugated with FITC by addition of 10 μg antibody into the cell suspension (100 μl) and incubated at 4°C for 30 min. The cell mixtures were washed, and sorted by FACS as described in section 2.2.26. The cells in positive (as shown in Figure 6-3, arrowed peak) and negative fractions were recovered to assess the effectiveness of CD147 for the cell separation of fetal NRBCs from the mixture.

*Result*s. The separation of fetal NRBCs from adult RBCs was illustrated in Figure 6-3. Two distinct peaks were obtained in FACS based on fluorescence intensity. Cell population in “arrowed peak” was collected as positive fraction, and the rest was collected as negative fraction. Wright stain of a set of initial mixture, positive cells and negative cells was shown in Figure 6-4. Nearly pure cell population could be obtained after FACS sorting, suggesting that fetal NRBCs could be well

separated from adult RBCs by setting an appropriate gating (flurescence intensity (FL1-H) ~10^2 as shown in Figure 6-3).

Figure 6-3 Histograms of results from FACS cell sorting experiments
FACS cell sorting of NRBC and RBC mixture with anti-CD147 and without anti-CD147 (Control); the initial ratios of NRBC/RBC mixture are indicated in the picture and FL1-H is the logarithm of green fluorescence intensity.

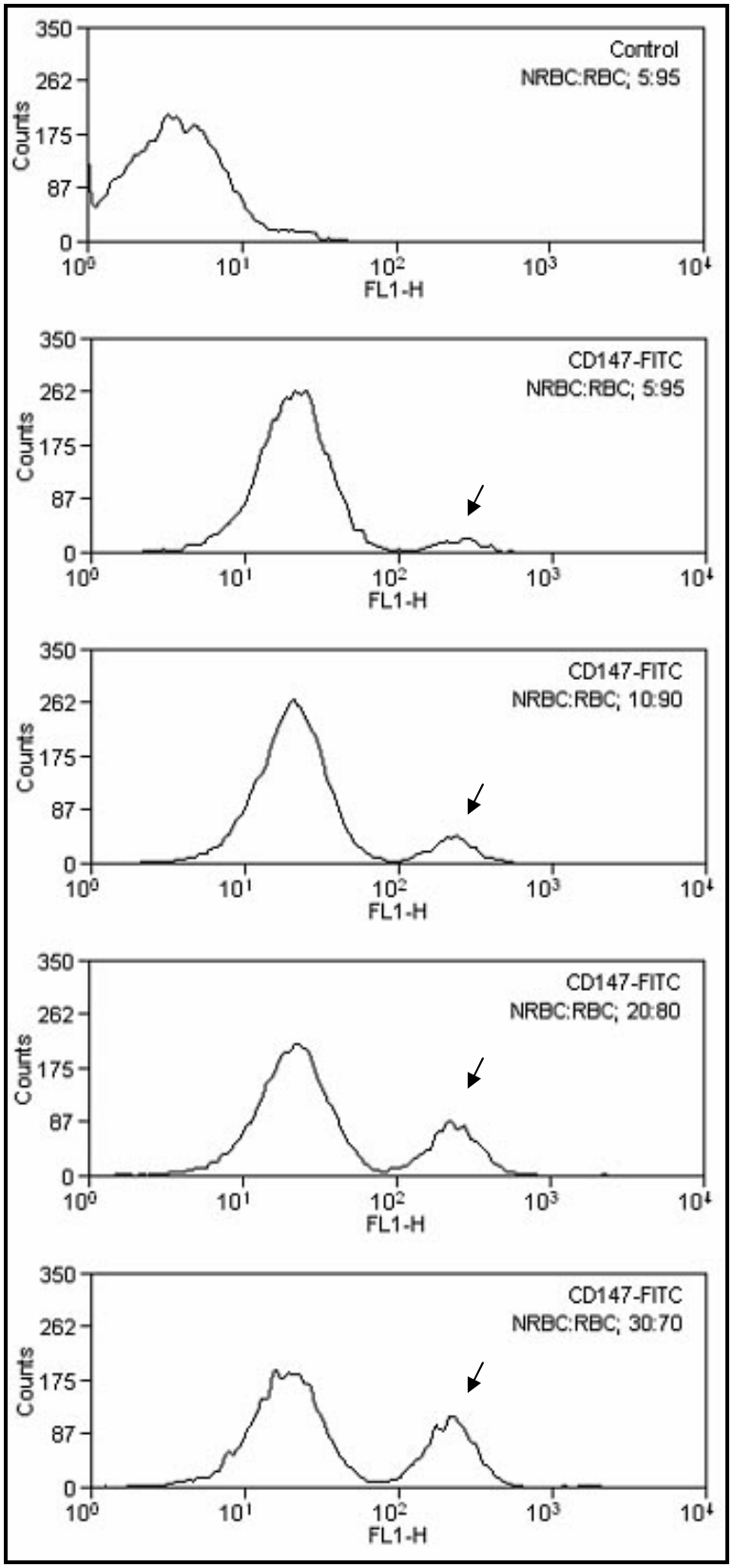
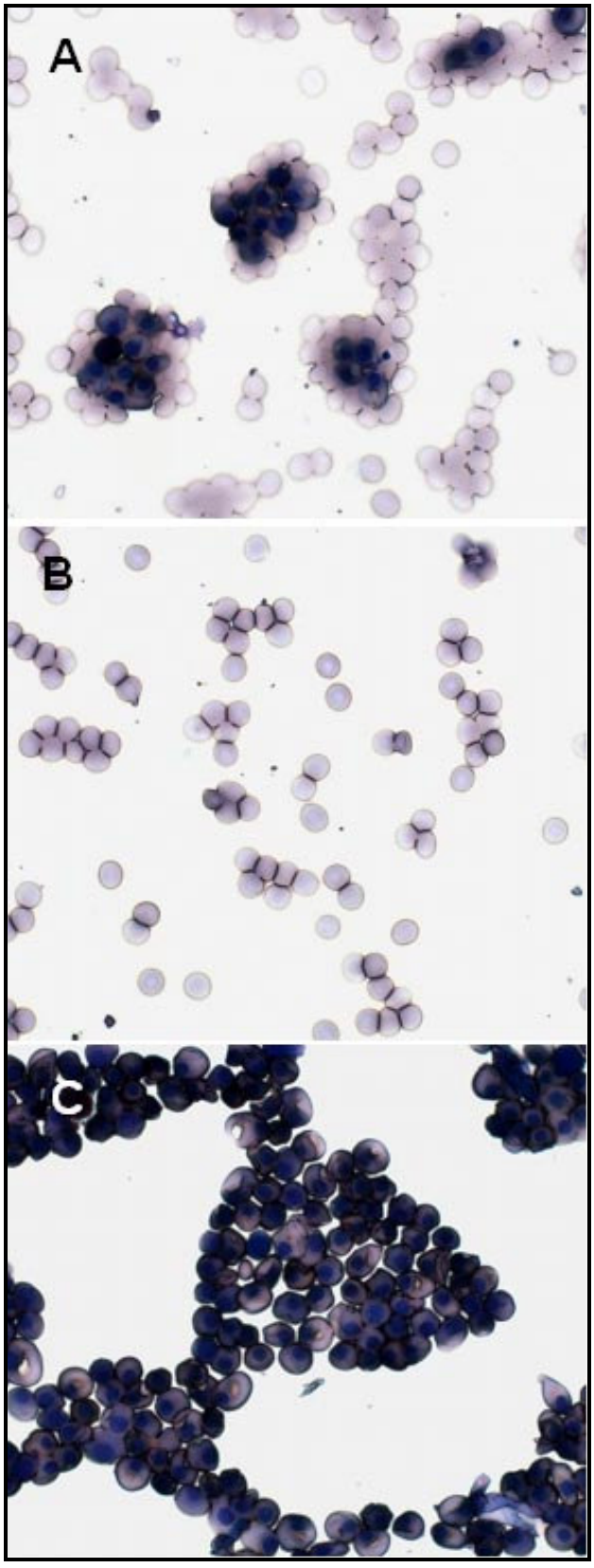


Figure 6-4 Wright stain of cell samples before and after FACS sorting
(A) initial mixture of fetal NRBCs and adult RBCs (10: 90), (B) Negative fraction from FACS sorting with anti-CD147 (FITC), (C) Positive fraction from FACS sorting with anti-CD147 (FITC).



In order to compare the efficiency of FACS separation with immunomagnetic cell sorting, fetal NRBCs from five sets of NRBC/RBC (1:50) mixture were separated following the gating set as above. The mean recovery of fetal NRBCs was 59.0% (range: 56.4-60.7%, n=5), which is comparable with Dynabeads sorting (56.2%) but lower than MACS sorting (79.9%) (Table 6-4). The purity of fetal NRBCs after FACS separation was more than 99%, which was significantly higher that that of immunomagnetic cell sorting (Dynabeads and MACS sorting).

Table 6-4 Fluorescence-activated cell sorting (FACS) with anti-CD147

Sample No.	Recovery (%)	NRBC in negative fraction (%)	Purity (%)	Cell loss (%)
1	60.7	<1.0	>99.0	39.3
2	58.1	<1.0	>99.0	41.9
3	56.4	<1.0	>99.0	43.6
4	59.6	<1.0	>99.0	40.4
5	60.4	<1.0	>99.0	39.6
Mean	59.0*	<1.0	>99.0	41.0

* (Mean recovery 95% CI, 56.8-61.3%)

Interpretation. Although CD147 was found to be expressed on both cell types, the expression on fetal NRBCs was much stronger as compared to that on adult RBCs. Thus, FACS that can efficiently separate cell populations based on the differential expression of their antigens should be an ideal technique for our purpose. Theoretically, each CD147 molecule could bind one molecule of anti-CD147 IgG conjugated with FITC. Thus, when enough anti-CD147 (FITC) was added, the fluorescence intensity on fetal NRBCs would be in proportion to the density of surface antigen CD147, and hence stronger than that on adult RBCs. The successful separation of fetal NRBCs from adult RBCs could then be achieved according to their difference in fluorescence intensity.

In order to determine an appropriate gating, fetal NRBCs and adult RBCs were mixed in different ratios and sorted using FACS. As shown in Figure 6-3 and Figure 6-4, fetal NRBCs were located in higher fluorescence intensity region, and could be separated from adult RBCs by an appropriate gating. Furthermore, it was shown that FACS separation gave a comparable recovery (59.0%) as in immunomagnetic cell sorting, but had a significantly higher fetal NRBC purity (nearly 100%). This suggested that the application of CD147 in FACS sorting could be very useful for the separation of fetal NRBCs from maternal blood.

In conclusion, the usefulness of CD147 was experimentally demonstrated in the enrichment of fetal NRBCs from model mixture with an encouraging recovery of fetal NRBCs and depletion rate of adult RBCs, indicative of its potential use for the development of an enrichment protocol for isolating fetal NRBCs from maternal blood.

6.5 Conclusion

From immunocytochemical screening of surface antigens on fetal NRBCs and adult RBCs, I have found three antigens (CD164, CD147, and MCT1) that were expressed differentially on fetal NRBCs and adult RBCs. Erythroid progenitor antigens CD164 were expressed on some of fetal NRBCs, but almost undetectable on adult RBCs. Similarly, MCT1 was expressed on some of fetal NRBCs but undetectable on adult RBCs. CD147, on the other hand, was strongly expressed on all fetal NRBCs, and also expressed on adult RBCs but with lower and/or negligible level. Interestingly, it has been suggested that CD147 was necessary for the translocation and/or correct localisation of MCT1 to plasma membrane, and in most cases closely interact with each other. Data from this study indicated a similar “co-expression” in only some of human erythrocytes,

as MCT1 was undetectable on more than half of fetal NRBCs despite with high level expression of CD147 in fetal NRBCs.

The usefulness of CD147 for immuno-cell separation was experimentally demonstrated in the enrichment of fetal NRBCs from model mixture. Although different yields and purities were obtained from three types of separation techniques (Dynabeads, MACS and FACS), all of them are very encouraging and promising. Thus, this study provided potentially useful markers that could be used for the development of an efficient enrichment protocol to isolate first trimester fetal NRBCs from maternal blood for early non-invasive prenatal diagnosis.

Chapter 7 General Discussion

Current prenatal diagnosis involves invasive procedures which carry a small but definitive risk of fetal loss, and limits the uptake of at-risk pregnant women for prenatal diagnosis of chromosomal and monogenic disorders. Over the last 20 years there has been much interest in the development of non-invasive techniques. Use of fetal nucleated erythroblasts (NRBCs) in maternal blood during early pregnancy (e.g. at first trimester) represents the state of art technique and offers great potential to replace existing methods. However, challenges preventing this from becoming clinically applicable are the technical difficulties in isolating the very rare fetal cells, which is mainly due to the lack of a specific surface marker that could be used to isolate fetal NRBCs from maternal red blood cells (RBCs).

7.1 Hypothesis

The aim of this thesis was to examine whether there is/are difference(s) of the surface membrane protein expression between fetal NRBCs and adult RBCs. My primary hypothesis is that there is/are difference(s) and that the difference(s) could be identified by systematically analysing their membrane proteins either by proteomic profiling or immunocytochemical screening. In order to logically address this hypothesis, I have restated it as following:

1. That the most comprehensive membrane protein profiles of fetal NRBCs and adult RBCs can be obtained by developing an efficient proteomic strategy for the membrane protein analysis.
2. That it is possible to identify unique surface protein(s) on fetal NRBCs by comparing the two sets of membrane profiling data obtained by proteomic studies.

3. That differential expression of surface antigen(s) is/are present in primitive fetal NRBCs, and could be identified by immunocytochemical screening using commercially available antibodies.

7.2 Research findings

Development of an efficient proteomic strategy for the analysis of fetal NRBC membrane proteome. As only a small number of fetal NRBCs could be recovered from one sample of termination of pregnancy (~10^6 cells per sample), an efficient proteomic method has to be used in order to maximise the recovery of the membrane proteome. Current methods for protein sample preparation use detergents, chaotropes and organic acids for membrane protein extraction that require sample clean-up or pH adjustment, and are associated with significant sample loss. As such, these methods are not suitable for membrane proteome analysis in a situation when a sample is limited. In this study, I have developed a very efficient proteomic strategy for the analysis of membrane proteins from a small number of fetal NRBCs (5×10^7), that is, sequential use of two organic solvents MeOH and TFE to recover both hydrophilic and hydrophobic peptides, and identification of proteins using 2-D LC-MALDI-TOF/TOF-MS (Chapter 3). This method not only allowed us to present the first relatively comprehensive fetal NRBC membrane proteome (Chapter 4), but also is applicable to other similar situations where only a limited sample is available.

Identification of differentially expressed membrane proteins between fetal NRBCs and adult RBCs. In this thesis, twenty-three unique fetal NRBC membrane proteins were identified by proteomic strategy. Of these, twenty-two proteins were validated by RT-PCR or immunocytochemistry (Chapter 5). In addition, three differentially/uniquely expressed surface antigens were identified using

immunocytochemical screening (Chapter 6). Of these potentially useful markers, the most promising target CD147 was tested and demonstrated to be very useful in the separation of fetal NRBCs from the model mixture of fetal NRBCs and adult RBCs by using both immunomagnetic cell sorting (MACS and Dynal system) and fluorescence-activated cell sorting (FACS) techniques (Chapter 6).

7.3 Implications and limitations of this research

Implications. Early non-invasive prenatal diagnosis using fetal cells in maternal blood is hampered by the difficulty in enrichment of these rare cells from maternal blood. This is due to the lack of unique/specific surface antigens that could be used to efficiently separate primitive fetal NRBCs from adult RBCs. Current separation techniques including density gradient centrifugation and selective RBC lysis are accompanied with significant cell loss and adversely affect fetal NRBC morphology, rendering the overall sensitivity and specificity of enrichment of fetal NRBCs from maternal blood as yet to be clinically useful.

This study offered markers (e.g. CD147) that could be used to effectively separate fetal NRBCs from adult RBCs by commonly used techniques (Dynabeads, MACS and FACS). These markers, therefore, could be potentially useful for the development of an optimal protocol for the separation of fetal NRBCs from maternal blood by modifying or replacing the density gradient centrifugation and/or selective RBC lysis, thereby minimising fetal NRBC loss and improving the overall efficiency of the enrichment of fetal NRBCs from maternal blood.

Limitations. The proteomic study of fetal NRBCs was limited by the small amount of sample available from trophoblast villi obtained at elective termination of

pregnancy. Duplicate or triplicate of LC-MS/MS experiment and/or study of glycoproteins simultaneously, if more sample available, would not only increase overall membrane protein identification, but also could provide more information (e.g. relative abundance) that helps to yield more likely protein targets for further investigation. Fetal NRBC number could be increased if more invasive techniques such as direct intracardiac puncture were performed under ultrasound guidance immediately before termination of pregnancy. However, this procedure is not under routine practice in Singapore.

The second limitation is that there is no commercially available antibody to many of the unique proteins identified from our proteomic study. As such, antibodies have to be generated and assessed for their reactivity to fetal NRBCs and adult RBCs, which is time-consuming and very costly.

Third, immunochemical screening is an alternative and complementary to proteomic analysis, which allowed us to identify differential expression and unique protein targets that were probably missed out in proteomic study. However, due to constraints of cost and the availability of antibody for immunocytochemical stain, only those more likely to show up difference between fetal NRBCs and adult RBCs were tested, which could result in inability to identify some differentially/uniquely expressed surface proteins.

7.4 Directions of future study

Investigation of identified targets. In this thesis, potential markers have been identified. It would be therefore logical to extend this study by exploring the usefulness of the identified markers, which can include: (1) development of an optimal enriment protocol for isolating fetal NRBCs from maternal blood by using

the identified surface marker CD147. CD147 has been demonstrated to be very useful for the separation of fetal NRBCs from adult RBCs in a model mixture using both immunomagnetic cell sorting (MACS, Dynabeads) and fluorescence-activated cell sorting (FACS) techniques. I envisage that the use of CD147 for fetal NRBC enrichment would be able to enhance the recovery of these cells from maternal blood; (2) investigation of other markers. As three antigens (CD71, CD164 and MCT1) were found to be expressed in some of fetal NRBCs but undectable on adult RBCs, use of one of them for fetal NRBC separation would result in a loss of some target cells. However, use of a combination of them for fetal NRBC separation might be able to minimise fetal NRBC loss, and is thus worthwhile to be investigated. In addition, for many potential markers, there is no commercially available antibody. Thus there is a need to generate antibodies and to assess their usefulness for the separation of fetal NRBCs from adult RBCs. Antibodies to the most promising potential maker(s) could then be tested in the separation of fetal NRBCs from maternal blood.

Exploration of new surface marker: As only limited sample was available for my study, some proteins (in particular glycosylated proteins) could be missed out despite the use of a sensitive, high throughput proteomic strategy. The recently developed antibody array (Sigma-Aldrich) could test more than 725 protein targets in a single assay, and it is suitable for cell assay. Another antibody array GlycoChip® (Glycomind) that specifically designed for studying glycoprotein could also be used to detect the difference of protein glycosylation between fetal NRBCs and adult RBCs. These newly developed techniques are high throughput, fast, reliable, and more importantly require a relatively small amount of sample. Thus, these techniques have their place for the identification of new surface biomarkers that could be used to separate fetal NRBCs from adult RBCs.

7.5 Conclusion

Fetal primitive erythroblasts (NRBCs) in the maternal blood provide an ideal source of fetal genetic material for early non-invasive prenatal diagnosis. However, these cells are rare and it is technically challenging in recovering them from maternal blood due to the lack of a specific surface marker that could be used to separate them from adult RBCs.

In this thesis, I have studied the membrane protein expressions of fetal NRBCs and adult RBCs using proteomic profiling and immunocytochemical screening, in order to identify differentially/uniquely expressed surface membrane proteins. I have shown that both approaches yielded potentially useful markers that can be used to differentiate fetal NRBCs from adult RBCs. Most of these markers were validated by RT-PCR and immunocytochemistry. Moreover, one of identified markers (CD147) has been tested and demonstrated to be very useful in the isolation of fetal NRBCs from the model mixture by both immunomagnetic cell sorting and fluorescence-activated cell sorting. While this was adequate for the purposes of this thesis, it is not yet to apply it to isolate fetal NRBCs from clinical samples. This is because that the usefulness of this marker for clinical application can only be concluded when well-designed clinical trials with an optimised protocol and a large sample size are conducted. The effort from our group, which applies the identified marker for the enrichment of fetal NRBCs from clinical samples, is currently on-going and an efficient enrichment protocol is anticipated.

In conclusion, I have achieved the primary aim of this thesis: to identify differentially/uniquely expressed membrane proteins in fetal NRBCs and adult RBCs. I envisage that after all the potential targets found in our study have been

further investigated, an optimal enriching protocol based on targeting identified markers could be developed to isolate fetal NRBCs from maternal blood for early non-invasive prenatal diagnosis.

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Appendix tables

Appendix table 1 Total proteins identified from extended LC elution gradient (60 min) from MeOH-based extraction and digestion method

No.	Protein Name	Accession Number	Protein Mw	Protein p*I*	TMD	Peptide Count	Best Ion Score
1	Spectrin, alpha, erythrocytic 1	IPI00641363	279501.69	4.95	0	83	192
2	Spectrin, alpha, erythrocytic 1 (elliptocytosis 2)	IPI00220741	280883.88	4.98	0	83	192
3	Spectrin beta isoform a	IPI00216704	267589.56	5.24	0	71	212
4	Ankyrin 1 isoform 1	IPI00216697	206136.92	5.65	0	52	196
5	Band 3 anion transport protein	IPI00022361	101727.41	5.08	11	35	200
6	Flotillin-2	IPI00029625	41659.24	5.23	0	18	224
7	Splice Isoform Short of Erythrocyte membrane protein band 4.2	IPI00028614	76793.61	8.27	0	19	150
8	55 kDa erythrocyte membrane protein	IPI00215610	52263.65	6.91	0	16	210
9	Splice Isoform 4 of Protein 4.1	IPI00218699	66357.31	6.81	0	18	164
10	Solute carrier family 2, facilitated glucose transporter member 1	IPI00220194	54082.52	8.93	12	16	164
11	Flotillin-1	IPI00027438	47325.62	7.08	0	11	176
12	Erythrocyte band 7 integral membrane protein	IPI00219682	31579.70	7.90	1	10	142
13	Alpha 2 globin variant	IPI00410714	15270.94	8.72	0	6	215
14	Beta-globin gene from a thalassemia patient, complete cds	IPI00382950	18918.59	6.28	0	8	150
15	Splice Isoform XB of Plasma membrane calcium-transporting ATPase 4	IPI00217169	133845.70	6.04	8	9	148
16	Aquaporin-1	IPI00024689	28377.02	7.15	6	6	179
17	Calpain-5	IPI00742984	73122.66	7.57	0	10	126
18	Complement component (3b/4b) receptor 1, including Knops blood group	IPI00640083	223560.88	6.57	1	9	120
19	Hemoglobin delta chain	IPI00473011	15914.25	7.97	0	6	121
20	Splice Isoform Glycophorin D of Glycophorin C	IPI00218128	11491.61	4.76	1	3	255
21	Kell blood group glycoprotein	IPI00220459	82770.92	8.09	1	7	147
22	Hypothetical protein	IPI00456429	14718.96	9.87	0	4	159
23	Calnexin precursor	IPI00020984	67525.85	4.47	1	5	109
24	Multidrug resistance-associated protein 4	IPI00006675	149445.70	8.48	11	5	124
25	Splice Isoform 2 of Cell division control protein 42 homolog	IPI00016786	21245.02	6.15	0	5	103

Appendix table 1. Continued

26	ATP-binding cassette half-transporter	IPI00465442	99649.17	9.26	9	7	124
27	Acetylcholinesterase precursor/ACHE protein	IPI00220026	69665.78	6.45	0	8	82
28	Splice Isoform B of Ras-related C3 botulinum toxin substrate 1	IPI00219675	23452.31	8.87	0	7	85
29	Rhesus blood group-associated glycoprotein	IPI00024094	44169.66	6.19	12	4	128
30	Splice Isoform 2 of Neutral alpha-glucosidase AB precursor	IPI00011454	109369.04	5.82	1	7	92
31	Probable endonuclease KIAA0830 precursor	IPI00001952	54981.26	5.55	3	5	100
32	Ras-related protein Rap-2b	IPI00018364	20491.21	4.73	0	6	105
33	Equilibrative nucleoside transporter 1	IPI00550382	58924.58	8.49	11	5	78
34	Splice Isoform 2 of Disheveled associated activator of morphogenesis 1	IPI00337801	122229.07	6.81	0	5	84
35	Erythroblast membrane-associated protein	IPI00647116	42800.59	8.93	1	4	111
36	Ras-related protein Rab-10	IPI00016513	22526.59	8.59	0	4	89
37	Protein FAM38A	IPI00006093	232892.56	6.51	25	7	64
38	Solute carrier family 40	IPI00005547	62473.32	6.08	10	2	151
39	Semaphorin 7A precursor	IPI00025257	74776.17	7.57	0	4	89
40	Ras-related protein Rab-21	IPI00007755	24201.21	8.16	0	3	100
41	Glycophorin A precursor	IPI00298800	16263.40	5.47	1	2	150
42	Splice Isoform Long of Dematin	IPI00292290	45486.22	8.94	0	4	101
43	Hypothetical protein DKFZp686P17114	IPI00478210	202466.06	6.21	0	4	75
44	Splice Isoform 3 of Alpha adducin	IPI00220158	84250.40	5.67	0	4	89
45	Splice Isoform 1 of Duffy antigen/chemokine receptor	IPI00215964	35654.63	5.84	7	2	126
46	Urea transporter, erythrocyte	IPI00298337	48309.66	6.11	8	2	141
47	Rhesus blood group, CcEe antigens, isoform 1	IPI00465155	45421.20	9.40	12	4	66
48	NADH-cytochrome b5 reductase	IPI00328415	34081.68	7.31	0	4	72
49	Splice Isoform Alpha-S1 of Guanine nucleotide-binding protein G(s)	IPI00219835	44238.32	5.92	0	5	64
50	Hypothetical protein DKFZp564D0478	IPI00030236	21164.34	6.28	3	2	159
51	Phosphatidylinositol 4-kinase type II	IPI00020124	53988.61	8.51	0	2	106
52	Splice Isoform 2 of Syntaxin-7	IPI00552913	27383.66	5.02	0	3	100
53	Actin, cytoplasmic 2	IPI00021440	41765.79	5.31	0	3	81
54	Stromal cell-derived receptor-1 alpha	IPI00011578	31271.86	6.40	1	3	97

Appendix table 1. Continued

55	28 kDa protein	IPI00069985	28213.33	8.52	1	2	127
56	Polyposis locus protein 1	IPI00024670	21118.89	8.25	2	4	63
57	Similar to mouse 1500009M05Rik protein	IPI00166865	15268.45	9.66	1	2	99
58	Vesicle-associated membrane protein-associated protein A isoform 1	IPI00374657	32592.62	9.03	1	3	72
59	Splice Isoform Short of Intercellular adhesion molecule-4 precursor	IPI00396335	25914.66	10.19	0	3	87
60	Splice Isoform 1 of Choline transporter-like protein 2	IPI00549521	80098.56	8.93	11	3	84
61	CD59 glycoprotein precursor	IPI00011302	14167.79	6.02	0	2	113
62	Ras-related protein Rab-8A	IPI00028481	23653.19	9.15	0	3	60
63	RAP1A, member of RAS oncogene family	IPI00640287	20939.72	6.38	0	2	104
64	Zinc transporter 1	IPI00002483	55292.08	6.02	6	2	77
65	Splice Isoform 2 of Zinc finger DHHC domain containing protein 3	IPI00216069	34147.21	8.54	4	1	134
66	Splice Isoform H14 of Myeloperoxidase precursor	IPI00236554	73806.61	9.30	0	4	34
67	Splice Isoform 1 of Beta adducin	IPI00019904	80803.43	5.67	0	3	57
68	Thioredoxin domain containing protein 4 precursor	IPI00401264	46941.40	5.09	0	1	122
69	Acyl-Coa synthetase long-chain family member 6 isoform A	IPI00296333	80477.23	6.79	1	3	58
70	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D	IPI00419930	45565.47	5.80	0	2	67
71	Solute carrier family 2, facilitated glucose transporter member 4	IPI00027281	54752.36	6.47	12	2	74
72	Calreticulin precursor	IPI00020599	48111.82	4.29	0	2	83
73	Mesenchymal stem cell protein DSCD75	IPI00550002	23849.87	9.71	1	1	110
74	Similar to Ras-related protein Rab-5C	IPI00016339	31028.31	8.48	0	2	74
75	Glyceraldehyde-3-phosphate dehydrogenase, liver	IPI00219018	35899.36	8.58	0	2	60
76	DKFZP564J0863 protein	IPI00550523	60503.42	5.43	2	2	62
77	Splice Isoform 1 of Choline transporter-like protein 1	IPI00221393	73253.26	8.93	9	2	63
78	Chromosome 9 open reading frame 19	IPI00479010	14204.11	9.55	0	1	101
79	Splice Isoform CD44 of CD44 antigen precursor	IPI00305064	81503.40	5.13	1	1	100
80	Lutheran blood group glycoprotein isoform 2 precursor	IPI00554618	63653.97	5.74	1	2	64
81	Splice Isoform 2 of Complement decay-accelerating factor precursor	IPI00292069	41373.58	7.79	0	2	65
82	Ras-related protein Ral-A	IPI00217519	23551.97	6.66	0	1	96
83	78 kDa glucose-regulated protein precursor	IPI00003362	72288.43	5.07	0	2	59

Appendix table 1. Continued

84	Suppressor of actin 1	IPI00022275	66908.01	6.66	2	2	53
85	Transforming protein RhoA	IPI00027500	21754.07	5.83	0	1	88
86	Ras-related protein Rab-35	IPI00300096	23010.77	8.52	0	1	85
87	Vesicular integral-membrane protein VIP36 precursor	IPI00009950	40203.10	6.46	1	2	48
88	Splice Isoform OA3-323 of Leukocyte surface antigen CD47 precursor	IPI00374740	35190.74	6.82	6	2	52
89	Flavin reductase	IPI00219910	22579.64	7.12	0	2	44
90	Hypothetical protein FLJ14347	IPI00022300	28266.57	8.70	1	2	45
91	Splice Isoform Short of Lymphocyte function-associated antigen 3 precursor	IPI00219549	26643.16	5.85	2	1	78
92	Solute carrier family 12 member 5	IPI00301180	123415.32	5.91	12	1	78
93	Myeloblastin precursor	IPI00027409	27789.27	8.72	0	2	43
94	Splice Isoform 1 of Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1	IPI00552514	96350.11	6.02	7	1	74
95	Splice Isoform 1 of Solute carrier family 2 (facilitated glucose transporter member 14	IPI00152505	56283.47	8.00	10	1	73
96	Hypothetical protein MGC13057	IPI00031140	10963.21	4.84	0	1	70
97	Guanine nucleotide binding protein	IPI00639998	16396.27	9.27	0	1	70
98	ABC transporter ABCA7	IPI00293895	234275.16	6.85	11	2	40
99	Splice Isoform 3 of Cyclin fold protein 1	IPI00413625	33224.14	7.62	0	1	69
100	Reticulon 3 isoform a variant	IPI00555783	28803.32	8.88	3	2	36
101	Protein kinase C substrate 80K-H isoform 1	IPI00419384	59387.82	4.33	0	1	65
102	Protein disulfide-isomerase A3 precursor	IPI00025252	56746.75	5.98	0	2	39
103	Splice Isoform 1 of Disheveled associated activator of morphogenesis 2	IPI00514893	123420.36	6.36	0	1	64
104	Guanine nucleotide binding protein, q polypeptide	IPI00288947	42115.31	5.48	0	2	39
105	UDP-glucose:glycoprotein glucosyltransferase 1 Precursor	IPI00619903	174867.02	5.40	1	2	38
106	Endoplasmin precursor	IPI00027230	92411.34	4.76	0	1	63
107	EVIN2	IPI00302538	81589.02	9.49	8	1	62
108	Cathepsin G precursor	IPI00028064	28819.07	11.19	0	1	61
109	Surfeit 4	IPI00399142	21115.80	8.88	2	1	60
110	ARFRP2 protein	IPI00005163	22843.55	5.41	0	1	60
111	Splice Isoform Short of Sodium/potassium-transporting ATPase alpha-1 chain precusor	IPI00414005	74092.64	5.73	4	1	59
112	Galphai2 protein	IPI00465121	41521.62	5.66	0	2	32

Appendix table 1. Continued

113	17 kDa protein	IPI00642218	16710.62	6.49	3	1	58
114	Phospholipid scramblase 1	IPI00005181	35025.85	4.83	0	1	57
115	Splice Isoform Delta of Poliovirus receptor related protein 1 precursor	IPI00003648	57122.48	5.77	2	2	29
116	Microsomal glutathione S-transferase 3	IPI00639812	18404.62	9.99	3	1	56
117	Casein kinase I gamma 1 isoform	IPI00465058	50312.43	8.91	0	1	56
118	Protein BAT5	IPI00033075	63072.05	8.40	2	1	56
119	Splice Isoform 2 of ADP-ribosyl cyclase 1	IPI00395006	13779.04	9.01	1	1	56
120	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 2	IPI00003348	37176.02	5.60	0	1	54
121	Hypothetical protein 327024.1	IPI00178934	44565.75	7.24	1	1	53
122	Splice Isoform 1 of Multiple inositol polyphosphate phosphatase 1 precursor	IPI00293748	55016.08	7.93	0	1	52
123	Phospholipid scramblase 4	IPI00016776	36939.60	5.53	0	1	51
124	Intermediate conductance calcium-activated potassium channel protein 4	IPI00032466	47664.55	9.87	5	1	51
125	Signal peptidase complex subunit 1	IPI00219755	11797.11	9.33	2	1	50
126	Growth-inhibiting protein 12	IPI00646848	78334.00	8.54	0	1	52
127	FCGR3A protein	IPI00639988	29038.68	8.20	0	1	48
128	Epidermal growth factor receptor pathway substrate 15	IPI00385325	83602.79	4.56	0	1	48
129	Thioredoxin domain containing protein 1 precursor	IPI00395887	31770.80	4.92	3	1	48
130	101 kDa protein	IPI00514990	101264.14	6.17	8	1	48
131	Transmembrane protein Tmp21 precursor	IPI00028055	24960.02	6.97	2	1	48
132	ATP-binding cassette, sub-family C, member 1 isoform 6	IPI00008463	166576.64	7.27	15	1	47
133	Leukocyte elastase precursor	IPI00027769	28499.79	9.71	1	1	44
134	Histidine decarboxylase	IPI00290368	74092.53	8.30	0	1	44
135	Splice Isoform 2 of Large neutral amino acids transporter small subunit 3	IPI00479732	61011.13	8.26	11	1	43
136	Splice Isoform 2 of Transmembrane protein 55B	IPI00332278	30165.25	9.29	2	1	43
137	SPATS2 protein	IPI00329345	59507.56	9.01	0	1	41
138	Ras-related protein Rab-7	IPI00016342	23474.84	6.40	0	1	40
139	Membrane protein	IPI00026111	21161.16	9.77	2	1	40
140	Protein transport protein SEC61 gamma subunit	IPI00006072	7736.17	10.01	1	1	39
141	Solute carrier family 43, membrane 3	IPI00301100	54494.25	8.71	11	1	38

Appendix table 2 Comprehensive membrane protein list summarised from various studies on human adult RBCs

(Adjacent proteins in Italic were identified from same peptide but in different studies, and were counted as one protein)

No.	Protein Name	Accession Number	TMD	Subcellular localisation
1	101 kDa protein	IPI00514990	8	Integral membrane protein
2	17 kDa protein	IPI00642218	3	Integral membrane protein
3	22 kDa protein	IPI00478755	0	Plasma membrane
4	27 kDa protein	IPI00641837	3	Integral membrane protein
5	28 kDa protein	IPI00069985	1	Integral membrane protein
6	55 kDa erythrocyte membrane protein	IPI00215610	0	Membrane associated protein
7	64 kDa protein	IPI00479914	0	Membrane; Cytoplasmic
8	ABC transporter ABCA7	IPI00293895	11	Integral membrane protein
9	Acetylcholinesterase precursor/ACHE protein	IPI00220026	0	Membrane; Extracellular
10	Splice isoform 1 of P22303 Acetylcholinesterase precursor	IPI00026103	0	Integral membrane protein
11	Acyl-Coa synthetase long-chain family member 6 isoform A	IPI00296333	1	Membrane
12	*Splice isoform 1 of P35611 Alpha adducin*	*IPI00019901*	*0*	*Membrane; Cytoskeleton*
	Adducin subunit (alpha), erythrocyte	*Gi12644231*	*0*	*Membrane; Cytoskeleton*
	Adducin 1 (alpha) isoform c	*Gi7710117*	*0*	*Membrane; Cytoskeleton*
13	Splice Isoform 3 of Alpha adducin	IPI00220158	0	Membrane; Cytoskeleton
14	*Splice isoform 1,3 of Beta adducin*	*IPI00019904*	*0*	*Membrane; Cytoskeleton*
	Beta adducin	*P35612*	*0*	*Membrane; Cytoskeleton*
15	Adipocyte plasma membrane-associated protein, Low molecular weight phosphotyrosine protein phosphatase	IPI00031131	1	Membrane associated protein
16	Splice Isoform 2 of ADP-ribosyl cyclase 1	IPI00395006	1	Integral membrane protein
17	Splice isoform APP770 of P05067 Amyloid beta A4 protein precursor	IPI00006608	1	Type I membrane protein
	Ankyrin 1 isoform 1	*IPI00216697*	*0*	*Membrane; Cytoskeleton*
18	*Similar to ankyrin 1*	*Gi13645508*	*0*	*Membrane; Cytoskeleton*
	Ankyrin 1	*P16157*	*0*	*Membrane; Cytoskeleton*
19	*Ankyrin 1, splice form 2*	*Gi105337*	*0*	*Membrane; Cytoskeleton*
	Ankyrin 1, isoform 2, erythrocytic	*Gi10947042*	*0*	*Membrane; Cytoskeleton*

Appendix table 2. Continued

20	Annexin A11 protein	IPI00001856	1	Membrane; Cytoskeleton
21	Annexin I	IPI00218918	0	Membrane associated protein
22	Annexin VII isoform 2	IPI00002460	0	Integral membrane protein
23	ANXA4 protein	IPI00221225	0	Membrane; Cytoskeleton
24	Apolipoprotein E precursor	IPI00021842	0	Extracellular binding RBCs
25	Aquaporin 1	IPI00024689	6	Integral membrane protein
26	Aquaporin 1 splice variant 2	IPI00428490	1	Integral membrane protein
27	*ATP-binding cassette half-transporter*	*IPI00465442*	*9*	*Integral membrane protein; Inner mitochondrial*
	ATP-binding cassette, sub-family B, member 6, mitochondrial precursor	*IPI00014555*	*9*	*Mitochondrial membrane*
28	ATP-binding cassette, subfamily C, member 6	Gi6715561	12	Integral membrane protein
29	ATP-binding cassette, sub-family C, member 1 isoform 6	IPI00008463	15	Integral membrane protein
30	ATP-binding cassette, sub-family G, member 2	IPI00298214	6	integral membrane protein
31	Band 3 anion transport protein	IPI00022361	11	integral membrane protein
32	Splice Isoform 2 or 1 of P35613 Basigin precursor	IPI00019906	2	Type I membrane protein
33	C4B1	IPI00418163	0	Extracellular protein
34	Calcium and integrin-binding protein 1	IPI00018451	0	Membrane associated protein
35	Calcium binding protein 39	IPI00032561	0	Integral membrane protein
36	Calnexin precursor	IPI00020984	1	Integral membrane protein; ER
37	Calreticulin precursor	IPI00020599	0	ER; Extracellular; Cytosolic protein
38	Carnitine O-palmitoyltransferase I, mitochondrial liver isoform	IPI00032038	2	Mitochondrial outer membrane.
39	Cathepsin G precursor	IPI00028064	0	Extracellular binding RBC
	CD44 antigen	*IPI00297160*	*1*	*Type I membrane protein*
40	*Cell surface glycoprotein CD44*	*Gi7512338*	*1*	*Integral membrane protein*
	Splice Isoform CD44 of CD44 antigen precursor	*IPI00305064*	*1*	*Integral membrane protein*
41	CD59 glycoprotein precursor	IPI00011302	0	Membrane associated protein
42	Splice Isoform 2 of P60953 Cell division control protein 42 homolog	IPI00016786	0	Membrane associated protein
43	Channel-like integral membrane protein	1314306	3	Integral membrane protein
44	Chloride intracellular channel protein 3	IPI00000692	0	Integral membrane protein
45	Splice Isoform 1 of Choline transporter-like protein 1	IPI00221393	9	Integral membrane protein

Appendix table 2. Continued

46	Splice Isoform 1 of Choline transporter-like protein 2	IPI00549521	11	Integral membrane protein
47	CGI-26 protein	IPI00219677	0	Extracellular protein
48	Clathrin coat assembly protein AP180	IPI00006612	0	Membrane associated protein
49	Clusterin precursor	IPI00291262	0	Extracellular binding RBC
50	*Splice Isoform 1 of P08174 Complement decay-accelerating factor precursor*	*IPI00216550*	*0*	*Membrane associated protein*
	Splice Isoform 2 of Complement decay-accelerating factor precursor	*IPI00292069*	*0*	*Integral membrane protein*
51	Complement C3b	IPI00164623	0	Extracellular binding RBC
52	*Complement component (3b/4b) receptor 1, including Knops blood group system (Complement receptor 1)*	*IPI00640083*	*1*	*Integral membrane protein*
	Complement receptor 1	*IPI00412546*	*1*	*Integral membrane protein*
	Complement receptor type 1 precursor	*IPI00018287*	*1*	*Type I membrane protein*
53	Cop-coated vesicle membrane protein p24 precursor	IPI00016608	2	Integral membrane protein
54	Copine III	IPI00024403	0	Membrane associated protein
55	Splice Isoform 4 of Q04656 Copper-transporting ATPase 1	IPI00028610	7	Golgi; Plasma membrane
56	*Splice Isoform CNPI of 2',3'-cyclic-nucleotide 3'-phosphodiesterase*	*IPI00220993*	*0*	*Membrane associated protein*
	2',3'-cyclic-nucleotide 3'-phosphodiesterase	*Gi7435185*	*0*	*Membrane associated protein*
57	Cytochrome b5 reductase membrane-bound isoform	IPI00328415	0	Membrane associated protein
58	Splice Isoform 2 or 1 of Q9H3Z4 DNAJ homolog subfamily C member 5	IPI00023780	1	Membrane associated protein
59	DC-TM4F2 protein	IPI00165394	4	Integral membrane protein
60	*Splice Isoform Long of Dematin*	*IPI00292290*	*0*	*Membrane associated protein*
	Dematin 52 kDa subunit	*Gi4503581*	*0*	*Membrane associated protein*
	Dematin (Erythrocyte membrane protein band 4,9)	*Q08495*	*0*	*Membrane associated protein*
	Splice Isoform Short of Q08495 Dematin	*IPI00216633*	*0*	*Integral membrane protein*
61	DKFZP564J0863 protein	IPI00550523	2	Unclassified
62	Down syndrome cell adhesion molecule 2	IPI00154755	1	Type I membrane protein
63	*Splice Isoform 1 of Duffy antigen/chemokine receptor*	*IPI00215964*	*7*	*Integral membrane protein*
	Splice Isoform 2 of Q16570 Duffy antigen/chemokine receptor	*IPI00002940*	*7*	*Integral membrane protein*
64	*Duodenal cytochrome b sequence coverage:10%*	*IPI00432050*	*1*	*Integral membrane protein*
	Duodenal cytochrome b	*Gi13376257*	*6*	*Integral membrane protein*

Appendix table 2. Continued

65	Ecto-ADP-ribosyltransferase 4 precursor	IPI00004065	0	Integral membrane protein
66	Endoplasmin precursor	IPI00027230	0	Membrane; ER
67	Alpha enolase	IPI00215736	0	Cytosolic; Plasma membrane
68	ENSEMBL:ENSP00000309219 Tax_Id=9606	IPI00156774	0	Membrane associated protein
69	Eosinophil granule major basic protein precursor	IPI00010341	0	Extracellular protein
70	Equilibrative nucleoside transporter 1	IPI00550382	11	Integral membrane protein
71	Erythroblast membrane-associated protein	IPI00647116	1	Integral membrane protein
72	Erythroid membrane-associated protein	IPI00044556	2	Membrane associated protein
	Erythrocyte membrane protein band 4.2 (pallidin)	*P16452*	*0*	*Membrane associated protein*
73	*Splice Isoform Short of Erythrocyte membrane protein band 4.2*	*IPI00028614*	*0*	*Membrane associated protein*
	Erythrocyte membrane protein band 4.2	*IPI00028120*	*0*	*Membrane associated protein*
74	EVIN2	IPI00302538	8	Integral membrane protein
75	Similar to expressed sequence AA536743	IPI00216890	2	Integral membrane protein
76	Splice Isoform 1 Of Fibronectin precursor	IPI00022418	0	Integral membrane protein
77	Flotillin-1	IPI00027438	0	Membrane associated protein
78	*Flotillin-2*	*IPI00029625*	*0*	*Membrane associated protein*
	Similar to flotillin 2	*IPI00386741*	*0*	*Membrane associated protein*
79	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 2	IPI00221205	1	Integral membrane protein; Golgi
80	Galectin-3	IPI00465431	0	Extracellular binding RBC
81	Splice isoform Long of O00182 Galectin-9	IPI00010477	0	Extracellular protein
82	Gene rich cluster, C3f gene	IPI00306419	7	Unclassified
83	*Glycophorin A precursor*	*IPI00298800*	*2*	*Type I membrane protein*
	Similar to glycophorin A	*Gi13529077*	*2*	*Type I membrane protein*
84	Glycophorin A	Gi106140	2	Type I membrane protein
85	Glycophorin Erik I-IV precursor	IPI00384414	1	Integral membrane protein
	Glycophorin C, isoform 1	*Gi4504229*	*1*	*Integral membrane protein*
86	*Splice Isoform Glycophorin C of P04921 Glycophorin C*	*IPI00026299*	*1*	*Integral membrane protein*
	Splice Isoform Glycophorin D of Glycophorin C	*IPI00218128*	*1*	*Integral membrane protein*
87	Gp25L2 protein	IPI00023542	2	Integral membrane protein

Appendix table 2. Continued

88	Guanine nucleotide binding protein	IPI00639998	0	Extrinsic plasma membrane
89	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	IPI00220578	0	Inner surface of plasma membrane
90	Guanine nucleotide binding protein (G protein), q polypeptide	IPI00288947	0	Inner surface of plasma membrane
91	Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-5 (like) subunit	IPI00000060	0	Inner surface of plasma membrane
92	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	IPI00026268	0	Inner surface of plasma membrane
93	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 2	IPI00003348	0	Inner surface of plasma membrane
94	Guanine nucleotide-binding protein, alpha-13 subunit	IPI00290928	0	Inner surface of plasma membrane
95	Guanine nucleotide-binding protein, alpha-14 subunit	IPI00000695	0	Inner surface of plasma membrane
96	*Splice Isoform Alpha-S1 of Guanine nucleotide-binding protein G(s)* *Guanine nucleotide-binding protein G(S), alpha subunit*	*IPI00219835* *P04895*	*0* *0*	*Membrane associated protein* *Membrane associated protein*
97	Splice Isoform Alpha-S2 of Guanine nucleotide-binding protein G(s)	IPI00025899	0	Inner surface of plasma membrane
98	HGTD-P	Gi9295192	1	Integral membrane protein
99	Hypothetical protein XP_092517	gi18552304	1	Unclassified
100	High mobility group protein 1 - AMIGO2 protein	IPI00219096	0	Integral membrane protein
101	Hypothetical protein	IPI00029002	4	Integral membrane protein
102	Hypothetical protein	IPI00031697	5	Unclassified
103	Hypothetical protein	IPI00385392	0	Integral membrane protein
104	Hypothetical protein CGI-109 precursor	IPI00032825	1	Integral membrane protein
105	Hypothetical protein DKFZp564J0863	IPI00383828	2	Unclassified
106	Hypothetical protein 327024.1	IPI00178934	1	Integral membrane protein
107	Hypothetical protein DKFZp564D0478	IPI00030236	3	Integral membrane protein
108	Hypothetical protein DKFZp564E227	IPI00100199	6	Integral membrane protein
109	Hypothetical protein DKFZp686P17114	IPI00478210	0	Membrane; Cytoskeleton
110	Hypothetical protein DKFZp762A227	IPI00032013	11	Integral membrane protein
111	Hypothetical protein FLJ14347	IPI00022300	1	Unclassified
112	Hypothetical protein FLJ16766	IPI00442030	7	Integral membrane protein
113	Hypothetical protein FLJ31842	IPI00043429	6	Integral membrane protein
114	Hypothetical protein FLJ40269	IPI00167359	2	Integral membrane protein

Appendix table 2. Continued

115	Hypothetical protein MGC34680	IPI00171004	12	Integral membrane protein
116	Hypothetical protein ORF9 precursor	IPI00003441	1	Integral membrane protein
117	Hypothetical protein PSEC0098	IPI00171421	1	Unclassified
118	Ig gamma-1 chain C region	IPI00332161	0	Extracellular binding RBC
119	Ig kappa chain C region	IPI00385058	0	Extracellular binding RBC
120	Ig heavy chain V-V region	Gi87863	0	Extracellular binding RBC
121	Integrin beta-2 precursor	P05107	1	Integral membrane protein
122	*Splice Isoform Long of Intercellular adhesion molecule-4 precursor*	*IPI00000118*	*1*	*Integral membrane protein*
	Splice Isoform Short of Intercellular adhesion molecule-4 precursor	*IPI00396335*	*0*	*Plasma membrane*
123	B-CAM protein	Gi2134798	1	Integral membrane protein
124	Intermediate conductance calcium-activated potassium channel protein 4	IPI00032466	5	Integral membrane protein
125	Ion transporter protein	IPI00415077	9	Integral membrane protein
126	Junctional adhesion molecule 1 precursor	IPI00001754	2	Type I membrane protein
127	JWA protein regulates intracellular concentrations of taurine and glutamate	IPI00007426	3	Integral membrane protein
128	Kell blood group glycoprotein	IPI00220459	1	Integral membrane protein
129	KIAA0830 protein	IPI00001952	3	Integral membrane protein
130	KIAA0851 protein	IPI00022275	2	Membrane associated protein
131	KIAA1363 protein	IPI00002230	1	Membrane associated protein
132	KIAA1617 protein	IPI00010970	0	Integral membrane protein
133	Lactotransferrin precursor	IPI00298860	0	Extracellular binding RBC
134	LanC-like protein 1	IPI00005724	0	Integral membrane protein
135	Leukemia inhibitory factor receptor	P42702	1	Integral membrane protein
136	Leukocyte elastase precursor	IPI00027769	1	Extracellular protein
137	*Splice Isoform OA3-293 of Leukocyte surface antigen CD47 precursor*	*IPI00216514*	*0*	*Integral membrane protein*
	Splice Isoform OA3-323 of Leukocyte surface antigen CD47 precursor	*IPI00374740*	*6*	*Integral membrane protein*
138	LFA-3	IPI00000059	2	Integral membrane protein
139	LGALS3 protein	IPI00219220	0	Integral membrane protein
140	Long-chain-fatty-acid--CoA ligase 3	IPI00031397	1	Type III membrane protein
141	Splice Isoform Short of Q9UKU0 Long-chain-fatty-acid--CoA ligase 6	IPI00218718	0	Type III membrane protein

Appendix table 2. Continued

142	Low affinity immunoglobulin gamma Fc region receptor III-B precursor	IPI00023858	1	Integral membrane protein
	Lutheran blood group glycoprotein precursor	*IPI00002406*	*1*	*Type I membrane protein*
143	*Lutheran blood group*	*IPI00328869*	*1*	*Integral membrane protein*
	Lutheran blood group glycoprotein isoform 2 precursor	*IPI00554618*	*1*	*Integral membrane protein*
144	Similar to Lutheran blood group	Gi18589892	0	Integral membrane protein
145	Splice Isoform Short of Lymphocyte function-associated antigen 3 precursor	IPI00219549	2	Integral membrane protein
146	Lysozyme C precursor	IPI00019038	0	Extracellular protein
147	Membrane associated progesterone receptor component 2	IPI00005202	1	Integral membrane protein
148	Membrane protein	IPI00026111	2	Unclassified
149	Membrane transport protein XK	IPI00020896	9	Integral membrane protein
150	Mesenchymal stem cell protein DSCD75	IPI00010292	1	Unclassified
151	Microsomal glutathione S-transferase 3	IPI00639812	3	Integral membrane protein
152	Moesin	IPI00219365	0	Membrane; Cytoskeleton
153	Monocarboxylate transporter 1	IPI00024650	11	Integral membrane protein
154	Splice Isoform Delexon-17 of P33527 Multidrug resistance-associated protein 1	IPI00008338	16	Integral membrane protein
155	Multidrug resistance-associated protein 4	IPI00006675	11	Integral membrane protein
156	Multidrug resistance-associated protein 5	IPI00385383	11	Integral membrane protein
157	Myeloblastin precursor	IPI00027409	0	Extracellular protein
158	Splice Isoform 1 of Q92542 Nicastrin precursor	IPI00021983	0	Type I membrane protein
159	Neuropathy target esterase	IPI00217600	1	Unclassified
160	Splice Isoform 2 of Q14697 Neutral alpha-glucosidase AB precursor	IPI00011454	1	ER and Golgi
161	Splice Isoform 2 of Large neutral amino acids transporter small subunit 3	IPI00479732	11	Integral membrane protein
162	Novel protein	IPI00513701	3	Integral membrane protein
163	Splice Isoform 1 of Q16563 Pantophysin	IPI00009507	3	Integral membrane protein
164	PB39	IPI00021075	12	Integral membrane protein
165	Phosphatidylinositol 4-kinase type II	IPI00020124	0	Integral membrane protein
166	*Phosphatidylinositol-4-phosphate 5-kinase type II alpha*	*IPI00009688*	*0*	*Integral membrane protein*
	Phosphatidylinositol-4-phosphate 5 kinase, type III	*Gi1730569*	*0*	*Integral membrane protein*

Appendix table 2. Continued

167	Splice Isoform 1 Of Phosphofurin acidic cluster sorting protein 1	IPI00376229	0	Membrane associated protein
168	Phospholipid scramblase 1	IPI00005181	0	Type II membrane protein
169	Phospholipid scramblase 4	IPI00016776	0	Type II membrane protein
170	PICALM protein	IPI00472438	0	Membrane associated protein
171	Splice isoform B of P20020 Plasma membrane calcium-transporting ATPase 1	IPI00021695	7	Integral membrane protein
	Splice Isoform XB of Plasma membrane calcium-transporting ATPase 4	*IPI00217169*	*8*	*Integral membrane protein*
177	*Splice isoform XD of P23634 Plasma membrane calcium-transporting ATPase 4*	*IPI00012490*	*8*	*Integral membrane protein*
178	Splice Isoform Delta of Poliovirus receptor related protein 1 precursor	IPI00003648	2	Integral membrane protein; Cell junction
179	Polyposis locus protein 1	IPI00024670	2	Integral membrane protein
180	Potassium channel subfamily K member 5	IPI00029507	6	Integral membrane protein
181	Presenilin-associated protein	Gi6409316	2	Integral membrane protein
182	Prolactin	IPI00022974	0	Extracellular binding RBC
	Splice Isoform 1 of Protein 4.1	*IPI00003921*	*0*	*Membrane; Cytoskeleton*
183	*Protein 4.1*	*P11171*		*Membrane; Cytoskeleton*
	Protein band 4.1, erythrocytic	*Gi14916944*	*0*	*Membrane; Cytoskeleton*
184	*Splice Isoform 4 of P11171 Protein 4.1*	*IPI00218699*	*0*	*Membrane; Cytoskeleton*
	Protein band 4.1 (elliptocytosis 1, RH-linked)	*Gi4758274*	*0*	*Membrane; Cytoskeleton*
185	Splice Isoform 2 of P11171 Protein 4.1	IPI00218697	0	Membrane; Cytoskeleton
186	Band 4.1-like protein 4A	IPI00030794	0	Membrane; Cytoskeleton
187	Erythroid protein 4.1 isoform B	Gi182074	0	Membrane; Cytoskeleton
188	Protein BAT5 (HLA-B-associated transcript 5)	IPI00033075	2	Integral membrane protein
189	Protein disulfide-isomerase A2 precursor	IPI00011571	0	Membrane associated protein
190	Protein disulfide-isomerase precursor	IPI00010796	0	ER; Extracellular region
191	Protein FAM38A	IPI00006093	25	Membrane associated protein
192	Protein transport protein SEC61 gamma subunit	IPI00006072	1	Integral membrane protein
193	PREDICTED: similar to RAB1B, member RAS oncogene family subset match by rab-10 and 8A	IPI00374519	0	Membrane associated protein

Appendix table 2. Continued

194	Ras-related C3 botulinum toxin substrate 4	IPI00001352	0	Membrane associated protein
195	Ras-related protein Rab-10	IPI00016513	0	Membrane associated protein
196	Ras-related protein Rab-14	IPI00291928	0	Membrane associated protein
197	Ras-related protein Rab-2A	IPI00031169	0	Membrane associated protein
198	Ras-related protein Rab-21	IPI00007755	0	Membrane associated protein
199	Splice Isoform 1 of P62820 Ras-related protein Rab-1A	IPI00005719	0	Membrane associated protein
200	Ras-related protein Rab-33B	IPI00021475	0	Membrane associated protein
201	*Ras-related protein Rab-35*	*IPI00300096*	*0*	*Membrane associated protein*
	RAB 35, RAS oncogene family	*Gi5803135*	*0*	*Membrane associated protein*
202	Ras-related protein Rab-5B	IPI00017344	0	Membrane associated protein
203	Ras-related protein Rab-5C	IPI00016339	0	Membrane associated protein
204	Ras-related protein Rab-7b	IPI00016342	0	Membrane associated protein
205	Ras-related protein Rab-8A	IPI00028481	0	Membrane; Cytosolic protein
206	Ras-related protein Rab-8B	IPI00024282	0	Membrane; Cytosolic protein
207	Ras-related protein Ral-A	IPI00217519	0	Membrane; Cytosolic protein
208	*Ras-related protein Rap-1A*	*IPI00019345*	*0*	*Membrane associated protein*
	RAP1A, member of RAS oncogene family	*Gi4506413*	*0*	*Membrane associated protein*
209	*Ras-related protein Rap-1B*	*IPI00015148*	*0*	*Membrane associated protein*
	RAP1B	*Gi7661678*	*0*	*Membrane associated protein*
210	*Ras-related protein Rap-2b*	*IPI00018364*	*0*	*Membrane associated protein*
	RAP2B, member of RAS oncogene family	*Gi11433346*	*0*	*Membrane associated protein*
211	Splice Isoform 1 of P59190 Ras-related protein Rab-15	IPI00394882	0	Membrane associated protein
212	*Ras-related C3 botulinum toxin substrate 1*	*P15154*	*0*	*Membrane associated protein*
	Splice isoform A of P15154 Ras-related C3 botulinum toxin substrate 1	*IPI00010271*	*0*	*Membrane associated protein*
213	Splice Isoform B of Ras-related C3 botulinum toxin substrate 1	IPI00219675	0	Membrane associated protein
214	Similar to RAS-related protein RAL-A	Gi14740792	0	Membrane associated protein
215	Similar to RAS-related protein RAB-15	Gi18596861	0	Membrane associated protein
216	RECS1 protein homolog	IPI00290452	7	Integral membrane protein
217	Splice Isoform 2 Of Reticulon 4	IPI00298289	1	Integral membrane protein

Appendix table 2. Continued

	Reticulon protein 3	*IPI00028946*	*3*	*Integral membrane protein*
218	*Reticulon 3 isoform a variant*	*IPI00555783*	*3*	*Integral membrane protein*
	RTN3-A1	*IPI00398795*	*3*	*Integral membrane protein*
	PREDICTED: similar to Reticulon protein 3	*IPI00177423*	*1*	*Integral membrane protein*
219	Rh blood CE group antigen polypeptide	IPI00039665	12	Integral membrane protein
	RhD protein	*IPI00329565*	*10*	*Integral membrane protein*
220	*Rhesus blood group D antigen*	*IPI00478119*	*10*	*Integral membrane protein*
	Rh blood D group antigen polypeptide	*10800054*	*10*	*Integral membrane protein*
221	Rhesus D category VI type III protein	Gi2765839	12	Integral membrane protein
222	Rhesus blood group-associated glycoprotein	IPI00024094	11	Integral membrane protein
223	Rhesus blood group, CcEe antigens, isoform 1	IPI00465155	12	Integral membrane protein
224	Splice Isoform RHVIII of P18577 Blood group Rh(CE) polypeptide	IPI00221017	10	Integral membrane protein
225	Hypothetical protein FLJ45640 (Rhesus blood group, CcEe antigens)	IPI00444375	10	Integral membrane protein
226	Similar to RIKEN cDNA 1500009M05 gene	IPI00166865	1	Unclassified
227	PREDICTED: similar to RIKEN cDNA C730027E14	IPI00373867	1	Integral membrane protein
228	Secretory carrier-associated membrane protein 4	IPI00056310	4	Integral membrane protein
229	Semaphorin 7A precursor	IPI00025257	0	Integral membrane protein
230	Serum albumin precursor	IPI00022434	0	Extracellular protein
231	Signal peptidase complex subunit 1	IPI00219755	2	Integral membrane protein
232	Alpha-2,8-sialyltransferase 8C	Q64689	1	Inner cell membrane
233	Similar to SWAP-70	IPI00307200	0	Membrane associated protein
234	Splice Isoform 2 of Sodium channel protein type I alpha subunit	IPI00216029	19	Integral membrane protein
235	Splice Isoform Long of P05023 Sodium/potassium-transporting ATPase alpha-1 chain precursor	IPI00006482	10	Integral membrane protein
236	Sodium/potassium-transporting ATPase alpha-2 chain precursor	IPI00003021	8	Integral membrane protein
237	Splice Isoform Short of Sodium/potassium-transporting ATPase alpha-1 chain precursor	IPI00414005	4	Integral membrane protein
238	Solute carrier family 1 (glutamate transporter), member 7	IPI00100081	7	Integral membrane protein
239	Solute carrier family 12 member 5	IPI00301180	12	Integral membrane protein
240	solute carrier family 19 member 1 isoform b	IPI00299186	9	Integral membrane protein

Appendix table 2. Continued

241	Solute carrier family 2, facilitated glucose transporter, member 3 or 14	IPI00003909	10	Integral membrane protein
242	Solute carrier family 2, facilitated glucose transporter, member 4	IPI00027281	12	Integral membrane protein
243	*Solute carrier family 2, facilitated glucose transporter, member 1*	*IPI00220194*	*12*	*Integral membrane protein*
	Glucose transporter type I	*P11166*	*12*	*Integral membrane protein*
	Glucose transporter glycoprotein	*Gi3387905*	*8*	*Integral membrane protein*
244	Splice Isoform 1 of Q9Y666 Solute carrier family 12 member 7	IPI00008616	11	Integral membrane protein
245	Solute carrier family 27 (fatty acid transporter), member 4	IPI00021089	2	Integral membrane protein
246	Solute carrier family29 (nucleoside transporters), member 1	IPI00412547	11	Integral membrane protein
247	Solute carrier family 40, member 1	IPI00005547	10	Integral membrane protein
248	Solute carrier family 43, member 3	IPI00301100	11	Integral membrane protein
249	Spectrin alpha chain, erythrocyte	IPI00220741	0	Membrane, cytoskeleton
250	Spectrin, alpha, erythrocytic 1	IPI00641363	0	Membrane, cytoskeleton
251	*Spectrin beta isoform a*	*IPI00216704*	*0*	*Membrane, cytoskeleton*
	*Splice Isoform 1 **2** of P11277 Spectrin beta chain, erythrocyte*	*IPI00004501*	*0*	*Membrane, cytoskeleton*
252	Splice Isoform 1 of Q9H254 Spectrin beta chain, brain 3	IPI00018829	0	Membrane, cytoskeleton
253	Splice Isoform Long of Q01082 Spectrin beta chain, brain 1	IPI00005614	0	Membrane, cytoskeleton
254	Stomatin	IPI00377081	1	Membrane, cytoskeleton
255	*Stomatin isoform a*	*IPI00219682*	*1*	*Integral membrane protein*
	Erythrocyte band 7 integral membrane protein (stomatin) (protein 7.2B)	*P27105*	*1*	*Integral membrane protein*
256	*Stromal cell-derived receptor-1 alpha*	*IPI00011578*	*1*	*Integral membrane protein*
	Stromal cell-derived receptor-1 beta	*IPI00018311*	*1*	*Integral membrane protein*
257	Surfeit 4	IPI00399142	2	Integral membrane protein
258	Splice Isoform SNAP-23a of Synaptosomal-associated protein 23	IPI00010438	0	Membrane associated protein
259	Syntaxin 4	IPI00029730	1	Type IV membrane protein
260	*Syntaxin 7*	*IPI00289876*	*1*	*Type IV membrane protein*
	Splice Isoform 2 of Syntaxin-7	*IPI00552913*	*0*	*Membrane; Cytosolic protein*
261	Splice Isoform I of P14209 T-cell surface glycoprotein E2 precursor	IPI00253036	2	Integral membrane protein
262	TGF-beta receptor type I precursor	P36897	2	Integral membrane protein
263	Thioredoxin domain containing protein 1 precursor	IPI00395887	3	Integral membrane protein

Appendix table 2. Continued

264	Thioredoxin-like protein KIAA1162 precursor	IPI00100247	1	Type I membrane protein
265	Thrombospondin 1 precursor, glycoprotein IV	IPI00296099	0	Extracellular region
266	Splice Isoform 1 Of Thyrotropin receptor precursor	IPI00028642	0	Integral membrane protein
267	Titin	IPI00179357	0	Cytoskeleton
268	Titin isoform novex-1	IPI00375498	0	Cytoskeleton
269	TPM1 protein	IPI00384369	0	Cytoskeleton
270	TPR repeat containing protein	IPI00007052	1	Integral membrane protein
271	Transforming protein RhoA	IPI00027500	0	Cytoskeleton
272	Splice Isoform 2B of P01116 Transforming protein p21	IPI00423570	0	Membrane associated protein
273	Translocon-associated protein, delta subunit precursor	IPI00019385	0	ER membrane
274	Transmembrane protein 24	IPI00394781	1	Integral membrane protein
275	Transmembrane protein Tmp21 precursor	IPI00028055	2	Type I membrane protein
276	Splice Isoform 2 of Transmembrane protein 55B	IPI00332278	2	Integral membrane protein
277	Triadin	IPI00220272	1	Integral membrane protein
	Tropomodulin 1	*IPI00002375*	*0*	*Cytoskeleton*
278	*Tropomodulin*	*Gi4507553*	*0*	*Cytoskeleton*
	Tropomodulin	*P28289*	*0*	*Cytoskeleton*
279	*Splice Isoform 1 of P09493 Tropomyosin 1 alpha chain*	*IPI00014581*	*0*	*Cytoskeleton*
	Tropomyosin, cytoskeletal type	*P12324*	*0*	*Cytoskeleton*
	Splice isoform 2 of P06753 Tropomyosin alpha 3 chain	*IPI00218319*	*0*	*Cytoskeleton*
280	*Tropomyosin 3, cytoskeletal*	*Gi136096*	*0*	*Cytoskeleton*
	Similar to Tropomyosin	*Gi18590249*	*0*	*Cytoskeleton*
281	*Splice Isoform 1 of P07226 Tropomyosin alpha 4 chain*	*IPI00010779*	*0*	*Cytoskeleton*
	Similar to Tropomyosin 4	*Gi14729747*	*0*	*Cytoskeleton*
282	Tropomyosin isoform	Gi1082876	0	Cytoskeleton
283	Tropomyosin chain (smooth muscle)	Gi136100	0	Cytoskeleton
284	UDP-glucose:glycoprotein glucosyltransferase 1precursor	IPI00024466	1	Integral membrane protein
285	Uncharacterised hematopoietic stem/progenitor cells protein MDS032	IPI00020515	1	Type II membrane protein
286	UPF0198 protein CGI-141	IPI00007061	3	Integral membrane protein

Appendix table 2. Continued

287	Urea transporter, erythrocyte	IPI00298337	8	Integral membrane protein
288	Vacuolar ATP synthase 16 kDa proteolipid subunit	IPI00018855	4	Integral membrane protein
289	Splice Isoform 1 of Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1	IPI00552514	7	Integral membrane protein
290	Vesicle trafficking protein SEC22b	IPI00006865	1	Type IV membrane protein
291	Vesicle-associated membrane protein-associated protein A isoform 2	IPI00170692	1	Type IV membrane protein
292	Vesicle-associated membrane protein-associated protein A isoform 1	IPI00374657	1	Membrane; Cytoskeleton
293	Splice Isoform 1 of O95292 Vesicle-associated membrane protein-associated protein B/C	IPI00006211	1	Type IV membrane protein
294	Vesicle-associated membrane protein 2	GI7657675	1	Type IV membrane protein
295	Vesicular integral-membrane protein VIP36 precursor	IPI00009950	1	Type I membrane protein
296	WNT-3 proto-oncogene protein [precursor]	P56703	1	Integral membrane protein
297	Splice Isoform 2 of Zinc finger DHHC domain containing protein 3	IPI00216069	4	Integral membrane protein
298	Zinc transporter 1	IPI00002483	6	Integral membrane protein
299	Zona pellucida binding protein	Gi5902116	1	Integral membrane protein

Appendix table 3 Comprehensive RBC membrane proteins with potential surface domain(s)

(Adjacent proteins in Italic were identified from same peptide but in different studies, and were counted as one protein)

No.	Protein Name	Accession Number	TMD	Subcellular localisation
1	101 kDa protein	IPI00514990	8	Integral membrane protein
2	17 kDa protein	IPI00642218	3	Integral membrane protein
3	22 kDa protein	IPI00478755	0	Plasma membrane
4	27 kDa protein	IPI00641837	3	Integral membrane protein
5	28 kDa protein	IPI00069985	1	Integral membrane protein
6	ABC transporter ABCA7	IPI00293895	11	Integral membrane protein
7	Acetylcholinesterase precursor/ACHE protein	IPI00220026	0	Membrane; extracellular
8	Splice Isoform 1 of P22303 Acetylcholinesterase precursor	IPI00026103	0	Integral membrane protein
9	Acyl-Coa synthetase long-chain family member 6 isoform A	IPI00296333	1	Membrane
10	Adipocyte plasma membrane-associated protein, Low molecular weight phosphotyrosine protein phosphatase	IPI00031131	1	Membrane associated protein
11	Splice Isoform 2 of ADP-ribosyl cyclase 1	IPI00395006	1	Integral membrane protein
12	Splice Isoform APP770 of P05067 Amyloid beta A4 protein precursor	IPI00006608	1	Type I membrane protein
13	Annexin A11 protein	IPI00001856	1	Membrane associated protein
14	Apolipoprotein E precursor	IPI00021842	0	Extracellular binding RBC
15	Aquaporin 1	IPI00024689	6	Integral membrane protein
16	Aquaporin 1 splice variant 2	IPI00428490	1	Integral MP
17	*ATP-binding cassette half-transporter*	*IPI00465442*	*9*	*Integral membrane protein; Inner mitochondrial*
	ATP-binding cassette, sub-family B, member 6, mitochondrial precursor	*IPI00014555*	*9*	*Mitochondrial membrane*
18	ATP-binding cassette, subfamily C, member 6	Gi6715561	12	Integral membrane protein
19	ATP-binding cassette, sub-family C, member 1 isoform 6	IPI00008463	15	Integral membrane protein
20	ATP-binding cassette, sub-family G, member 2	IPI00298214	6	Integral membrane protein
21	Band 3 anion transport protein	IPI00022361	11	Integral membrane protein
22	Splice isoform 2 or 1 of P35613 Basigin precursor	IPI00019906	2	Type I membrane protein
23	C4B1	IPI00418163	0	Extracellular

Appendix table 3. Continued

24	Calnexin precursor	IPI00020984	1	Type I membrane protein
25	Calreticulin precursor	IPI00020599	0	ER; Extracellular; Cytosolic protein
26	Carnitine O-palmitoyltransferase I, mitochondrial liver isoform	IPI00032038	2	Mitochondrial outer membrane.
27	Cathepsin G precursor	IPI00028064	0	Extracellular binding RBC
	CD44 antigen	*IPI00297160*	*1*	*Type I membrane protein*
28	*Cell surface glycoprotein CD44*	*Gi7512338*	*1*	*Integral membrane protein*
	Splice Isoform CD44 of CD44 antigen precursor	*IPI00305064*	*1*	*Integral membrane protein*
29	CD59 glycoprotein precursor	IPI00011302	0	Membrane associated protein
30	Channel-like integral membrane protein	Gi1314306	3	Integral membrane protein
31	Splice Isoform 1 of Choline transporter-like protein 1	IPI00221393	9	Integral membrane protein
32	Splice Isoform 1 of Choline transporter-like protein 2	IPI00549521	11	Integral membrane protein
33	CGI-26 protein	IPI00219677	0	Extracellular
34	Clusterin precursor	IPI00291262	0	Extracellular binding RBC
35	*Splice Isoform 1 of P08174 Complement decay-accelerating factor precursor*	*IPI00216550*	*0*	*Membrane associated protein*
	Splice Isoform 2 of Complement decay-accelerating factor precursor	*IPI00292069*	*0*	*Membrane associated protein*
36	Complement C3b	IPI00164623	0	Extracellular binding RBC
	Complement component (3b/4b) receptor 1, incl. Knops blood group	*IPI00640083*	*1*	*Integral membrane protein*
37	*Complement receptor 1*	*IPI00412546*	*1*	*Integral membrane protein*
	Complement receptor type 1 precursor	*IPI00018287*	*1*	*Type I membrane protein*
38	Cop-coated vesicle membrane protein p24 precursor	IPI00016608	2	Integral membrane protein
39	Splice Isoform 4 of Q04656 Copper-transporting ATPase 1	IPI00028610	7	Golgi, Plasma membrane
40	Splice Isoform 2 or 1 of Q9H3Z4 DNAJ homolog subfamily C member 5	IPI00023780	1	Membrane associated protein
41	DC-TM4F2 protein	IPI00165394	4	Integral membrane protein
42	DKFZP564J0863 protein	IPI00550523	2	Unclassified
43	Down syndrome cell adhesion molecule 2	IPI00154755	1	Type I membrane protein
44	*Splice Isoform 1 of Duffy antigen/chemokine receptor*	*IPI00215964*	*7*	*Integral membrane protein*
	Splice Isoform 2 of Q16570 Duffy antigen/chemokine receptor	*IPI00002940*	*7*	*Integral membrane protein*
45	*Duodenal cytochrome b sequence coverage:10%*	*IPI00432050*	*1*	*Integral membrane protein*
	Duodenal cytochrome b	*Gi13376257*	*6*	*Integral membrane protein*

Appendix table 3. Continued

46	Ecto-ADP-ribosyltransferase 4 precursor	IPI00004065	0	Integral membrane protein
47	Eosinophil granule major basic protein precursor	IPI00010341	0	Extracellular
48	Equilibrative nucleoside transporter 1	IPI00550382	11	Integral membrane protein
49	Erythroblast membrane-associated protein	IPI00647116	1	Integral membrane protein
50	Erythroid membrane-associated protein	IPI00044556	2	Membrane associated protein
51	EVIN2	IPI00302538	8	Integral membrane protein
52	Similar to expressed sequence AA536743	IPI00216890	2	Integral membrane protein
53	Splice Isoform 1 Of Fibronectin precursor	IPI00022418	0	Integral membrane protein; Extracellular
54	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 2	IPI00221205	1	Integral membrane protein; Golgi
55	Galectin-3	IPI00465431	0	Extracellular binding RBC
56	Splice Isoform Long of O00182 Galectin-9	IPI00010477	0	Extracellular
57	gene rich cluster, C3f gene	IPI00306419	7	Unclassified
58	*Glycophorin A precursor*	*IPI00298800*	*2*	*Type I membrane protein*
	Similar to Glycophorin A	*Gi13529077*	*2*	*Type I membrane protein*
59	Glycophorin A	Gi106140	2	Type I membrane protein
60	Glycophorin Erik I-IV precursor	IPI00384414	1	Integral membrane protein
	Glycophorin C, isoform 1	*Gi4504229*	*1*	*Integral membrane protein*
61	*Splice Isoform Glycophorin C of P04921 Glycophorin C*	*IPI00026299*	*1*	*Integral membrane protein*
	Splice Isoform Glycophorin D of Glycophorin C	*IPI00218128*	*1*	*Integral membrane protein*
62	Gp25L2 protein	IPI00023542	2	Integral membrane protein
63	HGTD-P	Gi9295192	1	Integral membrane protein
64	Hypothetical protein XP_092517	gi18552304	1	Integral membrane protein
65	Hypothetical protein	IPI00029002	4	Integral membrane protein
66	Hypothetical protein	IPI00031697	5	Unclassified
67	Hypothetical protein CGI-109 precursor	IPI00032825	1	Integral membrane protein
68	Hypothetical protein DKFZp564J0863	IPI00383828	2	Unclassified
69	Hypothetical protein 327024.1	IPI00178934	1	Integral membrane protein
70	Hypothetical protein DKFZp564D0478	IPI00030236	3	Integral membrane protein
71	Hypothetical protein DKFZp564E227	IPI00100199	6	Integral membrane protein

Appendix table 3. Continued

72	Hypothetical protein DKFZp762A227	IPI00032013	11	Integral membrane protein
73	Hypothetical protein FLJ14347	IPI00022300	1	Unclassified
74	Hypothetical protein FLJ16766	IPI00442030	7	Integral membrane protein
75	Hypothetical protein FLJ31842	IPI00043429	6	Integral membrane protein
76	Hypothetical protein FLJ40269	IPI00167359	2	Integral membrane protein
77	Hypothetical protein MGC34680	IPI00171004	12	Integral membrane protein
78	Hypothetical protein ORF9 precursor	IPI00003441	1	Integral membrane protein
79	Hypothetical protein PSEC0098	IPI00171421	1	Unclassified
80	Ig gamma-1 chain C region	IPI00332161	0	Extracellular binding RBC
81	Ig kappa chain C region	IPI00385058	0	Extracellular binding RBC
82	Ig heavy chain V-V region	Gi87863	0	Extracellular
83	Integrin beta-2 precursor	P05107	1	Integral membrane protein
84	*Splice Isoform Long of Intercellular adhesion molecule-4 precursor*	*IPI00000118*	*1*	*Integral membrane protein*
	Splice Isoform Short of Intercellular adhesion molecule-4 precursor	*IPI00396335*	*0*	*Membrane*
85	B-CAM protein	Gi2134798	1	Integral membrane protein
86	Intermediate conductance calcium-activated potassium channel protein 4	IPI00032466	5	Integral membrane protein
87	Ion transporter protein	IPI00415077	9	Integral membrane protein
88	Junctional adhesion molecule 1 precursor	IPI00001754	2	Type I membrane protein
89	JWA protein regulates intracellular concentrations of taurine and glutamate	IPI00007426	3	Integral membrane protein
90	Kell blood group glycoprotein	IPI00220459	1	Integral membrane protein
91	KIAA0830 protein	IPI00001952	3	Integral membrane protein
92	KIAA0851 protein	IPI00022275	2	Integral membrane protein
93	KIAA1363 protein	IPI00002230	1	Integral membrane protein
94	Lactotransferrin precursor	IPI00298860	0	Extracellular binding RBC
95	Leukemia inhibitory factor receptor	P42702	1	Integral membrane protein
96	Leukocyte elastase precursor	IPI00027769	1	Extracellular
97	*Splice Isoform OA3-293 of Leukocyte surface antigen CD47 precursor*	*IPI00216514*	*0*	*Integral membrane protein*
	Splice Isoform OA3-323 of Leukocyte surface antigen CD47 precursor	*IPI00374740*	*6*	*Integral membrane protein*

Appendix table 3. Continued

98	LFA-3	IPI00000059	2	Integral membrane protein
99	Long-chain-fatty-acid--CoA ligase 3	IPI00031397	1	Type III membrane protein
100	Splice Isoform Short of Q9UKU0 Long-chain-fatty-acid--CoA ligase 6	IPI00218718	0	Type III membrane protein
101	Low affinity immunoglobulin gamma Fc region receptor III-B precursor	IPI00023858	1	Integral membrane protein
	Lutheran blood group glycoprotein precursor	*IPI00002406*	*1*	*Type I membrane protein*
102	*Lutheran blood group*	*IPI00328869*	*1*	*Integral membrane protein*
	Lutheran blood group glycoprotein isoform 2 precursor	*IPI00554618*	*1*	*Integral membrane protein*
103	Similar to Lutheran blood group	Gi18589892	0	Integral membrane protein
104	Splice Isoform Short of Lymphocyte function-associated antigen 3 precursor	IPI00219549	2	Integral membrane protein
105	Lysozyme C precursor	IPI00019038	0	Extracellular
106	Membrane associated progesterone receptor component 2	IPI00005202	1	Integral membrane protein
107	Membrane protein	IPI00026111	2	Unclassified
108	Membrane transport protein XK	IPI00020896	9	Integral membrane protein
109	Mesenchymal stem cell protein DSCD75	IPI00010292	1	Unclassified
110	Microsomal glutathione S-transferase 3	IPI00639812	3	Integral membrane protein; Microsome
111	Monocarboxylate transporter 1	IPI00024650	11	Integral membrane protein
112	Splice Isoform Delexon-17 of P33527 Multidrug resistance-associated protein 1	IPI00008338	16	Integral membrane protein
113	Multidrug resistance-associated protein 4	IPI00006675	11	Integral membrane protein
114	Multidrug resistance-associated protein 5	IPI00385383	11	Integral membrane protein
115	Myeloblastin precursor	IPI00027409	0	Extracellular
116	Splice isoform 1 of Q92542 Nicastrin precursor	IPI00021983	0	Type I membrane protein
117	Neuropathy target esterase	IPI00217600	1	Unclassified
118	Splice Isoform 2 of Q14697 Neutral alpha-glucosidase AB precursor	IPI00011454	1	ER; Golgi
119	Splice Isoform 2 of Large neutral amino acids transporter small subunit 3	IPI00479732	11	Integral membrane protein
120	Novel protein	IPI00513701	3	Integral membrane protein
121	Splice isoform 1 of Q16563 Pantophysin	IPI00009507	3	Integral membrane protein
122	PB39	IPI00021075	12	Integral membrane protein

Appendix table 3. Continued

123	Phosphatidylinositol 4-kinase type II	IPI00020124	0	Integral membrane protein
124	*Phosphatidylinositol-4-phosphate 5-kinase type II alpha*	*IPI00009688*	*0*	*Integral membrane protein*
	Phosphatidylinositol-4-phosphate 5 kinase, type III	*Gi1730569*	*0*	*Integral membrane protein*
125	Phospholipid scramblase 1	IPI00005181	0	Type II membrane protein
126	Phospholipid scramblase 4	IPI00016776	0	Type II membrane protein
127	Splice Isoform B of P20020 Plasma membrane calcium-transporting ATPase 1	IPI00021695	7	Integral membrane protein
	Splice Isoform XB of Plasma membrane calcium-transporting ATPase 4	*IPI00217169*	*8*	*Integral membrane protein*
128	*Splice Isoform XD of P23634 Plasma membrane calcium-transporting ATPase 4*	*IPI00012490*	*8*	*Integral membrane protein*
129	Splice Isoform Delta of Poliovirus receptor related protein 1 precursor	IPI00003648	2	Integral membrane protein
130	Polyposis locus protein 1	IPI00024670	2	Integral membrane protein
131	Potassium channel subfamily K member 5	IPI00029507	6	Integral membrane protein
132	Presenilin-associated protein	Gi6409316	2	Integral membrane protein
133	Prolactin	IPI00022974	0	Extracellular binding RBC
134	Protein BAT5	IPI00033075	2	Integral membrane protein
135	Protein disulfide-isomerase precursor	IPI00010796	0	ER lumen, extracellular region
136	Protein FAM38A	IPI00006093	25	Unclassified
137	Protein transport protein SEC61 gamma subunit	IPI00006072	1	Integral membrane protein
138	RECS1 protein homolog	IPI00290452	7	Integral membrane protein
	Reticulon protein 3	*IPI00028946*	*3*	*Integral membrane protein*
139	*Reticulon 3 isoform a variant*	*IPI00555783*	*3*	*Membrane; Extracellular*
	RTN3-A1	*IPI00398795*	*3*	*Integral membrane protein*
	PREDICTED: similar to Reticulon protein 3	*IPI00177423*	*1*	*Integral membrane protein*
140	Splice Isoform 2 Of Reticulon 4	IPI00298289	1	Integral membrane protein
141	Rh blood CE group antigen polypeptide	IPI00039665	12	Integral membrane protein
	RhD protein	*IPI00329565*	*10*	*Integral membrane protein*
142	*Rhesus blood group D antigen*	*IPI00478119*	*10*	*Integral membrane protein*
	Rh blood D group antigen polypeptide	*Gi10800054*	*10*	*Integral membrane protein*
143	Rhesus D category VI type III protein	Gi2765839	12	Integral membrane protein

Appendix table 3. Continued

144	Rhesus blood group-associated glycoprotein	IPI00024094	11	Integral membrane protein
145	Rhesus blood group, CcEe antigens, isoform 1	IPI00465155	12	Integral membrane protein
146	Splice Isoform RHVIII of P18577 Blood group Rh(CE) polypeptide	IPI00221017	10	Integral membrane protein
147	Hypothetical protein FLJ45640 (Rhesus blood group, CcEe antigens)	IPI00444375	10	Integral membrane protein
148	Similar to RIKEN cDNA 1500009M05 gene	IPI00166865	1	Unclassified
149	PREDICTED: similar to RIKEN cDNA C730027E14	IPI00373867	1	Integral membrane protein
150	Secretory carrier-associated membrane protein 4	IPI00056310	4	Integral membrane protein
151	Semaphorin 7A precursor	IPI00025257	0	Integral membrane protein
152	Serum albumin precursor	IPI00022434	0	Extracellular
153	Signal peptidase complex subunit 1	IPI00219755	2	Integral membrane protein
154	Alpha-2,8-sialyltransferase 8C	Q64689	1	Inner cell membrane
155	Splice Isoform 2 of Sodium channel protein type I alpha subunit	IPI00216029	19	Integral membrane protein
156	Splice Isoform of Sodium/potassium-transporting ATPase alpha-1 chain	IPI00006482	10	Integral membrane protein
157	Sodium/potassium-transporting ATPase alpha-2 chain precursor	IPI00003021	8	Integral membrane protein
158	Splice Isoform Short of Sodium/potassium-transporting ATPase alpha-1 chain precursor	IPI00414005	4	Integral membrane protein
159	Solute carrier family 1 (glutamate transporter), member 7	IPI00100081	7	Integral membrane protein
160	Solute carrier family 12 member 5	IPI00301180	12	Integral membrane protein
161	solute carrier family 19 member 1 isoform b	IPI00299186	9	Integral membrane protein
162	Solute carrier family 2, facilitated glucose transporter, member 3, or 14	IPI00003909	10	Integral membrane protein
163	Solute carrier family 2, facilitated glucose transporter, member 4	IPI00027281	12	Integral membrane protein
	Solute carrier family 2, facilitated glucose transporter, member 1	*IPI00220194*	*12*	*Integral membrane protein*
164	*Glucose transporter type I*	*GiP11166*	*12*	*Integral membrane protein*
	Glucose transporter glycoprotein	*Gi3387905*	*8*	*Integral membrane protein*
165	Splice Isoform 1 of Q9Y666 Solute carrier family 12 member 7	IPI00008616	11	Integral membrane protein
166	Solute carrier family 27 (fatty acid transporter), member 4	IPI00021089	2	Integral membrane protein
167	Solute carrier family29 (nucleoside transporters), member 1	IPI00412547	11	Integral membrane protein
168	Solute carrier family 40, member 1	IPI00005547	10	Integral membrane protein
169	Solute carrier family 43, member 3	IPI00301100	11	Integral membrane protein
170	Stomatin	IPI00377081	1	Cytoskeleton

Appendix table 3. Continued

171	*Stomatin isoform a*	*IPI00219682*	*1*	*Integral membrane protein*
	Erythrocyte band 7 integral membrane protein (stomatin) (protein 7.2B)	*P27105*		*Integral membrane protein*
172	*Stromal cell-derived receptor-1 alpha*	*IPI00011578*	*1*	*Extracellular; Integral membrane protein*
	Stromal cell-derived receptor-1 beta	*IPI00018311*	*1*	*Extracellular; Integral membrane protein*
173	Surfeit 4	IPI00399142	2	Integral membrane protein; ER membrane
174	Syntaxin 4	IPI00029730	1	Type IV membrane protein
175	*Syntaxin 7*	*IPI00289876*	*1*	*Type IV membrane protein*
	Splice Isoform 2 of Syntaxin-7	*IPI00552913*	*0*	*Membrane; Cytoplasmic*
176	Splice Isoform I of P14209 T-cell surface glycoprotein E2 precursor	IPI00253036	2	Integral membrane protein
177	TGF-beta receptor type I precursor	P36897	2	Integral membrane protein
178	Thioredoxin domain containing protein 1 precursor Protein disulfide-isomerase A6 precursor	IPI00395887	3	ER lumen
179	Thioredoxin-like protein KIAA1162 precursor	IPI00100247	1	Type I membrane protein
180	Thrombospondin 1 precursor, glycoprotein IV, also in mature RBCs	IPI00296099	0	Extracellular region
181	Splice Isoform 1 Of Thyrotropin receptor precursor	IPI00028642	0	Integral membrane protein
182	TPR repeat containing protein	IPI00007052	1	Integral membrane protein
183	Transmembrane protein 24	IPI00394781	1	Integral membrane protein
184	Transmembrane protein Tmp21 precursor	IPI00028055	2	Type I membrane protein
185	Splice Isoform 2 of Transmembrane protein 55B	IPI00332278	2	Integral membrane protein
186	Triadin	IPI00220272	1	Integral membrane protein
187	UDP-glucose:glycoprotein glucosyltransferase 1precursor	IPI00024466	1	Integral membrane protein
188	Uncharacterised hematopoietic stem/progenitor cells protein MDS032	IPI00020515	1	Type II membrane protein
189	UPF0198 protein CGI-141	IPI00007061	3	Integral membrane protein
190	Urea transporter, erythrocyte	IPI00298337	8	Integral membrane protein
191	Vacuolar ATP synthase 16 kDa proteolipid subunit	IPI00018855	4	Integral membrane protein
192	Splice Isoform 1 of Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1	IPI00552514	7	Integral membrane protein

Appendix table 3. Continued

193	Vesicle trafficking protein SEC22b	IPI00006865	1	Type IV membrane protein
194	Vesicle-associated membrane protein-associated protein A isoform 2	IPI00170692	1	Type IV membrane protein
195	Vesicle-associated membrane protein-associated protein A isoform 1	IPI00374657	1	Membrane; Cytoskeleton
196	Splice Isoform 1 of O95292 Vesicle-associated membrane protein associated protein B/C	IPI00006211	1	Type IV membrane protein
197	Vesicle-associated membrane protein 2	Gi7657675	1	Type IV membrane protein
198	Vesicular integral-membrane protein VIP36 precursor	IPI00009950	1	Type I membrane protein
199	WNT-3 proto-oncogene protein [precursor]	P56703	1	Integral membrane protein
200	Splice Isoform 2 of Zinc finger DHHC domain containing protein 3	IPI00216069	4	Integral membrane protein
201	Zinc transporter 1	IPI00002483	6	Integral membrane protein
202	Zona pellucida binding protein	Gi5902116	1	Integral membrane protein

Appendix table 4 Total proteins identified from fetal NRBC membrane

(PM: Plasma membrane; MIM: mitochondrial inner membrane; MOM: mitochondrial outer membane; IMP: integral membrane proteins)

						TFE		MeOH		
No.	Protein Name	Accession Number	Protein MW	Protein PI	TMD	Peptide Count	Best Ion Score	Peptide Count	Best Ion Score	Subcellular location
1	Band 3 anion transport protein	IPI00022361	101727.41	5.08	11	15	149	13	178	PM
2	Histone H4	IPI00453473	11229.34	11.36	0	12	122	11	116	Nuclear
3	Ankyrin 1 isoform 1	IPI00216697	206136.92	5.65	0	9	104	6	81	PM
4	Hemoglobin epsilon chain	IPI00217471	16061.43	8.68	0	5	164	6	170	Cytosolic
5	Histone H2B.n	IPI00152785	13766.52	10.32	0	6	146	9	128	Nuclear
6	ADP/ATP translocase 3	IPI00291467	32714.15	9.76	2	7	101	5	102	MIM; PM
7	Ferritin light chain	IPI00375676	28399.25	6	0	4	134	3	104	Cytosolic
8	Solute carrier family 2, facilitated glucose transporter member 1	IPI00220194	54082.52	8.93	12	6	83	8	92	PM
9	Transferrin receptor protein 1	IPI00022462	84847.95	6.18	1	5	96	1	55	PM
10	Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor	IPI00305383	48412.88	8.74	0	4	129	2	127	MIM
11	Microsomal glutathione S-transferase 3	IPI00639812	18404.62	9.99	3	4	117	4	91	Microsome membrane; ER
12	Calnexin precursor	IPI00020984	67525.85	4.47	1	5	103	4	89	ER membrane
13	25 kDa protein	IPI00646289	25141.15	8.93	1	4	113	3	77	Unclassified
14	Splice Isoform Short of Erythrocyte membrane protein band 4.2	IPI00028614	76793.61	8.27	0	4	100	4	89	PM
15	Histone H2A.z	IPI00218448	13413.51	10.58	0	4	87	4	83	Nuclear
16	H3 histone, family 3B	IPI00219038	15318.50	11.27	0	5	85	6	147	Nuclear
17	Splice Isoform B of Phosphate carrier protein	IPI00215777	39932.64	9.43	2	4	111	1	41	MIM
18	Hypothetical protein	IPI00646240	7390.90	9.86	0	3	112	4	88	Nuclear
19	NAD(P)H: quinone oxidoreductase type 3, polypeptide A2 variant	IPI00470674	34073.18	9.41	1	4	98	3	106	ER; MOM; Membrane; Cytosolic
20	Splice Isoform H14 of Myeloperoxidase precursor	IPI00236554	73806.61	9.3	0	4	79	8	77	Lysosome; Nuclear
21	40S ribosomal protein S7	IPI00013415	22113.26	10.09	0	3	100	6	89	Ribosomal

Appendix table 4. Continued

22	Ribosomal protein L5 variant	IPI00647085	34340.69	9.73	0	3	131	5	72	Ribosomal
23	Fibrillarin	IPI00025039	33763.42	10.18	0	3	94	6	72	Nuclear
24	NADH-ubiquinone oxidoreductase 39 kDa subunit, mitochondrial precursor	IPI00003968	42482.57	9.81	0	3	100	2	91	Mitochondrial
25	PREDICTED: similar to ribosomal protein L18a	IPI00176629	20753.89	10.73	0	4	71	4	49	Ribosomal
26	60S ribosomal protein L26	IPI00027270	17247.53	10.55	0	3	84	1	44	Ribosomal
27	H2B/t variant	IPI00454695	21458.16	10.71	0	3	84	4	83	Nuclear
28	Coproporphyrinogen III oxidase, mitochondrial precursor	IPI00093057	50119.97	8.59	0	4	56	4	59	Mitochondrial
29	Splice isoform short of Heterogenous nuclear ribonucleoprotein U	IPI00386491	88890.16	5.6	0	3	86	6	123	Nuclear
30	Cytochrome c oxidase polypeptide Va, mitochondrial precursor	IPI00025086	16763.72	6.3	0	2	85	2	56	MIM
31	Lamin B receptor variant	IPI00645733	70651.06	9.41	8	3	89	3	67	Nuclear inner membrane.
32	NADH-cytochrome b5 reductase	IPI00328415	34081.68	7.31	0	3	86	2	76	ER; MOM; membrane; Cytosolic
33	HNRPC protein	IPI00552125	27804.46	4.55	0	2	80	1	59	Nuclear
34	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 1	IPI00554481	71079.20	4.84	1	2	85	4	64	PM
35	ATP-binding cassette half-transporter	IPI00465442	99649.17	9.26	9	4	52	2	59	PM
36	Equilibrative nucleoside transporter 1	IPI00550382	58924.58	8.49	11	2	79	2	52	PM
37	Mitochondrial 2-oxoglutarate/malate carrier protein	IPI00219729	33908.81	9.92	0	2	84	1	61	MIM
38	Nucleolar protein Nop56	IPI00411937	66194.78	9.21	0	2	86	3	61	Nuclear
39	Cytochrome c1, heme protein, mitochondrial precursor	IPI00029264	35367.00	9.15	0	2	110	1	75	Mitochondrial membrane
40	60S ribosomal protein L27	IPI00219155	15656.71	10.56	0	2	68	3	63	Ribosomal
41	Spectrin beta isoform a	IPI00216704	267589.56	5.24	0	2	90	5	62	PM
42	Ribosomal protein L27a	IPI00456758	16468.03	11	0	2	76	2	93	Ribosomal
43	Leukocyte elastase precursor	IPI00027769	28499.79	9.71	1	1	123	1	101	PM
44	Splice isoform 2 of Reticulon 4	IPI00298289	40292.95	4.71	2	1	117	1	52	ER membrane
45	60S ribosomal protein L3	IPI00550021	45948.72	10.19	0	2	73	6	96	Ribosomal
46	Membrane protein	IPI00026111	21161.16	9.77	2	1	110	1	65	Unclassified
47	40S ribosomal protein S4, X isoform	IPI00217030	29448.01	10.16	0	2	73	2	56	Ribosomal
48	H2A histone family, member Y isoform 1	IPI00148096	39159.18	9.83	0	1	103	3	97	Nuclear
49	Ubiquinol-cytochrome c reductase complex 14 kDa protein	IPI00220416	13390.94	8.75	0	2	59	3	81	MIM

Appendix table 4. Continued

50	CAAX prenyl protease 1 homolog	IPI00027180	54777.53	7.12	7	1	97	1	93	ER/Golgi; PM membrane; Cytosolic;
51	Splice Isoform 2 of Heat shock cognate 71 kDa protein	IPI00037070	53887.70	5.74	0	2	51	2	46	Nuclear
52	18 kDa protein	IPI00514123	17639.75	10.86	0	2	61	3	58	Ribosomal
53	Membrane associated progesterone receptor component 2	IPI00005202	23803.73	4.76	1	1	94	1	44	Microsome membrane
54	Splice Isoform 2 of 40S ribosomal protein S24	IPI00219486	15059.24	10.89	0	1	91	1	79	Ribosomal
55	Growth-inhibiting protein 12	IPI00646848	78334.00	8.54	0	1	91	1	84	PM
56	Thioredoxin domain containing protein 1 precursor	IPI00395887	31770.80	4.92	3	1	85	1	65	ER membrane
57	ATP synthase beta chain, mitochondrial precursor	IPI00303476	56524.60	5.26	0	1	82	1	49	MOM
58	Ubiquinol-cytochrome c reductase complex ubiquinone-binding protein QP-C	IPI00024742	9769.08	10.08	0	1	82	1	42	MIM
59	60S ribosomal protein L28	IPI00182533	15606.63	12.02	0	1	81	1	74	Ribosomal
60	Ubiquinol-cytochrome-c reductase complex core protein I, mitochondrial precursor	IPI00013847	52585.42	5.94	0	2	48	4	58	MIM
61	RAB14, member RAS oncogene family	IPI00646415	20396.31	5.94	0	1	78	1	38	Universal
62	Novel protein	IPI00640179	11673.18	10.21	0	1	74	1	69	Unclassified
63	Cathepsin G precursor	IPI00028064	28819.07	11.19	0	1	73	1	45	PM; Intermediate filament
64	Eosinophil peroxidase precursor	IPI00006690	80989.13	10.31	0	2	38	2	41	Lysosome; Nuclear ;
65	Thioredoxin-like protein KIAA1162 precursor	IPI00100247	38927.68	4.31	1	1	70	1	90	PM
66	Splice Isoform short of Splicing factor, proline-and glutamine-rich	IPI00216613	72217.75	9.26	0	1	68	1	72	Nuclear
67	Splice Isoform 4 of Plasminogen activator inhibitor 1 RNA-binding protein	IPI00412714	42401.27	8.42	0	1	66	2	49	Unclassified
68	ATP synthase g chain, mitochondrial	IPI00027448	11421.24	9.65	0	1	62	1	58	Mitochondrial
69	Cytochrome c	IPI00465315	11610.09	9.59	0	1	62	1	38	MIM
70	GSTK1 protein	IPI00440703	31545.51	8.85	0	1	61	1	75	Mitochondrion
71	Kell blood group glycoprotein	IPI00220459	82770.92	8.09	1	1	53	2	93	PM
72	50 kDa protein	IPI00396485	50138.50	7.26	0	1	69	1	54	Nuclear; Cytosolic
73	Histone H1x	IPI00021924	22473.53	10.76	0	1	52	1	79	Nuclear
74	DEK protein	IPI00020021	42647.92	8.69	0	1	49	3	53	Nuclear

Appendix table 4. Continued

75	Splice isoform 1 of Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1	IPI00552514	96350.11	6.02	7	1	47	1	54	Vesicle membrane; PM
76	Olfactory receptor 11H4	IPI00168981	36861.19	9.05	7	1	47	1	39	PM
77	Solute carrier family 43, member 3	IPI00301100	54494.25	8.71	11	1	47	1	54	ER; PM
78	ATP-binding cassette sub-family D member 3	IPI00002372	75427.57	9.41	3	1	44	2	64	Perixisome membrane
79	PRA1 family protein 3	IPI00007426	21600.41	9.77	3	1	43	1	49	ER Membrane
80	Suppressor of actin 1	IPI00022275	66908.01	6.66	2	1	45	1	39	ER membrane; Golgi
81	HSPC051	IPI00100810	14391.31	10.96	0	1	41	1	40	Mitochondrial membrane
82	Ribosomal protein S29 isoform 2	IPI00639942	8081.99	10.08	0	1	39	1	39	Ribosomal
83	H2A histone family, member J, isoform 2	IPI00220855	14010.93	10.9	0	7	132			Nuclear
84	Histone H2A.q	IPI00339274	13848.80	10.9	0	7	121			Nuclear
85	ADP/ATP translocase 2	IPI00007188	32743.13	9.76	2	7	101			MIM
86	ATP-binding cassette sub-family B member 10, mitochondrial precursor	IPI00015826	79048.95	9.91	5	5	72			MIM
87	B-cell receptor-associated protein BAP37	IPI00027252	33275.92	9.83	0	5	56			Mitochondrial membrane
88	HP1-BP74	IPI00549250	61169.27	9.69	0	3	111			Nuclear
89	SAM50-like protein CGI-51	IPI00412713	51929.30	6.44	0	3	93			MOM
90	40S ribosomal protein S8	IPI00216587	24059.12	10.32	0	4	88			Ribosomal
91	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor	IPI00025874	68526.81	5.96	1	3	93			ER membrane
92	Tubulin beta-2 chain	IPI00011654	49638.97	4.78	0	3	95			Cytosolic
93	Splice Isoform 3 of Mitochondrial inner membrane protein	IPI00470829	79977.51	6.31	0	3	69			MIM
94	Protoporphyrinogen oxidase	IPI00031357	50733.65	8.44	0	3	81			Mitochondrial
95	Splice Isoform 2 of Voltage-dependent anion-selective channel protein 3	IPI00294779	30770.33	8.85	0	2	100			MOM
96	Splice Isoform 2 of Voltage-dependent anion-selective channel protein 2	IPI00216024	35111.16	5.87	0	2	117			MOM
97	PREDICTED: similar to ribosomal protein S3a	IPI00472119	29951.83	9.78	0	3	64			Ribosomal
98	ATP synthase alpha chain, mitochondrial precursor	IPI00440493	59713.59	9.16	0	3	69			MOM
99	Splice Isoform 2 of Protein 4.1	IPI00218697	93181.33	5.41	0	3	58			PM
100	16 kDa protein	IPI00334432	15532.11	8.76	0	3	144			Cytosolic

Appendix table 4. Continued

101	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b isoform 2	IPI00456746	17451.13	9.36	0	2	95	Mitochondrial
102	Heterogeneous nuclear ribonucleoprotein M isoform b	IPI00383296	73572.36	8.94	0	2	109	Nuclear
103	Mitochondrial substrate carrier family protein	IPI00383240	29874.04	9.66	0	3	59	MIM
104	ATP synthase, H+ transporting, subunit f isoform 2a variant	IPI00220300	10910.70	9.7	1	2	103	Mitochondrial
105	Splice Isoform 2 of Retinol dehydrogenase 11	IPI00339385	33981.68	8.95	0	2	101	ER membrane
106	Probable DNA dC->dU editing enzyme APOBEC-3C	IPI00555878	22810.96	7.52	0	2	79	Unclassified
107	Similar to Cytochrome b5 outer mitochondrial membrane isoform precursor	IPI00641334	14163.00	5.06	0	2	86	MOM
108	Splice Isoform A of Protein C20orf108	IPI00096986	20411.22	10.45	3	2	89	ER membrane; mitochondrial; Microsome
109	OTTHUMP00000028841	IPI00478327	22617.43	10.43	0	2	96	Ribosomal
110	Hypothetical protein FLJ35097	IPI00554589	48583.91	8.96	0	1	133	Mitochondrial
111	Transmembrane protein 14C	IPI00009346	11556.97	9.87	4	2	93	ER/Golgi membrane
112	Tricarboxylate transport protein, mitochondrial precursor	IPI00639810	35972.12	10.12	0	3	60	MIM
113	NADH-ubiquinone oxidoreductase 30 kDa subunit, mitochondrial precursor	IPI00025796	30222.71	6.99	0	2	74	MIM
114	60S ribosomal protein L10	IPI00554723	24429.82	10.11	0	2	65	Ribosomal
115	21 kDa protein	IPI00398234	20886.74	9.46	0	2	60	Ribosomal
116	50 kDa protein	IPI00180730	50121.92	9.06	0	2	79	Ribosomal
117	Rhesus blood group, CcEe antigens, isoform 1	IPI00465155	45421.20	9.4	12	2	61	PM
118	Mitochondrial carrier homolog 2	IPI00003833	33308.86	8.25	0	2	55	MIM
119	HLA-B associated transcript 1 variant	IPI00641829	48987.98	5.51	0	2	55	Nuclear
120	Splice Isoform Heart of ATP synthase gamma chain, mitochondrial precursor	IPI00395769	32860.24	9.31	0	2	55	MIM
121	40S ribosomal protein S11	IPI00025091	18418.99	10.31	0	2	60	Ribosomal
122	10 kDa protein	IPI00186338	9733.91	5.81	0	1	90	Nuclear
123	17 kDa protein	IPI00642218	16710.62	6.49	3	1	92	ER membrane
124	Mitochondrial transmembrane GTPase FZO-2	IPI00293073	86938.53	5.99	0	2	61	Mitochondrial membrane
125	Cytochrome c oxidase subunit 2	IPI00017510	25548.21	4.67	2	1	90	MIM
126	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d isoform b	IPI00456049	15763.17	6.6	0	2	57	Mitochondrial

Appendix table 4. Continued

127	Myeloblastin precursor	IPI00027409	27789.27	8.72	0	2	52	PM; Cytosolic
128	Steroid dehydrogenase homolog	IPI00007676	34328.24	9.34	3	1	87	Unclassified
129	15 kDa protein	IPI00477513	15095.99	7.9	0	4	134	Cytosolic
130	40S ribosomal protein S26	IPI00186712	12876.04	11.01	0	1	87	Ribosomal
131	SM-11044 binding protein	IPI00644458	29918.72	5.52	1	2	48	Endosome membrane
132	Voltage-dependent anion-selective channel protein 1	IPI00216308	30622.53	8.63	0	2	50	MOM; PM
133	Ras-related protein Rab-7	IPI00016342	23474.84	6.4	0	1	79	Endosome; Golgi membrane
134	Splice Isoform GN-1S of Glycogenin-1	IPI00216115	29070.36	4.73	0	1	78	Cytosolic
135	Optic atrophy 1 isoform 5	IPI00107750	113445.91	7.63	0	2	43	Mitochondrial
136	40S ribosomal protein S16	IPI00221092	16304.00	10.21	0	1	75	Ribosomal
137	KIAA0102 protein	IPI00452747	14159.28	7.85	1	2	38	ER/Microsome membrane
138	Protein expressed in T-cells and eosinophils in atopic dermatitis	IPI00172656	52590.54	5.46	0	1	73	Cytosolic
139	Azurocidin precursor	IPI00022246	26868.65	9.75	0	1	75	PM; Cytosolic
140	Pre-mRNA processing splicing factor 8	IPI00007928	273426.56	8.95	0	2	40	Nuclear
141	Protein	IPI00411968	17775.75	9.13	0	1	64	Nuclear
142	Vitamin K epoxide reductase complex subunit 1-like protein 1	IPI00166079	19822.68	9.28	2	1	73	Unclassified
143	CDNA FLJ90376 fis, clone NT2RP2004794, highly similar to Butyrate- induced transcript 1	IPI00385976	21254.36	9.74	4	1	72	ER membrane
144	60S ribosomal protein L15	IPI00470528	24000.04	11.62	0	2	43	Ribosomal
145	Sodium/potassium-transporting ATPase beta-3 chain	IPI00008167	31492.09	8.58	1	1	71	PM
146	55 kDa erythrocyte membrane protein	IPI00215610	52263.65	6.91	0	1	70	PM
147	Splice isoform XB of Plasma membrane calcium-transporting ATPase 4	IPI00217169	133845.70	6.04	8	2	41	PM
148	Heterogeneous nuclear ribonucleoprotein L isoform b	IPI00465225	50528.51	7.22	0	1	70	Nuclear
149	12 kDa protein	IPI00644559	12027.62	6.81	0	1	70	Mitochondrial
150	NADH dehydrogenase	IPI00643646	52526.70	7.21	0	1	69	Mitochondrial
151	PREDICTED: similar to hypothetical protein	IPI00455976	27850.92	10.36	0	1	69	Ribosomal
152	Ras-related protein Rab-5A	IPI00023510	23643.82	8.32	0	1	69	Golgi membrane;
153	Neutral amino acid transporter B	IPI00019472	56576.27	5.34	9	1	68	PM
154	Splice isoform 2 of Protein GPR107 precursor	IPI00382815	61936.41	6.72	7	1	68	PM

Appendix table 4. Continued

155	High mobility group protein 2	IPI00219097	23887.68	7.77	0	1	67	Nuclear
156	PREDICTED: similar to RPL7L1 protein	IPI00455892	59708.63	8.4	0	2	36	Ribosomal
157	Golgi-associated plant pathogenesis-related protein 1	IPI00007067	17076.48	9.44	0	1	66	Golgi membrane; Extracellular Endosome; Golgi
158	Ras-related protein Rab-10	IPI00016513	22526.59	8.59	0	1	65	membrane;
159	ATP synthase F0 subunit 6	IPI00549893	24735.88	10.09	6	1	65	MIM
160	Thyroid hormone receptor-associated protein complex 150 kDa component	IPI00104050	108629.04	10.16	0	1	65	Nuclear
161	Phosphatidylserine synthase 1	IPI00010746	55491.10	8.71	9	2	33	Mitochondrial membrane
162	Splice Isoform 2 of Synaptophysin-like protein	IPI00335277	26394.49	6.79	3	1	64	Vesicle membrane; PM
163	Ras-related protein Rab-21	IPI00007755	24201.21	8.16	0	1	62	Endosome; Golgi membrane
164	Splice Isoform 2 of DNA-dependent protein kinase catalytic subunit	IPI00376215	465202.00	6.81	0	1	64	Nuclear
165	Splice Isoform 2 of Translocon-associated protein alpha subunit precursor	IPI00449669	29355.03	4.52	1	1	63	ER membrane
166	SLC27A2 protein	IPI00447606	64574.28	8.73	1	1	61	ER/peroxisome membrane
167	PREDICTED: similar to ribosomal protein S2	IPI00165486	29940.93	10.01	0	1	61	Ribosomal
168	Hypothetical protein DKFZp586C1924	IPI00031064	21513.48	9.36	2	1	61	Unclassified
169	Hypothetical protein DKFZp686L18234	IPI00414000	37566.79	9.45	0	1	59	Unclassified
170	Synaptosomal-associated protein 29	IPI00032831	28952.59	5.56	0	1	59	PM; Synaptosome
171	60S ribosomal protein L9	IPI00031691	21849.80	9.96	0	1	58	Ribosomal
172	Zn-finger, RING domain containing protein	IPI00414712	35574.19	8.54	6	1	57	Microsomal membrane
173	Mitochondrial carrier homolog 1 isoform b	IPI00386258	41517.29	9.4	2	1	57	Mitochondrial membrane; Cytosolic
174	13 kDa protein	IPI00374249	12579.21	10.85	0	1	57	Ribosomal
175	Splice Isoform 2 of NADH-ubiquinone oxidoreductase 51 kDa subunit, mitochondrial precursor	IPI00221298	49836.33	8.51	0	1	56	MIM

Appendix table 4. Continued

176	Hypothetical protein FLJ14938	IPI00024642	55838.31	4.76	0	1	56	Unclassified
177	FLJ00144 protein	IPI00383085	28437.22	5.75	0	1	54	Unclassified
178	Prohibitin	IPI00017334	29785.90	5.57	0	1	54	mitochondrial membrane
179	Aquaporin 1 splice variant 2	IPI00428490	14913.58	5.48	1	1	53	PM
180	BCG induced integral membrane protein BIGM103	IPI00034208	49598.38	5.71	7	1	53	PM
181	48 kDa protein	IPI00471915	47536.39	11.07	0	2	52	Ribosomal
182	Hypothetical protein FLJ90397	IPI00171459	36994.10	8.88	0	1	52	Unclassified
183	FLJ12442 protein	IPI00100938	54142.43	6.47	0	1	52	Cytosolic
184	Actin, cytoplasmic 1	IPI00021439	41709.73	5.29	0	1	52	Cytosolic
185	RCC2 protein	IPI00465044	56049.20	9.02	0	1	51	Nuclear
186	Calreticulin precursor	IPI00020599	48111.82	4.29	0	1	52	Cytosolic
187	Similar to Translocon-associated protein, delta subunit precursor	IPI00644824	13270.66	6.48	1	1	51	ER membrane
188	Proliferation-associated 2G4, 38kDa	IPI00299000	43785.20	6.13	0	1	50	Nuclear
189	ATPase, H+ transporting, lysosomal V0 subunit a isoform 2	IPI00419696	98018.34	6.18	6	1	49	Vacuolar membrane; lysosome
190	Ubiquitin-specific protease 31	IPI00328815	118956.15	5.75	1	1	48	Nuclear
191	Der1-like domain containing protein	IPI00643745	21238.77	9.69	4	1	48	ER membrane
192	Phytanoyl-coa hydroxylase	IPI00514332	34049.12	9.02	0	1	49	Peroxisome
193	130 kDa leucine-rich protein	IPI00329745	158825.66	5.91	0	1	48	Nuclear; Cytosolic
194	Hypothetical protein FLJ13940	IPI00017454	27533.68	7.71	0	1	47	Cytosolic
195	ATP synthase e chain, mitochondrial	IPI00218848	7797.28	9.34	1	1	47	Mitochondrial membrane
196	PREDICTED: similar to dJ753D5.2	IPI00397441	13749.95	9.36	0	1	47	Ribosomal
197	Erythrocyte band 7 integral membrane protein	IPI00219682	31579.70	7.9	1	1	47	PM
198	Hypothetical protein MGC14288	IPI00176708	6595.25	9.58	1	1	46	Unclassified
199	Peroxisomal membrane protein 2	IPI00221002	22106.98	10.58	4	1	46	Peroxisome membrane
200	ALEX3 protein variant	IPI00604615	42443.90	8.75	1	1	46	Unclassified
201	Adaptor-related protein complex 2, mu 1 subunit isoform b	IPI00619900	49357.91	9.57	0	1	46	Vesicle/Golgi membrane; Cytosolic;
202	Mitochondrial aspartate-glutamate carrier protein	IPI00007084	74256.70	8.79	0	1	45	MIM; PM

Appendix table 4. Continued

203	NADH dehydrogenase subunit 5	IPI00221391	66968.42	9.14	15	1	45			MIM
204	Isoform 2 of Solute carrier family 22, member 11	IPI00418660	48269.11	8.88	6	1	44			PM
205	Splice isoform Glycophorin D of Glycophorin C	IPI00218128	11491.61	4.76	1	1	44			PM
206	Isocitrate dehydrogenase [NADP], mitochondrial precursor	IPI00011107	50876.86	8.88	0	1	44			Mitochondrial
207	Novel protein	IPI00513768	52673.49	9.76	0	1	43			Unclassified
208	Heparan sulfate 2-O-sulfotransferase 1	IPI00549891	41844.40	8.83	0	1	43			Nuclear; Golgi; IMP
209	9 kDa protein	IPI00376529	9459.86	6.75	1	1	43			Mitochondrial membrane
210	NADH-ubiquinone oxidoreductase B15 subunit	IPI00220059	15068.03	9.85	1	1	42			MIM
211	Metaxin 1	IPI00013678	51444.59	9.8	1	1	42			MOM; IMP
212	Guanine nucleotide-binding protein, alpha-13 subunit	IPI00290928	44021.66	8.11	0	1	42			PM
213	13kDa differentiation-associated protein	IPI00604532	17073.62	9.63	0	1	42			Mitochondrial
214	Full-length cDNA clone CS0DC006YH13 of Neuroblastoma of Homo sapiens	IPI00009247	8609.50	9.94	1	1	41			Mitochondrial
215	Splice isoform 1 of Heterogeneous nuclear ribonucleoprotein D0	IPI00028888	38410.30	7.62	0	1	41			Nuclear
216	Hypothetical protein FLJ32830	IPI00065287	65726.57	9.35	0	1	41			Unclassified
217	Protein p65	IPI00014168	68575.97	9.67	0	1	41			Nuclear
218	46 kDa protein	IPI00335130	46266.42	7.28	0	1	41			Unclassified
219	Cleft lip and palate transmembrane protein 1	IPI00396411	76048.46	5.88	5	1	40			PM
220	Splice isoform 1 of Protein C9orf5	IPI00607576	100881.01	9.03	14	1	40			PM
221	Novel protein	IPI00642244	109351.93	7.94	0	1	40			ER
222	Mitochondrial ribosomal protein L41	IPI00217553	15372.99	9.58	0	1	40			Ribosomal
223	Splice isoform B of Chloride channel protein 6	IPI00305252	35910.30	8.03	3	1	41			PM
224	Splice isoform 1 of Protein C20orf22	IPI00394779	45068.18	8.87	1	1	40			Unclassified
225	Kelch domain containing protein 1	IPI00383231	46661.73	5.67	0	1	39			Nuclear; Cytosolic
226	P450-like protein	IPI00419579	52399.01	5.84	0	1	39			Cytosolic
227	Histone H2A.o	IPI00216457	13955.85	10.9	0			7	125	Nuclear
228	Histone H1.2	IPI00217465	21220.71	10.94	0			6	111	Nuclear
229	Histone H1.4	IPI00217467	21720.96	11.03	0			5	113	Nuclear
230	Histone H1.5	IPI00217468	22435.43	10.91	0			5	112	Nuclear
231	Ubiquitin and ribosomal protein S27a	IPI00179330	17893.44	9.65	0			5	159	Ribosomal
232	Histone H1.3	IPI00217466	22205.28	11.02	0			5	103	Nuclear

Appendix table 4. Continued

233	Histone H2A	IPI00031562	14112.93	11.05	0	7	121	Nuclear
234	Histone H2B.j	IPI00303133	13752.50	10.32	0	9	128	Nuclear
235	Histone H1.1	IPI00217469	21697.83	10.99	0	4	112	Nuclear
236	Ribosomal protein S8	IPI00645201	21866.01	10.37	0	4	104	Ribosomal
237	Hemoglobin gamma-2 chain	IPI00554676	15985.25	6.71	0	4	146	Cytosolic
238	Alpha 2 globin variant	IPI00410714	15270.94	8.72	0	5	106	Cytosolic
239	Hemogen	IPI00464963	55278.74	4.82	0	4	101	Golgi; Nuclear
240	60S ribosomal protein L19	IPI00025329	23451.25	11.48	0	4	89	Ribosomal
241	HNRPR protein	IPI00644055	71170.40	8.23	0	4	73	Nuclear
242	Ribosomal protein L10 variant	IPI00641164	24526.87	10.11	0	5	67	Ribosomal
243	60S ribosomal protein L17	IPI00413324	21252.29	10.18	0	3	71	Ribosomal
244	OTTHUMP00000016816	IPI00402185	62617.34	7.18	0	3	73	Nuclear; Cytosolic
245	Splice Isoform 2 of Bromodomain adjacent to zinc finger domain protein 1B	IPI00216695	170340.38	8.7	0	4	55	Nuclear
246	ATP synthase, H+ transporting, mitochondrial F0 complex	IPI00220487	18479.50	5.21	0	3	90	MIM
247	25 kDa protein	IPI00413986	24735.28	9.8	0	2	81	Ribosomal
248	Novel protein similar to histone 2, H3c	IPI00455457	15420.55	11.27	0	3	47	Nuclear
249	PREDICTED: similar to 60S ribosomal protein L32	IPI00455900	52270.86	10.1	0	1	118	Ribosomal
250	Splice Isoform 2 of Syntaxin-7	IPI00552913	27383.66	5.02	0	2	66	Endosome membrane
251	60S ribosomal protein L35	IPI00412607	14411.52	11.04	0	1	108	Ribosomal
252	OTTHUMP00000016319	IPI00176681	12179.15	9.3	0	2	76	MOM
253	50 kDa protein	IPI00174849	49540.60	4.75	0	2	80	Mitochondrial
254	Cytochrome c oxidase polypeptide VIb	IPI00216085	10054.68	6.78	0	2	75	Mitochondrial Membrane;
255	Hypothetical protein DKFZp564K247	IPI00295621	10137.19	9.52	2	1	100	IMP
256	ATP-dependent RNA helicase DDX18	IPI00301323	75359.44	9.52	0	2	60	Nuclear
257	Splice Isoform 2 of U1 small nuclear ribonucleoprotein 70 kDa	IPI00219483	50587.32	9.9	0	2	53	Nuclear
258	Histone H1.0	IPI00550239	20719.15	10.84	0	1	91	Nuclear
259	PREDICTED: similar to 60S ribosomal protein L21	IPI00374234	10973.01	10.97	0	2	51	Ribosomal
260	18 kDa protein	IPI00176755	17696.17	10.44	0	2	44	Ribosomal
261	SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily A me	IPI00297211	121828.36	8.27	0	1	81	Nuclear

Appendix table 4. Continued

262	Beta-globin gene from a thalassaemia patient, complete cds	IPI00382950	18918.59	6.28	0	2	50	Cytosolic
263	Hemoglobin delta chain	IPI00473011	15914.25	7.97	0	2	50	Cytosolic
264	CAG-ISL 7	IPI00069693	23417.02	10.94	0	2	45	Ribosomal
265	Hypothetical protein FLJ32119	IPI00065554	62438.95	9.43	0	1	73	Cytosolic
266	Splice Isoform 2 of H/ACA ribonucleoprotein complex subunit 1	IPI00607820	20821.40	10.45	0	1	71	Nuclear
267	60S ribosomal protein L7	IPI00030179	29207.20	10.66	0	2	102	Ribosomal
268	60S ribosomal protein L22	IPI00219153	14646.76	9.22	0	1	70	Ribosomal
269	HEAT repeat containing protein	IPI00549664	105626.65	9.45	0	1	69	Nuclear
270	Ribosomal protein L15	IPI00375511	24071.05	11.62	0	2	36	Ribosomal
271	Splice Isoform short of Prostaglandin G/H synthase 1 precursor	IPI00298268	64440.72	7.9	0	2	39	Microsome membrane; Cytosolic;
272	PREDICTED: similar to 40S ribosomal protein S16	IPI00397701	16401.85	10.05	0	1	64	Ribosomal
273	NADH-ubiquinone oxidoreductase MWFE subunit	IPI00005695	8067.13	8.93	1	1	64	Mitochondrial Membrane
274	Splice Isoform 2 of Chromodomain helicase-DNA-binding protein 4	IPI00455210	220694.56	5.7	0	1	64	Nuclear
275	NADH-ubiquinone oxidoreductase 15 kDa subunit	IPI00220063	12378.35	9.29	0	1	63	Mitochondrial Membrane
276	High mobility group protein 4	IPI00217477	22834.40	8.5	0	2	31	Nuclear
277	NADH dehydrogenase	IPI00604684	30141.58	6.25	0	1	60	Mitochondrial
278	Stromal cell-derived receptor-1 beta	IPI00018311	44359.50	8.11	1	1	59	PM
279	EH-domain containing protein 3	IPI00021458	61857.09	6.06	0	1	57	Nuclear
280	Protein C10orf70	IPI00020510	12191.20	9.19	1	1	56	Mitochondrial
281	Splice Isoform 2 of Putative RNA-binding protein Luc7-like 2	IPI00216804	46396.71	10.1	0	1	56	Nuclear
282	Antibacterial protein FALL-39 precursor	IPI00292532	19578.27	9.48	1	1	55	PM
283	Flap endonuclease-1	IPI00026215	42566.10	8.8	0	2	27	Nuclear
284	Hypothetical protein FLJ31842	IPI00043429	30020.92	9.4	6	1	54	PM
285	40S ribosomal protein S15	IPI00216153	16898.13	10.39	0	2	27	Ribosomal
286	Hypothetical protein FLJ14347	IPI00022300	28266.57	8.7	1	1	53	Unclassified
287	NADH-ubiquinone oxidoreductase B22 subunit	IPI00255052	21685.80	8.59	0	1	54	Mitochondrial Membrane
288	Splice Isoform 2 of N-acylneuraminate cytidylyltransferase	IPI00479958	29543.15	8.59	0	1	52	Unclassified
289	NADH-ubiquinone oxidoreductase 23 kDa subunit, mitochondrial precursor	IPI00010845	23689.62	6	0	1	51	Mitochondrial Membrane

Appendix table 4. Continued

290	Dihydroorotate dehydrogenase isoform 2 precursor	IPI00607764	22006.30	9.27	1	1	50	MIM
291	Vesicle-associated membrane protein 2	IPI00553138	12509.63	7.82	1	1	50	Vesicle membrane; Synapse; Mitochondrial
292	NLN protein	IPI00025564	70259.06	8.42	0	1	50	Mitochondrial
293	Splice Isoform 2 of Myb-binding protein 1A	IPI00607584	149273.88	9.34	0	1	49	Nucleus; Cytosolic
294	Splice Isoform A of Band 4.1-like protein 3	IPI00032230	120603.14	5.09	0	1	48	PM protein; Cytoskeleton
295	65 kDa protein	IPI00478631	64667.14	9.8	0	1	47	Nuclear
296	ATPase subunit 8	IPI00641145	7972.15	9.93	1	1	47	Mitochondrial membrane
297	58 kDa protein	IPI00556310	57968.46	10.11	0	1	47	Ribosomal
298	Hypothetical protein DKFZp313B047	IPI00013452	170483.09	7.02	0	1	45	Cytosolic
299	Splice Isoform 1 of Voltage-dependent anion-selective channel protein 3	IPI00031804	30639.28	8.85	0	1	46	MOM
300	8 kDa protein	IPI00639803	8078.35	9.01	2	1	45	Unclassified
301	TUBA6 protein	IPI00166768	36624.66	8.2	0	1	45	Cytosolic
302	Splice Isoform long of Dematin	IPI00292290	45486.22	8.94	0	1	45	PM; Cytoskeleton
303	40S ribosomal protein S19	IPI00215780	15919.49	10.31	0	1	44	Ribosomal
304	Stomatin	IPI00377081	13466.03	7.82	1	1	43	PM
305	ATP synthase, H+ transporting, mitochondrial F1 complex	IPI00549805	59771.60	9.07	0	1	42	MIM
306	Sec61 alpha 1 subunit	IPI00218466	52230.51	8.3	10	1	41	ER membrane
307	Ferritin heavy chain	IPI00419501	25207.52	6.33	0	1	41	PM
308	Vesicular integral-membrane protein VIP36 precursor	IPI00009950	40203.10	6.46	1	1	41	ER membrane; Golgi
309	Cell death-regulatory protein GRIM19	IPI00219685	25804.38	9.82	1	1	40	MIM
310	40S ribosomal protein S9	IPI00221088	22446.51	10.66	0	1	40	Ribosomal
311	Pol protein	IPI00386255	97621.79	8.89	0	1	39	Cytosolic
312	Similar to Telomeric repeat binding factor 2	IPI00216552	32277.72	9.6	0	1	37	Nuclear
313	Ras-related protein Rap-1A	IPI00019345	20973.71	6.38	0	1	36	Unclassified
314	NDUFC2 protein	IPI00555919	14164.40	9.04	1	1	36	MIM
315	Serine/threonine kinase 22B (Spermiogenesis associated)	IPI00289715	40971.18	8.95	0	1	36	Cytosolic

Appendix table 5 Total identified fetal NRBC membrane proteins with potential surface domain(s)

No.	Protein Description	Accession No.	TMD	Sub-cellular localisation
1	Membrane associated progesterone receptor component 2	IPI00005202	1	Microsome membrane; Plasma membrane
2	PRA1 family protein 3	IPI00007426	3	ER Membrane; Plasma membrane
3	Steroid dehydrogenase homolog	IPI00007676	3	Unclassified
4	Vesicular integral-membrane protein VIP36 precursor	IPI00009950	1	ER/Golgi membrane; Plasma membrane
5	Stromal cell-derived receptor-1 beta	IPI00018311	1	Plasma membrane
6	Neutral amino acid transporter B	IPI00019472	9	Plasma membrane
7	Calnexin precursor	IPI00020984	1	ER membrane; Plasma membrane
8	Azurocidin precursor	IPI00022246	0	Plasma membrane
9	Suppressor of actin 1	IPI00022275	2	ER/Golgi membrane; Plasma membrane
10	Hypothetical protein FLJ14347	IPI00022300	1	Unclassified
11	Band 3 anion transport protein	IPI00022361	11	Plasma membrane
12	Transferrin receptor protein 1	IPI00022462	1	Plasma membrane
13	Membrane protein	IPI00026111	2	Unclassified
14	CAAX prenyl protease 1 homolog	IPI00027180	7	ER/Golgi membrane; Plasma membrane
15	Leukocyte elastase precursor	IPI00027769	1	Plasma membrane
16	Cathepsin G precursor	IPI00028064	0	Plasma Membrane
17	Hypothetical protein DKFZp586C1924	IPI00031064	2	Unclassified
18	BCG induced integral membrane protein BIGM103	IPI00034208	7	Plasma membrane
19	Hypothetical protein FLJ31842	IPI00043429	6	Plasma membrane
20	Thioredoxin-like protein KIAA1162 precursor	IPI00100247	1	Plasma membrane
21	Vitamin K epoxide reductase complex subunit 1-like protein 1	IPI00166079	2	Unclassified
22	Olfactory receptor 11H4	IPI00168981	7	Plasma membrane
23	Hypothetical protein MGC14288	IPI00176708	1	Unclassified
24	Splice Isoform XB of Plasma membrane calcium-transporting ATPase 4	IPI00217169	8	Plasma membrane
25	Splice Isoform Glycophorin D of Glycophorin C	IPI00218128	1	Plasma membrane
26	Erythrocyte band 7 integral membrane protein	IPI00219682	1	Plasma membrane
27	Solute carrier family 2, facilitated glucose transporter member 1	IPI00220194	12	Plasma membrane

Appendix table 5. Continued

28	Kell blood group glycoprotein	IPI00220459	1	Plasma membrane
29	Antibacterial protein FALL-39 precursor	IPI00292532	1	Plasma membrane
30	Hypothetical protein DKFZp564K247	IPI00295621	2	Unclassified
31	Splice Isoform 2 of Reticulon 4	IPI00298289	2	ER membrane; Plasma membrane
32	Solute carrier family 43, member 3	IPI00301100	11	ER membrane; Plasma membrane
33	Splice Isoform B of Chloride channel protein 6	IPI00305252	3	Plasma membrane
34	Splice Isoform 2 of Synaptophysin-like protein	IPI00335277	3	Vesicle membrane; Plasma membrane
35	Stomatin	IPI00377081	1	Plasma membrane
36	Splice Isoform 2 of Protein GPR107 precursor	IPI00382815	7	Plasma membrane
37	Splice Isoform 1 of Protein C20orf22	IPI00394779	1	Unclassified
38	Thioredoxin domain containing protein 1 precursor	IPI00395887	3	ER membrane; Plasma membrane
39	Cleft lip and palate transmembrane protein 1	IPI00396411	5	Plasma membrane
40	Isoform 2 of Solute carrier family 22, member 11	IPI00418660	6	Plasma membrane
41	Aquaporin 1 splice variant 2	IPI00428490	1	Plasma membrane
42	Rhesus blood group, CcEe antigens, isoform 1	IPI00465155	12	Plasma membrane
43	ATP-binding cassette half-transporter	IPI00465442	9	Plasma membrane
44	Equilibrative nucleoside transporter 1	IPI00550382	11	Plasma membrane
45	Splice Isoform 1 of Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1	IPI00552514	7	Vesicle membrane; Plasma membrane
46	Splice Isoform 2 of Syntaxin-7	IPI00552913	0	Endosome membrane; Plasma membrane
47	Vesicle-associated membrane protein 2	IPI00553138	1	Vesicle membrane; Integral membrane protein; Synapse; Mitochondria
48	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	IPI00554481	1	Plasma membrane
49	ALEX3 protein variant	IPI00604615	1	Unclassified
50	Splice Isoform 1 of Protein C9orf5	IPI00607576	14	Plasma membrane
51	8 kDa protein	IPI00639803	2	Unclassified
52	Microsomal glutathione S-transferase 3	IPI00639812	3	ER/Microsome membrane; Plasma membrane
53	17 kDa protein	IPI00642218	3	ER membrane; Plasma membrane
54	25 kDa protein	IPI00646289	1	Unclassified

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