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Expression and Localization of StarD7 in Trophoblast Cells

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Abstract

The StAR-related lipid transfer (START) domain is defined as a motif of around 200 amino acids implicated in lipid/sterol binding. In a previous study, we identified the StarD7 transcript encoding one of the 15 family members with START domain present in the human genome. This transcript was found to be overexpressed in choriocarcinoma JEG-3 cells. In addition, we demonstrated that the recombinant StarD7 protein forms stable Gibbs and Langmuir monolayers at the air-buffer interface, showing marked surface activity and interaction with phospholipid monolayers, mainly with phosphatidylserine, cholesterol and phosphatidylglycerol. This study was undertaken to evaluate the expression and localization of StarD7 protein in trophoblastic samples. Here, we show for the first time the presence of StarD7 protein in human trophoblast cells. Western blot assays revealed a unique specific 34 kDa protein in JEG-3 cell line, choriocarcinoma tissue, complete hydatidiform mole, early and normal term placenta. Immunohistochemical data from early and normal term placentas and complete hydatidiform moles showed that this protein is abundant in the syncytiotrophoblasts, mainly at the apical side of the syncytium, with a weak and focal reaction in the cytotrophoblast cells. Furthermore, an increased StarD7 mRNA and protein expression, as well as a change in its sub-cellular localization was observed in *in vitro* differentiating cytotrophoblast isolated from normal term placenta. Taken together, these findings support and allow future studies to explore the possibility that StarD7 protein mediates transplacental lipid transport and/or is involved in syncytialization.

Keywords: Lipid-transport; StarD7; START domain; Trophoblast

1. Introduction

Trophoblast performs the majority of the absorptive, immunoprotective and endocrinological functions of the placenta, regulating the exchange of nutrients, gases, and other factors between the maternal and fetal circulations. The trophoblast differentiates in two ways: the villous and the extravillous trophoblast. In the villous phenotype, the cytotrophoblastic cells of the floating villi remain attached to the villous basement membrane, forming a monolayer of epithelial cells. These cells proliferate and differentiate, by fusion, to form a syncytiotrophoblast that covers the entire surface of the villous. Cytotrophoblast-syncytiotrophoblast fusion is clearly an event that starts with modifications of the plasma membranes of both partners such as expression of syncytin, cadherin 11, phosphatidylserine and connexin 43 [1]. Differentiation of the villous cytotrophoblast results in redistribution of plasma membrane phospholipids with an enrichment of phosphatidylserine on the syncytiotrophoblast surface [2].

Lipid movement across and between the two membrane layers is a biochemical event, in which the transport process can be attributed to the functions of specific proteins. A large list of structurally different protein families that can transport lipid monomers across the aqueous phase have been discovered [3]. Among these proteins, the StAR-related lipid transfer domain superfamily encompasses a 200-amino acid globular domain which has been implicated in lipid/sterol binding [4,5]. We have previously reported the cloning and characterization of a new member of this gene family up-regulated in the choriocarcinoma JEG-3 cell line, initially denominated GTT1 (gestational trophoblastic tumour 1, later renamed

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StarD7) [6]. StarD7 transcript is widely expressed in human cells with highest levels in choriocarcinoma tissue and HepG2, JEG-3 and HT-29 cell lines [6]. Nucleotide sequence analysis of the cDNA and computer-assisted homology search of the deduced amino acid sequence showed approximately 25% identity and 49% similarity with human, bovine and mouse phosphatidylcholine transfer protein (PCTP/StarD2) [6]. StarD7 shares a conserved central region from amino acids 66 to 250 with the START domain proteins proposed to bind lipids and to interact with membranes. The human and mouse genomes each have 15 genes encoding proteins containing a START domain and phylogenetic analysis divides the family into six subfamilies [7]. Reported data include StarD7 into the PCTP subfamily together with PCTP, StarD10, and Goodpasture antigen-binding protein/CERT/StarD11 [7,8].

Ligands have been only demonstrated for some START domain proteins. The mammalian StAR (StarD1), metastatic lymph node 64 (MLN64/StarD3) [9], StarD4, and StarD5 proteins bind cholesterol [10,11], the PCTP binds phosphatidylcholine [12], StarD11 binds ceramide [13] and the carotenoid binding protein (CBP1) from silkworm binds the carotenoid lutein [14]. In addition, StarD10 was recently found to function as a phospholipid transfer protein by binding to phosphatidylcholine and phosphatidylethanolamine [15]. Although the endogenous lipid ligand for StarD7 remains to be identified, we demonstrated that StarD7 recombinant protein forms stable Gibbs and Langmuir monolayers at the air-buffer interface, showing marked surface activity and interaction with phospholipid monolayers, mainly with phosphatidylserine, cholesterol and phosphatidylglycerol [16].

In addition to participating in lipid transport, START domain containing proteins may target proteins to membranes [8] and/or perform certain catalytic functions [17]. Ligand binding by the START domain can also regulate the activities of other domains that co-occur with the START domain in multidomain proteins [17,18].

The presence of START domains in evolutionarily distant species such as animals and plants suggests a conserved mechanism for interaction of proteins with lipids/sterols [8,11,19]. Human StarD7 orthologous genes have been annotated in the Ensembl and GenBank databases in different genomes, suggesting a conserved physiological function [20]. In addition, StarD7 protein has been predicted in most animal phyla, vertebrates and invertebrates, as well as in plants, underscoring its functional role.

The NCBI database points out the presence of two putative human StarD7 protein isoforms of 34.5 and 43.9 kDa (Accession number Q9NQZ5 and NP_064536, respectively). The smaller StarD7 isoform is predicted to localize at the cytoplasm, meanwhile the larger one is expected to be a mitochondrial protein.

Although we have previously determined the expression of StarD7 mRNA in trophoblast samples and different cell lines [6], nothing is known about the expression, localization and function of StarD7 protein. The aim of this study was to evaluate the expression and localization of StarD7 protein in human trophoblastic samples. Utilizing newly generated polyclonal antibodies to human StarD7, this study demonstrates the presence of a 34 kDa protein in JEG-3 cells, choriocarcinoma tissue, and complete hydatidiform moles, as well as in early and normal term placentas. Immunolocalization of StarD7 in syncytiotrophoblast and cytotrophoblast was demonstrated in complete hydatidiform mole, early and normal term placentas. Furthermore, an increased StarD7 expression level and a change in its localization were observed in *in vitro* differentiating cytotrophoblast cells.

2. Materials and methods

2.1. Tissue collection

Tissues from five normal term placentas (37–41 weeks of pregnancy), two normal early placentas from spontaneous abortions (10–12 weeks), five complete hydatidiform moles, and one choriocarcinoma were collected from unidentified patients with the approval of the Human Studies Committee of Faculty of Medicine of the National University of Córdoba, Argentine. The diagnosis of the complete hydatidiform moles and choriocarcinoma was established before the evacuation time according to biochemical (human chorionic gonadotrophin, hCG, value), echographic and clinical parameters [21]. Normal early placentas, complete hydatidiform moles and choriocarcinoma were identified on the basis of gross morphology and histopathology. Written consent for the collection of tissue samples was obtained according to a protocol approved by the Human Studies Committee of Faculty of Medicine of the National University of Córdoba, Argentine. After removal of the cord, amniochorion, and decidual layer, the tissue was cut into small pieces and washed with 154 mmol/l NaCl to remove blood.

2.2. Protein expression and purification

The StarD7 cDNA encoding a 295-amino-acid polypeptide (Accession number Q9NQZ5) [6] was obtained as previously reported [16]. Briefly, total RNA of JEG-3 choriocarcinoma cell line was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi [22]. Single-stranded cDNA was synthesized with StarD7 antisense primer (5′-GTGATAACGGACTGAGACAGGGCTAGAAGCA-3′) and PCR amplification was performed with one set of PCR primers, including the sense (5′-GCCTCTggtaccATGGCGGCGTTA-3′) and antisense (5′-TATCCCAAAgggtaccAAGCATACTCAATCCG-3′) primers incorporating KpnI flanking sites (small letters). In order to express and purify the recombinant StarD7 protein, the amplified fragment was cloned into the pGEMTeasy vector (Promega) and subcloned into the KpnI site of the pRSET expression vector (Invitrogen), which carries NH2-terminal His-tag/EK configuration. pRSET-StarD7 construct was selected and transformed into the BL21(DE3)-competent *Escherichia coli* cells. StarD7 construct was verified by DNA sequencing using the dideoxy chain termination method (Macrogen Inc., Seoul, Korea). The optimal expression condition of recombinant StarD7 was 1 mM isopropyl-1-thio-β-D-galactopyranoside induction for 4 h. All subsequent manipulations were performed at 4 °C. Bacteria were lysed by sonication and the homogenate was centrifuged at 10,000 × *g* for 15 min. The supernatant was collected and used to purify the StarD7 protein by Ni^{+2}-charged resin chromatography (Novagen). The protein was eluted with a linear gradient of 10–500 mM imidazole, 100 mM KCl, and 20 mM Tris–HCl, pH 7.8, in the presence of protease inhibitors. The purity of the His6-StarD7 recombinant protein was estimated by SDS-PAGE, being more than 95% (data not shown). Purified protein was stored in 20% glycerol (v/v) at −20 °C.

2.3. Generation and immunoaffinity purification of anti-StarD7 antibodies

Rabbit polyclonal antibodies were raised against the recombinant purified StarD7 protein (StarD7T) and against the synthetic peptide SSERKNEGSCGPARIEYA, corresponding to carboxyterminal residues (aa 278–295) of human

StarD7 (StarD7Ct), coupled to keyhole limpet hemocyanin (KLH). For the production of polyclonal anti-StarD7T and anti-StarD7Ct, purified His6-StarD7 (100 μg/animal) or StarD7Ct peptide-KLH (50 μg/animal) were emulsified in Freund's complete adjuvant. Two different New Zealand White rabbits were injected intradermally with each antigen preparation, respectively. At 2-week intervals, the rabbits received booster doses of the corresponding antigen in Freund's incomplete adjuvant. Seven days after the final injection, blood was collected and serum was separated by centrifugation at 1000 × *g* for 20 min at 4 °C.

The antibodies were immunopurified from rabbit serum. Briefly, the purified His6-StarD7 protein (100 μg) was loaded onto a 10% SDS-PAGE gel and electrotransferred to a nitrocellulose membrane. The StarD7 protein was visualized by Ponceau staining and the membrane containing the protein was cut and incubated with anti-StarD7T or anti-StarD7Ct at 4 °C overnight. Then, the membrane was washed three times with TBST (20 mM Tris−HCl [pH 8] containing 150 mM NaCl and 0.2% v/v Tween 20) during 5 min and incubated with 200 μl of 0.1 M glycine [pH 2.7]. After two minutes, the solution was neutralized with 20 μl of 1 M Tris−HCl [pH 8] with shaking. The eluted IgG molecules were used for Western blot, immunofluoresence, and immunohistochemical analysis. Specificity of anti-StarD7T and anti-StarD7Ct antibodies was verified as described below.

2.4. Expression of StarD7-myc fusion construct in COS-7 cells

The cDNA encoding human wild type StarD7 was cut from the pGEMTeasy vector with KpnI restriction enzyme and fused in-frame at the N-terminal of the c-myc tag, into the KpnI site of the expression vector pcDNA/TO/c-myc (Invitrogen, La Jolla, CA), and the resulting plasmid was named as StarD7-myc. COS7 cells were seeded in 37-mm wells of six-well plates at a density of 5×10^5 cells/well and grown overnight. The following day, 2200 ng of StarD7-myc and 300 ng of pCMV-*LacZ* construct (control of transfection efficiency) were resuspended in 250 μl of serum-free/antibiotic-free DMEM for 5 min at room temperature. This was added to a mixture containing 2 μl of LipofectAMINE 2000 (Invitrogen) in 250 μl of serum-free/antibiotic-free DMEM. After 5 min, this volume together with 1.5 ml of antibiotic-free DMEM supplemented with 10% serum was added to a monolayer that had been washed twice with PBS (20 mM Tris [pH 8], 150 mM NaCl). Following incubation for 5 h at 37 °C, cells were supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin. Transfected cells were cultured for 48 h and protein extracts were prepared and used in Western blot assays. Rab1 wt-myc protein (27 kDa), a generous gift from Dr. Cecilia Alvarez (Dpto. Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina), was used as a control [23]. Immunofluorescence studies were also performed in StarD7-myc-transfected COS7 cells.

2.5. SDS-PAGE and Western blotting

Cultured cells and tissue samples were washed with PBS and homogenized on ice for 1 min in RIPA lysis buffer (50 mM Tris−HCl [pH 7.5], 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin). The homogenates were centrifuged (10,000 × *g* at 4 °C) for 15 min, and the supernatants were collected. Protein concentrations were determined using the Bradford assay. Proteins were loaded onto a 10% SDS-PAGE gel. After migration, proteins were electrotransferred to nitrocellulose (Amersham Bioscience UK limited). The membrane was blocked in PBS containing 0.2% Tween 20 and 5% non-fat dry milk, washed and incubated with each one of the following primary antibodies: anti-StarD7T (0.25 μg/ml), anti-StarD7Ct (0.5 μg/ml), mouse monoclonal anti-α-tubulin (1:1000, Sigma-Aldrich, St. Louis, USA) and mouse monoclonal anti-c-myc (1:1000, Delta Biolabs, San Franciso, USA), for 1 h at room temperature with shaking. After washing, the blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibodies (Amersham Bioscience UK limited) at room temperature for 1 h. Protein-antibody complexes were visualized by an enhanced chemiluminescence detection system (SuperSignal West Pico; Pierce). Blots were quantified by densitometry using Scion Image programme (Scion Corporation, http://www.scioncorpor.com). Protein expression was normalized to the α-tubulin expression.

In order to establish the specificity of the generated StarD7 antibodies, competitive inhibition experiments were performed. In these experiments the following primary antibodies were used: anti-StarD7T antibody (0.25 μg/ml) preincubated with 2 μM of the recombinant purified His6-StarD7 protein for 2 h at room temperature or anti-StarD7Ct antibody (0.5 μg/ml) preincubated with 2 mM of the StarD7Ct peptide-KLH for 2 h at room temperature.

2.6. Quantitative real-time reverse transcription-PCR

Single-stranded cDNAs were synthesized from three μg of total RNA with random primers (Invitrogen) and M-MLV reverse transcriptase in a 20 μl reaction, as previously described [6]. StarD7 expression was quantified by real-time PCR (ABI 7500, Applied Biosystems) with Sequence Detection Software v1.3.1. Experiments were performed using 1× SYBR Green PCR Master Mix (Applied Biosystems) with 100 nM of each primer. The cycling conditions included a hot start at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Specificity was verified by melting curve analysis and agarose gel electrophoresis. Each sample was analyzed in triplicate. Transcript levels were normalized to those of Cyclophilin A and relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [24]. Amplification efficiency for both primers was near 98%. RNA samples incubated without reverse transcriptase during cDNA synthesis, as well as PCR reactions using water instead of template showed no amplification. Primers, designed with the Primer Express software (ABI), were as follows: StarD7 forward: 5′-GGTAATCAAGCTGGAGGTGATTG-3′; StarD7 reverse: 5′-GAGTACATTGGATAAGGAAAATGGGT-3′; Cyclophilin A forward: 5′-GTCAACCCCACCGTGTTCTT-3′; Cyclophilin A reverse: 5′-CTGCTGTCTTTGGGACCTTGT-3′.

2.7. Cytotrophoblast culture

Cytotrophoblasts were isolated as described previously by Kliman et al. [25]. Briefly, the tissue was thoroughly washed in 50 ml of cold sterile Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin, until the supernatant was nearly free of blood. Areas rich in chorionic villi were selected and minced into small pieces between scalpels. Tissue was incubated in HBSS containing 2.5 mg/ml trypsin (Difco), 4.2 mM $MgSO_4$, 25 mM HEPES and 50 U/ml Dnase I type IV (Sigma-Aldrich) for 20 min at 37 °C with shaking. After tissue sedimentation, the supernatant was discarded and the chorionic villi were submitted to three 30 min trypsin 2.5 mg/ml and 50 U/ml Dnase I type IV digestions. Enzymatic activity in the disaggregated supernatant was stopped with 10% fetal calf serum (FCS) (GBO, Argentine S.A.). After a further centrifugation at 850 × *g* for 10 min, the pellet was resuspended in DMEM supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cytotrophoblasts were separated using density gradient centrifugation at 1200 × *g* for 35 min on discontinuous Percoll (5−70%). Cells between the 40% and 50%-Percoll bands were collected, washed with DMEM, and plated onto 20 cm^2 culture dishes at a density of 3×10^6 cells/dish. Cell culture experiments were performed at 37 °C. The cells were grown in keratinocyte growth medium (Invitrogen), supplemented with 10% FCS and 100 U/ml penicillin, 0.1 mg/ml streptomycin and 5 ng/ml Epidermal Growth Factor (EGF), in 5% CO2 humidified atmosphere. The cells were maintained in culture for up to 96 h. hCG was determined in the supernatant using a clinical direct agglutination kit (Wiener Laboratories). The purity of the isolated cell population was assessed by staining with antibodies against cytokeratin 7 (DAKO). At least 95% of the isolated cells were cytokeratin 7 positive.

2.8. Immunofluorescence

Isolated cytotrophoblasts and StarD7-transfected COS7 cells were cultured on coverslips using supplemented medium as described above. The cells were fixed 10 min in cold methanol and incubated 10 min with 1 mM ammonium chloride to inhibit quenching. The cells were

permeabilized for 7 min with 0.01% Triton X-100 in PBS (PBST). The cells were then rinsed with PBS three times, blocked with 2.5% normal goat serum in PBST and with 0.2% fish skin gelatin in PBST, and then incubated at room temperature with the primary antibodies, rabbit purified anti-StarD7T (5 μg/ml), mouse monoclonal anti-c-myc (1:50, Delta Biolabs), and mouse anti-desmosomal protein (0.045 mg/ml, ZK-31, Sigma Chemical Co., St. Louis, MO). Cells were washed with PBST and incubated 1 h with the appropriate species specific secondary antibodies, either Alexa Fluor 594-conjugated goat anti-mouse IgG or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR). Cells were washed with PBST and nuclei were counterstained with Hoechst 33258 dye. Slides were mounted in PBS 90% glycerol containing 5 ng/ml *p*-phenylene-diamine (Sigma-Aldrich).

Observations of cells were made in a LSM5 Pascal laser scanning confocal microscope equipped with an argon/helium/neon laser (Zeiss, Jena, Germany), a ×63 Plan-Apochromat oil immersion objective with appropriate filters.

2.9. Immunohistochemical analysis

Tissue samples were fixed overnight in 4% paraformaldehyde and washed in saline buffer and 70% ethanol until processing and paraffin embedding. Representative blocks of paraffin-embedded placental tissue were cut to 4-μm thick sections and processed by the standard avidin-biotin peroxidase method. Briefly, the sections were dewaxed in xylene and rinsed in ethanol and a graded series of ethanol in water, and the endogenous peroxidase activity was blocked with 6% hydrogen peroxide. Antigen retrieval was performed using 1% antigen-unmasking solution (Vector laboratories, Burlingame, CA, USA) incubated at 96 °C for 60 min. The tissue sections were then blocked 20 min in PBS containing 0.8% horse serum (GIBCO) and then incubated with purified anti-StarD7T (5 μg/ml), followed by incubation with biotinylated anti-rabbit IgG and horseradish peroxidase-streptavidin (DAKO), before detection with diaminobenzidine (DAKO). All antibody incubations were carried out in PBS containing 0.1% albumin. Slides were counterstained with hematoxylin. For the above immunohistochemical procedures, controls were performed by replacing the primary antibody by PBS-albumin or using the anti-StarD7T antibody preincubated with recombinant purified StarD7 protein. Observations were made with a Zeiss Axioskop optical microscope (Carl Zeiss, USA).

3. Results

3.1. Expression of StarD7 protein in trophoblastic samples

Although we have previously determined the expression of StarD7 mRNA in trophoblastic samples and different cell lines [6], nothing is known about the expression, localization and function of the StarD7 protein. The aim of this study was to evaluate the expression and localization of StarD7 protein in trophoblastic samples. For these purpose two different polyclonal anti-StarD7 antibodies were generated. As part of their characterization, we performed Western blot and immunofluorescence assays in COS7 cells transfected with a StarD7-myc fusion construct. Protein extracts of transfected cultures were analyzed by Western blotting using anti-StarD7T, anti-StarD7Ct, and anti-c-myc antibodies. Both polyclonal anti-StarD7 antibodies revealed a single polypeptide band with the expected molecular weight of the StarD7-myc fusion protein (Fig. 1A, panel a and b, lane 2). Furthermore, the same band was detected with anti-c-myc antibody (panel c, lane 2). In the immunofluorescence assays, the same staining pattern for anti-StarD7T and anti c-myc antibodies was observed (Fig. 1B). In addition, anti-StarD7T revealed a weak signal corresponding to endogenous StarD7, observed in some non-transfected cells. These results indicate that the generated antibodies are useful to detect the recombinant StarD7 protein in eukaryotic cells by Western and immunofluorescence techniques.

To evaluate the endogenous StarD7 protein expression in trophoblast cells, protein extracts from JEG-3 cell line, complete hydatidiform mole and normal term placenta were checked in Western blot experiments. As shown in Fig. 2 (lanes 8–12), the anti-StarD7Ct was able to detect a predominant 34 kDa band in all samples, although faint bands of higher molecular weight were evident in some cellular extracts. In order to confirm the specificity of the signals observed, we performed competition experiments adding StarD7Ct peptide-KHL into the StarD7Ct antibody solution. As shown (Fig. 2, lanes 2–5) the 34 kDa band was completely abolished meanwhile the higher molecular weight bands persisted, suggesting that the later are non-specific. The same results were observed using StarD7T antibody (data not shown).

In order to extend these findings, protein extracts from three normal term placentas, two early placentas, five complete hydatidiform moles and one choriocarcinoma tissue were analyzed. These Western blot assays revealed the presence of the 34 kDa band in all trophoblastic samples (Fig. 3A). StarD7 signals normalized to that of α-tubulin revealed slightly elevated levels of StarD7 in the normal term placenta compared to normal early placenta samples. Meanwhile the highest StarD7 protein expression was found in pathological samples: JEG-3 cell line, complete hydatidiform moles and choriocarcinoma tissue, with an evident variability in complete hydatidiform moles (Fig. 3B). Nevertheless, a larger number of samples need to be analyzed to confirm the StarD7 expression pattern in normal and pathological trophoblastic tissues.

3.2. Cellular localization of StarD7 in trophoblastic tissues

To determine which type of cells in human trophoblast tissues expresses StarD7 protein, immunohistochemistry was applied. Human placental sections from early and normal term placentas and complete hydatidiform moles were immunostained with anti-StarD7T antibody. Positive immunostaining of StarD7 protein was localized to the villous syncytiotrophoblast and cytotrophoblast layers, mainly at the apical side of syncytiotrophoblast (Fig. 4). In normal early placentas, StarD7 was consistently expressed by villous syncytiotrophoblast, meanwhile the reactivity of villous cytotrophoblast was focal and weak (Fig. 4A,B). In normal term placentas and complete hydatidiform moles StarD7 immunostaining was markedly expressed by villous syncytiotrophoblast (Fig. 4C–E). Besides, a well-defined signal was detected in the endothelia of fetal blood vessels of normal term placenta. No staining was observed in controls in which the primary antibody was blocked with the recombinant purified His6-StarD7 protein or substituted by PBS (Fig. 4F).

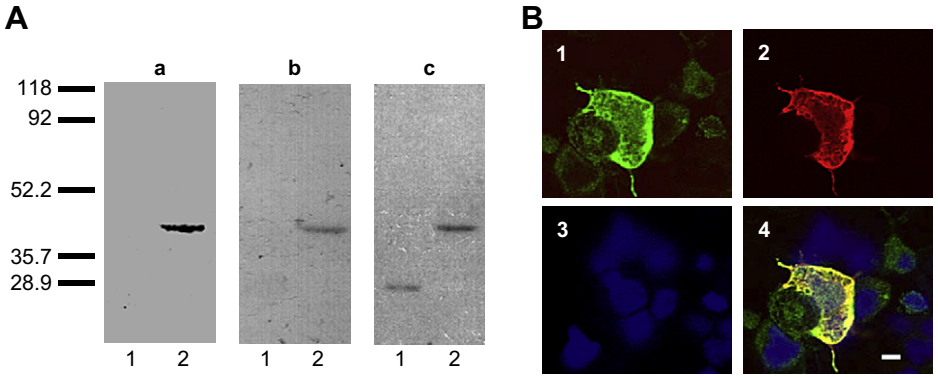

Fig. 1. Purified StarD7T and StarD7Ct antibodies specifically recognize recombinant StarD7-myc fusion protein. (**A**) Western blot analysis of StarD7-myc fusion protein. Extracts of COS7 cells transfected with the StarD7-myc plasmid were prepared 48 h after transfection and subjected to Western blotting. Blots show that the StarD7-myc recombinant protein (lane 2) was detected with anti-StarD7T (a), anti-StarD7Ct (b) or anti-c-myc (c) antibodies at the predicted molecular mass of 40 kDa. Control Rab1 wt-myc protein (27 kDa) was detected with anti-c-myc antibody (panel c, lane 1). Positions of molecular weight markers (kDa) are shown on the left. (**B**) StarD7-myc-transfected COS7 cells were fixed, permeabilized, and then incubated with anti-StarD7T (panel 1, green) and anti-c-myc (panel 2, red) antibodies. All images were obtained using a confocal microscope. Nuclei counterstained with Hoechst 33258 dye (blue) and overlay are shown in the panels 3 and 4, respectively. The yellow staining indicates co-localization of both antibodies in the cytoplasm. Bar = 10 μm. Original magnification, ×630.

3.3. Expression of StarD7 in in vitro differentiating cytotrophoblasts

Considering that the immunohistochemical data pointed to the villous syncytiotrophoblast as the main site of StarD7 expression, we decided to explore StarD7 protein levels in *in vitro* differentiated cytotrophoblast. Morphological and biochemical differentiation were observed in cytotrophoblasts purified from human term placenta and cultured *in vitro*. As a biochemical marker of trophoblast differentiation, hCG production was assessed in the cell culture supernatant, showing maximal concentration at 96 h of culture (above 5000 U/L). Morphological differentiation was monitored microscopically.

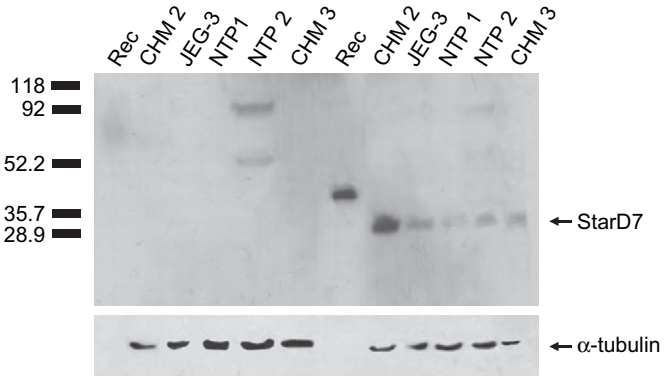

Fig. 2. Analysis of anti-StarD7Ct antibody specificity by competition experiments. Representative Western blot of StarD7 expression in homogenates of JEG-3 cells, complete hydatidiform mole (CHM) and normal term placenta (NTP). Protein extracts (100 μg/lane) from JEG-3 cells (lanes 3 and 9), two CHMs (lanes 2, 6, 8, and 12), two NTPs (lanes 4, 5, 10, and 11) and recombinant His6-StarD7 protein (Rec, lanes 1 and 7) were electrophoresed on a 7.5% SDS polyacrylamide gel and transferred to a nitrocellulose filter. Filters were incubated with anti-StarD7Ct (lanes 7–12) or with anti-StarD7Ct preincubated with the antigenic protein (lanes 1–6). A specific 34 kDa band is indicated (arrow). Molecular size markers on the left-hand side are given in kDa. Protein loading was confirmed blotting the membrane with anti-α-tubulin antibody (*bottom*).

As illustrated in Fig. 5A–I, purified mononuclear cytotrophoblasts aggregated, fused, and formed a multinucleated syncytium. At six hours, when most of the culture consisted of isolated mononuclear cells, staining for desmoplakin revealed a diffuse fluorescence (Fig. 5B), meanwhile desmoplakin staining was readily detectable at areas of cell–cell contact after 48 h (Fig. 5E) and 96 h (Fig. 5H) in culture.

Immunofluorescence assays performed with anti-StarD7T antibody revealed a cytoplasmic staining with a predominant perinuclear signal in isolated cytotrophoblasts after 6 h in culture (Fig. 5A). Meanwhile, with aggregation and differentiation StarD7 expression became more evident at the borders of adjoining mononuclear or multinucleated trophoblast cells (Fig. 5D,G).

RNA and total proteins were isolated from trophoblast cells and StarD7 levels were determined by quantitative real time-PCR and immunoblot analysis, respectively. Western blot analysis performed with total cell lysates of trophoblast cells cultured during 6 h and 96 h, revealed the presence of the 34 kDa StarD7 band (Fig. 6A). The intensity of the StarD7 signal was higher in the protein extracts from differentiated cultures than those of the undifferentiated ones (Fig. 6A, lane 2 vs. lane 1). This protein increase correlated with mRNA StarD7 expression revealed by quantitative real time PCR (Fig. 6B, lanes 1 and 2).

4. Discussion

In this study, we examined the protein expression and localization of StarD7 in trophoblastic tissues. For this purpose, two different polyclonal antibodies against StarD7 were generated and used in immunoblot, immunohistochemistry, and immunofluorescence assays.

StarD7 protein was detected in JEG-3 cell line, choriocarcinoma and complete hydatidiform mole, as well as in early and normal term placenta tissues. This is the first evidence of the presence of the endogenous StarD7 protein in any

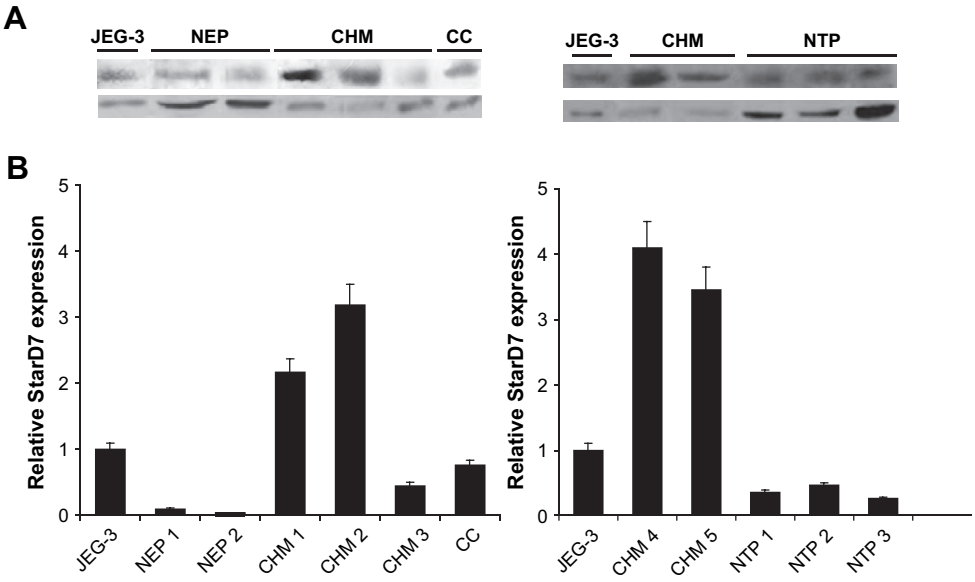

Fig. 3. Western blot analysis of StarD7 in trophoblastic samples. (**A**) Protein extracts (100 μg/lane) from JEG-3 cells, two normal early placentas (NEPs), three normal term placentas (NTPs), five complete hydatidiform moles (CHMs), and one choriocarcinoma (CC) were electrophoresed on a 7.5% SDS polyacrylamide gel and transferred to a nitrocellulose filter. Filters were incubated with anti-StarD7Ct antibody (*top*) and with the monoclonal anti-α-tubulin antibody (*bottom*). Two representative experiments that were repeated three times with similar results are shown. (**B**) StarD7 levels were normalized to α-tubulin and plotted as fold changes relative to JEG-3 cells (mean ± SEM).

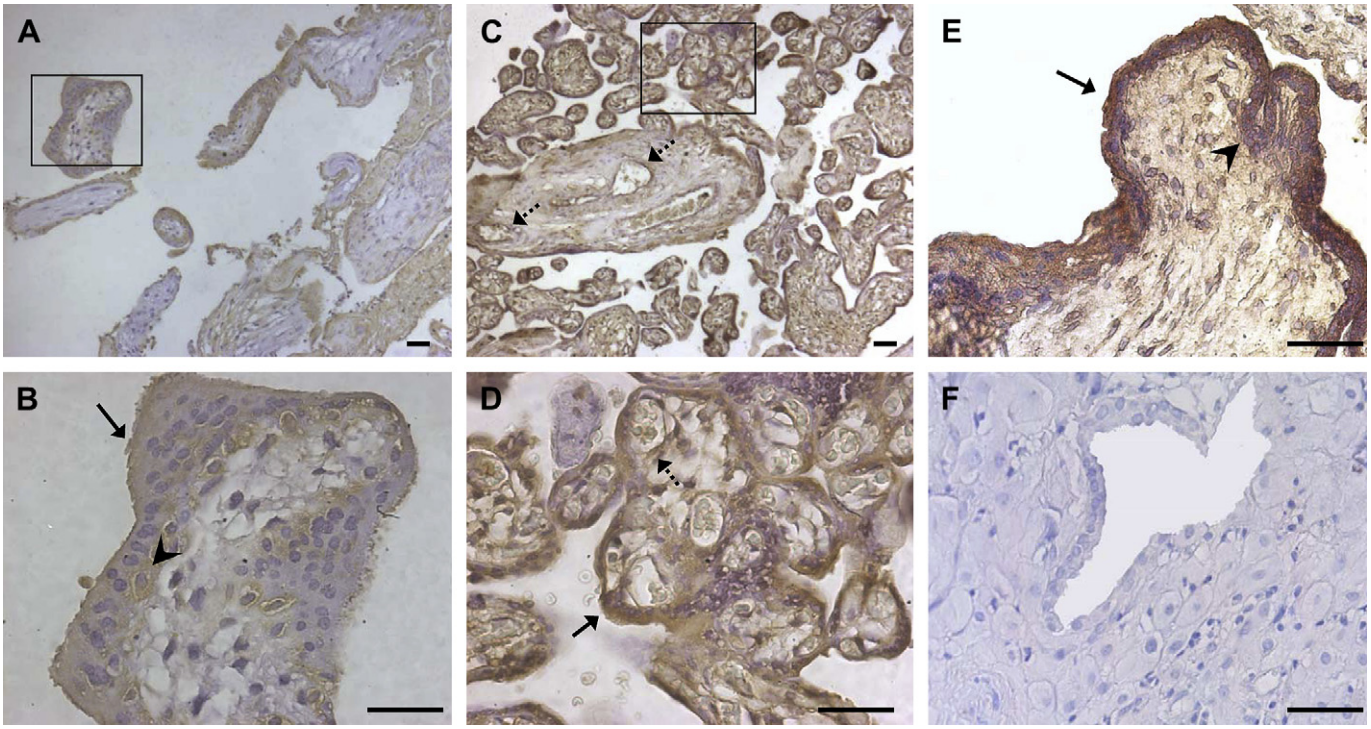

Fig. 4. Immunolocalization of StarD7 in early and normal term placenta, and complete hydatidiform mole. Paraffin sections of early (A and B) and normal term placenta tissue (C and D) and complete hydatidiform mole (E) were immunostained with purified anti-StarD7T antibody. StarD7 was detected in cytotrophoblast cells (arrowheads), in the syncytiotrophoblast layer of the villi (solid arrows), and in the endothelial fetal blood vessels (dashed arrows). In B and D, a higher magnification of the zone delimitated by the frame in A and C are shown. Samples were counterstained with hematoxylin. A representative example of three normal term placentas, two normal early placentas and two complete hydatidiform moles is depicted. A representative negative control performed with normal early placenta is shown (F). A and C: (×100); B, D, E, and F: (×400). Bars = 50 μm.

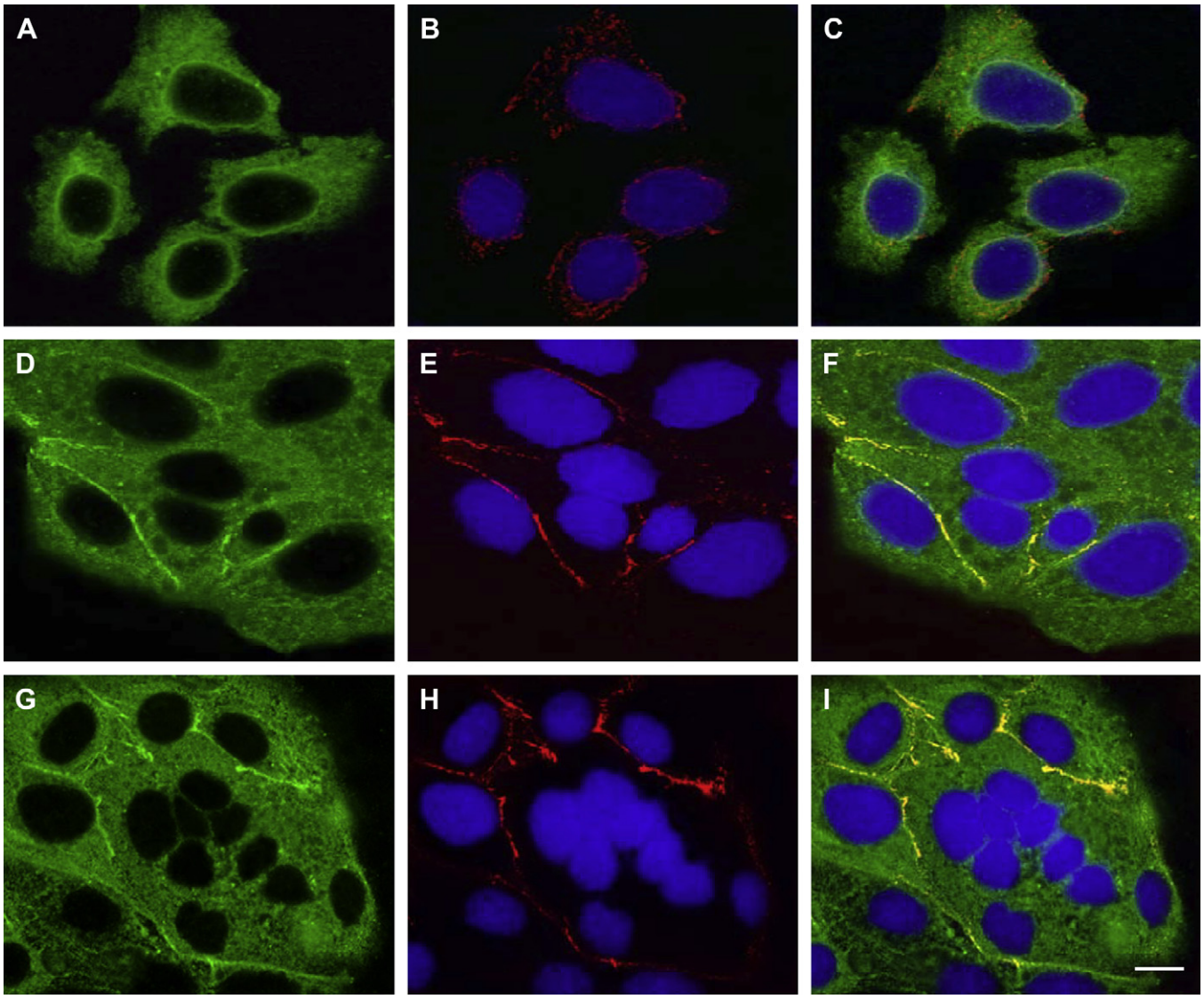

Fig. 5. Detection of StarD7 in *in vitro* differentiating cytotrophoblast cells. Isolated mononuclear cytotrophoblast cells after six hours (**A**, **B**, and **C**), forty-eight hours (**D**, **E**, and **F**), and ninety-six hours of culture (**G**, **H**, and **I**) were fixed and stained for detection of StarD7 with the purified anti-StarD7T antibody (**A**, **D**, and **G**), and desmoplakin antibody (**B**, **E**, and **H**). The nuclei were counterstained with Hoechst 33258 dye (blue). The green (representing StarD7) and red (representing desmoplakin) fluorescent signals were merged by computer assistance (panels **C**, **F**, and **I**). The same fields of cells are shown in panels **A**–**C**, **D**–**F**, and **G**–**I**. Images were obtained using a confocal microscope. Bar = 10 μm. Original magnification, ×1000.

type of cell. Both newly StarD7 antibodies revealed a unique specific band of 34 kDa suggesting that the predicted 34.5 kDa StarD7 isoform and not the 43.9 kDa isoform is synthesized in human trophoblastic tissues.

Additionally, the anti-StarD7T antibody displayed a well defined signal in immunohistochemistry and immunofluorescence assays. In normal term placenta and complete hydatidiform mole, the immunostaining signal was localized in both cytotrophoblast and syncytiotrophoblast cells, with a clear staining in the vicinity of the syncytium apical side. In normal early placenta, StarD7 was consistently expressed by villous syncytiotrophoblast, meanwhile the reactivity of villous cytotrophoblast was focal and weak. Besides, a well-defined signal was detected in the vascular endothelial cells of the placental villi of normal term placenta.

Immunofluorescence microscopy demonstrated endogenous StarD7 protein in the cytoplasm of cytotrophoblasts purified from human term placenta. This localization agrees with that predicted by bioinformatics primary sequence analysis of the 34.5 kDa isoform (Psort, http://psort.ims.u-tokyo.ac.jp). A clear and partial StarD7 relocalization towards plasma membrane was seen after the syncytialization process. In addition, mRNA and StarD7 protein expression increased in *in vitro* differentiated cytotrophoblasts. These data are in line with Western blot results which revealed a slightly higher StarD7 level in the analyzed normal term placenta compared to the normal early placenta samples.

Taking into account that syncytiotrophoblast represents the main trophoblast cell type in human term placenta; altogether the immunochemical, immunofluorescence, and Western blot

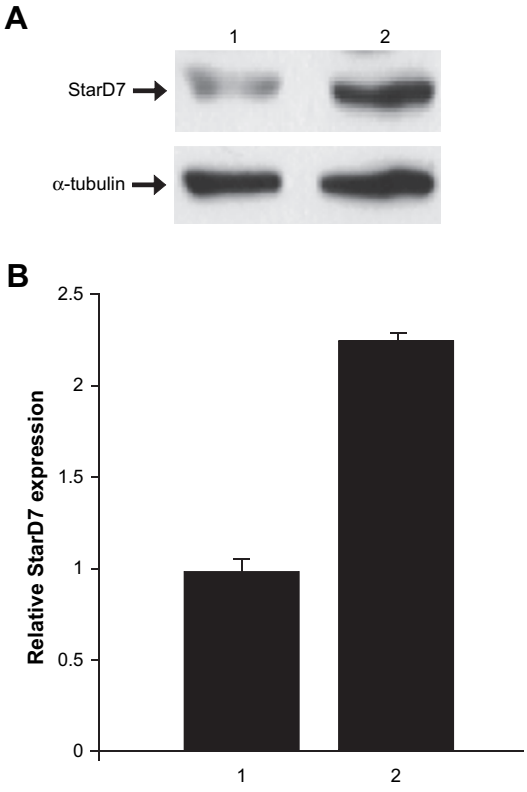

Fig. 6. Protein and mRNA StarD7 levels in *in vitro* differentiating cytotrophoblast cells. (**A**) Protein extracts (100 μg/lane) from isolated mononuclear cytotrophoblast cells after six hours (1) and ninety-six hours (2) of culture were electrophoresed on a 7.5% SDS polyacrylamide gel and transferred to a nitrocellulose filter. Filter was incubated with anti-StarD7Ct antibody (*top*), and with the monoclonal anti-α-tubulin antibody (*bottom*). (**B**) Quantitative real-time reverse transcription-PCR StarD7 analysis was performed using cDNA derived from three μg of total RNA extracted from isolated mononuclear cytotrophoblast cells after six hours (1) and ninety-six hours (2) of culture. The results are expressed as fold changes in StarD7 mRNA expression after normalizing to cyclophilin A. The values represent the mean ± SD of triplicate experiments performed with cytotrophoblast purification from three different normal term placentas.

data suggest that StarD7 expression may be regulated as a function of villous tissue development.

Finally, considering that StarD7 shows marked surface activity and interaction with phospholipid monolayers, mainly with phosphatidylserine, cholesterol and phosphatidylglycerol [16], bears a START domain, implicated in lipid/sterol binding, and modifies its expression and localization upon syncytialization, it is possible to hypothesize a StarD7 functional role in the intraplacental lipid transport and distribution. Particularly, StarD7 may be involved in phophatidylserine delivery to the syncytiotrophoblast surface, required for trophoblast differentiation [2].

In conclusion, these findings unequivocally demonstrate for the first time StarD7 protein expression and localization in placental tissues and stimulate to perform further studies to clarify StarD7 function related to the process of trophoblast differentiation. Additionally, the newly generated antibodies will be helpful for pursuing investigations into the clinical significance of StarD7 in normal and malignant trophoblast cells.

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