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Title: IFITM proteins inhibit placental syncytiotrophoblast formation and promote fetal demise

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Abstract:

A critical step of placental development is the fusion of trophoblast cells into a multinucleated syncytiotrophoblast (ST) layer. Fusion is mediated by Syncytins, proteins deriving from ancestral endogenous retroviral envelopes. Elevated levels of type-I Interferons (IFN) during pregnancy are associated with intrauterine growth retardation, preterm birth, and fetal demise, but the mechanisms are not well understood. Using cultures of human trophoblasts or mouse cells, we show that IFN-induced transmembrane proteins (IFITMs), a family of restriction factors blocking the entry step of many viruses, impair ST formation and inhibit Syncytin-mediated fusion. Moreover, the IFN inducer Poly-IC promotes fetal resorption and placental abnormalities in wild-type, but not in *Ifitm*-deleted mice. Thus, excessive levels of IFITMs may mediate pregnancy complications observed during congenital infections and other IFN-induced pathologies.

Main Text:

The placenta is the primary maternal-fetal barrier, achieving metabolic exchanges, hormone production as well as protection from pathogens and the maternal immune system. The placental tissue stems from embryonic cytotrophoblasts. The structural and functional unit of human placenta is the chorionic villous, that includes proliferative mononuclear villous cytotrophoblasts (VCT) at the basement and the syncytiotrophoblast (ST) layer at the surface. The multinuclear ST arises from the differentiation and fusion of VCT. In humans, an abnormal ST is observed in pregnancy pathologies including intrauterine growth retardation (IUGR), preeclampsia, lupus, and Down syndrome (1-3). In contrast to the human placenta, the mouse placenta has a labyrinthic structure and exhibits two ST layers that separate fetal capillaries from maternal blood (4). In gestating mice, IFN- β triggered by Zika or other viruses, or by administration of Poly-IC, promotes IUGR and fetal demise (2, 5-7). IFN- β

signalling leads to abnormal labyrinth and ST structures, with the presence of many unfused cells and reduced fetal blood vessels (7).

Cytotrophoblast fusion is mediated by envelope glycoprotein (env)-derived genes of endogenous retroviruses (ERV) that have been domesticated by mammals (3). These genes were termed syncytins based on their fusogenic capacity. The capture of syncytin genes, sometimes as pairs (Syncytin-1 and -2 in humans and Syncytins-A and -B in mice) (3, 8, 9), occurred independently from different ERV in diverse mammalian lineages 10 to 85 million years ago. Knocking out Syncytin-A or both Syncytin-A/B genes leads to fetal growth restriction and mid-gestational lethality (8, 9).

The immune-related interferon-induced transmembrane proteins IFITM1, IFITM2, and IFITM3 are restriction factors that protect uninfected cells from viral infection. They block the entry into host cells of many enveloped viruses by inhibiting virus–cell fusion at the hemifusion stage (10-12). They act by altering the biophysical properties or cholesterol content of the cellular membranes in which they are found (11-13). IFITM modify the rigidity and/or curvature of the membranes without evidence of a direct interaction with fusogenic viral envelopes (11, 12). Due to different sorting motifs, IFITM1 mostly resides at the plasma membrane, whereas IFITM2/3 accumulate in the endo-lysosomal compartment after transiting at the cell surface. IFITM proteins are expressed at various basal levels in different cell types and are upregulated by IFNs and other cytokines (11). Besides their antiviral activity, the physiological cellular functions of IFITM proteins remain poorly characterized. Transgenic mice in which the cluster of *Ifitm* genes is knocked out (thereafter *IfitmDel* mice) are more sensitive to various viral infections (12), but exhibit no overt abnormalities apart from being a little over-weight (14). Not much is known about the impact of IFITMs on fusion events mediated by cellular proteins or by ERV-derived Env proteins. Here, we examined whether IFITMs impair Syncytin-mediated fusion and thus impact fetal development.

We first investigated whether IFITMs inhibit cell fusion mediated by exogenously-expressed human Syncytins. We generated 293T cells carrying a GFP-Split complementation system, in which

two cells separately produce half of the reporter protein, generating a signal only upon fusion (Fig. 1A). The extent of fusion was quantified by measuring the GFP-positive area (Fig. 1A). Transfection of Syncytin-1 induced the appearance of multinucleated GFP+ cells. Fusion was significantly decreased when Syncytin-1 was co-transfected with FLAG-tagged IFITM1, 2, or 3, but not with a control plasmid (Fig. 1A). The extent of inhibition of fusion varied between individual IFITMs. The IFITM3 Δ 1-21 and IFITM3 Δ ub mutants, which lack endocytic and ubiquitination motifs, respectively, and accumulate in the cell (15, 16), were more active at inhibiting fusion than WT IFITM3. In contrast, an IFITM3 Δ palm mutant, whose levels are reduced (15, 16), was inactive. The co-expression of three IFITMs strongly inhibited fusion (Fig. 1A). IFITMs similarly inhibited fusion mediated by Syncytin-2 (Fig. S1A). IFITMs did not decrease the levels of Syncytin-1 in transfected cells (Fig. S1B). To distinguish the effect of IFITMs in Syncytin-1+ (donor) cells from that in target cells (expressing SLC1A5, the Syncytin-1 receptor), we used an HIV Tat and LTR-luc transactivation co-culture system, where cell fusion generates luciferase activity (13). IFITMs inhibited fusion when present in either donor and/or target cells (Fig. S1C).

We next examined the effect of IFITMs on endogenous Syncytins. In trophoblast-like BeWo human choriocarcinoma cells, addition of the adenylate cyclase activator forskolin triggers Syncytin-1 production and syncytium formation (17). To quantify fusion, we generated BeWo cells carrying two complementation systems, based on either β -galactosidase- α/ω (β -Gal- α/ω) (Fig. 1B) or GFP-Split (Fig. S2C). These cells were then transduced with lentiviral vectors to express a control protein or FLAG-tagged IFITM1-3 (Fig. S2A and D). Forskolin triggered fusion and production of β -Gal, detected in fixed cells and quantified in cell lysates (Fig. 1 B and Fig. S2B). The fusion efficacy was significantly reduced by IFITMs. The presence of IFITMs did not modify BeWo cell growth or alter their ability to produce syncytia-independent β -Gal (Fig. S3A-D). Similar results were observed with the GFP-based system. A video-microscopy analysis showed delayed and reduced fusion with each IFITM (Fig. S2C-E, Movie S1).

We next tested the capacity of endogenous IFITMs to block fusion in mouse embryonic fibroblasts (MEFs) derived from WT or *IfitmDel* mice (14). To this aim, MEFs were co-transfected with Syncytin-A and GFP plasmids (Fig. 1C, Fig. S4A). Syncytin-A induced numerous and large GFP+ syncytia (with up to 20 nuclei) in *IfitmDel* MEFs, and fewer and smaller syncytia in WT MEFs. Transduction of *IfitmDel* MEFs with a murine *Ifitm3* vector (Fig. S4B) restored resistance to fusion (Fig. 1C, Fig. S4A). That WT MEFs were poorly sensitive to fusion was likely due to high basal IFITM3 levels (Fig. S4B).

We then evaluated the effect of type-I IFN and IFITMs in primary human trophoblasts. We first asked whether IFITMs were upregulated by type-I IFN in human placental explants. It has been reported that in human mid-term placental chorionic villi explants, addition of IFN- β , but not IFN- λ , leads to defects including cellular damage, decreased production of human chorionic gonadotropin (hCG), and appearance of syncytial knots (7). In such explants, IFN- β upregulated hundreds of Interferon Stimulated Genes (ISGs) including IFITM1 (7). We took advantage of this large set of published RNA data to investigate the expression of IFITMs, Syncytins, and their receptors. IFITMs were upregulated by IFN- β , when compared to mock and IFN- λ treated explants, whereas Syncytins and their receptors were not modulated (Fig. S5).

These observations were made in whole placental explants where non-trophoblastic cells are also present. We thus studied the impact of IFN- β on primary VCT isolated from 8 term human placentas. VCT can be cultured up to three days and spontaneously differentiate in multinucleated ST (18). Treating VCT with IFN- β (100 or 1000 IU/mL for 48 h) strongly upregulated IFITM1 and 2/3 proteins, as shown by immunofluorescence and western blot (Fig. S6A, C). IFN- β slightly upregulated RNA levels of Syncytin-1 but did not impact those of Syncytin-2 and Syncytin receptors (Fig. S6D). We next quantified VCT fusion, using a method based on the detection of the transcription factor GATA3 by immunofluorescence and its down-regulation upon ST differentiation (18). Cells were also stained for E-cadherin, to visualize plasma membranes. IFN- β inhibited VCT fusion, to the same extent than

GW9662, a PPAR γ antagonist known to block this process (18) (Fig. 2A,B). As it acts independently of IFN signalling, GW9662 did not induce IFITM (Fig. S6A,C). The effect of IFN- β on ST differentiation was supported by a decreased release of hCG, secreted by ST but not by VCT (Fig. 2C). The reduction of fusion was not due to a cytotoxic effect of IFN- β , since levels of an apoptosis marker (cCK18), were not augmented (Fig. S6B, C).

To further assess the effect of IFITMs in this system, VCTs were transfected with a FLAG-tagged IFITM3 or a control GFP plasmid. The poor efficiency of transfection (10% of the cells expressing IFITM or GFP) precluded precise quantification of syncytia. However, a visual examination indicated that IFITM3+ cells remained mononucleated, even in vicinity of large syncytia, whereas GFP-transfected cells were able to fuse (Fig. S7). Altogether, these results demonstrate that IFN- β induces IFITMs in VCT and that both IFN- β and IFITMs inhibit ST formation.

We then evaluated the role of IFITM in pregnant mice. Administration of Poly-IC has been extensively used as a model of type-I IFN induction, triggering fetal growth retardation and resorption (2, 6, 7). We thus tested the effect of Poly-IC in gestating WT, *Ifnar*^{-/-} or *IfitmDel* dams that have been mated to males of the same genotypes (Fig. 3A). Poly-IC was injected at E7.5, a time point that shortly precedes ST formation (about E8.5) (4). A dose of 200 μ g, that results in resorption of almost all fetuses within 48h (7) was injected. With Poly-IC, WT fetuses, but neither *Ifnar*^{-/-} nor *IfitmDel* fetuses were resorbed at E9.5 (Fig. 3 B). The fetus size was similar in *Ifnar*^{-/-} and *IfitmDel* mice (Fig. 3 C, S8) and slightly reduced compared to untreated animals (Fig. 3C, S8). We checked that *IfitmDel* and WT mice similarly responded to Poly-IC, as shown by the induction of various ISGs (*Irf7*, *Oas1a*, *Stat1*) measured in the liver at 14h post-injection, indicative of a systemic inflammation (Fig. S9B). A local response was also observed in placental extracts of WT mice, which up-regulated *ifitm1*, *ifitm3* and *Irf7* RNAs. As expected, *IfitmDel* placentas did not express *Ifitm* genes but up-regulated *Irf7* (Fig. S9 C). Of note, levels of Syncytin A and B RNAs were not significantly impacted by Poly-IC (Fig. S9C).

We next performed histological analysis of the placentas to examine the consequences of Poly-IC treatment. Placentas from WT and IfitmDel fetuses were stained for E-cadherin to label trophoblasts (Fig. 3D). As expected, ST formation was detectable in untreated placenta from both WT and IfitmDel fetoplacental units (arrows). In contrast, with Poly-IC, less ST was detected in WT placentas, whereas it was still present in IfitmDel placentas. We next triple stained placenta 14h post Poly-IC injection for IFITM3, E-cadherin, and CD31 (a marker of endothelial cells) (Fig. S9D). As expected, IFITM3 was upregulated in placentas of Poly-IC treated WT mice, but not in IfitmDel mice. IFITM3 overexpression upon Poly-IC injection was noticed in CD31 positive E-cadherin negative cells (endothelium), and CD31 negative E-cadherin positive cells (trophoblasts), demonstrating that IFITM3 is induced in trophoblasts in response to Poly-IC, consistent with *in vitro* results.

Altogether, these results strongly suggest that IFITM upregulation in trophoblasts inhibits ST formation *in vivo*, which likely contributes to type-I IFN-associated placental dysfunction and fetal demise in mice.

In summary, we have uncovered a function for the IFITM family of antiviral restriction factors. We report that IFITMs impair the fusogenic activity of Syncytins required for ST formation and maintenance. Our results provide a possible molecular explanation for placental dysfunctions associated with IFN-mediated disorders, such as IUGR and “TORCH” infections (Toxoplasmosis, Other, Rubella, Cytomegalovirus, and Herpes) (19). In addition to infection, genetic and autoimmune interferonopathies such as Aicardi-Goutières syndrome and systemic lupus erythematosus (SLE) are associated with pregnancy complications (2). High serum IFN level is a marker of poor pregnancy outcome in SLE (20). Down syndrome in trisomy 21 (T21) patients is also associated with serious birth defects (1). *In vitro*, VCT from T21 patients poorly fuse into ST (1). This may be due to the location of the IFN receptor on chromosome 21, rendering T21 cells hyper-responsive to IFN (21) and thus potentially expressing high amounts of IFITMs.

Like other restriction factors, *lfitm* genes are polymorphic in humans and primates (11, 12). It will be worth determining whether placental pathologies of unknown etiology, such as certain preeclampsia or early spontaneous abortions, implicate IFITM proteins and variants. It is also tempting to suggest that blockade of IFITMs may represent a possible intervention strategy to prevent pregnancy complications linked to interferonopathies.

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Author contribution: JB, DAD, TH, XM, TF, ML and OS: designed experimental strategy. JB, QN, DAD, FP, MA and KR: Designed and performed experiments with cell lines. SAD and TF designed and performed experiments with primary trophoblasts. JB, TC, CM, QN, OD and XM designed and performed mouse experiments. EP and KHH performed the bioinformatic analysis. AD and TH provided vital materials and expert advice. JB and OS wrote the manuscript. All authors agreed on the final version.

Competing interests: Authors declare no competing interests. **Data and materials availability:** All data is available in the manuscript or the supplementary materials.

Supplementary Materials

Materials and Methods

Fig S1 – S9

References (22 – 37)

Movie S1

Figure Legends

Figure 1. IFITMs inhibit Syncytin-mediated cell fusion. (A) Left: 293T-GFP1-10 and -GFP11 cells were co-cultured at a 1:1 ratio and co-transfected with Syncytin-1 and IFITM or control plasmids. Cell fusion was quantified by measuring the GFP+ area at 18h. Middle: Representative image. White lines display the GFP area. Right: Quantification of GFP areas. Results are mean \pm sd from 4 independent experiments. Bar: 200 μ m. **(B)** Left: BeWo β -Gal- α and - ω were transduced with IFITM or control vectors. Cells were co-cultured at a 1:1 ratio and fusion was induced by Forskolin for 48h. Right: Fusion index (β -Gal activity) measured with a colorimetric (CPRG) assay. Results are mean \pm sd from 4 independent experiments. **(C)** Left: WT or IfitmDel MEFs were co-transfected with GFP and Syncytin-A plasmids. Syncytia were quantified after 24h. Right: Quantification of syncytia (cells with >3 nuclei) per well. Results are mean \pm sd from 3-6 independent experiments. Statistical analysis: One-Way ANOVA, ns: non-significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Figure 2. IFN- β inhibits fusion in primary human villous cytotrophoblasts (VCTs). (A) Quantification of fusion. Mononucleated VCTs express the nuclear GATA3 protein, downregulated after fusion. After 48h, nuclei are visualised by DAPI and plasma membranes with E-Cadherin. Representative images are shown. Syncytia, defined as large cells containing multiple GATA3-negative nuclei, have been delimited in white. Bar: 50 μ m. **(B)** Fusion index was quantified and calculated as $(100 - \% (\text{number of GATA3}^+ \text{ nuclei} / \text{total number of DAPI nuclei}))$ **(C)** hCG levels in culture supernatants at 72h. Results are mean \pm sd from 8 independent experiments. Statistical analysis: One-Way ANOVA, **** p < 0.0001.

Figure 3. IFITMs are key mediators of IFN induced fetal demise in mice. (A) Gestating dams were injected intra-peritoneally with Poly-IC or PBS at E7.5. Number, size and resorption of the

embryos were assessed at E9.5. Number of litters: WT PBS: 3, WT Poly-IC: 6, Ifnar^{-/-} PBS: 3, Ifnar^{-/-} Poly-IC: 3, IfitmDel PBS: 3, IfitmDel Poly-IC: 7. **(B)** Percentage of resorption for the indicated conditions. Numbers in brackets indicate the number of resorptions/total number of feto-placental units. Statistical analysis: Mann-Whitney, ns: non-significant, ****p < 0.0001. **(C)** Representative images of E9.5 embryos. Bar: 500 μm. **(D)** WT and IfitmDel placentas (E9.5) stained for E-cadherin (red), and Hoechst (blue). White arrows indicate ST. Representative images from 3 independent experiments are shown. Bar: 50 μm.