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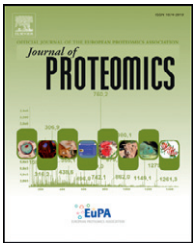

Membrane proteins of human fetal primitive nucleated red blood cells

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ABSTRACT

In humans, primitive fetal nucleated red blood cells (FNRBCs) are thought to be as vital for embryonic life as their counterpart, adult red blood cells (adult RBCs) are in later-gestation fetuses and adults. Unlike adult RBCs, the identity and functions of FNRBC proteins are poorly understood owing to a scarcity of FNRBCs for proteomic investigations. The study aimed to investigate membrane proteins of this unique cell type. We present here, the first report on the membrane proteome of human primitive FNRBCs investigated by two-dimensional liquid chromatography coupled with mass-spectrometry (2D-LCMS/MS) and bioinformatics analysis. A total of 273 proteins were identified, of which 133 (48.7%) were membrane proteins. We compared our data with membrane proteins of adult RBCs to identify common, and unique, surface membrane proteins. Twelve plasma membrane proteins with transmembrane domains and eight proteins with transmembrane domains but without known sub-cellular location were identified as unique-to-FNRBCs. Except for the transferrin receptor, all other 19 unique-to-FNRBC membrane proteins have never been described in RBCs. Reverse-transcriptase PCR (RT-PCR) and immunocytochemistry validated the 2D-LCMS/MS data. Our findings provide potential surface antigens for separation of primitive FNRBCs from maternal blood for noninvasive prenatal diagnosis, and to understand the biology of these rare cells.

1. Introduction

In humans, primitive fetal nucleated red blood cells (FNRBCs, fetal primitive erythroblasts) generated in the yolk sac mesoderm remain the predominant blood cell type in the embryonic circulation [1] until 10 weeks post-conception [2]. Studies on this cell type in humans have been limited owing to limited access to pure populations of these cells for laboratory investigations;

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only recently has it been shown that these cells may enucleate within the first trimester human placenta [3], suggesting that may be terminally differentiated. Primitive erythroblasts differ from fetal definitive erythroblasts not only in their anatomical site of origin, but also in the types of hemoglobins contained within them [4]. Whereas, much is already known about embryonic, fetal and adult-type hemoglobins within red blood cells, and developmental hemoglobin switching [5], very little is known about all other proteins within FNRBCs.

Adult red blood cells (adult RBCs) are smaller, discoid, readily deformable cells that are produced in the long bone marrow. Owing to their ready availability, these cells have been extensively studied in recent years. Using mass spectrometry, adult RBC membrane and cytoplasmic proteins have been characterized [6–12], and differences demonstrated between mouse and human adult RBCs [13].

Cell surface membrane proteins have an integral role in maintaining health: when altered structurally or functionally, they are responsible for the more commonly known diseased states such as spherocytosis [14] and sickle cell disease [15], and also the less commonly recognized conditions such as elliptocytosis [16], familial pseudohyperkalaemia [17], dehydrated hereditary stomatocytosis [18,19] and membrane defects in β-thalassemia [20]. Knowledge about cell membrane proteins and their functions in health and disease could lead to understanding mechanisms of disease processes such as the invasion of the malaria parasite into human erythrocytes [21] and the possibility of developing therapeutic interventions [22].

In contrast to the large amount of information already available on the adult RBC membrane proteome [23,24], no information is currently available on the proteome of human fetal primitive erythroblasts. Only very limited data on their cell surface antigens such as CD71 [25] and Glycophorin A [2] and some information on their cytoplasmic hemoglobin are known. We anticipate that knowledge on the membrane proteome of the FNRBC would be useful in two ways: to facilitate a deeper understanding of primitive erythropoiesis in humans, and to identify specific surface antigen(s) for the enrichment of ε-globin-positive fetal primitive erythroblasts from maternal blood for noninvasive prenatal diagnosis. It has been suggested that the ε-globin-positive fetal primitive erythroblast is the ideal fetal cell type for non-invasive prenatal diagnosis [2,26–30] and we hope to identify unique membrane proteins on either FNRBC or adult RBC that could be exploited for noninvasive prenatal diagnosis in the future. To our knowledge, differences between human FNRBCs and adult RBCs have never been studied systematically to date.

We planned to profile membrane proteins of FNRBCs by mass spectrometry, and compare this profile with that of the adult RBC membrane proteome as previously published by others [6–10] and ourselves [11]. A shot-gun proteomics approach, two-dimensional liquid chromatography coupled with MALDI-TOF/TOF-MS (2D-LCMS/MS) was used to characterize the membrane proteome of fetal primitive erythroblasts. This is the first report on the membrane proteome of the fetal primitive erythroblasts. We present details of all 273 proteins identified including their annotated sub-cellular locations, molecular functions and number of transmembrane domains. 133 (48.7%) proteins were membrane proteins, of which 37 were plasma membrane proteins. The biological significance of plasma membrane proteins unique to fetal primitive erythroblasts is discussed.

2. Materials and methods

2.1. Tissues

Placental tissue collection from women undergoing elective first trimester surgical termination of pregnancy was approved by the Institutional Review Board, and all patients gave written informed consent.

2.2. Extraction of FNRBCs from placental villi

Placental tissues were collected at the termination of pregnancy (7^{+0} to 9^{+3} weeks amenorrhea). FNRBCs were extracted from placental villi as per our protocol [30]: placental villi were digested in trophoblast digestion buffer (146.3 ml HBSS containing 0.182 g trypsin and 3.75 ml 1 M Hepes (Gibco®-Invitrogen-Life-Technologies, NY, USA)) for 30 min at 37 °C in a shaking-water-bath, and digestion was stopped using fetal calf serum (Pierce, IL, USA) (5 ml/45 ml digestion buffer). Single cell suspensions were centrifuged (3000 rpm, 20 °C, 10 min). Red cell pellets containing FNRBCs were suspended in PBS, and separated using Percoll 1083 (GE Healthcare, Uppsala, Sweden) (3000 rpm, 20 °C, 20 min). FNRBC purity was determined by basic staining of cytospun slides. Samples were stored for membrane preparation (if purity ≥90% FNRBCs) in HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA and 250 mM sucrose) with protease-inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at −80 °C. Morphologies of FNRBCs and adult RBCs are shown in Fig. 1.

2.3. Membrane protein preparation and digestion

Membranes from pooled FNRBCs (5×10^7 cells) were prepared as described earlier by us [11]: cells stored in HES buffer were lysed by thawing and sonication, and ultra-centrifuged at 100,000 x*g* 4 °C (1 h) to obtain the membrane pellet which was then washed using high pH solution (0.1 M Na_2CO_3, pH11), and twice with Milli-Q water. Proteins were extracted from FNRBC membranes using methanol (MeOH)/50 mM NH_4HCO_3 (60:40,vol/vol), and protein reduction, alkylation and digestion were carried out as described by Blonder and others [31]. Tryptic digestion was carried out using sequencing grade modified trypsin (Promega, Southampton, UK). Digested sample was centrifuged and the pellet washed in MeOH solution (60%MeOH in 50 mM NH_4HCO_3) twice. Supernatants were pooled (MeOH-derived digests), while the pellet was re-suspended in Trifluoroethanol (TFE)/50 mM NH_4HCO_3 (50:50vol/vol) and the proteins extracted were then diluted 10 times with 50 mM NH_4HCO_3 for a second trypsin digestion to obtain supernatants (TFE-derived digests). Both digests were lyophilized and stored at −80 °C.

2.4. Two-dimensional liquid chromatography and mass spectrometry (2D-LCMS/MS)

2D-LCMS/MS was essentially the same as described earlier by us [11]. Briefly, lyophilized digests were re-suspended in

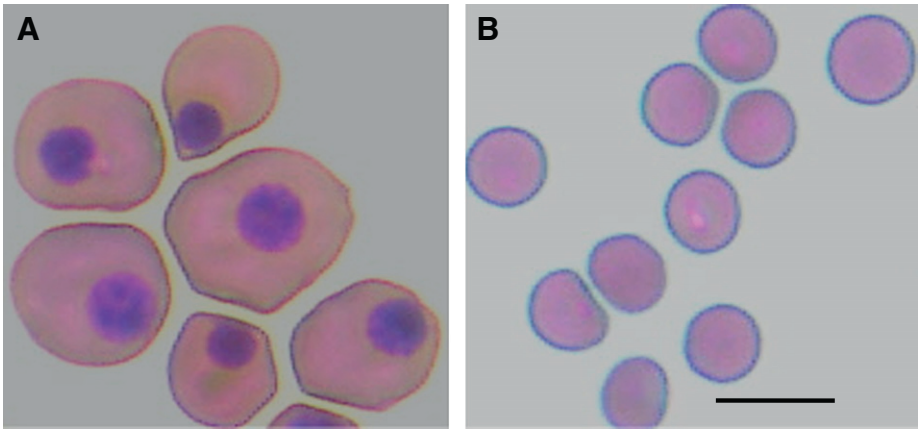

Fig. 1 – Wright's stained FNRBCs and adult RBCs. FNRBCs extracted from placental villi, and adult RBCs prepared from volunteer blood sample. (A) FNRBCs (nucleated). (B) adult RBCs without nuclei. Bright field images were captured using 20×/0.40PhPobjective lens of CKX41 Olympus microscope. Bar represents 10 μm.

solvent [(98%H_2O, 2% acetonitrile (CAN) and 0.05% trifluoroacetic acid (TFA))], and after centrifugation supernatants were separated using an Ultimate-Dual-HPLC system (Dionex, Sunnyvale, CA, USA). All samples were first separated on a strong cation exchange (SCX) column (300 μm i.d.,×15 cm, packed with 10 μm POROS 10S) and eluted fractions were captured on the PepMap trap column (300 μm i.d.,×1 mm, packed with 5 μm C18 100 Å), and eluted by gradient elution to a reversed-phase column (Monolithic Capillary Column, 200 μm i.d.,×5 cm). LC fractions were mixed with matrix-assisted laser desorption/ionization (MALDI) matrix (7 mg/ml α-cyano-4-hydroxycinnamic acid and 130 μg/ml ammonium citrate in 75% CAN) at a flow rate of 5.4 μl/min through a 25 nl mixing-tee (Upchurch Scientific, Oak Harbor, WA, USA) before being spotted onto 192-well stainless steel MALDI target plates (AB SCIEX, Foster City, CA, USA), at a rate of one well per 5 s, using a Probot Micro Fraction collector (Dionex).

Samples on the MALDI target plates were analyzed using an ABI 4700 Proteomics Analyzer (AB SCIEX) with a MALDI source and time of flight analyzer TOF/TOF™ optics. For MS analysis, typically 1000 shots were accumulated for each sample well. Tandem-MS_(MS/MS) analyses were performed using nitrogen, at collision energy of 1 kV and a collision gas pressure of ~3.0×10^{-7} Torr. 3000 to 6000 shots were combined for each spectrum depending on the quality of the data.

2.5. *Database searching*

MASCOT search engine (v2.0; Matrix Science) was used to search tandem mass spectra. GPS Explorer™ software (v3.6; AB SCIEX) 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 (v3.10) was used for the search of tryptic peptides [32] and 57,478 entries were searched. All 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 missed cleavages were allowed. Precursor error tolerance was set to 200 ppm and MS/MS fragment error tolerance was 0.4 Da.

2.6. *Estimation of false positive rate*

The false positive rate was calculated by comparing the search results from a randomized database versus the actual database. The minimum ion score C.I. percent was set such that no more than 5% false discovery rate (FDR) was achieved and was used as the cut-off threshold at the peptide level. All the proteins identified from random database search were single peptide-matched. Proteins identified by this method from IPI human database were color coded as red, green or black: those red colored proteins are matched to at least two peptides and hence are statistically confident (FDR is zero); proteins that are green colored are identified by single peptide where match scores are higher than the highest score in the decoy database and essentially the FDR is zero; black colored proteins were identified based on single peptide match fall within the set threshold of 5% FDR. Top ranked peptides with Best Ion scores ≥33 and ≥36 for TFE and MeOH extractions, respectively, were included for analysis as peptides counted for each protein. All the MS/MS spectra were further validated manually.

2.7. *Annotation*

Sub cellular and functional categories of the identified proteins were obtained based on annotations of Gene Ontology using GoFig.(http://udgenome.ags.udel.edu/gofigure/index.html). Swiss-prot and TrEMBL data base were also used for functional annotation of unique proteins of FNRBCs. The number of transmembrane domains (TMD) of the identified proteins was predicted using TMHMM Server (v2.0) (http://www.cbs.dtu.dk/services/TMHMM/).

2.8. *Evaluation of the identified unique proteins*

2.8.1. *Reverse transcriptase PCR (RT-PCR) for mRNA expression of unique proteins*

2.8.1.1. RNA extraction. RNA from FNRBCs was isolated using an RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Briefly, FNRBCs (3×10^6 cells) were

resuspended in 350 μl lysis buffer and passed through QIAshredder spin column. The lysate was mixed with 350 μl of 70% ethanol and pipetted onto an RNeasy mini column, and centrifuged at 15,000×*g* for 15 s. RNA trapped in the column was washed using 350 μl buffer RW1 and incubated with 10 μl of DNase in 70 μl RDD buffer at room temperature for 15 min. RNA was then washed twice with 350 μl of buffer RW1 and once with 500 μl buffer RPE and recovered by the addition of 50 μl RNase-free water onto the column and centrifugation at 15,000×*g* for 1 min.

2.8.1.2. RT-PCR. cDNA template was synthesized using Sensiscript RT Kit (Qiagen, Germany). Briefly, 5 μl of RNA was mixed with oligo-dT, RNase inhibitor, dNTP mix and RNase-free water (as per manufacturer's instructions) and incubated at 70 °C for 5 min and chilled on ice. RT buffer and RT enzyme were added to the mixture and incubated at 25 °C (15 min), 42 °C (60 min) and 72 °C (15 min), and cooled on ice. PCR mixture contained 5 μl cDNA, 1×PCR buffer, 1 mM dNTP, 8 mM $MgCl_2$, 2.5U Taq polymerase and 0.6 μM primers. Denatured (94 °C 2 min) mixture was amplified by 45 cycles of 94 °C for 15 s, ~60 °C (depends on primer pairs) for 15 s, 72 °C for 1 min. A final extension at 72 °C for 4 min was performed for each gene. RT control (no enzyme in RT step) and PCR control (Water-blanks) were also included. PCR products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 g/ml) and visualized under UV light. The images were captured using a digital imager (Alpha Innotech Corp., San Leandro, CA). Primer pairs (Sigma-Proligo) used for the amplification for individual gene are listed in Supplemental Table 6.

2.8.2. Localisation of unique proteins on FNRBCs by alkaline phosphatase immunocytochemistry

Eight commercially available antibodies against unique proteins of FNRBCs annotated to be on plasma membrane, and also in other membranes or unique proteins with unknown sub-cellular location were used to localize their antigens in both FNRBCs and adult RBCs: Neutral amino acid transporter B (SLC1A5) (Chemicon-International, Temecula, CA, USA), Solute carrier family 3, member 2, isoform A (SLC3A2), Olfactory receptor 11H4 (OR11H4) and Antibacterial protein FALL-39 precursor (Cathelicidin antimicrobial peptide, CAP-18) (all from Abcam, Cambridge, UK), Cleft lip and palate transmembrane protein1 (CLPTM1), Armadillo Repeat-Containing X-linked protein 3 (ARMCX3/ALEX3), CAAX prenyl protease1 homolog (FACE1) (all from Novus-Biologicals, Littleton, CO), and Chloride channel protein 6 (CLCN6) (Santa-Cruz Biotechnology,Inc., CA, USA). Cells were fixed for 10 min either with 4% paraformaldehyde for SLC1A5, SLC3A2, OR11H4, CLCN6, CLPTM1, ARMCX3 or ice-cold methanol:acetone (1:1) for CAP-18 and FACE1; following steps were common for all slides: Briefly, nonspecific binding was inhibited with diluted goat serum (Sigma-Diagnostics, MO, USA) (1:10 in PBS) for 120 min which was followed by incubation with respective primary-antibodies (1:100) for 60 min at room temperature or over night at 4 °C. Slides were then incubated with corresponding mouse or rabbit biotinylated secondary-antibody (1:100) for 60 min (Vector-Laboratories, CA, USA). This was followed by incubation with streptavidin conjugated alkaline phosphatase (Vector-Laboratories) (1:100). Immunoreaction was detected with freshly prepared Vector-Blue-substrate (Vector-Laboratories) for 10 min in dark. All incubations were performed in a humidifying chamber at room temperature and washes between incubations were in 1XPBST (5 min). Slides were rinsed in water and nuclei stained with nuclear-fast-stain (10 min), slides were rinsed in water and dehydrated with 100% ethanol (30 s each). Air dried slides were mounted with Vectashield (Vector-Laboratories) and analyzed by light microscopy. The staining intensity for each antibody tested was calculated as described by Lehr et al. [33]. Mean pixel intensities calculated from the luminosity histogram function on Adobe Photoshop CS4 software (Adobe Systems, Mountain View, CA) were compared for statistical significance.

2.9. Statistical analysis

Mean staining intensities (Mean±SD) between FNRBCs and adult RBCs were compared using Mann–Whitney *U* test (GraphPad Prism software, GraphPad Prism Inc, CA). Differences were considered significant when *P* values were <0.05.

3. Results

3.1. FNRBC membrane proteins

Cell membrane protein extraction is challenging because many of these proteins have hydrophobic side chains [34,35]. Furthermore, the significant quantity of protein needed for detailed proteomic analysis restricts studies on limited-access cells such as the human FNRBCs. To overcome these difficulties, we collected and pooled cell membrane protein material harvested from several trophoblastic villi, and developed our own protocol for maximal cell membrane protein recovery [11]. We used two organic solvents, MeOH and TFE, and recovered both hydrophilic and hydrophobic proteins using pooled samples of FNRBCS. 144 and 199 proteins were identified from MeOH and TFE digests respectively, while 70 proteins were common to both (Supplemental Table 1; Fig. 2).

As FNRBCs are nucleated, and also contain other organelles, our protein identification found not only plasma membrane proteins, but also membrane proteins from the nucleus,

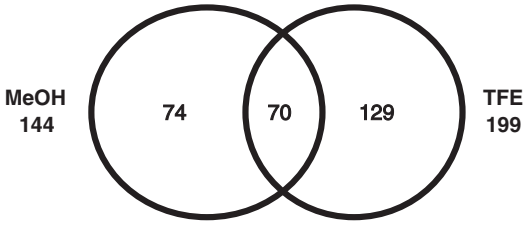

Fig. 2 – Identification of FNRBC proteins. A total of 273 proteins were identified. Two organic solvents, MeOH and TFE enabled to recover 144 and 199 proteins respectively and only 26% of total proteins identified were commonly identified from both the extractions, thus the recovery of proteins could be enhanced by the sequential use of two solvents with limited sample (5×10^7 cells).

mitochondria, endoplasmic reticulum, Golgi, microsomes and peroxisomes.

3.2. Location annotation of identified proteins

A total of 273 proteins were identified, and their locations within the cell annotated (Supplemental Table 2): 133 were membrane proteins (Supplemental Table 2) while 132 were non-membrane proteins including 16 that have been described as exclusively cytoplasmic (Supplemental Table 3). Locations of the remaining 8 are as yet unclassified (Supplemental Table 4).

Sub-cellular localizations of the 133 membrane proteins were analyzed: of these proteins, 37 were noted to localize to the plasma membrane, 48 mitochondrial membranes, 10 endoplasmic reticular membranes, and the remaining 38 membrane proteins were annotated to be localized in more than one location of the cell (Fig. 3A).

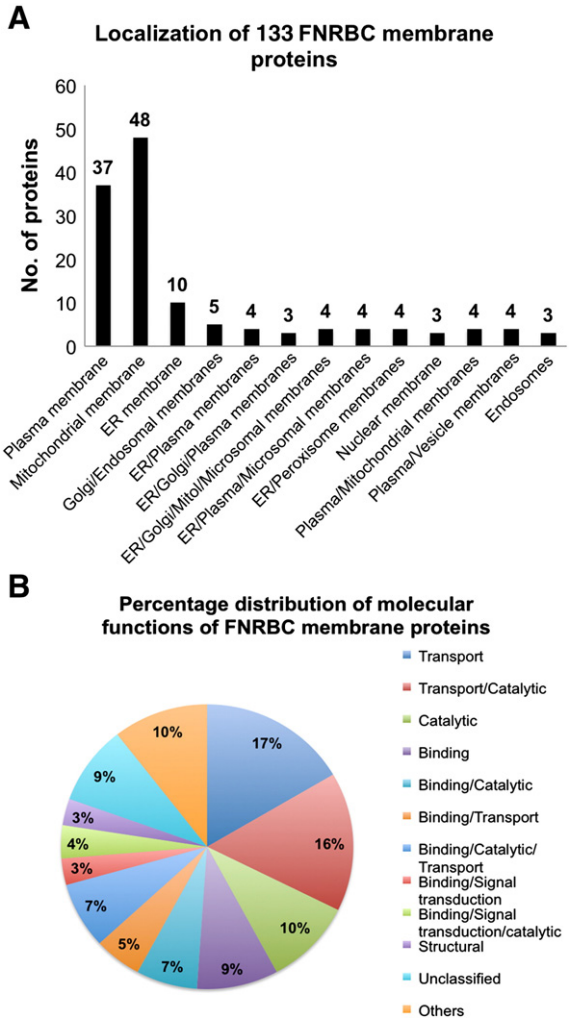

Fig. 3 – A–B—Sub-cellular localization (A) and molecular functions (B) of FNRBC membrane proteins. Sub-cellular localization and functional categories of the identified proteins were obtained based on the annotations of Gene Ontology using GoFig. (http://udgenome.ags.udel.edu/gofigure/index.html). Swiss-prot and TrEMBL data base were also used for the functional annotations of unique proteins of FNRBCs.

3.3. Functional annotation of membrane proteins

Molecular functions of the 133 membrane proteins identified are detailed in Fig. 3B. Some proteins were noted to have more than one function. Most were transport proteins (16.54%), 15.79% were both transport and catalytic, 9.77% catalytic, 9.02% binding, 6.77% binding and catalytic, 5.26% binding and transport, 7.51% binding/catalytic/transport, 3.76% binding/signal transduction/catalytic, 3.00% each for binding/signal transduction, and structural, 9.02% unclassified and 10.53% other functions.

3.4. Proteins with transmembrane domains

Transmembrane domains (TMDs) of all the proteins are provided in the Supplemental Table 1. The number of predicted transmembrane domains in the identified membrane proteins varied from 0 to 15: NADH dehydrogenase subunit 5 was found to possess the maximum number of TMD. Plasma membrane proteins of primitive FNRBCs with at least one TMD (25 proteins) and the plasma membrane proteins known to be present on other membranes as well (14 proteins) are presented in Tables 1 and 2, respectively.

3.5. Single peptide based identification of proteins

Color coding of proteins based on the number of peptides for their identification shown in Supplemental Table 1 indicated that only 23 of the total proteins (273) were black colored and were identified based on single peptide match which fall within the set threshold of 5% FDR, and the rest were red (≤2 peptides) or green colored (by single peptide) where FDR was zero. Proteins identified based on single peptides from TFE and MeOH extractions, their peptide sequence and ion score are presented in Supplemental dataset-II and MS/MS spectra of the peptides derived from Mascot search results for these identifications are provided in Supplemental dataset-III. Owing to the sample limitation of FNRBCs, we could not perform replicate mass-spectrometry analysis with more than the one pooled sample.

3.6. Comparison of plasma membrane proteins of FNRBCs and adult RBCs to identify unique membrane proteins

Mass spectrometry-based identification of membrane proteins of adult RBCs has so far been reported by only a few studies [6–11] including ours [11]. We curated, from the published literature, a comprehensive list of all adult RBC membrane proteins identified by mass spectrometry to date. In the final list, only those candidates annotated as membrane proteins by gene ontology using GoFig. were included. Redundant entries were removed by manually comparing the sequences of all membrane proteins. A total of 299 non-redundant adult RBC membrane proteins were finally short-listed (data not shown); we further short-listed them to 202 to include only membrane proteins with known- and potential surface domains (e.g. membrane-associated extracellular proteins and integral membrane proteins) (Supplemental Table 5). Membrane proteins of FNRBCs were compared manually with this final list of adult RBC membrane proteins to identify both common and unique membrane proteins.

Table 1 – Plasma membrane proteins of FNRBCs.

No	Protein description	IPI accession #	TMD	Sub-cellular location
1	Splice Isoform 1 of Protein C9orf5	IPI00607576	14	Plasma membrane
2	Solute carrier family 2, facilitated glucose transporter member 1	IPI00220194	12	Plasma membrane
3	Rhesus blood group, CcEe antigens, isoform 1	IPI00465155	12	Plasma membrane
4	Equilibrative nucleoside transporter 1	IPI00550382	11	Plasma membrane
5	Band 3 anion transport protein	IPI00022361	11	Plasma membrane
6	ATP-binding cassette half-transporter	IPI00465442	9	Plasma membrane
7	Neutral amino acid transporter B	IPI00019472	9	Plasma membrane
8	Splice Isoform XB of Plasma membrane calcium-transporting ATPase 4	IPI00217169	8	Plasma membrane
9	Olfactory receptor 11H4	IPI00168981	7	Plasma membrane
10	Splice Isoform 3 of Protein GPR107 precursor	IPI00184474	7	Plasma membrane
11	BCG induced integral membrane protein BIGM103	IPI00034208	7	Plasma membrane
12	Sodium/potassium-transporting ATPase beta-3 chain	IPI00008167	1	Plasma membrane
13	Hypothetical protein FLJ31842	IPI00043429	6	Plasma membrane
14	Cleft lip and palate transmembrane protein 1	IPI00396411	5	Plasma membrane
15	Splice Isoform A of Chloride channel protein 6	IPI00180121	3	Plasma membrane
16	Leukocyte elastase precursor	IPI00027769	1	Plasma membrane
17	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	IPI00554481	1	Plasma membrane
18	Thioredoxin-like protein KIAA1162 precursor	IPI00100247	1	Plasma membrane
19	Aquaporin 1 splice variant 2	IPI00428490	1	Plasma membrane
20	Kell blood group glycoprotein	IPI00220459	1	Plasma membrane
21	Erythrocyte band 7 integral membrane protein	IPI00219682	1	Plasma membrane
22	Splice Isoform Glycophorin D of Glycophorin C	IPI00218128	1	Plasma membrane
23	Stromal cell-derived receptor-1 beta	IPI00018311	1	Plasma membrane
24	Transferrin receptor protein 1	IPI00022462	1	Plasma membrane
25	Antibacterial protein FALL-39 precursor	IPI00292532	1	Plasma membrane

3.7. *Membrane proteins common to both adult RBCs and FNRBCs*

Thirty-one proteins were common to both cell types. These included: structural proteins such as the erythrocyte band 7 integral-membrane protein, ankyrin, spectrin, dematin, Protein 4.1; proteins with transport function such as band 3, aquaporin, calcium-transporting ATPase, sodium/potassium-transporting ATPase, solute carrier family 2, facilitated glucose transporter, member 1; and plasma membrane binding proteins like Kell blood group glycoprotein (CD238).

3.8. *Plasma membrane proteins unique to FNRBCs*

Comparison of membrane proteins revealed that 20 proteins were unique to FNRBCs, and 171 unique to adult RBCs, respectively (Fig. 4). Among membrane proteins unique to FNRBCs, 9 proteins were annotated as being present only on plasma membranes, and 3 others were noted to be present on plasma membranes as well as on ER/Golgi/vesicle membranes (Table 3); but, for 8 other membrane proteins found unique to FNRBCs, the exact sub-cellular localization was not available (Table 4).

Table 2 – Plasma membrane proteins of FNRBCs known to be present on other membranes.

No	Protein description	IPI accession #	TMD	Sub-cellular location
1	CDNA PSEC0252 fis, clone NT2RP3003258, highly similar to Likely ortholog of mouse embryo	IPI00301100	11	Plasma membrane/ER membrane
2	Splice Isoform 1 of Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1	IPI00552514	7	Plasma membrane/vesicle membrane
3	CAAX prenyl protease 1 homolog	IPI00027180	7	Plasma membrane/ER/golgi membrane
4	Splice Isoform 2 of Synaptophysin-like protein	IPI00335277	3	Plasma membrane/vesicle membrane
5	Microsomal glutathione S-transferase 3	IPI00639812	3	Plasma membrane/ER/microsome membrane
6	PRA1 family protein 3	IPI00007426	3	Plasma membrane/ER membrane
7	Thioredoxin domain containing protein 1 precursor	IPI00395887	3	Plasma membrane/ER membrane
8	17 kDa protein	IPI00642218	3	Plasma membrane/ER membrane
9	Splice Isoform 1 of Reticulon 4	IPI00021766	1	Plasma membrane/ER membrane
10	Suppressor of actin 1	IPI00022275	2	Plasma membrane/ER/golgi membrane
11	Vesicle-associated membrane protein 2	IPI00553138	1	Plasma membrane/vesicle membrane/synapse
12	Membrane associated progesterone receptor component 2	IPI00005202	1	Plasma membrane/microsome membrane
13	Vesicular integral-membrane protein VIP36 precursor	IPI00009950	1	Plasma membrane/ER/golgi membrane
14	Calnexin precursor	IPI00020984	1	Plasma membrane/ER membrane

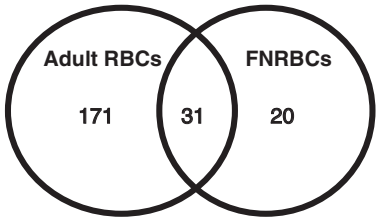

Fig. 4 – Membrane proteins with potential surface domains of adult RBCs and FNRBCs. A comparison of membrane proteins with potential surface domains (as annotated) indicated that only 31 proteins were common membrane proteins to adult RBCs and FNRBCs, and 171 and 20 were unique to them respectively.

Membrane proteins unique to FNRBCs fall mainly under broad functional groups such as (a) transporter proteins: neutral amino acid transporter B, solute carrier family 3 (activators of dibasic and neutral amino acid transport), splice isoform A of chloride channel protein 6 (chloride ion transport); (b) binding proteins: transferrin receptor protein, splice isoform 3 of Protein GPR107 precursor, olfactory receptor 11H4; and (c) catalytic proteins: CAAX prenyl protease 1 homolog, Vitamin K epoxide reductase complex subunit 1-like protein 1 (VKORC1 L1), Splice Isoform 1 of Protein C20orf22.

3.9. *Reverse transcriptase PCR (RT-PCR) to confirm expression of unique membrane proteins within FNRBCs*

It was challenging to obtain large numbers of FNRBCs from trophoblastic villi, and all were used to perform the mass spectrometry experiments. To determine if the proteins identified as unique to FNRBCs were indeed expressed within FNRBCs, we extracted total RNA from FNRBCs and performed a RT-PCR.

We evaluated the mRNA expression of 23 proteins including 13 proteins unique to FNRBCs (Fig. 5). mRNA expression of all the unique proteins on FNRBCs tested, except olfactory receptor 11H4 (OR11H4), was detected. The absence of amplification of olfactory receptor could probably be due to the low levels of mRNA accumulated as suggested by Feingold and his colleagues [36].

3.10. *Immunocytochemical localization of unique FNRBC proteins*

In situ localization of the putative unique FNRBC proteins was thought to be more informative than western blotting because the location of plasma membrane, cytoplasmic and nuclear proteins could be readily visualized. These were compared to adult RBCs. Intensities of immunostaining for the five antibodies tested, FACE-1, SLC1A5, CAP-18, ARMCX3 and OR11H4 were significantly higher ($\leq$0.001) on FNRBCs than on adult RBCs; in contrast, anti-CLCN6 antibody stained adult RBCs much more intensely than FNRBCs (<0.001). There was no significant difference in the staining between FNRBCs and adult RBCs for CLPTM1 and SLC3A2 (Fig. 6A–B).

4. Discussion

This is the first report on the membrane proteome of human FNRBCs. Identification of 133 membrane proteins from various sub-cellular locations with different functions would help to explore the importance of FNRBC in medicine. We also report 132 non-membrane proteins including a few known cytoplasmic proteins (for example, hemoglobin chains ε, γ, δ). Functions of FNRBCs and their proteins except for oxygen supply to the fast developing embryo are yet to be understood, and thus our findings are an important contribution to the literature.

Differential expression of proteins in pronormoblasts and poly/orthochromatic normoblasts during their development in vitro from CD34+ progenitors that were enriched from buffy coat material was investigated by Richardson and colleagues [37]. However, these erythroblasts presumably from adult blood

Table 3 – Unique membrane proteins of FNRBCs with transmembrane domain.

No	Protein description	IPI accession #	TMD	Sub-cellular location	Molecular function
1	Neutral amino acid transporter B (SLC1A5)	IPI00019472	9	Plasma membrane	Transporter-amino acid
2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2, isoform A (SLC3A2)	IPI00554481	1	Plasma membrane	Transporter-amino acid
3	Splice Isoform A of Chloride channel protein 6	IPI00180121	3	Plasma membrane	Transporter-chloride ion
4	Transferrin receptor protein 1	IPI00022462	1	Plasma membrane	Binding and transport —iron
5	Splice Isoform 3 of Protein GPR107 precursor	IPI00184474	7	Plasma membrane	Binding receptor —hormone and neurotransmitter
6	Olfactory receptor 11H4	IPI00168981	7	Plasma membrane	Binding receptor—odor
7	Splice Isoform 1 of Protein C9orf5	IPI00607576	14	Plasma membrane	Signaling pathways
8	Cleft lip and palate transmembrane protein 1	IPI00396411	5	Plasma membrane	Unknown
9	BCG induced integral membrane protein BIGM103	IPI00034208	7	Plasma membrane	Antimicrobial
10	Antibacterial protein FALL-39 precursor	IPI00292532	1	Plasma membrane/ extracellular	Antibacterial
11	CAAX prenyl protease 1 homolog	IPI00027180	7	Plasma/ER/golgi membrane	Catalytic
12	Splice Isoform 2 of Synaptophysin-like protein	IPI00335277	3	Plasma/vesicle membrane	Vesicle recycling

Table 4 – Unique membrane proteins of FNRBCs with transmembrane domain but location unknown.

No	Protein description	IPI accession #	TMD	Sub-cellular location	Molecular function
1	Vitamin K epoxide reductase complex subunit 1-like protein 1	IPI00166079	2	Unclassified membrane (potential)	Catalytic
2	Splice Isoform 1 of Protein C20orf22	IPI00394779	1	Unclassified membrane (by similarity)	Catalytic
3	Hypothetical protein DKFZp564K247 (hypoxia induced gene 1 protein)	IPI00295621	2	Unclassified membrane (potential)	Unclassified
4	Hypothetical protein DKFZp586C1924	IPI00031064	2	Unclassified membrane (potential)	Unclassified
5	ALEX3 protein variant	IPI00604615	1	Unclassified single pass membrane (potential)	Unclassified
6	Hypothetical protein MGC14288	IPI00176708	1	Unclassified membrane (potential)	Unclassified
7	8 kDa protein	IPI00639803	2	Unclassified	Unclassified
8	25 kDa protein	IPI00646289	1	Unclassified	Unclassified

samples differ from the yolk-sac derived fetal primitive erythroblasts extracted from chorionic villi in our study, although both types of erythroblasts may appear morphologically identical with higher cytoplasmic-nuclear ratio. In addition, of the 21 differentially expressed proteins in two stages of erythroblasts reported by them, only one protein (cytochrome b5) appears to closely match with our protein identification if not identical (similar to cytochrome b5 outer mitochondrial membrane isoform precursor). We presume that this observation may probably due to the difference in the origin of erythroblasts in addition to the proteomic strategies followed in both studies.

Proteomic analyses of FNRBCs had not been attempted previously owing to the difficulty to obtain sufficient number of cells. Our access to placental villi from patients undergoing termination of pregnancy enabled to pool cells for 2D-LCMS/MS analysis. In addition, the extraction of membrane proteins is yet another challenge in proteomics; recovery of more membrane proteins (48.7% of total) from a limited sample (5×10^7 cells) than those from adult RBCs using similar protocol (11) is encouraging, which also explains the structural complexity of these nucleated cells. Yolk-sac derived fetal primitive erythroblasts enter fetal circulation and they do enucleate in the first trimester human placenta [3]. Trophoblast and PDGF-B signaling as key

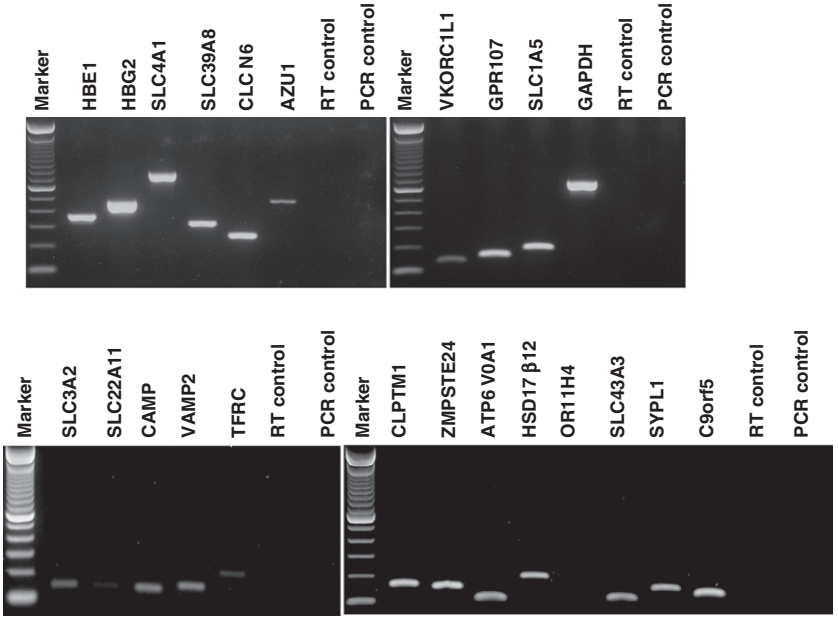

Fig. 5 – Validation of unique membrane proteins of FNRBCs by reverse transcriptase-PCR (RT-PCR). mRNA expression of unique proteins of FNRBCs using total RNA extracted from FNRBCs and by RT-PCR using primers specific for genes tested (Supplemental Table 6). RT control: no RT enzyme; PCR control: water blank in place of template. Top panel: HBE1, hemoglobin epsilon chain; HBG2, hemoglobin gamma-2 chain; SLC4A1, solute carrier family 4 member 1; SLC39A8, solute carrier family 39 member 8; CLCN6, chloride channel protein 6; AZU1, Azurocidin precursor; VKORC1L1, vitamin K epoxide reductase complex subunit 1-like protein 1; GPR107, protein GPR107 precursor; SLC1A5 neutral amino acid transporter B; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; Bottom panel: SLC3A2, solute carrier family 3 member 2, isoform A; SLC22A11, solute carrier family 22 member 11, isoform 2; CAMP, antibacterial protein FALL-39 precursor; VAMP2, vesicle-associated membrane protein 2; TFRC transferrin receptor protein 1; CLPTM1, cleft lip and palate transmembrane protein 1; ZMPSTE24, CAAX prenyl protease 1 homolog; ATP6VOA1, vacuolar proton translocating ATPase 116 kDa subunit a isoform 1; HSD17β12, steroid dehydrogenase homolog; OR11H4, olfactory receptor 11H4; SLC43A3, solute carrier family 43 member 3; SYPL1, synaptophysin-like protein; and C9orf5, protein C9orf5.

components of the placental hematopoietic niche has recently been demonstrated [38]. We extracted primitive erythroblasts from chorionic villi dissected out from placental tissue which were of high purity (≥90% purity, Fig. 1A) as presented earlier. Thus, the primitive erythroblasts used in the present study differ from recent proteomic investigations by Smith's group [39,40] where differential protein expression in developing erythroblasts in vitro from CD34+ progenitor cells that were enriched from normal and thalassemic patient blood samples was used.

Sub-cellular localization and molecular functions annotated for most of the proteins of FNRBCs are novel for this cell type, which may be useful for protein/developmental/structural biologists, pathologists, hematologists and others. Identified FNRBC membrane proteins show diverse physiological functions varying from transport, catalytic, binding to structural, while about 32% were transport and/or catalytic. Among the membrane proteins, most were identified from mitochondria (48 proteins) and plasma membrane (37 proteins).

The present study makes the first attempt to explore unique membrane proteins of FNRBCs. We intended to focus only on plasma membrane proteins in order to understand the potential physiological function through literature search and to find out potential candidates as surface antigens for future separation of this cell type by antibody based techniques. The comprehensive list of human adult RBC membrane proteins we prepared based on publications [6–11] was used for comparison of membrane proteins of FNRBCs with that of adult RBCs: twelve membrane proteins annotated to be in plasma membranes and eight without known sub-cellular locations were found to be unique to FNRBCs. Proteins with transmembrane domains without known sub-cellular location and molecular function may contain novel antigens of biological significance. This comparison also revealed that 171 proteins are unique to adult RBCs which are not found in the data set of FNRBCs.

A few proteins (31) were found to be common in both the cell types, which included major structural and transport proteins of plasma membrane such as band 3, erythrocyte band 7, facilitated glucose transporter (SLCA2A1), Kell blood group glycoprotein (CD238), aquaporin, ATP-binding cassette half-

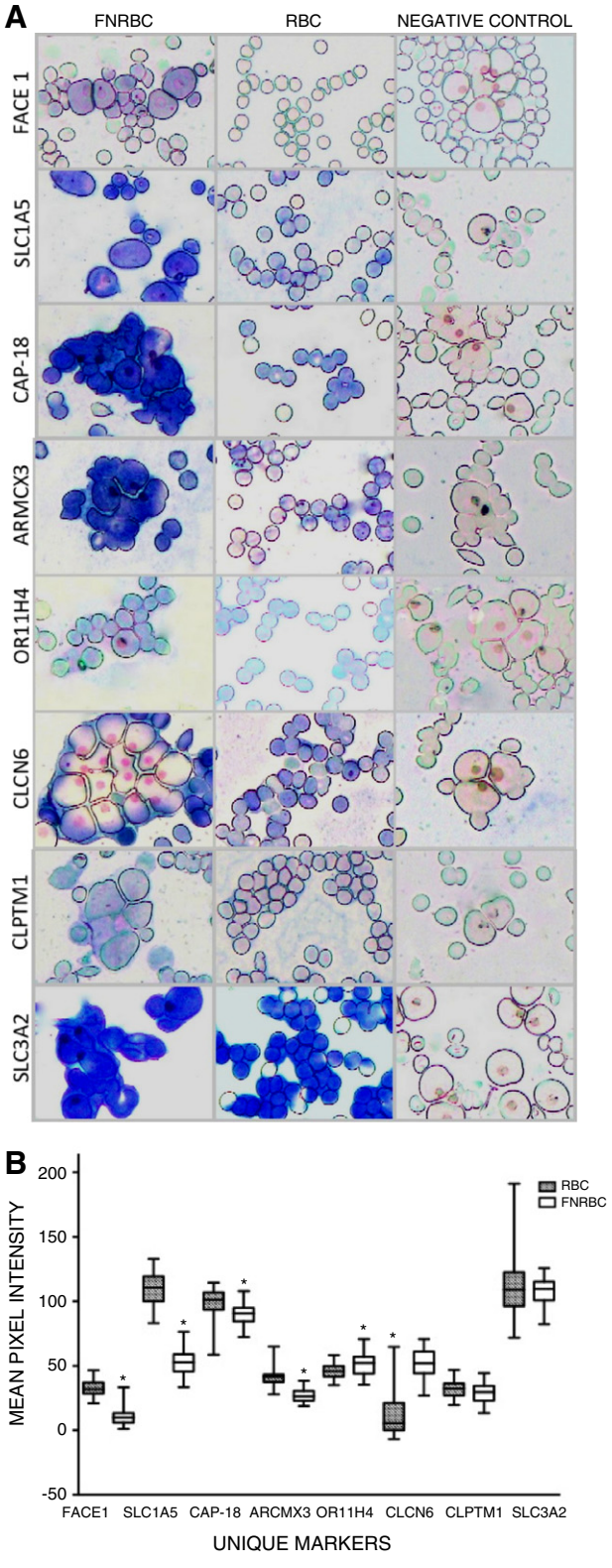

Fig. 6 – A—Immunolocalization of antigens on FNRBCs and adult RBCs. FNRBCs extracted from placental villi are relatively larger and identified by the presence of nuclei stained red by nuclear fast stain. FNRBCs and adult RBCs are shown in first and second panels respectively; negative controls (right extreme panel) by omitting the primary antibody were run in all experiments. Immunoreaction of the antibodies against FACE-1, SLC1A5, CAP-18, ARMCX3 and OR11H4 was significantly higher (≤0.001) on FNRBCs than on adult RBCs. In contrast, immunostaining of anti-CLCN6 antibody was opposite: intense on adult RBCs compared to FNRBCs (<0.001). There was no significant difference in the staining between the cells in the case of anti-SLC3A2 and CLPTM1. The Bar represents 10 μm. Bright field images were captured using 20×/0.7 UPlan APO objective lens of BX61 Olympus microscope with Evolution™ MP color Media Cybernetics CCD camera linked to Image-Pro Discovery software. B—Box plot showing the statistical significance (*) of intensities of immunoreaction by antibodies. Mean pixel intensities calculated from the luminosity histogram function on Adobe photoshop CS4 software (Adobe Systems, Mountain View, CA) were compared for statistical significance. Mean staining intensity values and intensity of immunoreaction are inversely related (Lehr et al. [33]).

transporter 1 and glycophorin C, suggesting similar functions for these proteins in FNRBCs as of their adult counterpart.

As anticipated, plasma membrane proteins which are developmental-stage specific to immature red cells but not to adult RBCs, such as transferrin receptor [25] and ferritin heavy chain [41] were identified unique to FNRBCs; similarly, absence of leukocyte specific antigen [42] in our data set also confirms the purity of the sample we used.

Association between erythroblasts and macrophages mediated by the erythroblast macrophage protein (Emp) is known to promote terminal maturation and enucleation of erythroblasts [43] while CD163, an erythroblast adhesion receptor to mediate adhesion to macrophages [44]. However, these proteins were not found to be present in our data set proteins of primitive erythroblasts. Instead, the proliferation-associated 2G4, 38 kDa, a nuclear protein was detected, but its functional role has not been elucidated in fetal primitive erythroblasts.

Indirect validation of unique proteins of FNRBCs by mRNA expression analysis using RT-PCR revealed the presence of all candidates tested except the olfactory receptor (OR11H4); and the reason for the failure of this protein could probably be due to the low level of the template present in the sample as reported by Feingold and his colleagues [36]. RT-PCR results for unique proteins confirm their identifications by mass spectrometry. Such validation is not possible for adult RBCs as they are mature cells without nuclei or RNA.

Proteomic identification followed by confirmation of their expression in tissues and cells by immunological techniques has been a useful tool in areas such as biomarker discovery, drug discovery and disease biology for example, tumor heterogeneity studies in bladder cancer [45,46]. Stronger expression levels of unique proteins of FNRBCs as identified by immunostaining for four of eight antibodies (FACE-1, SLC1A5, CAP-18 and OR11H4) on these cells compared to adult RBCs, do support their mass spectrometric identifications. However, expression of chloride channel protein (CLCN6) was found to be opposite (stronger in adult RBCs) and two other proteins (SLC3A2 and CLPTM1) did not reveal any difference in their immunostaining in the present study, and such observations may probably be due to the specificity and reactivity of the antibodies used [42] or due to the expression levels and the isoforms of proteins identified [23]. As mentioned earlier, FACE-1 and CAP-18 are also annotated to be present in other locations in addition to their presence in the plasma membrane.

One of our objectives was to identify a potential surface antigen for separation of FNRBCs from maternal blood for noninvasive prenatal diagnosis: these cells in maternal blood, can be separated easily from WBCs using leukocyte specific anti-CD 45 antibody, whereas, it is still challenging to select FNRBCs from overwhelming adult RBCs due to the absence of specific surface antigen present only in any one of these cell types. Identification of unique membrane proteins with transmembrane domains such as FACE-1, SLC1A5, CAP-18 and OR11H4 by mass spectrometry and their intense expressions in FNRBCs, as shown by immunocytochemistry are encouraging. These potential candidates may be explored further for separation of this cell type from adult RBCs by positive selection by means of immuno-cell sorting techniques such as magnetic activated cell sorting (MACS) or fluorescence activated cell sorting (FACS). Similarly, the absence of immunoreaction of the chloride channel protein in FNRBCs may also be useful for depletion from adult RBCs by such strategies.

4.1. *Biological significance of the unique plasma membrane proteins of FNRBCs*

We performed an in-depth literature search on the presence and the functional roles of identified unique plasma membrane proteins of FNRBCs in various human tissues and cells, including that of fetal origin (trophoblasts/placenta). Please refer to the Supplemental text detailing mainly their locations, and physiological roles (including those related to human fetal development), and diseases related to their mutations. We hope that this may provide an insight for future research on the biological roles of these unique proteins during fetal development and/or in fetal tissues such as placenta.

Briefly, 20 unique membrane proteins could be categorized under seven functional sub-groups: *Transportes/Channel molecules*: two amino acid transporting Solute Carrier (SLC) proteins, neutral amino acid transporter B0 (SLC1A5), SLC3A2; and an anion transporter, splice isoform A of chloride channel protein 6. *Binding proteins*: Transferrin receptor protein 1, Splice isoform 3 of protein GPR107 precursor and olfactory receptor 11H4. *Catalytic*: CAAX prenyl endopeptidase also known as farnesylated protein-converting enzyme (FACE), Vitamin K epoxide reductase complex subunit 1 like protein (VKORC1L1), Splice isoform 1 of protein C20orf22; *Signaling pathway*: Splice isoform 1 of Protein C9ORF5; *vesicle recycling*: Pantopysin; *Anti-microbial proteins*: BCG induced integral membrane protein BIGM 103 (BCG induced gene in monocyte, clone 103), FALL39; *Proteins with no known function*: Cleft lip and palate transmembrane protein 1.

Proteins of unknown location and function—To our knowledge, reports on protein expression or functional identity of five of the identified proteins of FNRBCs (with at least one transmembrane domain) are not available in any other cell/tissue; they are, Hypothetical protein DKFZp586C1924, Splice isoform 1 of protein C20orf22, Hypothetical protein MGC14288, 8 kDa protein and 25 kDa protein. Protein database searches (UniProtKB/Swiss-Prot) did not reveal much information for these proteins.

5. Conclusion

In conclusion, to date, studies on human fetal primitive erythroblasts were more focused to understand the biology of these cells, including hemoglobin switching and regulation of their expression, and, to some extent, on the enrichment of these ideal cells from maternal blood for noninvasive prenatal diagnosis. The proteomic information on the membrane proteins of these cells presented for the first time here would not only help to understand the biology and develop technology for enrichment of these cells from maternal blood for noninvasive prenatal diagnosis but will also attract investigators from different disciplines especially from human fetal development and diseases associated with membrane proteins.

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Author contribution

S.P. wrote the manuscript and participated in analysis and interpretation of data; H.Z. performed the proteomics, RT-PCR studies and analyzed the data, P.K. carried out immunocytochemistry; Q.L., T.K.L., helped in 2DLCMS studies and MS-data analyses; J.S.S., assisted in data analysis; M.C.M.C., N.K., A.P.M. S.B.J. and C-L.H. helped in proteomics study; A.B. and A.V. provided clinical samples; M.C. designed the research, provided overall direction and corrected the manuscript.

Conflicts of interest statement

All authors declare no conflicts of interest. A patent application has been submitted.

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