

Glypican-3 (GPC3) expression in human placenta: localization to the differentiated syncytiotrophoblast

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Summary. The expression of glypican-3 (GPC3), a heparan-sulfate proteoglycan associated with the Simpson-Golabi-Behmel fetal overgrowth syndrome, was studied in normal human placental tissue and cell lines derived from human placentae. Cytotrophoblasts derived from term placentae expressed GPC3 mRNA at low levels in culture. GPC3 mRNA expression increased markedly during trophoblast differentiation. By contrast, fibroblast cell lines derived from normal placentae did not express GPC3 in culture. Similarly, choriocarcinoma cell lines derived from human placentae (BeWo, JAR, and JEG) failed to express GPC3 mRNA. In situ hybridization confirmed the localization of GPC3 mRNA to the syncytiotrophoblast. Furthermore, immunohistochemical staining of paraffin imbedded placental tissue demonstrated intense staining of the syncytiotrophoblast cell layer and less intense staining of cytotrophoblasts. No staining of mesenchymal elements was noted. These data confirm the presence of GPC3 in human placenta and suggest it is expressed by the differentiated syncytiotrophoblast at term.

Key words: Placenta, Glypican-3, Trophoblast, Choriocarcinoma

Introduction

Glypican-3 (GPC3) is a recently identified heparan-sulfate proteoglycan associated with the Simpson-Golabi-Behmel (SGB) fetal overgrowth syndrome (Pilia et al., 1996; Pellegrini et al., 1998). GPC3 is located on the X chromosome and expressed primarily in embryonic tissue, including the placenta. SGB shares phenotypic similarities with the Beckwith-Weidemann and Perlman Syndromes, including the increased risk for the development of embryonal tumors (Neri et al., 1998; Veugelers et al., 1998). The physiological role of GPC3

has not been clearly defined but several important observations have provided some potentially significant insights. Pilia and colleagues (1996) originally demonstrated that GPC3 could bind IGF-II suggesting it could serve, in part, as an important modulator of local IGF-II levels and/or processing. Modulation of the biological activity of locally expressed IGF-II is intriguing given the phenotypic overlap of SGB with Beckwith-Weidemann Syndrome (BWS) and the overexpression of IGF-II associated with those patients (Hedborg et al., 1994). Subsequent reports looking specifically at OCI-5, the rat homologue of GPC3, failed to demonstrate IGF-II binding activity but did demonstrate its ability to bind FGF (Song et al., 1997). More recently, high levels of immunoreactive GPC3 capable of binding IGF-II have been demonstrated in human amniotic fluid, providing further support for a role at the maternal-fetal interface (Xu et al., 1998). However, the source of amniotic fluid GPC3 has not been identified.

Normal placental growth and function is essential for embryonic development. The syncytiotrophoblast is the placental cell associated with functions known to be critical to fetal well-being, i.e. nutrient transport, gas exchange, and hormone production. The expression of this functional activity is dependent on the normal growth of the cytotrophoblast and its differentiation into the mature syncytiotrophoblast. In addition, the interactions of the trophoblast with the adjacent stromal compartment and maternal decidua are important determinants of its developmental program. These interactions are dependent, in part, on the cell-specific expression of specific growth factors including the insulin-like growth factors. Both IGF-I and IGF-II are expressed by the placenta in a cell-specific manner throughout gestation suggesting important paracrine mechanisms of action (Fant et al., 1986; Wang et al., 1988; Ohlsson et al., 1989). We have developed *in vitro* models to study the role of IGFs in the regulation of placental growth utilizing both trophoblasts and placental fibroblasts (Fant, 1991; Cohran et al., 1996; Fang et al., 1997). Placental GPC3 is likely to play a

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significant role in placental growth, in part, by interacting with specific peptide growth factor signaling pathways. Nothing is known regarding the mechanisms of GPC3 action at the cellular level. We undertook these studies to determine the cell-specific expression of GPC3 in the human placenta in order to begin to study its role in placental development.

Materials and methods

Sources of materials

Human placentae were obtained from normal, uncomplicated pregnancies in accordance with a protocol approved by UT-Houston Health Science Center, Houston, Texas, and Washington University School of Medicine, St. Louis, MO. Rabbit, polyclonal anti-GPC3 antiserum was generated by Research Genetics against a polypeptide fragment of the GPC3 protein and affinity purified. Four distinct peptides from the predicted GPC3 primary amino acid sequence, N-RKMEEKYQLTAR, N-RHAKNYTNAMFK, N-EIDKYWREYIL, and N-AHSQQRQYRSAYY were produced by Research Genetics (Huntsville, AL) according to standard peptide synthesis protocols. All were conjugated to 8-MAP and injected into New Zealand white rabbits (3-9 months of age) in three subcutaneous dorsal sites at 0.5 mg peptide per immunization on day 0, week two, and week six, also according to standard protocols. Serum was collected every 2-4 weeks and tested for activity against the peptide pools. Antibody titer was determined using an ELISA with free peptide bound in solid phase (1 μ g/well). Those bleeds producing the highest titers were purified using a HiTrap protein G column (Pharmacia) according to the manufacturer's instructions. Epitope mapping was performed on this purified antibody pool using a slot blot containing all four individual peptides that comprised the initial immunogen. These slot blot results indicated that the polyclonal antibody was efficiently recognizing 3 of the 4 peptides. The antibody was then affinity purified using the peptide antigens immobilized on an activated support. The human GPC3 cDNA probe is a 2.1 kb insert containing the entire reading frame contained in a Bluescript transcription vector (Stratagene) at the EcoRI cloning site.

Isolation and culture of normal trophoblasts

Cytotrophoblasts were isolated from normal, human placentae by enzymatic dispersion with trypsin and isolated on a percoll gradient as previously described (Moe et al., 1991). Resulting trophoblast cultures were >95% pure as assessed by cytokeratin positivity. These cells spontaneously differentiate over 72 hours in culture to form syncytiotrophoblasts as assessed by characteristic morphological changes and the expression of differentiation state-specific markers, i.e. pregnancy-

specific β 1 glycoprotein (SP1) and human placental lactogen (Kliman et al., 1986). Placental fibroblasts were obtained from normal placental tissue by enzymatic dispersion and characterized by analysis of cell-specific markers to exclude the presence of differentiated endothelial cells, vascular smooth muscle cells, and trophoblasts. The isolation and characterization of these cells have been described in detail (Fant, 1991).

Northern analysis of GPC3

Total RNA was prepared from normal trophoblasts and choriocarcinoma cells by a modification of the method described by (Chomczynski and Sacchi, 1987). Poly A enriched RNA was obtained by oligo-dT chromatography. The RNA samples were size fractionated by electrophoresis on a 1.5% agarose gel containing 0.6M formaldehyde and transferred to Nylon + membranes (Amersham) for hybridization. Prior to hybridization the membranes were washed in 2 x SSC for 5 minutes.

A 2.1 kb insert of human GPC-3 was prepared and labeled by random primer hexamer method using [α - 32 P]dCTP (Amersham, Arlington Heights, IL). Prehybridization and hybridization were carried out for 30 minutes and 1 hour, respectively, in hybridization buffer (ExpressHyb - Clontech) for 1 hour at 65 $^{\circ}$ C. The membrane was then washed 4 x 10 min. at 22 $^{\circ}$ C in 2 x SSC, 0.05% SDS, and 2 x 20 min at 50 $^{\circ}$ C in 0.1 x SSC, 0.1% SDS. Thereafter the blots were stripped and rehybridized with cDNA probes for human β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In situ hybridization

Term placental tissue was collected and fixed overnight in 10% buffered formalin. After washing in fresh PBS, fixed tissues were dehydrated, cleared and embedded in paraffin by routine methods. Sections (5 μ m) were collected on Superfrost/plus positively-charged microscope slides (Fisher Scientific, Pittsburgh PA), deparaffinized, and in situ hybridization carried out according to established protocols (Albrecht et al., 1997). The full-length cDNA for human GPC3 was used to generate riboprobes used for detection. Riboprobes were labeled with [α - 35 S]UTP. To generate GPC3 the antisense riboprobe, the plasmid was linearized with XhoI and transcribed with T3 RNA polymerase. The sense riboprobe was generated by linearizing plasmid with XbaI followed by transcription with T7 RNA polymerase. Samples were overlaid with 4 million counts of antisense or sense riboprobe and hybridized overnight at 60 $^{\circ}$ C. Post-hybridization washes were carried out as described (Albrecht et al., 1997), and slides were dipped in Kodak NTB-2 emulsion and exposed for three days. Sections were viewed and photographed using an Olympus BX60 microscope equipped with a SPOT digital camera (Diagnostics Instruments, Sterling Heights, MI).

Placental glypican-3 expression

Immunohistochemistry

Human placental tissues were obtained from uncomplicated pregnancies at term. Small 0.5 x 0.5 cm. segments were cut and rinsed in ice cold PBS. The tissue was then placed in phosphate buffered formalin and imbedded in paraffin. Sections were cut, deparaffinized and washed x 3 in phosphate buffered saline, pH 7.4 (PBS) followed by 1% H₂O₂ in PBS to block endogenous peroxidase activity. The sections were then incubated for 30 minutes at 22 °C in normal goat serum (1:50 dilution in 1% bovine serum albumin). The tissue was then rinsed and incubated overnight with a 1:500 dilution of affinity purified anti-GPC3 antibody at 4 °C. Finally, the sections were washed and incubated with secondary antibody, biotinylated rabbit IgG 1:200 in 1:200 normal goat serum with 1% BSA, for 30 minutes at 22 °C. The sections were then washed and incubated with Vectastain (Vector Laboratories, CA) avidin: biotinylated enzyme complex (ABC) 30 minutes followed by 3,3'-diaminobenzidine (DAB) for 3 minutes at 22 °C per instructions. Finally, the sections were counterstained with hematoxylin. Tandem sections were incubated with 1:500 dilution of non-immune rabbit serum to identify non-specific staining.

Preparation of placental membranes

Basal membranes (BM) and microvillous membranes (MVM) were prepared essentially as described previously (Booth et al., 1980; Johnson and Smith, 1988; Fang et al., 1997). Briefly fresh placental tissue was dissected free of the basal and chorionic plates and washed in Earle's balanced salt solution. The tissue was passed through a meat grinder and stirred

vigorously in 1.5 vol of 150 mM NaCl. The tissue was removed by filtration. The filtrate was sequentially centrifuged at 800 x g for 10 min, 10,000 x g for 10 min, and 150,000 x g for 25 min. The pellet was resuspended in 300 mM D-mannitol, 2 mM HEPES-Tris, pH 7.5. The suspension was adjusted to 10 mM MgCl₂ for 10 min and centrifuged at 2200 x g for 12 min. The resulting MVM were enriched 19-20-fold as assessed by alkaline phosphatase activity.

Tissue remaining from filtration of the MVM described above were used to isolate BM (Kelley et al., 1983; Furesz et al., 1991). Briefly, the tissue was sonicated and stirred in hypotonic medium to remove residual MVM and cytoplasmic contents. The remaining tissue with attached BM was incubated in 10 mM EDTA and sonicated to remove the BM from the basal lamina. Free BM were obtained by differential centrifugation, resuspended in mannitol-HEPES buffer, pH 7.4, and stored under liquid nitrogen until assayed. BM were enriched 42-fold as assessed by [³H]-dihydroalprenolol binding and 0.63-fold for alkaline phosphatase activity, a marker for MVM.

Immunoblot analysis of placental membranes

40 µg aliquots of BM or MVM were subjected to 7.5% SDS-PAGE under non-reducing conditions followed by transfer to nitrocellulose membranes. The membranes were then blocked in 5% dried milk and incubated for 2 hours in anti-GPC3 antiserum (1:500 dilution) followed by washing and detection using a chemiluminescence detection system (Amersham) per instructions.

Results

GPC3 expression by placental cells in culture

To begin to examine the expression of GPC3 in human placenta, cytotrophoblasts were isolated from human placentae at term. The undifferentiated cytotrophoblast expressed low levels of GPC3 mRNA at 24 hours after plating. Expression increased markedly during 72 hours of culture while the cytotrophoblast underwent differentiation to form the mature syncytiotrophoblast (Fig. 1). The expression of two house-keeping genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are depicted, as well, to illustrate their expected and divergent expression during trophoblast differentiation. These data are representative of gene expression by trophoblasts derived from five different placentae. By contrast, GPC3 mRNA expression was not detected in placental fibroblast cell lines or in choriocarcinoma cell lines (JAR and JEG) derived from first trimester cytotrophoblasts (Fig. 2). The b30 clone of the BeWo choriocarcinoma cell line, another trophoblast-derived cell of human origin has been shown to differentiate in response to forskolin in a manner analogous to normal trophoblasts (Wice et al,

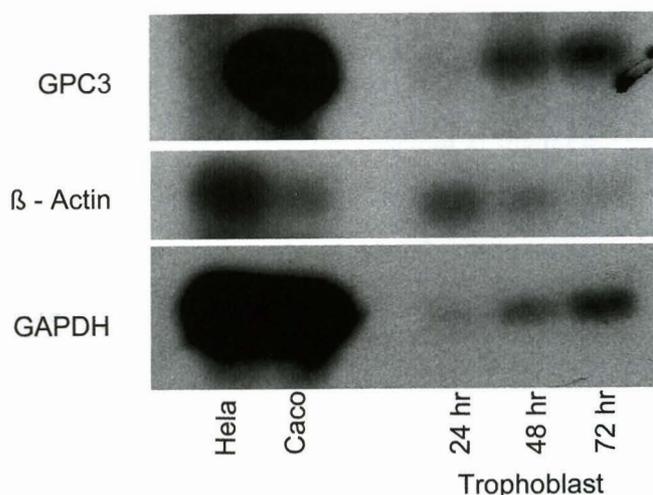


Fig. 1. Northern analysis of GPC3 mRNA expression during trophoblast differentiation. GPC3 mRNA expression by normal trophoblasts at 24, 48, and 72 hours in culture is shown. 5 µg poly A RNA were loaded at each time point. GPC3 expression by CaCo-2 cells (positive control) and HeLa cells (negative control) is also shown.

1990). Unlike normal trophoblasts, these cells failed to express GPC3 mRNA in the undifferentiated state or during forskolin-stimulated differentiation (Fig. 2). GPC3 mRNA expression in CaCo-2 and HeLa cells is included as positive and negative controls, respectively (Pilia et al., 1996). These results suggest that the normal syncytiotrophoblast expresses GPC3 and that expression is coupled to cell differentiation.

Localization of GPC3 mRNA by In Situ Hybridization

To further define the cellular localization of GPC3 transcripts in the term human placenta, in situ hybridization was carried out using an antisense riboprobe specific for GPC3 (Fig. 3). High levels of GPC3 transcript were detected in the syncytiotrophoblast layer of the chorionic villi of placentae hybridized with the antisense GPC3 riboprobe (Fig. 3A), whereas no hybridization signal was detected in a serial section hybridized with sense GPC3 riboprobe (Fig. 3B). The intensity of the signal appeared rather weak in some villi relative to others suggesting variable expression in some villous segments. These data are representative of three different placentae and confirm the expression of GPC3 mRNA by the syncytiotrophoblast and the lack of expression by mesenchymal elements.

Immunohistochemical localization of placental GPC3

Immunohistochemical analysis of paraffin-embedded tissue sections of term placentae also revealed specific staining confined to the syncytiotrophoblast in the chorionic villi (Fig. 4), consistent with the localization of GPC3 mRNA and expression in cultured cells. While the staining was intensely localized to the syncytiotrophoblast of the chorionic villi, its distribution was not uniform since neighboring villi appeared to be stained

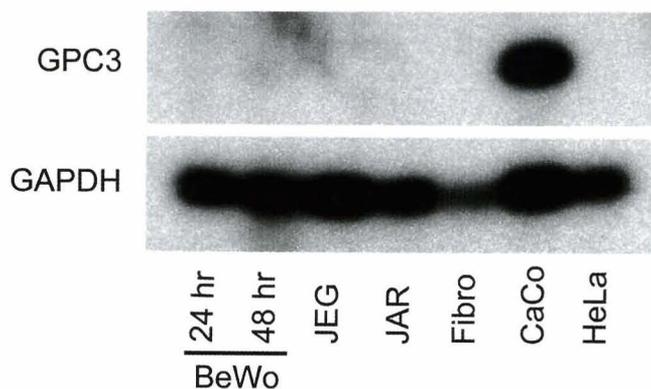


Fig. 2. Northern analysis of GPC3 mRNA expression by 10 week placental fibroblasts and choriocarcinoma cell lines. mRNA was obtained from each cell line just prior to reaching confluence. mRNA was obtained from the b30 clone of the BeWo cell line after 24 and 48 hours of forskolin (100 μ M) treatment to induce cell differentiation. JEG and JAR choriocarcinoma cell lines were not treated with forskolin. Fibro: placental fibroblast cell line. Analysis of CaCo-2 and HeLa cell expression is included as positive and negative controls, respectively.

less intensely (Fig. 4A). The underlying cytotrophoblasts exhibited less intense staining (Fig. 4A) consistent with the increased expression of GPC3 mRNA during cell differentiation observed in cell culture (Fig. 1). Intense staining was also noted in trophoblasts at the trophoblast-decidual interface (Fig. 4E). In addition, some endometrial cells also stained positively for GPC3 (Fig. 4E). The identity of these cells has not been determined.

GPC3 appears throughout the syncytial layer. The cellular staining is distributed throughout the cytoplasm and appears concentrated along the basal surface of the syncytiotrophoblast (Fig. 4A,C). Immunoblot analysis of plasma membranes isolated from the syncytiotrophoblast identified an immunoreactive species with molecular mass of approximately 230 kDa associated primarily with the fetal-facing basal membranes (Fig. 5). This is similar to the molecular mass of immuno-reactive GPC3 found in amniotic fluid by Xu et al. (1998) using the same antibody preparation. Furthermore, it is similar to the molecular mass of GPC3 protein "shed" from the CaCo-2 cell surface as initially described by Pilia et al. (1996). By contrast, the maternal-facing, microvillous membranes contained a less intense band migrating at 230 kDa associated with additional lower molecular mass species. The identities of these immunoreactive species have not been confirmed. However, these findings are consistent with the spatial polarization of GPC3 on the syncytiotrophoblast cell membrane or, alternatively, indicative of differential processing of GPC3 associated with distinct regions of the cell surface. These data are representative of membranes derived from two different placentae.

Discussion

GPC3 is a recently described member of the glypican family of heparan sulfate proteoglycans. Mutational deletions in the GPC3 gene have been shown to result in the Simpson-Golabi-Behmel fetal overgrowth syndrome (Pilia et al., 1996). Although its role in fetal development is unclear, it is expressed primarily in embryonic tissues and some tumor cells suggesting it serves to regulate specific aspects of embryonic and fetal development. Little is known concerning the cellular mechanisms by which GPC3 regulates fetal growth. Some insight has been obtained from recent studies examining the human gene product and its rat homologue, OCI-5. The initial report of the human gene demonstrated its ability to bind IGF-II. This was subsequently confirmed by demonstrating the IGF-II binding properties of immunoreactive GPC3 in human amniotic fluid (Xu et al., 1998). This suggested a possible role for GPC3 in the regulation of IGF-II by perhaps sequestering it or facilitating its turnover via the type 2 IGF receptor. SGB shares phenotypic similarities with other fetal overgrowth syndromes such as the Beckwith-Wiedemann Syndrome and Perlman Syndrome, including the propensity for embryonal

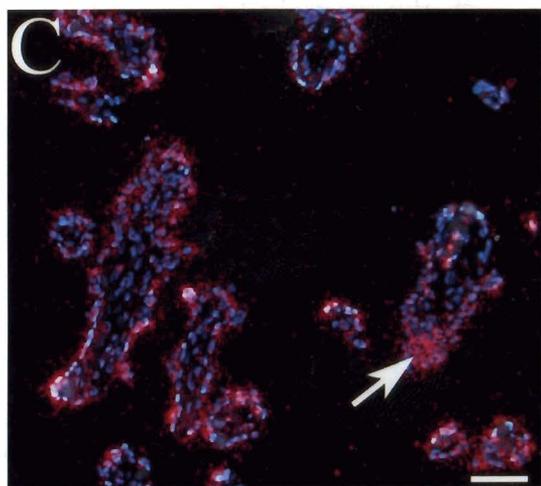
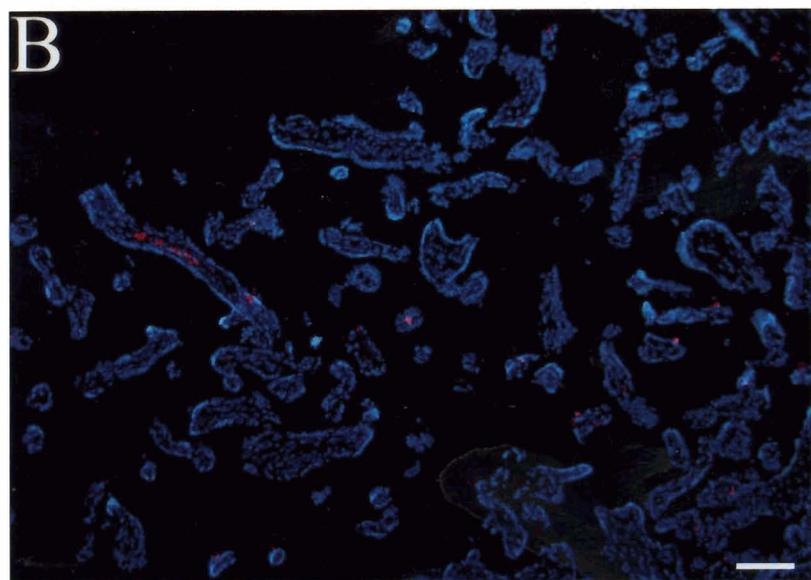
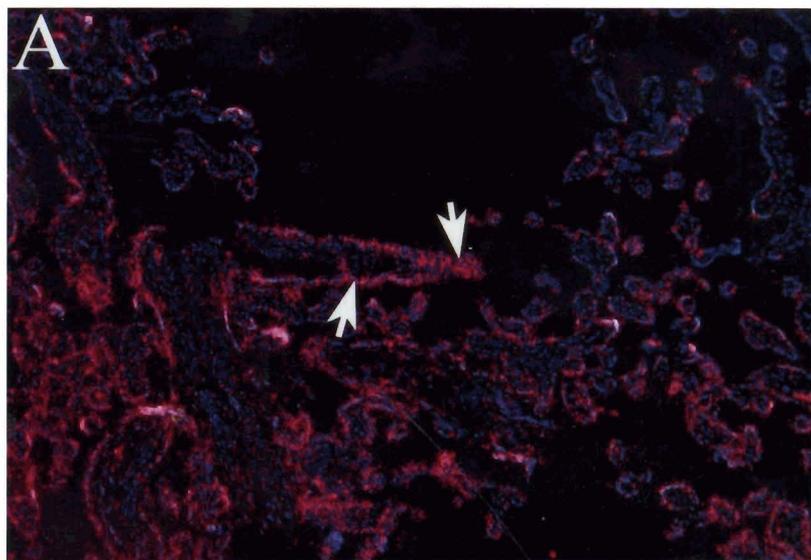


Fig. 3. Localization of GPC3 transcripts in the human placenta. **A.** Term placenta hybridized with 35 S-labeled antisense GPC3 riboprobe. Red pixels denote specific hybridization to GPC3 transcripts (arrows), while blue staining represents nuclear DNA stained with Hoechst 33258 (Albrecht et al., 1997). **B.** Term placenta hybridized with sense GPC3 riboprobe. Scale bar: 100 μ m for A and B. **C.** High magnification (x 20) of term human placenta hybridized with antisense GPC3 riboprobe demonstrating the localization of transcripts to the syncytiotrophoblast (arrow). Scale bar: 50 μ m.

tumors in certain organs (Coppin et al., 1997; Li et al., 1998; Neri et al., 1998). The role of increased IGF-II production in the pathophysiology of BWS has been well described (Hedborg et al., 1994). It has been speculated that in SGB, a disrupted GPC3 gene results in increased levels of biologically available IGF-II at the tissue level resulting in organ overgrowth and tumor progression. Supporting this hypothesis is data obtained from in situ hybridization revealing the co-localization of IGF-II, type 2 IGF receptor, and GPC3 gene expression in the developing mouse (Pellegrini et al., 1998). Interestingly, the rat homologue, OCI-5, was found to have binding activity towards FGF-2 but not IGF-II (Song et al., 1997) suggesting that GPC3 may interact with several growth factors but that there may be species differences in the binding specificity. These and other fundamental questions related to the basic cell

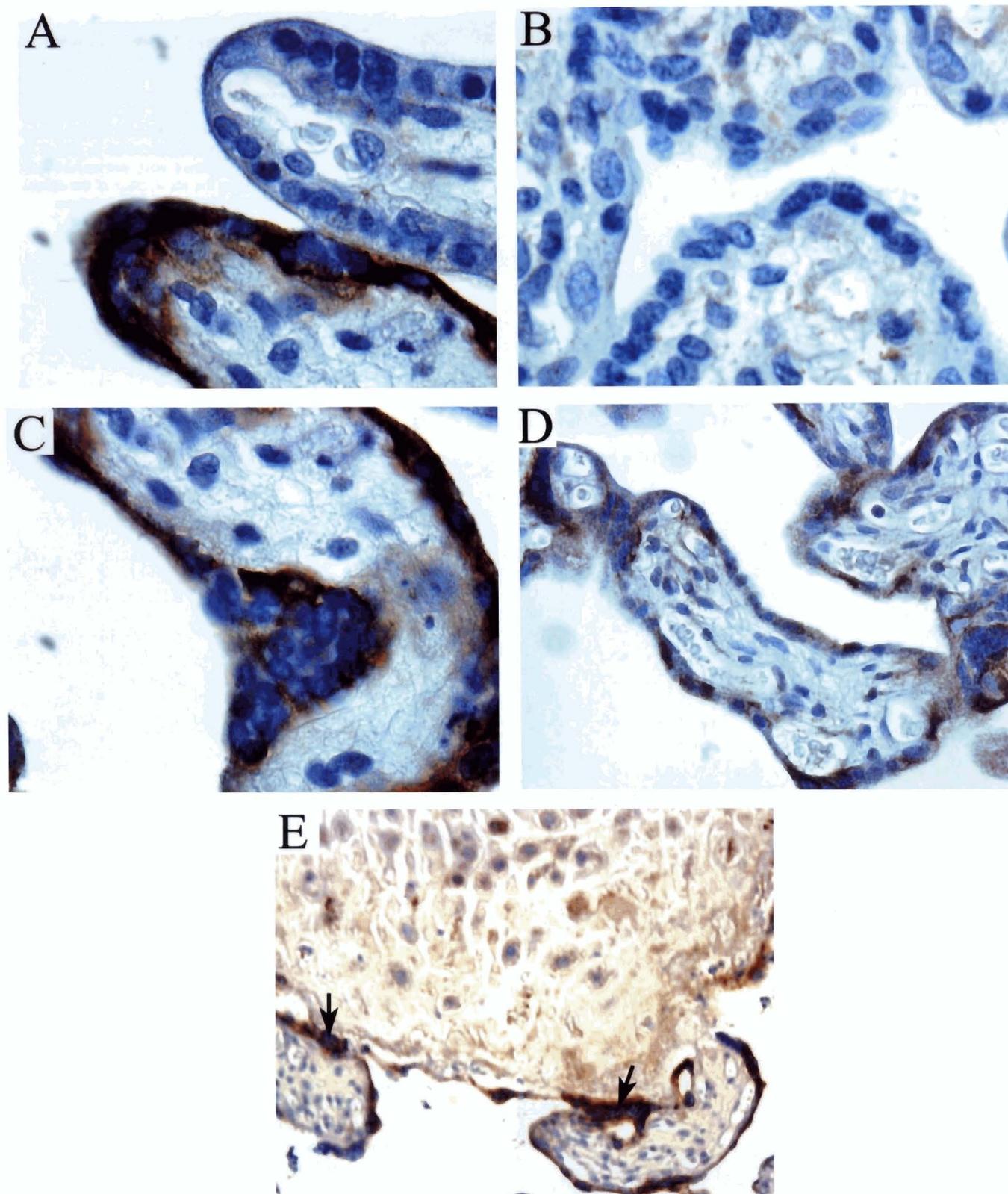
Placental glypican-3 expression

Fig. 4. Immunolocalization of GPC3 in term placental tissue. **A.** GPC3 staining is localized to the syncytiotrophoblast. An adjacent villus exhibits very faint staining. x 40. **B.** Non-immune serum. x 40. **C.** Staining relatively strong over nuclear aggregates. x 40. **D.** Chorionic villi. x 20. **E.** Specific staining was present in trophoblasts at the chorion/decidual junction and in some endometrial cells. x 4

biology of GPC3 action have not been resolved.

Given the importance of the insulin-like growth factors (IGFs) in regulating placental function we sought to identify the cell type responsible for GPC3 production in the placenta. This would permit us to begin to study its role at the cellular level and investigate, specifically, its interaction with the IGFs and other growth factor signaling pathways. The results of our study demonstrate that GPC3 is expressed by the syncytiotrophoblast in the term placenta. GPC3 mRNA expression in cultured cells increased markedly during the differentiation of cytotrophoblasts to form the syncytiotrophoblast. Its localization to the syncytium was confirmed by *in situ* hybridization as well as immunohistochemical techniques, and appears to localize preferentially to the basal surface of the villous syncytiotrophoblast. We have previously shown that the type I and the *unoccupied* type II IGF receptors are located primarily on the maternal-facing, microvillous surface of the syncytiotrophoblast

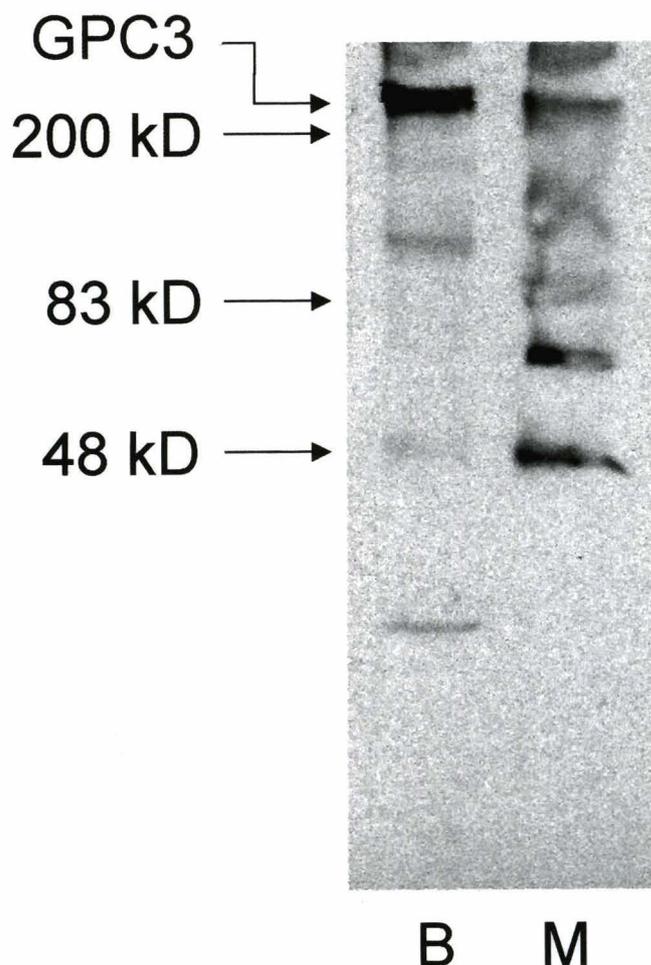


Fig. 5. Immunoblot analysis of GPC3 in placental membranes. Aliquots of plasma membranes isolated from the fetal-facing, basal surface (**B**) and the maternal-facing, microvillous surface (**M**) of the syncytiotrophoblast were subjected to SDS-PAGE under non-reducing conditions and immunoblot analysis using anti-GPC3 antiserum.

(Fang et al., 1997). By contrast, the basal surface has relatively greater amounts of immunoreactive type II IGF receptors that are almost all associated with phosphomannosyl-containing compounds, suggesting that they are actively involved in targeting proteins for lysosomal degradation and perhaps ongoing tissue turnover and remodeling. The presence of GPC3 on the basal surface of the syncytiotrophoblast could serve several important functions through both IGF-dependent and IGF-independent pathways. GPC3 could interact with IGF-II to localize them to the basal surface of the syncytiotrophoblast. There they could regulate trophoblast growth and metabolic function directly or, alternatively, regulate cell function in the stromal compartment via paracrine mechanisms by modulating the levels of IGF-II in the local environment. Interestingly, IGF-II is produced primarily by the stromal cells and is the predominant IGF present in the villous core (Wang et al., 1988). This would make it readily accessible to GPC3 on the basal membrane surface. In addition, a variety of peptide growth factors have been shown to bind to heparan-sulfate proteoglycans (HSPG) which serve to modulate their biologic activity, including fibroblast growth factor (FGF-2), hepatocyte growth factor (HGF), placental growth factor (PlGF), and heparin-binding epidermal growth factor-like factor (HB-EGF) (Song et al., 1997; Arkonac et al., 1998; Athanassiades and Lala, 1998; Hartmann et al., 1998; Lambrecht et al., 1998). These peptides presumably play significant roles in regulating both trophoblast growth and mesenchymal cell growth. The specificity of growth factor binding to GPC3, as well as the factors that influence its specificity, have not been characterized. The demonstrated associations of GPC-3 with IGF-II and its rat homologue with FGF-2, however, suggests that it may play multifunctional roles in regulating the trophoblast as well as activity within the stromal compartment, including angiogenesis and/or endothelial cell migration.

Finally, a recent study demonstrated that GPC3 expression induces apoptosis in a cell-type dependent manner (Gonzalez et al., 1998). A role for IGFs in protecting cells from apoptosis has been demonstrated in a variety of cell types (Basergera et al., 1997; O'Connor et al., 1997). It is intriguing to speculate that the coordinated expression of IGFs and GPC3 may also play a role in regulating the total cellular mass of the syncytiotrophoblast by influencing the cell's susceptibility to apoptotic stimuli.

In summary GPC3 is expressed primarily by embryonic tissues and has been shown to be necessary for normal fetal growth. We have demonstrated that it is expressed by the differentiated syncytiotrophoblast in the human placenta at term. GPC3 is therefore likely to regulate fetal growth, in part, by playing a significant role in placental development and/or function. Studies are currently underway to define the expression of GPC3 throughout gestation in normal and abnormal pregnancies.

Acknowledgements. These studies were supported, in part, by grant HD 20805 (M.E.F.).

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