Effects of individually silenced N-glycosylation sites and non-synonymous single-nucleotide polymorphisms on the fusogenic function of human syncytin-2

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ABSTRACT
The placental syncytiotrophoblast, which is formed by the fusion of cytotrophoblast cells, is indispensable for the establishment and maintenance of normal pregnancy. The human endogenous retrovirus envelope glycoprotein syncytin-2 is the most important player in mediating trophoblast cell-cell fusion as a fusogen. We constructed expression plasmids of wild-type and 21 single-amino-acid substitution mutants of syncytin-2, including 10 N-glycosylation sites individually silenced by mutagenizing N to Q, 1 naturally occurring single-nucleotide polymorphism (SNP) N118S that introduced an N-glycosylation site, and another 10 non-synonymous SNPs located within important functional domains. We observed that syncytin-2 was highly fusogenic and that the mutants had different capacities in merging 293T cells. Of the 21 mutants, N133Q, N312Q, N443Q, C46R (in the CXXC motif) and R417H (in the heptad repeat region and immunosuppressive domain) lost their fusogenicity, whereas N332Q, N118S, T367M (in the fusion peptide), V483I (in the transmembrane domain) and T522M (in the cytoplasmic domain) enhanced the fusogenic activity. We also proved that N133, N146, N177, N220, N241, N247, N312, N332 and N443 were all glycosylated in 293T cells. A co-immunoprecipitation assay showed compromised interaction between mutants N443Q, C46R, T367M, R417H and the receptor MFSD2A, whereas N118S was associated with more receptors. We also sequenced the coding sequence of syncytin-2 in 125 severe pre-eclamptic patients and 272 normal pregnant Chinese women. Surprisingly, only 1 non-synonymous SNP T522M was found and the frequencies of heterozygous carriers were not significantly different. Taken together, our results suggest that N-glycans at residues 133, 312, 332 and 443 of syncytin-2 are required for optimal fusion induction, and that SNPs C46R, N118S, T367M, R417H, V483I and T522M can alter the fusogenic function of syncytin-2.

KEYWORDS
cell-cell fusion; N-glycosylation; placenta; single-nucleotide polymorphism; syncytin-2

Introduction
The human placenta is a transient organ of embryonic origin whose main function involves the exchange of metabolic and gaseous products between the fetal and maternal circulation. It also acts as an important endocrine secretion organ for normal pregnancy and a barrier between the fetus and its mother for immune tolerance, ensuring the preservation of the fetus. The major constituent of human placental tissue is the trophoblast, which stems from the trophectoderm of the blastocyst and develops before any embryonic tissue arises. Villous cytotrophoblast cells (CTBs) have 2 distinct ways of differentiation: to form highly invasive extravillous trophoblast cells or to fuse and form a multinucleated syncytiotrophoblast (STB) layer, a process designated syncytialization. The STB comes into direct contact with maternal blood, secreting important hormones to maintain pregnancy and mediating gas, nutrient and waste exchange. Inadequate syncytialization is correlated with...
severe pregnancy disorders such as pre-eclampsia (PE), which is a leading cause of maternal and fetal mortality.1

Our understanding of the mechanisms underlying syncytialization in the placenta came from the identification of the human endogenous retrovirus (HERV) envelope proteins, syncytins, which are fusogens specifically expressed in the placenta.2–6 HERVs are remnants of infections of former exogenous retroviruses, occupying at least 8% of the human genome at various stages of “fossilization.”7 The 2 bona fide envelope glycoproteins, syncytin-1 and syncytin-2, are encoded by HERV-W (the official name is ERVW-1) and HERV-FRD (official name ERVFRD-1), and the retroviruses carrying them entered the primate lineage 25 and 40 million years ago, respectively.8,9 Indeed, these 2 HERV envelope genes have undergone purifying selection during primate evolution to play important functions in placental physiology.10,11 The existence of 2 murine endogenous retroviral envelope genes (syncytin-A and -B), homologous but not orthologous to the human syncytin genes and also critical for trophoblast cell fusion, have been reported.12 The syncytin-A-deficient mouse placenta shows specific disruption of the architecture of the syncytiotrophoblast layer I (ST-I).13 The syncytin-B null placenta exhibits impaired formation of the syncytiotrophoblast layer II (ST-II).14 Furthermore, syncytin genes with fusogenic activity and placenta-specific expression have been identified successively in the leporidiae, carnivora, ruminant, tenrecidae, rodentia squirrel-related clade and marsupial opossum, namely syncytin-Ory1,15 syncytin-Car1,16 syncytin-Rum1,17 syncytin-Ory1,15 syncytin-Car1,16 syncytin-Rum1,17 syncytin-Ten1,18 syncytin-Mari19 and syncytin-Opo1,20 respectively. This indicates that different syncytins have been acquired by different genomes and facilitate trophoblast cell-cell fusion in various animals.

Syncytin-1 has been visualized in almost all types of trophoblastic cells, including villous and extravillous trophoblast.3,5,6,21–24 Syncytin-1-induced cell-cell fusion is dependent on both ASCT1 and ASCT2 (alanine/serine/cysteine/threonine transporter type 1 and 2), also called SLC1A4 and SLC1A5, respectively, which are Na+-dependent transporters for polar neutral amino acids and a common cell surface receptor for retroviruses.6,25,26 Systematic ASC activity is present in basal but not in apical membranes of the STB,26,29 and ASCT2 appears to be ubiquitously expressed in most human placental tissues, including the CTBs and STB.28,30–32 By contrast, the expression of syncytin-2 is restricted to some of the CTBs throughout pregnancy, and more often at the interface between the CTBs and STB.33 The receptor for syncytin-2, major facilitator superfamily domain containing 2A (MFSD2A), is a carbohydrate transporter with 10–12 transmembrane domains and is specifically expressed in the STB layer.34 The highly specific localization of syncytin-2 and its receptor MFSD2A might argue for a major role of syncytin-2 in synergy with syncytin-1 during the fusion of the mononucleated CTBs into the multinucleated STB in vivo, therefore favoring the growth and renewal of the STB and precluding the fusion of CTBs between themselves.9

The 1,617 bp sequence of the syncytin-2 envelope (env) gene mapped at 6p24.1 is within the HERV-FRD locus and encodes a 73 kDa glycosylated precursor protein.4,33,35,36 Similar to a retroviral envelope protein, syncytin-2 is formed as a homotrimer connected by an intersubunit disulfide bond after posttranslational cleavage into the surface (SU) and transmembrane (TM) subunits by proteolytic furin, with the SU interacting with its receptor on the cell surface and the TM carrying the fusion activity and serving to mediate membrane fusion.36,37 The proper association of SU and TM subunits is necessary for the functional integrity of syncytin-1 and -2 because the chimeras of syncytin-1 and -2 swapping their subunits lose their fusogenicities.38 As shown in Fig. 1A, the syncytin-2 precursor polypeptide contains the following important regions: (a) a signal peptide (SP) in the amino-terminal end (1–15 aa); (b) a disulfide bond isomerase motif (43–46 aa), Cys-X-X-Cys (CXXC); (c) a furin cleavage site (347–350 aa), RVRR, which separates the surface (SU) (16–350 aa) and transmembrane (TM) subunits (351–538 aa); (d) a conserved hydrophobic domain as a putative fusion peptide (FP) (354–374 aa) mediating the fusion of cell membranes; (e) a highly conserved putative immunosuppressive domain (ISD) (414–430 aa); (f) a CX6CC motif (431–439 aa), in which the first 2 cysteines can form an intersubunit disulfide bond and the third cysteine can form an intrasubunit disulfide bridge with the CXXC motif; (g) 2 heptad repeat regions (HR1 and HR2) (384–422 aa and 447–456 aa); (h) a transmembrane domain (TMD) (479–499 aa); and (i) a 39-amino-acid cytoplasmic domain (CTM) (500–538 aa).36,38–40 Functional domain integrity and proper maturation conformation are required for the fusogenic activity of syncytin-2.

In the field of cell-cell fusion, interest in syncytins is emerging. Besides syncytins, only one another unrelated family of fusogens has been discovered, the F proteins in Caenorhabditis elegans.41 To date, critical functional characterization of single-amino-acid mutations of syncytins and their relevance to diseases are still lacking. In the present study, we investigated whether disruption of potential N-glycosylation sites and naturally occurring non-synonymous single-nucleotide polymorphisms (SNPs) (http://www.ncbi.nlm.nih.gov/snp) in functional domains affected the fusogenicity of syncytin-2, and whether naturally occurring SNPs that affected the fusogenic function of syncytin-2
were associated with pregnancy-related complications such as PE. We approached these issues by generating 21 site-specific mutants, including 10 potential N-glycosylation site mutants and 11 naturally occurring SNPs, and assessed their fusogenic activities in 293T and HeLa cells, which do not express syncytin-2 and serve as models for the cell-cell fusion assay. To define the receptor and other molecules interacting with syncytin-2, co-immunoprecipitation and protein mass spectrum analysis were utilized. Our results indicate that N-glycan and naturally occurring individual

**Figure 1.** Schematic of the syncytin-2 gene structure indicating the location of candidate N-glycosylation sites and non-synonymous single-nucleotide polymorphic (SNP) sites and the establishment of the fusion assay for functional evaluation of syncytin-2 and its mutants. (A) Upper panel: structure of a human endogenous retrovirus (HERV)-FRD gene locus and its encoded envelope protein syncytin-2. HERV is typically composed of 5′ and 3′ LTRs (long terminal repeats), gag (group-specific antigen gene), pol (polymerase), and env (envelope). Syncytin-2 encoded by the env gene is composed of SU and TM subunits. Lower panel: schematic diagram of syncytin-2 indicating the positions of 10 potential N-glycosylation sites (numbered 1–10) and 10 naturally occurring SNPs from SNP databases (http://www.ncbi.nlm.nih.gov/snp) in predicted functional domains (numbered 11–21). The positions of amino acids of different domains/motifs are indicated. SU, surface protein; TM, transmembrane protein; SP, signal peptide; FP, fusion peptide; HR, heptad repeat region; ISD, immunosuppressive domain; TMD, transmembrane domain. CTM, cytoplasmic domain. (B) Establishment of 293T and HeLa cell lines that stably express EmGFP. (C) Cell-cell fusion mediated by exogenous syncytin-2. The 293T-EmGFP cells were transfected with phCMV empty vector or phCMV-syncytin-2 for 36 h. HeLa-EmGFP cells were co-transfected with phCMV-syncytin-2 and pcDNA3.1-MFS2A-FLAG for 36 h. Single-plasmid transfection with either phCMV-syncytin-2 or pcDNA3.1-MFS2A-FLAG did not cause cell-cell fusion. All experiments were repeated 3 times. Bars = 100 μm.
SNPs have different implications in regulating syncytin-2 function and provide a better understanding of the structure-function relationship of syncytin-2.

### Results

**Searching for single mutation sites that might affect the fusogenicity of syncytin-2 and the establishment of a syncytin-2-mediated fusion assay in 293T and HeLa cells**

We first analyzed the putative N-glycosylation sites of syncytin-2 using the tool provided by the UniProt database. In total, there are 10 potential N-glycosylation sites, with the first 8 located in the SU and the last 2 located in the TM (Fig. 1A, numbered as 1–10). We also searched the SNP database and found that 162 naturally occurring non-synonymous SNPs in the open reading frame (ORF) of syncytin-2 had been recorded. We selected 10 sites that were located within important functional domains or motifs. Of these, N118S probably introduced a new N-glycosylation site, C46R was located in the CXXC domain, A359T and T367M were located in the CXXC domain, A359T and T367M were located in the SU subunit main domain, and N220Q, N241Q and N247Q in the SU subunit main domain (Fig. 1A, numbered as 11–21). To characterize the effects of these N-glycosylation sites and non-synonymous SNPs on the fusogenic function of syncytin-2, we established a fusion assay using 293T-EmGFP and HeLa-EmGFP cell lines that stably overexpress green fluorescent protein (Fig. 1B) to monitor cell-cell fusion. The syncytin-2 receptor, MFSD2A, is expressed in 293T cells. Therefore, syncytin-2-mediated cell-cell fusion could be observed 24 h after transfection with a syncytin-2 expression plasmid. As fusion proceeded, patches of green fluorescence were seen as the formation of multinucleated syncytia (Fig. 1C). Moreover, as HeLa cells do not express MFSD2A, syncytin-2 mediated cell-cell fusion only occurred when co-transfecting syncytin-2 and MFSD2A overexpression plasmids, but not either plasmid alone (Fig. 1C).

### Production and analysis of the effects of 21 single-amino-acid-substitution mutants that either disrupt N-glycosylation sites or are encoded by naturally occurring non-synonymous SNPs on the fusogenicity of syncytin-2

We first explored the requirement of each N-glycosylation site for the fusogenic function of recombinant syncytin-2 by N to Q substitution at N133, N146, N177, N220, N241, N247, N312, N332, N443 and N523 (Table 1). Meanwhile, the naturally occurring N118S, which probably introduces an N-glycosylation site, was also investigated. Of these mutants, N146Q, N177Q, N220Q, N241Q and N247Q in the SU subunit main domain maintained their original fusion efficiency compared to wild-type (WT) syncytin-2, whereas N133Q, N312Q and N443Q lost their fusogenicity and N118S and N332Q acquired enhanced fusogenic activity (Fig. S1). Importantly, as shown in Fig. 2, when the cell samples were subjected to Western blot analysis, all N to Q substitutions except for N523Q revealed bands with lower mobility compared to wild-type syncytin-2.

### Table 1. Nucleotide and amino acid changes introduced to generate syncytin-2 mutants by site-directed mutagenesis.

| Mutant site | Residue number | Forward Primer (5' - 3') | Allele change | Fusion |
|-------------|----------------|--------------------------|---------------|--------|
| 1           | 133            | GGTATGCGCCAAAAGAAAAACAGGGAAACAATGAGGCACTC | aat→cag N→Q | +     |
| 2           | 146            | CTCTCCAAATCACCTGTCAGGTCACTTCTGAGATTTA | aat→cag N→Q | +     |
| 3           | 177            | CAAGATCTCCAAACCTCCACAGTATCTTCTCAGGGGAA | aat→cag N→Q | +     |
| 4           | 220            | CCAGCTGTCGACACATTTCCAGCTCACTGAGGCAAAGTG | aac→cag N→Q | +     |
| 5           | 241            | GTGAAATCTTCTTTTTGGGAAACACAAAGGGGACCACTACAGCA | aat→cag N→Q | +     |
| 6           | 247            | GGGAAATAGCAACAGGGCTACAGAAGCGCACCACACTTGTCCGC | aac→cag N→Q | +     |
| 7           | 312            | CACCAATGCTCCCAGTCTCAATGTGAACTTACCC | aac→cag N→Q | +     |
| 8           | 332            | CTCTCTGCCCCTGCACTGCTCTTCTCAATACAT | aat→cag N→Q | +     |
| 9           | 443            | GAGAAATGTGGCTTTTGAGTACACAAAGAGATACACAGA | aat→cag N→Q | +     |
| 10          | 523            | GGGCTAAGAGGCTGAGGACCACGTACGAGGACCGGACCAC | aat→cag N→Q | +     |
| 11          | 46             | CCCAAGTGCTGAGGGAATCTGAGTCTCTTCCAC | tgt→cgt N→Q | ++    |
| 12          | 118            | CCATCTCTTATATAGCTCTCTAATGTTGAAGGTTAAGAC | aac→agc N→Q | ++    |
| 13          | 359            | CATTCTCTCTTCCTCACGGGAATCTGCCATCTAG | gcc→acg N→Q | +     |
| 14          | 367            | CGGGATTTGCTGAGTGGGAACCGAAAGGTGCTG | acg→atg N→Q | ++    |
| 15          | 417            | CAGCGTATGCTCCAAAATCAGGGAGCTAGCAGTAGTAA | cgt→cat N→Q | +     |
| 16          | 432            | GCAAGAGGAGATTTGTCGCTTAGTAAAAATG | ttc→ttg N→Q | ++    |
| 17          | 483            | GGAATGGCTGAGGGAATCTGAGTCTCTTCCAC | tgt→cgt N→Q | ++    |
| 18          | 503            | GCTCCCTTTCCTGTCTGCTAATGGCTTACTAATCC | ctc→ttc N→Q | +     |
| 19          | 518            | GTGCCCCTTCCCCTGCACTGAGGGAATCTGAGTCTCTACAGG | ata→gt N→Q | +     |
| 20          | 522            | GGCTAAAGAGGCTGAGGAGCTAGGAGGAGCAGG | acg→atg N→Q | ++    |
| 21          | 536            | CCTCCTAAATCCATGAGGTTCACTTCTAGTGCTGAG | tca→ttg N→Q | +     |

* Cell fusion was monitored 36 h after transfection. −, no fusion; +, moderate fusion; ++, excessive fusion.
molecular weight (arrowhead) than WT syncytin-2, indicating that N-glycosylation of syncytin-2 was widely distributed at these sites. Moreover, the naturally occurring N118S caused a higher molecular weight (arrow), indicating that the introduction of an extra N-glycosylation site occurred. Furthermore, the expression levels of the WT and mutant syncytins were consistent, showing that the transfection efficiency of the different plasmids and the exogenous expression of syncytin-2 were at a same level. Although the N118S immunostaining was reduced, it still significantly elevated the fusion efficiency. As a control, when all the N-glycans were removed by treatment with PNGase F N-glycanase, only a ~55 kDa band was seen, whereas the precursor of syncytin-2 protein appeared to be ~73 kDa, in accordance with previous results.33,35, 36

We also investigated the effects of the 10 selected non-synonymous SNPs located within important functional domains/motifs on the fusogenic function of syncytin-2. Of these, A359T, L432V, L503F, I518V and S536L had no impact on the fusion efficiency mediated by syncytin-2 (Fig. S1). Notably, C46R and R417H inhibited normal cell-cell fusion, whereas T367M, V483I and T522M enhanced fusogenic activity compared with the WT (Fig. S1). Again, the expression levels of these mutants were consistent with that of WT (Fig. S2).

Identification of dysfunctional syncytin-2 mutants that stimulate or inhibit syncytin-2-mediated fusion

We next wanted to further characterize the above dysfunctional mutants together with WT syncytin-2, and the effects of the different stimulatory mutants (including N118S, N332Q, T367M, V483I and T522M) and inhibitory mutants (including N133Q, N312Q, N443Q, C46R and R417H) were confirmed (Fig. 3A). N118 and N133 are close to each other and within the N-terminal half of the SU subunit. Gaining an extra N-glycosylation site at N118 and removal of the N-glycan at N133 stimulated and inhibited the fusion efficiency, respectively. However, although both N312 and N332 are located in the C-terminal region of the SU subunit, disruption of both sites individually caused contradictory effects. Furthermore, C46R within the CXXC motif, T367M within the FP, R417H within the HR1 and ISD, V483I within the TMD and T522M within the CTM all caused significantly altered fusogenic activity, indicating that these functional domains were sensitive to single-amino-acid substitution and that these sites were required for optimal fusion induction (Fig. 3A). Importantly, Western blot (Fig. 3B) and quantitative real-time PCR (Fig. 3C) analysis were used to ensure that all the WT and mutant syncytins were expressed at similar levels both at the mRNA and protein levels. Again, N118S stimulated the fusogenic activity of syncytin-2, despite the reduced immune-reactivity (Fig. 3B). Because its mRNA was expressed normally, one explanation for this could be the loss of an epitope due to the amino acid substitution.

We then tested whether the 5 inhibitory mutants (N133Q, N312Q, N443Q, C46R and R417H) blocked syncytin-2-mediated fusion in 293T-EmGFP cells. We found that 293T-EmGFP cells in 6-well plates transfected with 1.5 μg WT syncytin-2 expression vector for 36 h
formed obvious green multinucleated syncytia. The induction of cell-cell fusion upon syncytin-2 overexpression was dose-dependent, and higher doses of expression plasmids triggered cell-cell fusion to a larger extent (Fig. 4A and 4B). We then co-transfected the cells with 1.5 μg phCMV-syncytin-2 WT and 1.5 μg inhibitory mutant plasmids. As a control, the plasmids expressing the mutants were replaced with phCMV empty vector. As shown in Fig. 4C, the C46R and R417H mutants almost completely blocked the fusogenicity of WT
Mutants N133Q, N312Q and N443Q significantly suppressed the fusogenic properties of the native syncytin-2 protein. These results suggest that these mutants cause significant repressive effects on the fusogenic activity of syncytin-2 and therefore may serve as competitive inhibitors in WT syncytin-2-mediated cell-cell fusion.

The abnormal interaction between syncytin-2 mutant and its receptor MFSD2A during cell-cell fusion

To verify the interaction between syncytin-2 and its receptor MFSD2A, they were co-overexpressed in HeLa cells that express neither protein and analyzed by co-immunoprecipitation. We firstly detected the expression of FLAG-tagged MFSD2A in HeLa cells by Western blotting using an anti-FLAG antibody. No immunoreactive bands were detected in control mock-transfected cells and pEF6-MFSD2A-transfected cells (Fig. 5A). Monoclonal anti-FLAG antibody was then used for the co-immunoprecipitation of syncytin-2 in HeLa cells. The lysed cell samples precipitated by the anti-FLAG antibody contained significant amounts of syncytin-2 protein, indicating that full-length MFSD2A associates with syncytin-2. Syncytin-2 was not detected either in the co-transfection of pcDNA3.1-MFSD2A-FLAG and phCMV empty vector or of pcDNA3.1-GFP-FLAG and phCMV-syncytin-2, indicating the adequate protein expression in this system and that the antibodies in this expression system are largely devoid of nonspecific protein binding. To infer the sensitivity and specificity of syncytin-2 with its
receptor MFSD2A, we further examined the interaction between different syncytin-2 mutants and FLAG-tagged MFSD2A. The expression levels of WT syncytin-2 and syncytin-2 mutants were adjusted to similar levels. MFSD2A-FLAG strongly co-immunoprecipitated with WT syncytin-2 and mutants N133Q, N312Q, N332Q, V483I and T522M, whereas minimal amounts were detected in N443Q, C46R, T367M and R417H immunoprecipitated complexes. N118S was associated with more receptors than the WT (Fig. 5C). We also employed preliminary mass spectrometry studies to find syncytin-2 interacting proteins during membrane merging (see Supplementary data and Fig. S3).

**Identification of the T522M SNP in the coding region of syncytin-2 by genomic DNA sequencing in sPE patients and normal pregnant women**

We further set out to identify non-synonymous SNPs in the coding region of syncytin-2 in 125 sPE patients and 272 normal pregnant women by genomic DNA sequencing. Surprisingly, only 1 missense mutation (rs138651238; c.1565C>T and p.T522M) was discovered (Fig. 6). In total, 18 heterozygous carriers of T522M out of 272 normal pregnant women were found. However, only 4 heterozygous carriers out of 125 sPE patients were identified. The frequency of T522M heterozygous carriers was 0.0618 in the control group versus 0.0320 in sPE patients. A chi-square test was used to analyze the difference, revealing a chi-square of 0.167 and a P value of 0.124. The heterozygosity and MAF of T522M recorded by the 1000 Genomes Project are 0.009 and 0.0044, respectively. Therefore, the frequency of this SNP appeared to be higher in the Chinese population.

**Discussion**

We identified 10 single-amino-acid-substitution human syncytin-2 mutants with abnormalities in fusogenic function, including N133Q, N312Q, N332Q and N443Q, which silenced the N-glycosylation sites individually, and the naturally occurring SNPs C46R, N118S, T367M, R417H, V483I and T522M. N118S introduced a new N-glycosylation site. Of these 10 mutants, N133Q (in the N-terminal half of the SU), N312Q (in the C-terminal region of the SU), N443Q (in the TM, close to the CX6C motif and HR2 domain), C46R (in the CXXC motif) and R417H (in the HR1 and ISD domain) exerted inhibitory effects on the fusogenic activity of syncytin-2, whereas N118S (in the N-terminal half of the SU), N332Q (in the C-terminal region of the SU), T367M (in the FP), V483I (in the TMD) and T522M (in the CTM) were stimulatory.

Several lines of evidence suggest that the sequence polymorphisms of HERV family genes provide
susceptibility to human diseases. For example, ERVW-1 LTR SNPs (142 T>C and 277 A>G) are associated with syncytin-1 overexpression and may be an indicator of the risk of urothelial cell carcinoma of the bladder. However, there is also a report suggesting the conservation of the ERVW-1 locus, including the LTR transcriptional elements and the splice sites involved in env mRNA maturation in 155 individuals. The authors also sequenced the envelope ORF that encodes syncytin-1 in 24 individuals and found 4 non-synonymous mutations that occurred at a frequency of 25%, 4%, 2%, and 2%, respectively. However, these polymorphic variants are functionally preserved and do not affect the fusogenic function of WT syncytin-1 in a heterotypic cell-cell fusion assay. To our knowledge, this is the only published study related to the functional analysis of the non-synonymous SNPs in the coding region of syncytins. Despite the availability of public SNP databases in which 162 non-synonymous SNPs of syncytin-2 have been recorded, their functional study and relevance to diseases remain surprisingly elusive.

As one of the most common post-translational protein modifications, N-glycosylation is crucial for many elementary biological and pathological processes. Syncytins belong to the class I fusion proteins that include the envelope proteins of many viruses, such as the human immunodeficiency virus (HIV). Typically, class I fusion proteins are synthesized as glycosylated precursors in the endoplasmic reticulum and then modified by N-glycosylation and disulfide bond formation. In the case of the HIV envelope protein, N-glycosylation is critical for viral infectivity, immunogenicity, cytopathicity and transmission. In an extensive study, 23 N-glycosylation sites in the gp120 subunit of the simian immunodeficiency virus (SIV) envelope protein are individually silenced, and the mutations result in different effects on viral infectivity, replication and recovery. Therefore, we set out to screen all the N-glycosylation sites in syncytin-2 and to investigate whether they are necessary for the fusogenic function.

Using individual deglycosylation studies, we verified that except for N523, the other 9 predicted N-glycosylation sites, including N133, N146, N177, N220, N241, N247, N312, N332 and N443, were indeed glycosylated in 293T cells. Moreover, the naturally occurring N118S introduced an extra N-glycosylation site and produced a protein with a higher molecular weight. Of these 10 glycosylated sites, the first 9 were all located within the SU subunit. Interestingly, only the mutations of the first 2 and the last 2 sites (N118S, N133Q, N312Q and N332Q), but not of the middle 5 sites (N146Q, N177Q, N220Q, N241Q and N247Q) altered the fusogenicity of syncytin-2. The binding of the SU to the receptor is the prerequisite for the FP at the N-terminus of the TM to drive membrane fusion. Therefore, our results indicate that the N-glycans located at the N-terminal half and C-terminal arm of the SU subunit are important for syncytin-2-mediated cell-cell fusion probably by affecting the interaction between the SU and the receptor. The non-functional sites from N146 to N247 indicate that this region is flexible in conformation and tolerates losing the glycosylation. In the case of syncytin-1, the N-terminal 124 amino acids of the SU are the minimal receptor-

**Figure 6.** Identification of heterozygous carriers of syncytin-2 T522M (rs138651238) in normal pregnant women and severe pre-eclamptic (sPE) patients. (A) Frequency of T522M in normal pregnant women (n = 272) and sPE patients (n = 125). (B) Sequencing of syncytin-2 coding region revealed a C>T single-nucleotide polymorphism with amino acid substitution at 522 (T522M). MAF, minor allele frequency.
binding domain containing a conserved 18-residue motif essential for syncytin-1-ASCT2 interaction.\textsuperscript{48} Moreover, N443Q, which is in proximity to the CX6CC motif and HR2 domain in the central region of the ectodomain TM, also significantly suppressed the fusogenic property of the native syncytin-2 protein. As reported, the 2 heptad repeat regions HR1 and HR2 specifically form intramolecular interaction between each other and are necessary for syncytin-mediated cell-cell fusion.\textsuperscript{38} Our results add new evidence that the N443Q might destabilize the hairpin conformation of HR1 and HR2, or hinder the formation of the disulfide bond, and discourage cell-cell fusion. Taken together, our results indicate that syncytin-2 has 9 N-linked glycosylation sites, among which glycans at residues 133, 312, 443 and 332 are required for optimal fusion induction, and that appropriate sugar chain density over the specific region is preserved for functional integrity.

We also generated another 10 mutants carrying the naturally occurring SNPs C46R, A359T, T367M, L432V, R417H, V483I, L503F, I518V, T522M and S536L, which are located in important functional domains or motifs. Of these, 2 defective mutants (C46R and R417H) and 3 stimulatory mutants (T367M, V483I and T522M) were identified. C46R is located in the CXXC motif of the SU subunit, which is highly conserved among retroviral envelope proteins and participate in the formation of a labile intersubunit disulfide bond with the CX6CC motif of the TM subunit for isomerization.\textsuperscript{36,49} Therefore, the silencing effect of C46R on the fusogenic activity confirmed the significance of the CXXC motif for fusogenesis and the necessity of the cysteine for the formation of an intersubunit disulfide bridge. This agrees well with the effect of C46A from a previous extensive study, which shows mutations in the CXXC motif not only inhibit fusion but also can function as dominant-negative mutants.\textsuperscript{36} However, the naturally occurring SNP L432V located in the CX6CC motif in the TM subunit did not alter the fusion efficiency, indicating the flexibility of the 6 amino acids between the 3 cysteines. R417H is located in 2 domains, the HR1 domain discussed above and the 17-amino-acid ISD present in several retroviral envelope proteins that is highly immunosuppressive for inhibiting immune function.\textsuperscript{50,51} R417H led to a complete loss of its fusogenic activity, implying the significance of both domains for syncytin-2 fusion ability.

Of the 3 stimulatory mutants that can enhance the fusogenic activity of syncytin-2, the T367M is located within the FP. FP is a conserved hydrophobic domain located at the N-terminus of the TM subunit mediating cell membrane fusion.\textsuperscript{38} The SNP V483I is located at the hydrophobic stretch TMD domain. This suggests that the conformation of the hydrophobic transmembrane region might be sensitive to single-amino-acid substitution, although both valine and isoleucine are nonpolar amino acids. The CTM of syncytin-1 and syncytin-2 is essential for their fusogenic activities. The comparison of human ERVW-1 elements and the orthologous loci in simians reveals an syncytin-1-specific signature in the intracytoplasmic tail is crucial for the envelope fusogenic activity.\textsuperscript{10} A systematic study involves a series of C-terminal truncated mutants of syncytin-1 shows that cytoplasmic sequences immediately adjacent to the transmembrane region are necessary for inducing optimal cell-cell fusion, whereas the extreme C-terminus partially inhibits its fusogenic function.\textsuperscript{39} Similarly, different C-terminally truncated syncytin-2 mutants led to a decrease in the size and number of syncytia at different levels.\textsuperscript{36} Of the 4 SNPs (L503F, I518V, T522M and S536L) in the CTM tested, only T522M changed the fusogenic activity compared with the WT. This is also consistent with previous findings that some regions in the CTM of syncytin-1 are flexible in the conformation.\textsuperscript{38} Altogether, we have characterized 5 SNPs (C46R, T367M, R417H, V483I and T522M) in conserved domains as strong candidates that can alter the fusogenic function of syncytin-2.

To further define the functional properties of the 5 defective mutants, WT and mutant syncytin-2 expression plasmids were co-transfected. We found that the C46R and R417H mutants almost completely blocked syncytin-2-mediated cell-cell fusion, and N133Q, N312Q and N443Q mutants significantly suppressed the fusogenic properties of the native syncytin-2 protein. The molecular mechanism of inhibition is unclear, but it is likely that the mutants with single-amino-acid substitutions in important functional domains can efficiently compete with native syncytin-2 to bind the receptor or to trigger signaling pathways for merging membranes. We therefore employed co-immunoprecipitation experiments to study the sensitivity and specificity of the interaction between syncytin-2 and its receptor MFSD2A. Of the 5 defective mutants, N133Q and N312Q could be pulled down normally by MFSD2A, whereas N443Q, C46R and R417H showed compromised interaction with the receptor. The results with R417H and N443Q are quite unexpected because they are located within the TM subunit. This finding probably provides insight into the significance of the TM subunit serving as an indispensible component of the SU-TM homotrimer during the interaction between the SU and the receptor. Notably, the significantly induced fusion with the N118S mutant
correlated well with its higher ability to bind with the receptor. However, whether the T367M mutant that showed reduced interaction with the receptor while promoting fusion is related to the activation of other signaling pathways remains to be studied.

PE is a common, multi-system and serious complication of human pregnancy affecting 3–5% of all women. Characterized by new-onset hypertension and proteinuria after 20 weeks of gestation, PE is a major contributor to maternal morbidity and mortality worldwide, and unbalanced syncytialization has been implicated in its pathology. Expression of both syncytin-1 and syncytin-2 are significantly decreased in sPE placentas vs. normal control. In our attempts to identify polymorphic sites within the coding sequence of syncytin-2 in 125 sPE patients and 272 normal pregnant women, only 1 missense mutation [rs138651238 (T522M)] was found. This mutation enhanced syncytin-2-mediated cell-cell fusion in vitro and occurred at a frequency of 0.0320 in sPE patients and 0.0618 in the control group. However, the statistical analysis revealed no significant difference. Considering the large number of non-synonymous SNPs in the coding region of syncytin-2 in the SNP database, further studies in a larger sample size are warranted.

Taken together, we provided novel in vitro experimental evidence for the structure-function relationship of syncytin-2 through investigating 21 single-amino-acid mutants. We proved that N133, N146, N177, N220, N241, N247, N312, N332 and N443 played important roles in the fusogenic function of syncytin-2. The naturally occurring polymorphisms C46R, R417H, N118S, T367M, V483I and T522M led to abnormal fusogenicity. The binding between syncytin-2 and its receptor MFSD2A was compromised by the N443Q, C46R, T367M and R417H mutations, whereas N118S was associated with more receptors. We sequenced the syncytin-2 coding sequences in sPE patients and normal pregnant women and found 1 missense mutation T522M. The above results suggest some key residues in determining the fusogenic function of syncytin-2, and investigation of the mechanisms will help to better understand how this molecule merges the membranes of CTBs.

Materials and methods

Blood and DNA samples

A total of 397 blood samples were collected from 272 normal pregnant Han Chinese women and 125 sPE patients undergoing legal procedures in Beijing Obstetrics and Gynecology Hospital and Peking University Third Hospital. A woman was determined to have sPE when either severe hypertension (a systolic blood pressure \( \geq 160 \text{ mmHg} \) or a diastolic blood pressure \( \geq 110 \text{ mmHg} \)), severe proteinuria (more than 5 g of protein was collected in a 24 h urine specimen), or both, were present after 20 weeks of gestation. None of the sPE patients involved in this study had other maternal complications. All the sPE patients were aged 23–35 and delivered at 25 to 40 weeks. In all cases, blood samples were anonymized after routine analysis was completed.

Ethical approval and utilization of samples under standard experimental protocols was granted by the Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Peking University Third Hospital and the Institute of Zoology, Chinese Academy of Sciences. Genomic DNA from blood lymphocytes was extracted using a genomic DNA extraction kit (DN0113, Biomed Biotechnology Corp., Beijing, China).

Plasmid construction and site-directed mutagenesis

The human syncytin-1 expression construct phCMV-syncytin-114 is a kind gift from Dr. Francois Mallet, Laboratoire Commun de Recherche Hospices Civils de Lyon-bioMérieux, Cancer Biomarkers Research Group, Ecole Normale Supérieure, Lyon, France. The phCMV empty vector was generated by removing the syncytin-1 cDNA. The syncytin-2 cDNA fragment was amplified from pTM1-syncytin-236 (a kind gift from Dr. Hungwen Chen, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan) and cloned into phCMV. A full-length cDNA encoding the human MFSD2A with FLAG was amplified from pEF6-MFSD2A63 (a kind gift from Dr. Tommaso Dragani and Dr. Francesca Colombo, Department of Predictive and for Prevention Medicine, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy) and cloned into Xhol/XbaI-digested pcDNA3.1 to generate pcDNA3.1-MFSD2A-FLAG. The full-length EmGFP coding sequence was amplified from the pcDNA6.2-EmGFP vector (V355–20, Invitrogen) and cloned into the pQCXIP vector using the restriction enzymes NotI/BamHI to generate pQCXIP-EmGFP. The primers used for amplifying the above cDNAs are summarized in Table 2.

The 21 human syncytin-2 mutants, including 10 mutants (N133Q, N146Q, N177Q, N220Q, N241Q, N247Q, N312Q, N332Q, N443Q and N523Q) removing potential N-glycosylation sites, 1 naturally occurring SNP (N118S) introducing a new potential N-glycosylation site, and another 10 naturally occurring SNPs (C46R, A359T, T367M, R417H, L432V, V483I, L503F, I518V, T522M and S536L) located in predicted important functional domains were generated on the WT phCMV-syncytin-2 expression vector template using the
QuikChange II XL site-directed mutagenesis kit (200514, Stratagene) following the manufacturer’s instructions and verified by sequencing. The forward and reverse primers used for mutagenesis are summarized in Table 2. All constructs were sequenced to confirm that only the targeted mutations had occurred. The putative N-glycosylation sites in human syncytin-2 proteins were obtained from the UniProt database.

Cell lines

293T and HeLa cells were purchased from the American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 100 μg/ml streptomycin and 100 U/ml penicillin, and maintained in 95% air and 5% carbon dioxide at 37°C.

Establishment of stable GFP-expressing 293T and HeLa cell lines

293T and HeLa cells were transfected with pQCXIP-EmGFP using Lipofectamine 2000 (15338–100, Invitrogen) according to the manufacturer’s instructions. Transfected cells were subjected to puromycin (P8833; Sigma Chemical Co.) selection (1.5 μg/ml for 293 T cells and 0.125 μg/ml for HeLa cells), and the antibiotic-resistant clones were pooled for studies. The expression of EmGFP was confirmed under a fluorescence microscope.

Fusion assay of syncytin-2

Cell-cell fusion mediated by syncytin-2 and its mutants was monitored 36 h after expression plasmid transfection by tracing EmGFP-expressing 293T or HeLa cells. Fusion events were traced by fluorescence microscopy as the formation of green multinucleated syncytia in transfected cells.38 For the negative control, cells were transfected with phCMV empty vector.

Real-time quantitative-PCR

Total RNA was extracted and purified from cultured cells using TRIzol reagent (15596–018, Invitrogen) according to the manufacturer’s instructions. RNA concentration was determined with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific). Two micrograms of total RNA were reverse transcribed into single-stranded cDNA using Superscript II reverse transcriptase (18064022, Invitrogen). Real-time quantitative PCR was performed using SYBR Premix Ex Taq kit (DRR081A, Takara) with the Real-time PCR System (ABI PRISM 7500 Real-time PCR System, Applied Biosystems). Specific primers are indicated in Table 2. Data were analyzed using ΔCt method and normalized to GAPDH expression. All data were based on experiments performed at least in triplicates.

Western blotting and co-immunoprecipitation

The cells were homogenized in whole cell lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100). A total of 30 μg/well of proteins were resolved by SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (Pall Corporation). After incubation with blocking buffer (5% fat-free milk in phosphate-buffered saline containing 0.5% Tween 20), membranes were incubated overnight at 4°C with specific primary antibodies against syncytin-2 (P60508,
Abgent) and GAPDH (ab37187, Abcam), followed by incubation with appropriate secondary antibody. The blot was developed using the enhanced chemiluminescence (Pierce Chemical Co.). PNGase F N-glycanase (P0704S, Sigma Chemical Co.) was used to remove N-glycans in the protein samples following the manufacturer’s instructions.

For the co-immunoprecipitation assay, HeLa cells were co-transfected with 4 μg WT or mutant human syncytin-2 constructs and 4 μg pcDNA3.1-MFSD2A-FLAG in 60 mm culture dishes. Control experiments were performed by transfecting cells with appropriate empty vectors, phCMV empty vector or pcDNA3.1-GFP-FLAG. After transfection for 48 h, the cells were lysed by suspension in ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol and 0.5% Triton X-100) supplemented with protease inhibitor cocktail (1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin and 1 mM sodium orthovanadate) (04693132001, Roche Diagnostics) for 1 h. After centrifugation at 12,000 g for 20 min, the cell lysates containing FLAG-tagged MFSD2A proteins were immunoprecipitated with anti-FLAG M2 agarose beads (F2426, Sigma Chemical Co.) at 4°C overnight. After centrifuging and washing for 3 times, samples were subjected to Western blotting analysis with an anti-syncytin-2 antibody.

**Mass spectrometry analysis**

Immunoprecipitation of the concentrated proteins was carried out with protein A sepharose beads (10–1141, Invitrogen) essentially as described by the manufacturer. 293T cells were transfected with phCMV empty vector, phCMV-syncytin-2 WT, phCMV-syncytin-2 N312Q and phCMV-syncytin-2 T522M plasmids, respectively. After transfection for 48 h, the cells were lysed by suspension in ice-cold lysis buffer for 1 h. Lysates were collected after centrifugation at 12,000 g for 20 min and immunoprecipitated with 3 μg anti-syncytin-2 antibody. Following an overnight incubation, samples were added to 30 μl of protein A bead slurry and rotated for 4 hours at 4°C. After centrifuging and washing 3 times, samples were separated on an SDS-PAGE resolving gel and subjected to direct mass spectrometry (MS) analysis. Using the LTQ-orbitrap XL mass spectrometer (Thermo Fisher Scientific), 4 samples were run and analyzed by Scaffold (Proteome Software). The peptide identities were accepted when the Peptide Prophet algorithm specified probabilities were at >95.0%. Argot2 (Annotation Retrieval of Gene Ontology Terms; http://www.med-comp.medicina.unipd.it/Argot2) was used to identify the functional classifications of the total and specific expressed proteins. DAVID (DAVID Bioinformatics Resources 6.7; http://david.abcc.ncifcrf.gov/tools.jsp) was used to perform functional classification of the differentially expressed proteins (the fold change was greater than or equal to 1.5 and the spectrum count number was not less than 4). Cytoscape was used to draw programs.

**Genomic DNA sequencing of the human syncytin-2 coding region**

All PCR amplification reactions (50 μl) were performed using the TaKaRa LA Taq standard conditions. The 9.5 kb ERV-FRD locus including flanking non-retroviral sequences in chromosome 6, LTRs, gag, pol and env ORF was amplified by long-distance PCR according to the reported method using sense primer LF and antisense primer LR. Each reaction tube contained a maximum of 50 ng of DNA, 250 mM of each primer, 1.2 mM MgCl2, 500 μM dNTPs, and 5 units of TaKaRa LA Taq DNA polymerase in 50 μl of PCR buffer. Cycling conditions were 3 min at 94°C, followed by 30 cycles of PCR. Each cycle consisted of 30 sec at 94°C, 40 sec at 58°C, and a 10 min elongation step at 68°C. The 30th cycle included an additional 10 min elongation step at 68°C.

For template, 0.5 μl of the 9.5 kb long-distance amplification product was used to amplify the full-length syncytin-2 env gene using sense primer SF and antisense primer SR. The amplification fragment was sequenced using antisense primer S1, sense primer S2 and sense primer S3, which are located at 655–674 bp, 466–487 bp and 967–986 bp in the coding region of syncytin-2, respectively. At least 2 independent readings on each strand were performed. The primers used for amplifying and sequencing the full-length syncytin-2 ORF are indicated in Table 2.

A chi-square test was used to analyze the difference of the frequency of T522M between normal pregnant women and sPE patients. The analysis was conducted using Statistical Package for Social Science software (SPSS for Windows version 10.0; SPSS Inc.), and \( P < 0.05 \) was considered significant.

**Abbreviations**

- **ASCT**: alanine/serine/cysteine/threonine transporter
- **CTBs**: cytrophoblast cells
- **CTM**: cytoplasmic domain
- **DMEM**: Dulbecco’s modified Eagle’s medium
- **env**: envelope
- **FBS**: fetal bovine serum
- **FP**: fusion peptide
- **gag**: group-specific antigen gene
- **HERV**: human endogenous retrovirus
- **HR**: heptad repeat region
- **ISD**: immunosuppressive domain
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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