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SYNCYTINS

Retroviral proteins with potential role in the development of drug delivery systems.

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Syncytins: retroviral proteins with potential role in the development of drug delivery systems.

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Abstract

More than 40 millions of years ago, Human Endogenous retroviruses (HERV) introduced their envelope genes into the genome of primates. These genes were positively selected and they encode fusogenic glycoproteins now known as syncytin-1 (HER-W family) and syncytin-2 (HERV-FDR family). The syncytins were conserved in evolution to participate in the development of the placenta where they promote fusion, proliferation and differentiation of trophoblasts to form the syncytium layer that will connect foetus and mother. The fusogenic properties of the syncytins have been well-established in a variety of cells promoting homotypic and heterotypic cell fusion upon interaction with their respective membrane receptors: ASCT-2 (syncytin-1) and MFSD-2A (syncytin-2). Most of the information available regarding these proteins are related to their role in placentation and their fusogenic properties. Recent studies, however, focus on elucidating putative non-fusogenic properties (immune suppression) related to these proteins as well as their role in diverse pathologies (preeclampsia and cancer) in which their abnormal expression have been detected. We believe that both fusogenic and non-fusogenic properties of the syncytins hold great therapeutic potential in the field of diagnostics and drug delivery systems. Therefore, in this review, we compiled relevant data about syncytin-1 and synctin-2 to provide insight into their presently recognised functions in physiological and pathological conditions that could contribute to the use of these proteins for therapeutically purposes in the future.

Keywords: Syncytin-1, syncytin-2, cell fusion, placentation, immunosuppressive

1. Introduction

Syncytin-1 and -2 are endogenous fusion glycoproteins encoded by envelope (*env*) genes that once belonged to human endogenous retroviruses (HERV) of the W and FDR families, respectively[1,2]. These 'captured' proteins have conserved structural and functional properties comparable to those documented for the envelope viral proteins and, as a result, the syncytins can promote fusion between cell membranes upon interaction with a surface receptor[2–5].

The *HERV* genes were incorporated into the human genome millions of years ago and unlike most of the *HERV* genes, which have been deleteriously mutated, the *syncytin* genes were highly conserved during evolution[2,4,6,7]. These elements were most likely preserved because their function may have been converted for the host's benefit. Both proteins are specifically expressed in the placenta[1,2] and, in the past years, many studies have evidenced that their fusogenic properties play crucial role in placentation [3,8,9]. It has been proposed that the introduction of these genes into the genome of mammals may have been crucial to the development of the *Placentalia* group[3]. The syncytins are synergistically involved in cytotrophoblast differentiation into syncytium[3] (a multinuclear cell formed upon fusion of multiple uninuclear cells), which will later comprise the placenta, an essential organ in the development of the foetus also responsible for sustaining pregnancy[3,8,10–12].

In addition to their fusogenic properties, recent findings indicate that the syncytins present

immunosuppressive activity[13,14], which is supposedly related to the development of maternal tolerance to the foetus. Abnormal expression of these proteins has been linked to diverse pathological processes, such as preeclampsia and cancer[5,15–18]. When the first syncytin (syncytin-1) was identified, Mi *et al*. (2000) described the induced fusion of syncytin-1 expressing COS cells with liposomes. After their identification, these proteins and their encoding genes have received considerable attention as researchers aim to elucidate their role in many physiological and pathological conditions in which they are expressed. We believe that the discovery of these fusogenic proteins brings new therapeutic opportunities especially for the development of novel drug delivery systems. However, up to date, most of the research evaluated the fusogenic feature of these proteins and their role in placentation. Therefore, in this review, we summarise new relevant findings involving syncytin-1 and -2 that help understand their function and speculate on their future therapeutic applications.

2. Syncytin- 1 and syncytin-2

Identification of syncytin genes

Although the typical picture described in evolution involves viruses acquiring host`s genes, approximately 8% of the human genome comprises genetic material acquired from viruses[6,19]. Currently available data based on protein functional level and phylogenetic analyses illustrates that 'domesticated' *HERV* genes share numerous resemblances with *env* genes of present-day retroviruses, endorsing the viral origin of these genes present in the human genome[1,2].

The *HERV* genes (usually comprising *gag*, *pol* and *env* genes) were incorporated into the primate genome >40 million years ago[2,7,8]. In time, inhibitory mutations accumulated in the *gag* and *pol* genes[20], which encode for structural viral proteins (*gag* gene) and for viral enzymes (*pol* gene)[21]. The *env* gene encodes for envelope glycoproteins[20,21] responsible for targeting and entry of the virus into the host cell by endorsing fusion of the viral particle with the cell membrane during an infection[3]. Unlike the disrupted *gag* and *pol* genes, studies showed that the 'captured' *env* genes, namely *syncytin-1* and *syncytin-2*, are not in a process of extinction[20]. In contrary, they were positively selected during evolution probably because their fusogenic activity has been subverted for the benefit of the host with them now participating in reproduction/placentation (Figure 1).

The *syncytin-1* gene was first described by Mi *et al*. (2000) who also investigated its origin. Using syncytin-1 cDNA, they identified this gene as the envelope protein of a retrovirus member of the HERV-W family as they exhibited 100% match. The gene located in the chromosome 7q21.2 of the human genome was recognised as the *syncytin-1* gene since its DNA was identical to *syncytin-1* cDNA[1,2]. In addition, the gene at chromosome 7q21.2 contains two intact long terminal repeats (LTRs) and an open reading frame (ORF) that allow gene transcription. Many human tissues were analysed to detect syncytin-1 but the protein is particularly expressed in the placenta, with little expression in the testis[1]. Strick *et al*. (2007) exposed endometrial cancer cells to oestrogen and the result was increased syncytin-1 expression with enhanced cell proliferation[22]. Although definitive experimental data has not yet been provided, these results suggest that syncytin-1 detection in reproductive tissue can be associated with oestrogen stimulation[23].

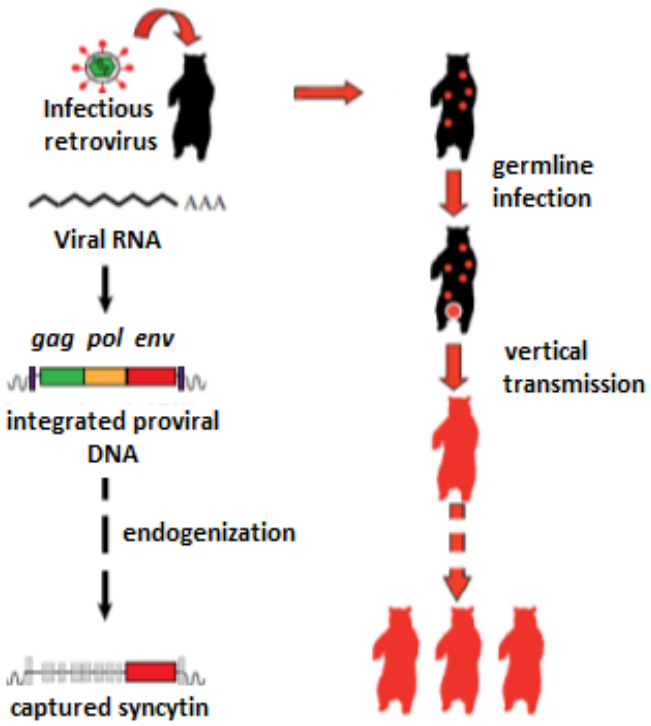

Figure 1. Schematic endogenization of retroviral *envelope* genes that resulted in the *syncytin* genes. These retroviral genes entered the genome of mammals and were transmitted to the offspring. In time, mutations and deletions accumulated in the *gag* and *pol* genes, while the *env* genes, namely *syncytin-1* and *syncytin-2*, were positively selected for their fusogenic activity to play a role in placentation. (Modified figure from Lavialle *et al*. 2013)[19].

A few years after the identification of the syncytin-1 gene, Blaise *et al.* (2003) scanned the human genome looking for other endogenous retroviral *env* genes. Their search led to the identification of a gene located in the

chromosome 6p24.1 matching an uncharacterized *env* gene originally from the HERV-FDR family[2]. Like syncytin-1, this gene presents two intact LTRs and an ORF, but also a putative TATA box sequence, which it has not been report for syncytin-1[5]. Because this gene and its encoded protein present various similarities with syncytin-1 such as expression restricted to the placental tissue, it was named syncytin-2[2].

After identification of *syncytin-1* and *-2* in the human genome, researchers screened the DNA of other mammals in search for other *syncytin* genes. These endogenous retroviral *env* genes are expressed in many groups of mammals as shown in Table 1.

Table 1. Summary of syncytin-like genes identified in mammals up to date.

Mammal group	Syncytin-like gene (reference)
Primates	Syncytin-1(1) and -2(2)
Muridae	Syncytin-A and –B (24)
Leporidae	Syncytin-Ory1 and Ory2 (25)
Carnivora	Syncytin-Car-1 (26)
Ruminantia	Syncytin-Rum1 (27)
Rodentia	Syncytin-Mar1 (28)

Fusogenic activity

Due their viral origin, the proteins syncytin-1 and -2 have been extensively investigated to determine whether they hold similar fusogenic activity as documented for viral envelope proteins. Mi *et al*. (2000) described a series of experiments in which they demonstrated *in vitro* fusion mediated by syncytin-1. Upon transfection with a plasmid encoding for syncytin-1, COS cells were not only able to form syncytia but also to fuse with HeLa and Chinese Hamster Ovarian (CHO) cells[1]. Other studies reported homotypic fusion of TELCeB6 and TE671 cells transfected with syncytin-1 as well as heterotypic fusion between TE671 cells and HeLa cells[29] and between BeWo cells transfected with syncytin-1 gene and COS cells[1]. Fusion, however, is hindered in the presence of anti-syncytin-1 serum or when the plasmid contained the gene in reversed orientation[1]. Inhibition of syncytin-1 expression also compromised trophoblast differentiation in medium containing specific antisense oligonucleotides[30]. Additionally, Cheynet *et al*. (2005) demonstrated that a small portion of the syncytin-1 protein present in the cytoplasm (namely intracytoplasmic tail) plays an important role in cell-cell fusion whereas the absence of this domain considerably reduces the fusogenic properties of this protein. However, only the first 16 amino acid residues are required in this process since mutations in latter fragments of the domain also resulted in actively fusogenic proteins[31].

The fusogenic activity of syncytin-2 was evaluated in some cells including in HeLa cells and compared to that reported for syncytin-1[2]. HeLa cells transfected with syncytin-2 displayed limited syncytium formation, inferior than that observed upon transfection with syncytin-1. Although fusogenic, syncytin-2 did not show the same performance as seen for syncytin-1[2]. The same study reported that the fusogenic index of syncytin-2 hardly altered upon transfection of these cells with ASCT-2, which is the identified receptor for syncytin-1 (described in the following section). These contrasts observed between syncytins led to the proposal of different receptors mediating cell fusion for each syncytin type[2]. Therefore, it is likely that the lower fusion rate reported by Blaise *et al*., (2003) is due to the lack of cells bearing the appropriate receptor for syncytin-2.

Vargas *et al*. (2009) performed a series of experiments using primary trophoblasts and BeWo cells to compare the fusogenic activity of syncytin-1 and -2. The culture of primary trophoblasts revealed that both syncytins mRNA are elevated already in 24 hours. Although the cells continue to fuse, the level of syncytin-1 mRNA slowly decreases in time but not the level of syncytin-2. More importantly, this study demonstrated that in the presence of siRNA directed against syncytin-1 or syncytin-2, the fusogenic activity of primary trophoblasts and BeWo cells was reduced but a more pronounced reduction was observed upon inhibition of the syncytin-2 gene. Altogether, these findings reveal that both proteins exhibit fusogenic properties; however, syncytin-2 seems to play a more relevant role in trophoblast fusion[5].

Receptors

The entry of a retrovirus into the target cell relies on the interaction between its envelope protein and a receptor to promote cell fusion. Similarly, the syncytins are required to interact with a receptor to endow cell-cell fusion, a process mediated by a receptor-binding domain on the extracellular surface of these proteins. Blond *et al*. (2000) showed that syncytin-1 exhibits its

fusogenic role by binding to the neutral amino acid transporter type 2 receptor (ASCT-2), also known as the type D mammalian retroviruses receptor[29] (Blond et al). This receptor, however, is universally expressed and its specific mRNA was detected in a large variety of tissues including placenta, brain, heart, lung, muscle, testis and cancers cells[32]. ASCT-2 is a sodium-dependent receptor comprising twelve transmembrane domains. It is involved in the transport of small neutral amino acids[32] and can mediate infection by replication-competent viruses of the RD114/ type D retrovirus interference group[29]. Cheynet *et al*. (2006) used truncated syncytin-1 surface domains to identify the region responsible for the interaction of this protein with ASCT-2. By means of binding assays, preserved residues in the N-terminal ($SDGGGX_2DX_2R$) were recognized as receptor-binding domain. Additionally, ASCT-1 has been reported as secondary receptor for syncytin-1[18,33].

In contrast, syncytin-2 interacts with only one receptor: major facilitator superfamily domain-containing protein-2a (MFSD-2A)[8]. MFSD-2A is a doubly glycosylated receptor containing 12 transmembrane domains[34,35] supposedly involved in the transport of carbohydrates[36]. Futhermore, MFSD-2A can mediate cellular entry of retroviruses bearing syncytin-2 like polypeptides during infection[8]. Differently from the ubiquitous ASCT-2, MFSD-2A shows more restricted expression, being detected in the intestine, testis but mainly in the placenta[8]. Considering the 'primary' function of this receptor as carbohydrate transporter, it is expected that its expression in the intestines and in the placenta aims to promote nutrient uptake and nutrient exchange in the foetal-mother barrier, respectively[8].

Non-fusogenic properties

Cell proliferation

In addition to its fusogenic activity (crucial in syncytium formation), syncytin-1 has been reported to play an important role in cell proliferation. Cellular proliferation occurs via mitosis, a process by which one cell generates two daughter cells with identical genetic material. This process requires the cell to grow (G1 phase) and to duplicate its content, from proteins and organelles (G1 and G2 phases) to DNA (S phase), prior its division (M phase). In order to ensure correct duplication and division of the cellular content, specific cellular factors are implicated in the regulation of two key phase transitions: G1/S and G2/M. Huang *et al*. (2013) evidenced the correlation between syncytin-1 expression and cellular proliferation by measuring changes in the levels of these regulators in BeWo and CHO cells.

Syncytin-1 expression in BeWo cells was supressed upon transfection with syncytin-specific siRNA resulting in lower proliferation rate characterized by a shift towards G1 phase with fewer cells in phases S and, especially, G2[16]. The levels of cellular checkpoint regulators were also altered in accordance with this shift. Transition regulators that should be activated to promote transition G1/S (PCNA, c-Myc and E2F1) were downregulated while inhibitors (p15) were upregulated. The effect of syncytin-1 overexpression was studied with CHO cells because they are devoid of ASCT-2 receptor and; thus, do not fuse. The overexpression of this protein led to a positive cell cycle shift towards G2 with fewer cells in G1 and S phases. Expression of CDK1, a regulator of S/G2 transition, was enhanced while the G1/S transition parameters showed an opposite pattern of expression compared to the first experiment (p15 levels decreased while PCNA, c-Myc and E2F1 levels were elevated). Although the mechanism by which syncytin-1 affects cell proliferation is not understood, these results suggest that cellular proliferation is hindered due to the lack of syncytin-1, which caused cell cycle halt in G1 phase. In addition, the results obtained with CHO cells indicate that syncytin-1 promotes cell proliferation regardless the presence of its receptor, ASCT-2[16].

Immunosuppression

The envelope protein of retroviruses has been shown to undermine the host's immune system and ensure viral infection[37]. These viruses then affect innate (natural killer cells) and/or adaptive immunity (T cells) hampering an immunological response that would compromise their propagation[37]. Leukocytes are inhibited by interaction with a highly conserved immunosuppressive domain (ISD) present in the envelope protein[37]. Mangeney *et al.*, (2007) identified the amino acid residues at position 14 and 20 of the ISD as crucial for immunosuppression. The amino acids residues on these positions, mainly residue 14, are essential to activate and deactivate this property without compromising its fusogenic activity. By substituting these amino acid residues on the *env* gene of Friend murine leukaemia virus, Schlecht *et al*. (2010) showed that immune competent mice generated a rapid immune response against the viral infection, which was efficiently cleared.

As the syncytins were originally HERV envelope proteins, they also possess the sequence of amino acids residues that compose such ISD[38]. However, experimental data collected by Mangeney *et al.*, (2007) indicated that only syncytin-2 (primates) and syncytin-B (murine) have conserved this immunosuppressive activity while syncytin-1 (primates) and syncytin-A (murine) have only conserved the fusogenic properties[37]. Tumour cells transfected with syncytin-2 ISD were tolerated *in vivo* while its absence led to antibody response[14]. At position 14, previously identified as essential for immune suppression, syncytin-1 presents an arginine residue (a positively charged group) while syncytin-2 has a glutamine residue (a polar but uncharged group)[14]. The interaction of residue 14 with residue 20 (phenylalanine in syncytin-1) provides correct folding required for fusogenic activity. Double mutations at positions 14 (R14Q) and 20 (F20A), however, restored syncytin-1 immunological properties without compromising its fusogenicity[14]. A more recent study performed by Tolosa *et al.* (2012), obtained different results for immunosuppression activity of syncytin-1. Whole blood exposure to syncytin-1 led to suppression of Th1 immunity and upregulation of IL-4, IL-5 and IL-10 proper of Th2 immunity[13]. The same pattern of cytokine expression is observed during normal pregnancy where Th1 immunity is silenced while Th2 immune response assures foetal development[13]. As a result, high levels of pro Th2 cytokines (IL-4, IL5 and IL10) overcome the downregulated pro-Th1 cytokines (TNF-α, IFN-γ)[13]. Both studies' findings do not afford conclusive information regarding the functionality of the ISD of syncytin-1. Therefore, it is not possible to determine whether this protein have preserved its immune suppression role, as reported for syncytin-2.

Moreover, the expression of endogenous retroviral elements has been reported to protect the host from infections of exogenous viruses[6] when the endogenous element highly resembles the exogenous viral element, which can lead to the production of defective viral particles[6]. In this context, syncytin expression may participate in preventing infection by exogenous retroviruses (possibly) expressing syncytin-like molecules. Infection with spleen necrosis virus and other viruses of the same family was hindered by syncytin-1 expression that cause cellular resistance[38] to viral infection while enhanced syncytin-1 expression in neuroblastoma cells in the presence of herpes simplex -1 (HSV-1) virus[21]. Furthermore, competition for receptor occupancy may also hinder viral infection. Altogether, these data suggests that the presence of syncytin may provide the host protection from exogenous viruses although not always directly related to its ISD.

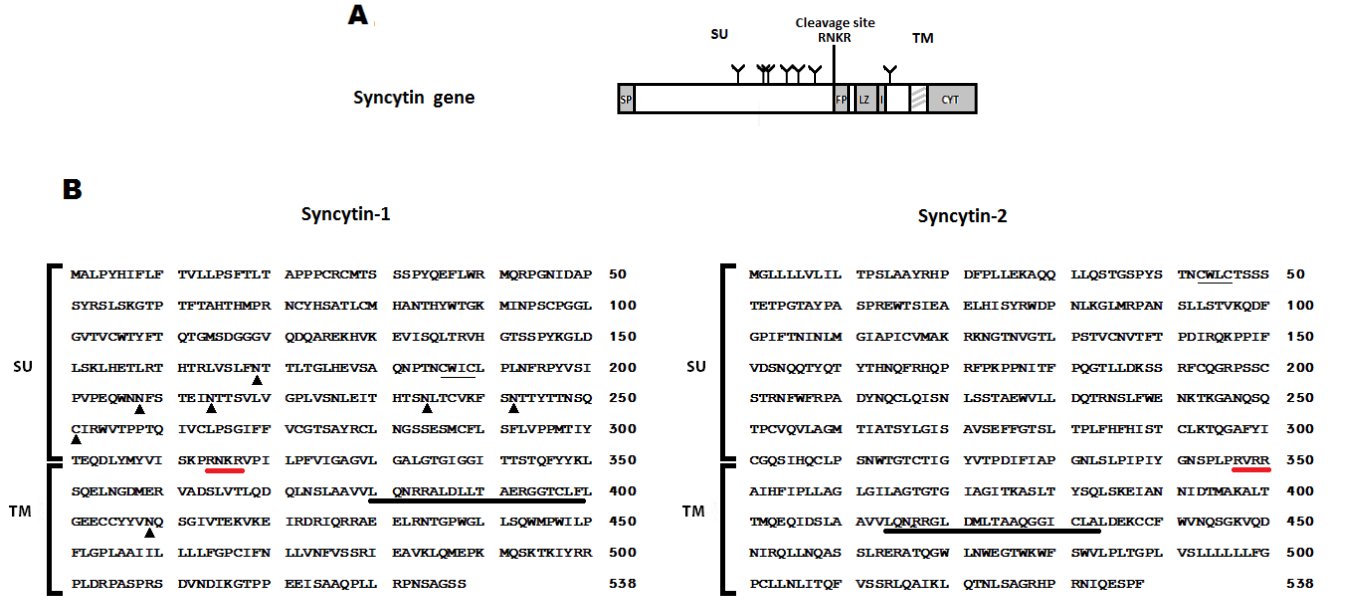

Figure 2. (A) Schematic representation of the main domains of a human syncytin gene (Modified figure from Cheyvet *et al.*, 2005)[31]. The gene encodes a cleavable protein containing 538 amino acid residues divided into surface (SU) and transmembrane (TM) subunits. The "Y" indicate putative glycosylation sites while RNKR designates the cleavage site. The SU subunit encloses the signal peptide (SP) and 6 glycosylation sites whereas the TM subunit contains the fusion peptide (FP), Leucine zipper (LZ), Immunosuppression domain (I), 1 glycosylation site, the transmembrane domain (area with diagonal lines) and the cytoplasmic tail (CYT). (B) The sequence of amino acid residues encoded by syncytin-1 (left) (Modified figure from Mi *et al.*, 2000)[1] and syncytin-2 (right) (Modified figure form Blaise *et al.*, 2003)[2]. The black thin line in each sequence indicates the domains that connects SU and TM after polypeptide cleavage. The red lines designates the cleavage sites while the thick black line shows the I domains. The arrows in the syncytin-1 sequence evidence the 7 assumed glycosylation sites.

Synthesis and structure

The *syncytin-1* gene encodes for a membrane glycoprotein (538 amino acid residues- Figure 2) with features that highly resemble envelope proteins from retroviruses. This protein originates from a glycosylated precursor (gPr73) synthesized in the endoplasmic reticulum (ER) that undergoes 7 predicted N-glycosylations (positions 169, 208, 214, 234, 242, 251 and 409)[31]. Next, the protein is transported to the Golgi complex where it is cleaved[10,31] at a specific furin cleavage site (RNKR) with subsequent formation of a surface subunit (gp50 SU) and a transmembrane subunit (gp24 TM)[31]. These subunits, however, remain linked due to a covalent bond between highly conserved regions of SU (CWIC motif) and TM (CX_6CC)[31]. The SU subunit (1-317 amino acids) is where six alleged glycosylations may occurs and it contains a signal peptide (SP) domain. The TM subunit (318-538 amino acids) comprises the following motifs: fusion peptide (FP), leucine zipper (LZ), immunosuppressive domain (I), transmembrane anchor domain and the intracytoplasmic tail (CYT) (Figure 2A). The only assumed glycosylation site in this subunit is located between the immunosuppressive and transmembrane domains. Mutations in the RNKR cleavage site involving amino acid substitution result in loss of its fusogenic activity[15,31] and no protein expression on the cellular membrane[39]. In addition, it has been shown that glycosylation of the predicted sites is crucial to ensure maturation, appropriate folding and, consequently, function of this protein[31]. After cleavage, syncytin-1 is carried to the cellular membrane where it is expressed as homotrimers (Figure 3)[31].

The synthesis of syncytin-2 has not been documented in details as for syncytin-1 but due to high similarity between the two genes/proteins, it is expected that they share a similar synthesis pathway. The *syncytin-2* gene product is a 538 amino acid polypeptide (Figure 2B) that after cleavage (cleavage site RNKR)[2] results into the SU and TM domains[4]. Like *syncytin-1*, *syncytin-2* encloses equivalent motifs in its SU and TM subunits; however, up to date, no glycosylation site has been reported. Renard *et al.* (2005) depicted the crystal structure of a long central domain present in the TM subunit between the FP motif and the transmembrane anchor, including the immunosuppressive domain. This domain is organized in homotrimers formed by its α-helices with a subsequent loop in the immunosuppressive domain (Figure 3A). In addition, their study showed that the crystal structure of this selected domain is quite similar to that observed for present-day retroviruses[4]. Therefore, this protein is most likely expressed on the membrane as a homotrimers as described for syncytin-1. Despite their numerous structural similarities, the subunits of the syncytins are not functionally equivalent and cannot be exchanged[34].

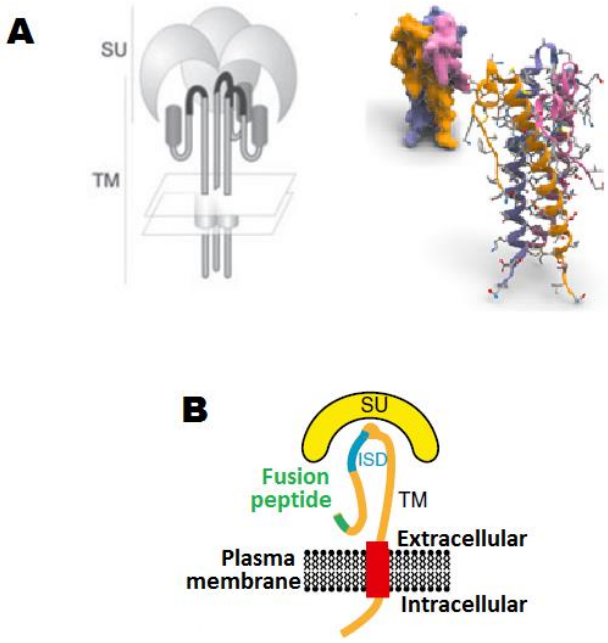

Figure 3. (A) General *env* protein of a retrovirus[37] and 3D homotrimer structure of syncytin-2 TM subunit showing a small central sequence of amino acid residues[40]. (B) General representation of an envelope glycoprotein expressed in the cell membrane[26].

Regulation of protein expression in placental and non-placental tissues

As any protein, the expression of the syncytins depends on the interaction of RNA polymerase with their promoter region of their genes, followed by transcription. Although the *syncytin-1 and syncytin-2* genes are present in all cells of the body, these proteins are specially expressed in the placenta. Controlled expression is required to avoid undesired syncytin expression that would lead to harmful cellular fusion endangering tissue integrity and, consequently, its organization[41]. Therefore, expression of these proteins is a consequence of tight epigenetic and non-epigenetic regulation of the gene in non- and placental tissues[41].

DNA methylation

A common epigenetic mechanism to regulate protein expression is based on (de-)methylation of CpG islands present in the promoter region of the gene. Because the promoter region of the *syncytin-1* gene is

located in the 5′ LTR, Matoušková *et al*. (2006) analysed *in vitro* the methylation status of the 5′LTR of this gene in diverse cells (*e.g*. HeLa, BeWo cells, and placenta tissues). The proviral 5′LTR in the host genome comprises the U3, R and U5 sequences and methylation sites of *syncytin-1* were identified within the U3 sequence (Figure 4). Their study demonstrated that the *syncytin-1* gene in placenta trophoblasts and BeWo cells have a low degree of methylation while non-placental tissues/cells (e.g. HeLa) are heavily methylated. In addition, HeLa cells were incubated with 5-azacytidine (inihibitor of DNA methylation) and trichostatin A (inhibitor of histone acetylation) to evaluate the stability of the promoter methylation; however, no significant increase in protein expression was observed. These findings support the hypothesis that CpG methylation of the promoter region in the 5′LTR affects syncytin-1 expression and the transcription of the gene is tightly controlled in non-placental tissues. Stable methylations hinders syncytin-1 expression in non-placental tissues were it could lead to harmful fusogenic activity, especially because the ASCT-2 receptor is present in various cell types[42]. Therefore, this indicates that the highly regulated expression of *syncytin-1* is the limiting factor for cell-fusion in these tissues. Despite the methylation pattern seen for 5′LTR, 3′LTR is constantly methylated probably to avoid competition between the LTRs. The U5 sequence in the 3′LTR contains the polyadenylation signal for a poly (A) tail required for mRNA transcription and stability[23].

Liang *et al*. (2010) reported a similar pattern of methylation for *syncytin-2*. In placental cells (BeWo cells and cytotrophoblasts), the CpG promoter region of the gene is hypomethylated while in non-placental cells (embryonic kidney 293T cells) it is hypermethylated displaying respectively high and low/undetectable levels of syncytin-2. As expected, non-placental cells that were demethylated displayed higher promoter activity. However, when BeWo cells were treated with SssI methylase to become methylated, the promoter activity did not reduce. These findings suggest that the methylation of the promoter region is an effective mechanism to regulate syncytin-2 expression in non-placental tissues while in the placenta other regulation mechanisms must play a more relevant role.

Histone modifications

Histones are important alkaline proteins involved in packing and ordering DNA into nucleosomes. Histone H3 lysine 9 (H3K9) is an active component implicated in the expression of the *syncytin* genes. Post-translational modifications comprising acetylation or methylation of H3K9 in the 5′LTR region are respectively translated into activation or suppression of *syncytins* transcription[42]. Trejbalova *et al*. (2011) studied the modification status of H3K9 and correlated it to the expression of these genes in BeWo and HeLa cells. The 5′LTR region of the *syncytin-1* gene in BeWo cells showed high acetylation and low trimethylation status whereas HeLa cells displayed the exact opposite pattern. Together with the data collected for CpG methylation, these findings explains the expression of syncytin-1 in BeWo cells but not in HeLa cells. In addition, *syncytin-2* was weakly acetylated in both cell types but strong methylation was observed in HeLa cells compared to BeWo cells[42]. Although these modifications are not associated with the expression of syncytin-2 in BeWo cells, it clearly indicates that in non-placental cells the expression of *syncytin* genes are highly supressed.

Alternative splicing

Alternative splicing is a process that occurs in the majority of mammalian genes generating various mRNA from one gene[43]. This process holds two great benefits: the possibility of exon shuffling (enhancing the chance to produce a novel and functional protein) and increased genome encrypting capacity (as various and non-identical mRNA are produced)[43]. The occurrence of alternative splicing in a gene is regulated by the previously mentioned histone modifications[43]. In retroviruses, the regulation of this process is important to assure the production of appropriate amount of protein that will compose the new viral particles[42].

Alternative splicing was reported for the syncytin-1 gene producing three mRNA (8.0, 3.1 and 1.3 kb); however, only the latter results in functional protein (Figure 4)[44]. BeWo cells display higher levels of the 3.1 kb isoform while HeLa cells showed low levels of the 3.1 and 8.0 kb isoforms indicating that the pattern of alternative splicing varies among cell types[23]. Although it is not fully understood the mechanism behind these different transcripts, they seem to be involved in *syncytin-1* regulation as in non-placental tissues there is prevalence of the 1.3 and 8.0 kb isoforms that do not result in protein expression[23]. In contrary, Vargas *et al*. (2009) used 5′-3′ RACE analysis to show that the *syncytin-2* gene encodes a singly spliced mRNA from 5′LTR to 3′ LTR. In addition, the transcription of this gene seems to start in a putative alleged TATA box[5]

whereas for *syncytin-1* it occurs by a CCAAT motif[45] containing 56 bp[46].

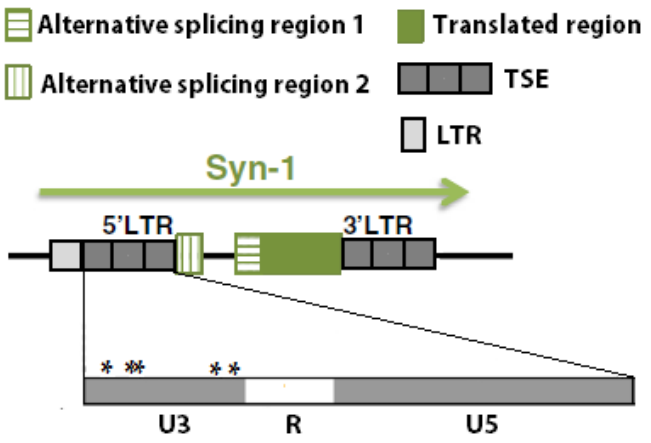

Figure 4. CpG islands (positions 32, 70, 86, 214, 235) in the U3 susceptible to methylation for regulation of syncybtin-1 gene transcription. In green, the putative regions where mRNA undergoes alternative splicing (Modified figures from Matoušková *et al*., 2006 and Huang *et al*., 2014)[23,41].

Glial cell missing a (GCMa) and cyclic adenosine monophosphate (cAMP)

GCMa is a transcriptional factor that binds to an upstream region of 5'LTR named trophoblast-specific enhanced (TSE). This factor was shown to induce *syncytin-1* transcription in cytotrophoblast and BeWo cells by inducing promoter activity. However, protein expression was barely detected unless these cells were induced to syncytiotrophoblast formation[41]. This suggests the existence of other active post-transcription factors that regulated protein expression and that may be involved in its synthesis and maturation as described by Cheynet *et al*. (2005)[41]. This mild effect of GCMa seems to be restricted to low methylated status since its overexpression in HeLa cells was not capable of overcoming epigenetic silencing to promote *syncytin-1* expression.

Regulation of *syncytin-1* expression by GCMa was also associated with cyclic adenosyl phosphate (cAMP) and Protein Kinase A (PKA) pathways. Treament of BeWo cells with cAMP demonstrated elevated levels of PKA, which up regulates GCMa. Upon phosphorylation, GCMa can be acetylated by CBP (CREB binding protein) at specific lysine residues (positions 367, 406 and 409)[47] and finally bind to the TSE region to increase promoter activity. A study performed by Tolosa *et al*. (2012) reported that this pathway could be induced by Cortico-releasing hormone (CRH), a placental peptide hormone. After interaction of the hormone with its receptor, a G protein activates the enzyme adenylyl cyclase (AC) increasing the intracellular levels of cAMP. High levels of cAMP generated by this pathway were reported to stimulate *syncytin-1* expression in exosomes produced in the placenta [13,23]. An opposite effect was observed when this hormone was administered in higher concentrations, although the mechanism responsible this downregulation is not yet elucidated.

In contrary to BeWo cells, MCF-7 cells (a breast cancer cell line) hardly express syncytin-2. Upon transfection with exogenous GCMa, both cell types exhibit higher levels of syncytin-2 mRNA; however, with different intensities. GCMa significantly increased mRNA transcription in BeWo cells but the most pronounced results were observed for MCF-7 cells. Nevertheless, these results indicate that GCMa actively participates in the regulation of syncytin-2 in placental and non-placental tissues[34]. Although these findings seem to contradict the stable regulation reported by promoter methylation in non-placental cells, it was shown that GCMa levels could influence the CpG methylation in syncytin-gene promoter region. Liang *et al*. (2010) also demonstrated that while sincyntin-1 methylation in placental tissue is barely susceptible to demethylation agents, syncytin-2 is not. Elevated levels of GCMa in MCF-7 cells led to lower methylation of the promoter region of this gene with its subsequent enhanced activity probably due to recruitment of proteins such as nucleotide excision repair (NER) and base excision repair (BER). In sum, both syncytins have their transcription regulated by methylation of their promoter regions; however, each gene exhibits a different susceptibility patterns[34].

Hypoxia

Appropriate levels of oxygen are crucial to promote placentogenesis. However, the oxygenation status varies during this process to assure cell differentiation at appropriate stage. In the beginning of placentogenesis, cytotrophoblasts show a high rate of differentiation and proliferation to promote trophoblast invasion and formation of placental vasculature[48]. At this stage, low oxygen levels (2.6%) are required to prevent oxidative harm to these cells[48]. However, if oxygenation level is excessively low, placenta development is compromised. BeWo cells in hypoxic environment displayed lower levels of syncytin-1 mRNA with additionally hindered fusogenic activity[49]. Studies have shown that oxygenation status can affect the methylation of some

genes[23]. Therefore, it is possible that when the cell is poorly oxygenated the gene undergoes epigenetic methylation leading to reduced transcription and consequent lower protein expression. Wich *et al*. (2009) demonstrated *in vitro* that GCMa and syncytim-1 are highly expressed in primary trophoblasts at oxygen levels of ≥ 3% when syncytium beings to form. As the placentation progresses, oxygenation gradually rises up to 8-10% and remains steady until delivery. When cytotrophoblast were exposed to normoxic (atmosphere) oxygen conditions (21%), these cells underwent enhanced fusion to form syncytium and increased levels of syncytin-1 were detected.

Receptor regulation

Additionally to protein expression, the appropriate receptor is required to promote intercellular fusion. Because ASCT-2 is widely expressed among diverse cell types, expression of syncytin-1 is expected to be the limiting factor to restrict fusogenic activity mediated by this protein. Therefore, the transcription of the syncytin-1 gene particularly in non-placental tissues is exceptionally controlled. In contrary, expression of MFSD2-A, the receptor for syncytin-2, is restricted to the placenta and this specific location suggests that the same factors that regulate *syncytin-2* gene expression may actively regulate its receptor. Liang *et al*. (2010) showed that upon stimulation with GCMa, the expression of MFSD2-A is increased. The opposite pattern of expression is reported when the cells are exposed to low oxygenation. It has been proposed that under hypoxic conditions, GCMa would be degradated and, therefore, receptor expression would decrease[34].

3. Physiological roles of Syncytins

Role in placentation

As previously mentioned, the syncytium is a multinucleated cell formed when multiple mononuclear cells fused together. This fusion is mediated by fusogenic proteins such envelope proteins in viruses and the syncytins in humans. For a virus, syncytium formation is important for viral propagation, as the infected cells bearing envelope protein on their surface fuses with health ones propagating the virus. In humans, syncytium formation is the first step to originate the placenta that will provide physical protective barrier between mother and foetus while allowing exchange of nutrients and other substances required to foetal development. Although transient, the placenta is an important organ in the embryogenesis mainly responsible for foetal implantation in the uterine wall and metabolic exchange between mother and foetus[11]. Briefly, trophoblast cells (the outer layer of cells of the blastocyst) undergoes differentiation to originate cytotrophoblast (inner monolayer of cells) and the syncytiotrophoblast (the outer layer of fused cells)[3]. The formation of the latter layer occurs upon homotypic fusion of cells of the trophoblast that will lead to syncytium formation[3,23]. This syncytial layer invades the uterus wall to achieve embryo implantation and differentiates to develop the chorionic villi[3]. Within a few days, the chorionic villi establishes direct contact of the syncytiotrophoblast with the maternal blood creating an exchange interface with the maternal uterus[3]. The placenta also has endocrine functions producing hormones, e.g. hCG (human Chorionic Gonadotropin) and estrogenic hormones, to ensure the development of a normal pregnancy[3,16].

The high and restrict expression of the syncytins in the placenta arose the query of their function in this organ. It has been speculated that the incorporation of these genes into the genome of mammals was crucial to the development of the *Placentalia* group[3] as the syncytins would play a similar fusogenic role in placentation promoting cellular fusion in the trophoblast[5,10,16,30]. Both proteins are found in trophoblasts where they induce cell proliferation and fusion that will result in the placenta. The expression of the syncytins through pregnancy increases from the first to the third trimester with remarkable decrease in mRNA at term, indicating that these proteins are required during placenta formation and foetal development. However, when gestation is completed (namely at term), their levels decrease, signalling the delivery moment. Pregnancy disorders and miscarriages have been reported upon abnormal expression of syncytins in placental tissues, which threatens both foetus and mother's lives (described in more details in section 4).

To elucidate the placentation process in humans, many studies use murine models as reference. As for any animal model, it is not possible to fully translate the results obtained with a murine model to humans[46]. However, human and murine placental tissues share several similar functional and structural features that help understand the placentation process[46]. DNA sequencing of mice revealed the existence of two genes encoding for retroviral envelope proteins: syncytin-A and syncytin-B[24]. Although different from the human

fusogenic proteins, mice and human syncytins share similar features[3]. Knockout of syncytin-A gene in this animal showed defective placental architecture (unfused cytotrophoblasts) and death of embryos at mid-gestation[50] whilst deletion of syncytin-B gene led to a decrease in the number of neonates[51]. Furthermore, embryos died earlier when both genes were deleted[51]. Although obtained from murine model, these data reveals that syncytin A and B act synergistically to promote placentation. As murine and human syncytins share commonly preserved features, these findings also suggest that both syncytin-1 and -2 collaborate to placentation as in humans, unbalanced expression of syncytin-1 or 2 is also linked with pathological conditions.

In addition, the placenta presents a putative role in generating immune tolerance to the foetus, a feature thought to be associated with the expression of syncytins in the placenta and its mechanisms are described in the following section.

Placental exosomes and immune tolerance towards the foetus

Placenta is an organ of embryogenic origin that displays both maternal and paternal antigens[3]. In order to allow healthy foetal development during pregnancy, the mother must tolerate the expression of paternal antigens in this organ[14]. Therefore, the placenta is also involved in generating maternal immune tolerance to the foetus[38]. Particular expression of syncytins in the placenta supported by well-established immune evasion provided to the viruses by envelope proteins suggest that the syncytins may participate in this immune modulation process.

During pregnancy, exosomes are shed from the placenta into maternal circulation[9,38,52,53]. Exosomes are vesicles (30-100 nm) secreted by a large variety of cells and that are involved in cell-cell communication by receptor activation/inhibition or by deliver of its cargo to a target cell[13,54]. The expression of HLA-G (an important factor for NK cell inhibition) and NKG2D (downregulator of NK, CD8 and T cells) in placenta-derived exosomes endorses their immunosuppression role[52]. In addition, these nanoparticles also express syncytin-1[13,53] and syncytin -2[9]. However, little has been published regarding syncytin-2 generating immune tolerance during pregnancy.

Holder *et al*. (2012) demonstrated that exosomes expressing syncytin-1 interact with macrophages and activated peripheral blood mononuclear cells resulting in increased the levels of IL-1β in culture. This indicates that syncytin-1 would display a pro-inflammatory activity instead of generating immune tolerance[53]. In this case, these results corroborates with those from published by Mangeney *et al*. (2007) in which they propose that the ISD of syncytin-1 has lost its immune suppression properties. However, Tolosa *et al*. (2012) described that presence of syncytin-1 in these exosomes led to immune tolerance as the immune response shifts from Th1 to Th2.

Due to these controversial results, it is not possible to draw a definite conclusion that explains the role of syncytins, mainly syncytin-1, in placenta derived-exosomes. Therefore, further investigation is required. Nevertheless, the immune tolerance provided by shedding of exosomes is crucial for a healthy pregnancy.

Fertilization

Fertilization in humans relies on membrane fusion between spermatozoa (male gamete) and cumulus-oocyte (female gamete). The presence of a fusogen molecule and its receptor is, therefore, essential in this process. The presence of the fusogenic protein syncytin-1 in testis was reported by Mi *et al*. (2000). However, more than a decade later Bjerregaard *et al*. (2014) showed by a series of experiments that syncytin-1 and its receptor (ASCT-2) are present in human male gametes (in the acrosome) while only the receptor identified in the female gamete (during the whole maturation of the oocyte). These findings led to the hypothesis that this fusogenic protein and its receptor could participate in the fertilization process by contributing to the fusion between gametes. However, the role of this protein in these cells remains unknown due to the absence of conclusive evidence.

4. Syncytin in pathologies

As previously mentioned, the expression of the syncytins is closely regulated in placental, but specially in non-placental tissues to avoid harmful cell fusion outside the placenta. However, despite strong regulation, studies have linked abnormal expression of syncytins with some pathological conditions related to pregnancy and cancer[5,15–18]. The role of the syncytins in these disturbances is not entirely elucidated but it has been justified by their fusogenic and immune

suppressive properties, as well as the impact of syncytin-1 in cell proliferation.

Pregnancy diseases

Placental abnormalities are regularly associated with pregnancy disorders such as preeclampsia and premature birth. Placenta has crucial functions to ensure normal embryogenesis, most of which are performed by the syncytium[16]. Due to high expression of syncytins in this tissue, many studies have investigated a putative association between abnormalities in the *syncytin* genes protein expression and pregnancy pathologies.

Preeclampsia is a pregnancy complication that threatens both mother and child's lives[48]. It is associated with hypertension, renal, immunological and systemic disturbances that may lead to premature birth or death of the child. It has been reported that recurrent hypoxic cycles contribute to the development of preeclampsia because it hinders cytotrophoblast differentiation and fusion[48]. The result is undifferentiated cytotrophoblasts that do not fuse together[23,46]. The lack of a functional syncytium compromises placental hormonal production, which leads to low levels of circulating human chorionic somatomammotropin (hCS)[23] and enhanced apoptotic rate in the placenta. As hCS acts 'antagonizing' insulin to promote appropriate energy supply to the embryo during the pregnancy, reduced levels of this hormone are potentially life threatening to the child. In addition, the presence of syncytium apoptotic fragments in maternal circulation leads to an inflammatory against the placenta[48]. All these physical and biochemical disturbances in preeclamptic placentas may result in intrauterine growth restrictions (IUGR).

Many studies have associated these disorders with abnormal levels of syncytins expression[5,36,46,48]. Lower levels of syncytin-1 mRNA (around 96% less) was reported with preeclampsia and IUGR[48] with mislocalization of the protein at apical instead of the basal microvillous membrane[46] (handwerger et al, 2010). Consequently, cell fusion in this tissue leads to unstable placental structure and shedding of apoptotic trophoblasts fragments into maternal circulation[46]. Hypoxic cycles in trophoblasts stimulates a pro-inflammatory response with increased TNF-α expression that has been reported to induce ICAM-1 expression[23]. It has been proposed that this inflammatory profile could be linked to lower levels of syncytin-1 in the placenta and in its exosomes. Furthermore, exosomes derived from preeclamptic placentas contain less syncytin-2 in their membranes[9] and their release into maternal blood is enhanced[53]. The reduced levels of syncytin-2 could be a contributing factor to the enhanced pro-inflammatory profile[53] detected in these placentas. In addition, the levels of MFSD2-A is also reduced in preeclampsia compared to normal placentas[36].

Cancer

Cancer cells demonstrate an invasive and undifferentiated phenotype because of activation and expression of embryonic genes that under normal conditions are not expressed in somatic cells[55]. The reactivation of embryonic genes in neoplasias is associated with genome demethylation followed by enhanced promoter activity and inhibition of tumour inhibitory genes[55]. Because multinuclear cells are commonly observed in diverse cancer types and the fusogenic activity of the syncytins has been well-established, the query arose about the activation of these placental/embryonic genes in cancer cells[23]. Studies showed unexpected syncytin-1 expression in various cancer such as leukaemia, breast and endometrial cancers[18,23]. In addition, it has been reported that this protein mediates fusion between cancer cells and between endothelial and cancer cells, which could associate syncytin-1 with an invasive cancer phenotype[18]. However, the role of this protein in these cells is under investigation, as currently available data does not provide definite conclusion. Up to date, most studies aim to verify syncytin-1 expression in cancer types and establish a direct prognostic based on protein levels[18].

In vitro studies using endometrial cancer cells have shown that misplaced syncytin-1 expression led to increased growth rate[22] while abnormal expression has been reported in breast and ovarian cancers[16]. Analysis of gene methylation status in cancer tissues shows low methylation of the syncytin-1 gene in cancer tissues compared to healthy tissues[23]. The expression of syncytin-1 in non-placental tissue is mainly regulated by stable methylation of its promoter region that prevents uncontrolled protein production and expression in the cell membrane. The hypo- or demethylation of this gene in cancer cells may be the main cause of syncytin-1 expression[23]. However, the clinical results of syncytin-1 expression vary depending to cancer type[18]. In some cancers, higher syncytin levels was linked to advanced carcinomas in initial pathological phases (endometrial carcinomas) while in others it was considered a

contributing factor for longer survival (colorectal carcinomas)[23] and positive prognostic phenotypes (breast cancer)[17]. In melanoma cells transfected with syncytin-1 (B16F10 cells), a remarkable reduction in invasive profile was observed suggesting that it could contribute for reducing metastases rates[18].

Some mechanisms have been suggested to explain the different phenotypes observed upon syncytin-1 expression in cancer tissues. As a fusogenic glycoprotein, syncytin-1 can mediate cell fusion in the presence of its receptors (ASCT-1 and -2). Fusion, however, may result in gain or loss of functional tumour suppressor gene that could lead to the development of a more or less malignant phenotype[17]. Additionally, the expression of FasL/Fas, well known apoptotic factors, is directly stimulated by cAMP in BeWo cells. Because the regulation of syncytin-1 via cAMP has been already elucidated, it is thought that syncytin expression in some cancer cells could stimulate apoptotic factors reducing proliferation[18]. Also, induction of apoptosis by means of increased levels of inflammatory mediators and nitric oxide has been suggested[17].

Fewer studies describe the part of syncytin-2 in neoplasias; however, its expression in cancer cells has been proposed to mediate fusion with host cells[38]. Moreover, an *in vivo* study, demonstrated that allogeneic mice did not develop an immune response to a tumour overexpressing syncytin-2[14]. This indicates that this the presence of syncytin-2 in cancer cells could be crucial in generating immune tolerance for these cells, as its immunosuppressive domain remains active[34]. Nevertheless, despite these findings indicating syncytin-1 expression in cancer cells, the real role of the syncytins in cancer remains incomprehensible due to the lack of conclusive experimental data.

5. Clinical applications

Many studies demonstrated that syncytin proteins are remarkably fusogenic. Transfection of cells with genes encoding for these proteins led to homotypic and heterotypic cell fusion in the presence of proper receptor. Although the fusogenic activity of the syncytins is dependent on its interaction with a receptor, Mi *et al*. (2000) described the fusion between cells transfected with syncytin-1 and liposomes composed of lipids isolated from heart. Up to date, the functionality of cell-liposome fusion was not further investigated though its fusogenic activity has been well established. The possibility of incorporating the syncytin proteins into novel drug delivery systems could endorse cargo delivery to target cells by promoting fusion mediated by the syncytins' receptor (ASCT-2 and MFSD-2A)and generating immune tolerance. The role of these proteins, at least syncytin-1, in immune responses is not yet clear and needs further investigation as experimental data provides contradictory information.

Recent studies involving mice demonstrated the expression of MFSD-2A, syncytin-2 receptor, in the brain and highly regulated expression in the liver and in brown adipose tissue (BAT)[35]. In the brain of mice, this receptor show high expression in the endothelial cells of the blood vessels comprising the 'blood-brain barrier' (BBB) in embryonic stages[56]. It participates actively in the formation of an intact BBB while its genetic deletion results in leaking BBB that starts in the embryo and persists until adult life[56]. Moreover, docosahexaenoic acid, an omega-3 fatty acid associated with brain growth and cognitive function, enters the central nervous systems via this receptor[57]. The presence of this receptor in the brain may represent new therapeutically approaches to target the central nervous system upon incorporation of this protein maybe in combination with other relevant molecules (e.g. docosahexaenoic acid) in new drug delivery systems. Interestingly, MFSD-2A expression was temporarily detected in the liver and in BAT of fasting mice followed by rapid removal of the cell surface after feeding[35]. These results indicates that MFSD-2A is involved in lipid metabolism although under tight regulation associated to glucagon signalling in the liver[35].

The fusogenic and non-fusogenic (cell proliferation and immune tolerance) properties of the syncytins are interesting features that could be subverted in cancer cells to favour the development of the pathology[38]. Currently available data regarding syncytin expression in cancer cells does not provide conclusive information about the role of these proteins in these tissues. However, present findings indicate that depending on cancer type, the level of protein expression could be used in the future as prognostic tool[18]. For that purpose, histopathological and clinicopathological studies are required to elucidate role played by the syncytins in neoplasias[38].

The function of syncytin-1 in human gametes is not fully understood; however, the detection of syncytin-1 and its receptor in human gametes may bring opportunities to improve the fertilization strategies, diagnostics and pharmaceutical interventions if proven

that this protein plays a fusogenic role in fertilization. An example is the possible to deliver pharmaceutical compounds to oocytes and spermatozoa using the ASCT-2 receptor[58].

6. Conclusions

The endogenous fusogenic proteins syncytin-1 and -2 are encoded by 'captured' env genes originally from HERV. They were incorporated into the primate genome millions of years ago where their functions were conserved and subverted to play crucial roles in placentation and supposedly in immunosuppression. In this review, we summarize currently available data about these two proteins, while emphasizing relevant findings related to their fusogenic and immunosuppressive properties. Many studies have reported unbalanced and/or unexpected expression of the syncytins in many pathologies such as preeclampsia and cancer. However, many aspects associated to the role played by these proteins in physiological and pathological conditions has not been entirely elucidated, as much of the experimental data is contradictory. Nevertheless, the fusogenic activity of both syncytins has been confirmed in a large variety of (syncytin-transfected) cells. This feature is of great value for the development of new drug delivery systems in which these proteins could be incorporated to promote fusion with the target cells with concomitant immune tolerance. However, further investigation is required before these proteins could be applied for such clinical application.

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