

Expression pattern of glypican-3 (GPC3) during human embryonic and fetal development

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Summary. Glypicans represent a family of cell surface proteoglycans. Loss-of-function mutations in the human *glypican-3* (GPC3) gene results in the Simpson-Golabi-Behmel syndrome, characterized by severe malformations and pre- and postnatal overgrowth. Because the expression of GPC3 during human embryonic and fetal periods remains largely unknown, we investigated by immunohistochemistry its pattern of expression during four periods of human development covering the embryonic period (P1) from 5 to 8 weeks of development, and the fetal periods (P2, P3 and P4) from 9 to 28 weeks of development.

Hepatocytes were homogeneously positive for GPC3 during the four periods while pancreatic acini and ducts showed a rather high staining only during P1.

GPC3 was also detected in several kidney structures and in the genital system where the sex cords were weakly positive in P1 and P2. In later developmental stages the male's genital system expressed GPC3 while the female's did not.

While the mesenchyme in the limbs showed positive staining in P1, GPC3 was not detected during the following stages. The mesenchymal tissue localized between the most caudal vertebrae was also positive in P1.

A strong GPC3 signal was observed in neurons of the spinal cord and dorsal root ganglia in P2 and P3, while the brain was negative.

In sum our studies revealed that GPC3 expression is highly tissue- and stage-specific during human development. The expression pattern of GPC3 is

consistent with the abnormalities seen in the Simpson-Golabi-Behmel syndrome.

Key words: Glypican-3 (GPC3), Heparan sulphate proteoglycans, Human embryo/fetus, Immunohistochemistry, Simpson-Golabi-Behmel syndrome

Introduction

Glypicans represent a family of cell surface heparan sulfate proteoglycans attached to the cell membrane through a glycosylphosphatidylinositol linkage (Fransson, 2003). Six members of this family have been identified in mammals (GPC1-6) and two in *Drosophila* (De Cat and David, 2001, Filmus and Selleck, 2001).

Loss-of-function mutations in the human *glypican-3* (GPC3) gene result in the Simpson-Golabi-Behmel syndrome (SGBS), an X-linked condition characterized by severe malformations and pre- and postnatal overgrowth. Characteristically, humans with this disorder display a wide range of abnormalities, including a distinct facial appearance, cleft palate, congenital heart and renal defects, supernumerary nipples, vertebral and rib defects, polydactyly and syndactyly. Other common features of this syndrome are macroglossia and abnormalities in the genitourinary tract (DeBaun et al., 2001). Furthermore, these patients have a high risk of developing embryonal tumors, mostly Wilms' kidney tumor and neuroblastoma (DeBaun et al., 2001).

Some of the clinical features of these patients and in vitro studies suggest that GPC3 inhibits cell proliferation and cell survival in specific tissues during development (Dueñas-Gonzalez et al., 1998). It has been recently reported that GPC3 regulates body size, at least in part, by acting as a negative regulator of Hedgehog signaling

(Capurro et al., 2008).

Interestingly, alterations in GPC3 function and/or expression could be associated with cell transformation and tumor progression (Dueñas-Gonzalez et al., 1998; Filmus and Selleck, 2001; Peters et al., 2003).

The expression profile of GPC3 during development was studied in several laboratories. These studies were performed mainly at the RNA level, and only in mice (Pellegrini et al., 1998; Chiao et al., 2002). However, there are several differences in the phenotypic features of GPC3-null mice and SGBS patients, suggesting that the pattern of expression of GPC3 may display significant differences between mice and humans (Cano-Gauci et al., 1999; Chiao et al., 2002; Viviano et al., 2005). Because the expression pattern of GPC3 during human embryonic and fetal periods remains largely unknown, we decided to investigate it by immunohistochemistry.

Materials and methods

Tissue collection and preparation

Human embryonic and fetal tissues were obtained from 13 anatomically normal spontaneously aborted embryos and fetuses, without visible autolysis, provided by the Hospital Municipal "J. M. Ramos Mejía" at Buenos Aires, under the approval of the Ethics Committee of the hospital and with the informed consent of the parents. The developmental age was determined by either foot-length measurement for the youngest embryos or by crown-rump measurement for the oldest fetuses. According to the developmental age (weeks) samples were grouped in four periods as follows: P1 (5 to 8 weeks, n=5), P2 (9 to 12 weeks, n=3), P3 (13 to 19 weeks, n=3) and P4 (20 to 28 weeks, n=2).

The samples were fixed in 4% paraformaldehyde for 24 hours after the delivery, dehydrated, embedded in paraffin and sectioned (4 µm).

Immunohistochemistry

Paraffin embedded tissue sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. An antigen retrieval technique was then performed by incubating the slides for 10 minutes in 0.01% trypsin (Sigma) at 37°C, washing with PBS and then heating in 0.01 M Tris-HCl buffer, pH 10, in a microwave oven (3 cycles of 10 minutes at 750 watts). The slides were rinsed with distilled water, dehydrated in a graded series of ethanol, and dipped in 10% hydrogen peroxide in methanol for 20 minutes to block the endogenous peroxidase. Then slides were rehydrated, incubated for 1 hour in PBS/5% non fat milk and incubated overnight at 4°C with a mouse monoclonal anti-GPC3 antibody 1:70, 1G121, isotype IgG1 K (Capurro et al., 2003). Next, slides were incubated with a biotinylated anti-mouse IgG 1:500 (Vector) for 1 hour at room temperature and antibody binding was detected

with the Vectastain Universal Elite ABC Kit (Vector Laboratories) as indicated by the vendor. The peroxidase activity was revealed in brown by diaminobenzidine and nuclei were counterstained with Mayer's Hematoxylin.

Human placenta served as positive control for GPC3 immunoreactivity and one slide was included in each staining series. Negative controls were performed by missing the primary antibody or by incubating with a mouse preimmune IgG. Additional adjacent sections of each sample were stained with hematoxylin-eosin for histological examination.

Results

General aspects of GPC3 pattern of expression

In general we found a variable expression of GPC3 depending on the tissue and the period of human embryonic and fetal development evaluated. In particular, a very high expression of GPC3 was observed in the parenchyma of the liver and kidney all along the four periods studied. The pericardium, pleura and peritoneum displayed a faint signal and the brain, thymus, thyroid, salivary glands, skin and spleen were negative in all cases. In the placenta, GPC3 staining was found in the cell membrane and cytoplasm of cells from the syncytiotrophoblast layer while the mesenchymal tissue was negative (Fig. 1).

GPC3 staining in the digestive tract and the annexed glands

Hepatocytes were homogeneously positive for GPC3 in all the samples along the four periods studied (Fig. 2A). In contrast the pattern of GPC3 expression in the exocrine pancreas changed significantly during

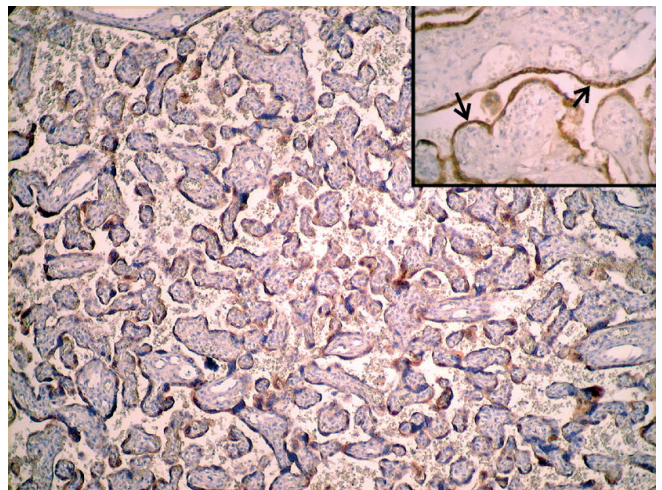


Fig. 1. Immunodetection of GPC3 in term human placenta. The inset shows a higher magnification of the same sample. The arrows point to the syncytiotrophoblast cells positive for GPC3. x 100 (inset, x 400).

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development. The acini and ducts showed high levels of GPC3 in two out of five of the embryos studied in the first period and all of the embryos studied in the second period, while the staining became less intense in the third period and disappeared by P4 (Fig. 2B-D). On the contrary, the endocrine pancreas was found to be always negative for GPC3 staining in the studied period (Fig. 2C).

In the developing intestine we only found high positive staining in the visceral smooth muscle at all the four periods in all the samples studied (Fig. 2E). Interestingly smooth muscle of body blood vessels and umbilical cord blood vessels was also positive for GPC3 in all samples studied (data not shown).

Noteworthy, in the gastric fundic glands, only parietal cells showed a strong positive staining for GPC3 in one of the three P2 fetuses studied and in all the cases of P3 and P4 (Fig. 2F).

The tongue expressed GPC3 in only one of the

embryos of the first period together with the palate and lips. Two of the three P2 fetuses and all P3 cases showed positive staining in the stratified epithelium and muscle of the tongue (Fig. 2G and 2H). The tongue at P4 was not studied.

GPC3 staining in the genitourinary and respiratory systems

GPC3 staining was detected at the urinary crest in P1 (data not shown). During P2 its expression was localized mostly at the cortex tubules and at the visceral and parietal epithelial cells of the Bowman's capsule of the glomeruli, all of them metanephric mesenchyme derivatives (Fig. 3A,B). Then, in late P2 and P3, GPC3 staining gradually disappeared from the renal corpuscles and only some proximal convoluted tubules remained positive in P4 (Fig. 3C,D).

In the genital system, we could observe that the

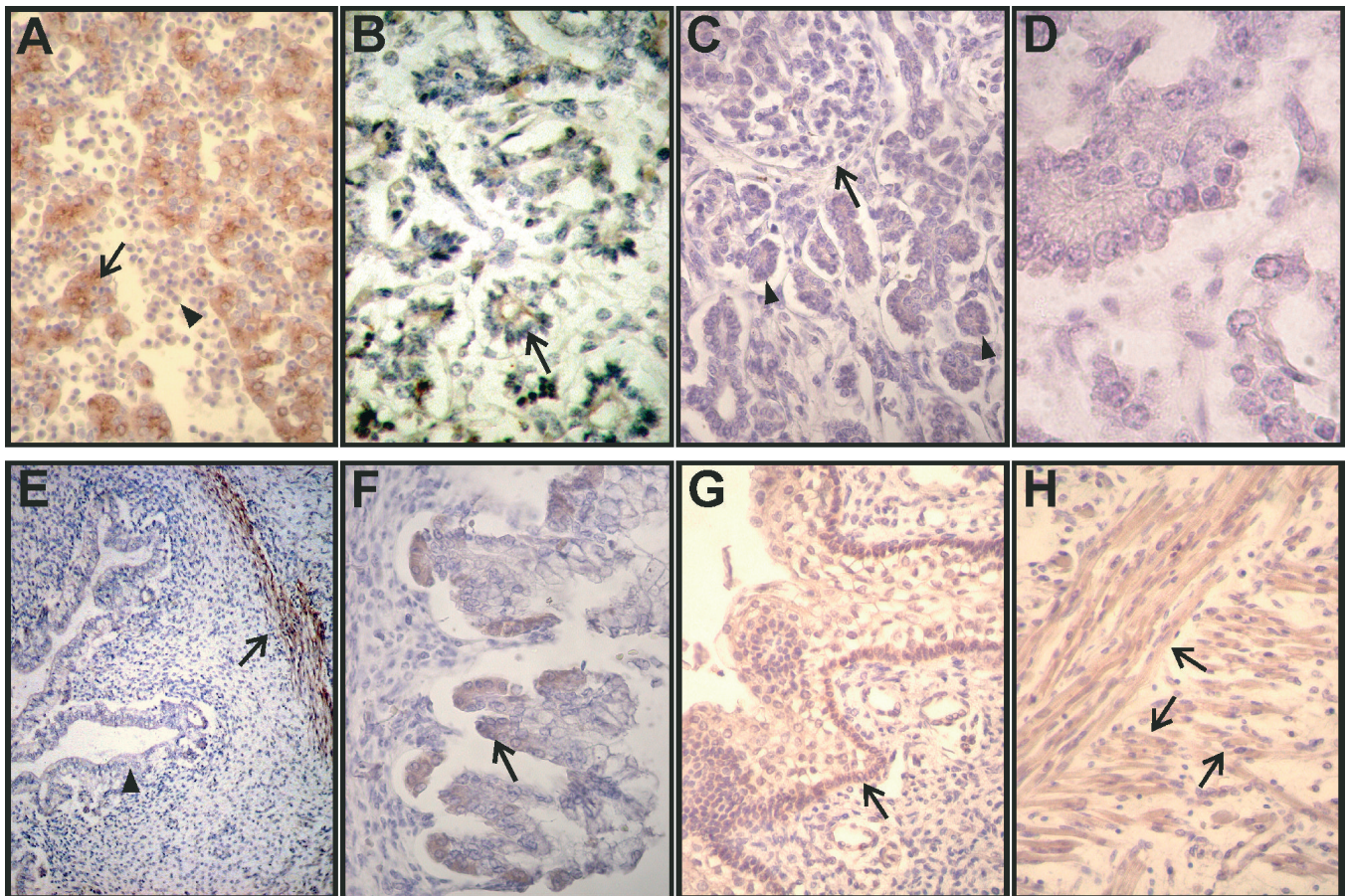


Fig. 2. GPC3 staining in the digestive tract and the annexed glands. **A.** Liver of a P1 embryo. The arrow points to the positive hepatocytes and the arrowhead points to the negative red blood cells still with nuclei. **B.** Pancreas of a P2 fetus. **C.** Pancreas of a P3 fetus. The arrow points to a negative Langerhans islet and the arrowhead points to a slightly positive acinus. **D.** Pancreas of a P4 fetus, negative for GPC3. **E.** Digestive tract of a P2 fetus. The arrow points to the positive smooth muscle and the arrowhead points to the negative epithelium. **F.** Stomach of a P3 fetus. The arrow is pointing to the positive parietal cells. **G.** Epithelium of the tongue of a P3 sample. The arrow is pointing to the positive basal cells. **H.** Striated muscle of the tongue of Figure 2G. The arrows point to positive muscle cells. A-C, F-H, x 200; D, x 400; E, x 100.

sexual cords preceding gonad development were poorly positive for GPC3 in the first period (three embryos analyzed) as well as in the second period (data not shown). In P3 male fetuses the seminiferous epithelium of the testis (Fig. 3E), and the epithelia of the epididymis, vas deferens and urethra (data not shown) were positive, while the ovary of the female fetus of the same group was negative (data not shown). Notably testis Leydig cells were positive in one of the samples of the second period (Fig. 3E)

In the respiratory system, we found that the trachea and all the respiratory epithelia were negative. On the contrary, lung mesenchyme showed a positive staining in three out of 4 lungs analysed in P1 and in 2 out of 3 samples in P2 (data not shown). Staining was gradually lost in the following weeks.

GPC3 staining in mesenchyme, skeletal tissue and nervous system

The mesenchyme of the limbs showed positive staining in all the embryos studied in the first period, when limb buds are developing (Fig. 4A). A detailed

analysis of the following periods revealed complete absence of GPC3 not only in the upper and lower limbs but also in the connective tissue, cartilage, striated muscle and skin of all the cases examined (Fig. 4B). The mesenchymal tissue localized between the most caudal vertebrae also had a similar pattern being only positive in P1 in all the embryos (Fig. 4C).

In the nervous system, the brain and the choroid plexi were negative along the whole period of study in all the samples with the exception of one P1 embryo (data not shown). In contrast, we found a strong and homogeneous GPC3 signal at the body of neurons and neuropilum of the spinal cord (Fig. 4D) and at the neurons of the sensory dorsal root ganglia in the second and third periods (Fig. 4E). The nervous system of P4 was not analysed.

Comparison between human and mice GPC3 expression patterns

In Table 1 a comparison between GPC3 expression along human (by IHC) and mouse (by ISH) development as well as the corresponding phenotypes in SGBS

Table 1. Glypican-3 expression patterns and phenotypes in humans and mice.

ORGAN	HUMAN STAINING (IHC)	MICE STAINING (ISH) (Pellegrini et al., 1998)	SGBS ABNORMALITIES (DeBaun et al., 2001)	GPC3 NULL MICE ABNORMALITIES (Cano-Gauci et al., 1999; Chiao et al., 2002)
Liver	Positive	Positive	Hepatomegaly	None
Pancreas	Positive (Exocrine in P1 and P2) Negative (Endocrine)	Not reported	Hyperplastic islets of Langerhans, hypoglycemia	Not reported
Digestive tract	Positive (smooth muscle and gastric parietal cells)	Positive (epithelium only at 8.5 days; submucosal layer)	Diastasis recti, omphalocele and hernias	Intestinal gas resulting in abdominal distension
Oral cavity	Positive (palate)	Positive (epithelium of oral cavity)	Cleft palate	None
Tongue	Positive (striated muscle, epithelium, connective tissue)	Positive (only connective tissue)	Macroglossia and midline groove	None
Gonads	Positive in male embryos (Leydig cells, seminiferous epithelium, urethra, deferent duct)	Positive	Cryptorchidism, small penis, risk for testicular gonadoblastoma	None
Kidney	Positive (cortex convoluted tubules and Bowman's capsule)	Positive (mesenchyme)	Renal dysplasia, nephromegaly and high risk of Wilm's tumors	Renal cysts and dysplasia
Respiratory system	Positive (only mesenchyme of the lungs in P1 and P2)	Positive (cartilage of trachea, larynx and major bronchi; mesenchyme of lungs)	Some of the patients develop pneumonia	Bacterial infections in respiratory tract
Limb buds	Positive mesenchyme in P1	Positive mesenchyme from 9.5 to 11.5 days post coitum. After, only in cartilage precursor of limb bones	Polydactyly, syndactyly	Minimal or no polydactyly
Vertebrae	Positive in P1 (mesenchyme between caudal vertebrae)	Positive (mesenchymal cells, cartilage and intervertebral discs)	Vertebral abnormalities	Some vertebral abnormalities
Nervous system	Positive (only spinal cord motoneurons and dorsal root ganglia neurons)	Negative	Hypotony, developmental delay, CNS malformations. High risk of neuroblastoma and medulloblastoma	None
Skin	Negative	Positive (dermis and hair follicles)	None	Pigmentation defects

IHC: Immunohistochemistry; ISH: In Situ Hybridization.

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patients and GPC3 null mice is outlined. While in general, GPC3 expression profile during human development is similar to that described in mice, several

differences were observed. Thus, we found expression of GPC3 in the gastric parietal cells, as well as a specific staining of visceral smooth muscle in the digestive tract,

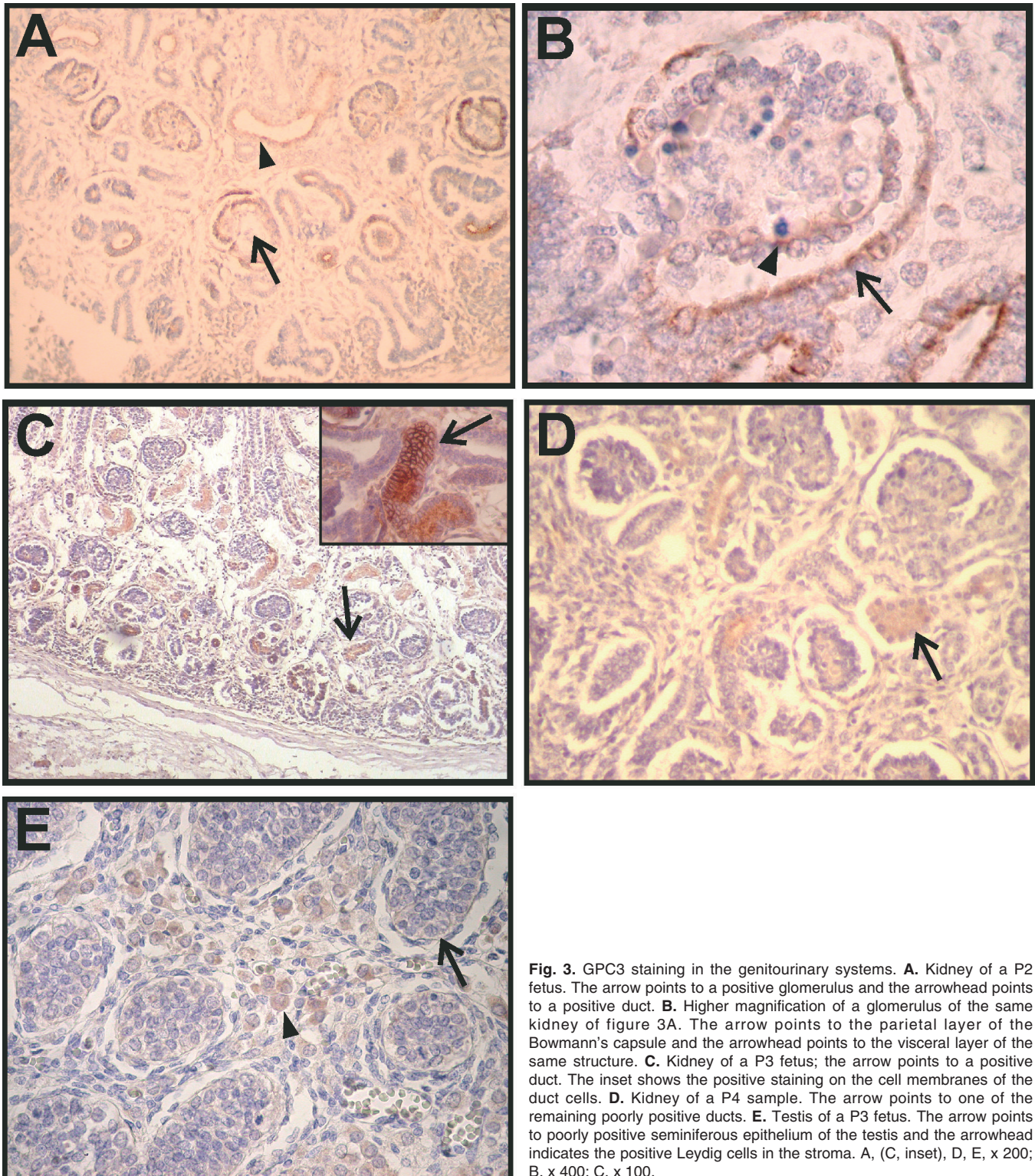


Fig. 3. GPC3 staining in the genitourinary systems. **A.** Kidney of a P2 fetus. The arrow points to a positive glomerulus and the arrowhead points to a positive duct. **B.** Higher magnification of a glomerulus of the same kidney of figure 3A. The arrow points to the parietal layer of the Bowman's capsule and the arrowhead points to the visceral layer of the same structure. **C.** Kidney of a P3 fetus; the arrow points to a positive duct. The inset shows the positive staining on the cell membranes of the duct cells. **D.** Kidney of a P4 sample. The arrow points to one of the remaining poorly positive ducts. **E.** Testis of a P3 fetus. The arrow points to poorly positive seminiferous epithelium of the testis and the arrowhead indicates the positive Leydig cells in the stroma. A, (C, inset), D, E, x 200; B, x 400; C, x 100.

that were not observed in mice. It is also important to mention the absence of staining in skin and in cartilage tissue, either at the skeletal precursors or in the trachea

and bronchi, of any of the human samples. Another remarkable difference was the specific GPC3 positivity found in neurons of the spinal cord and dorsal root

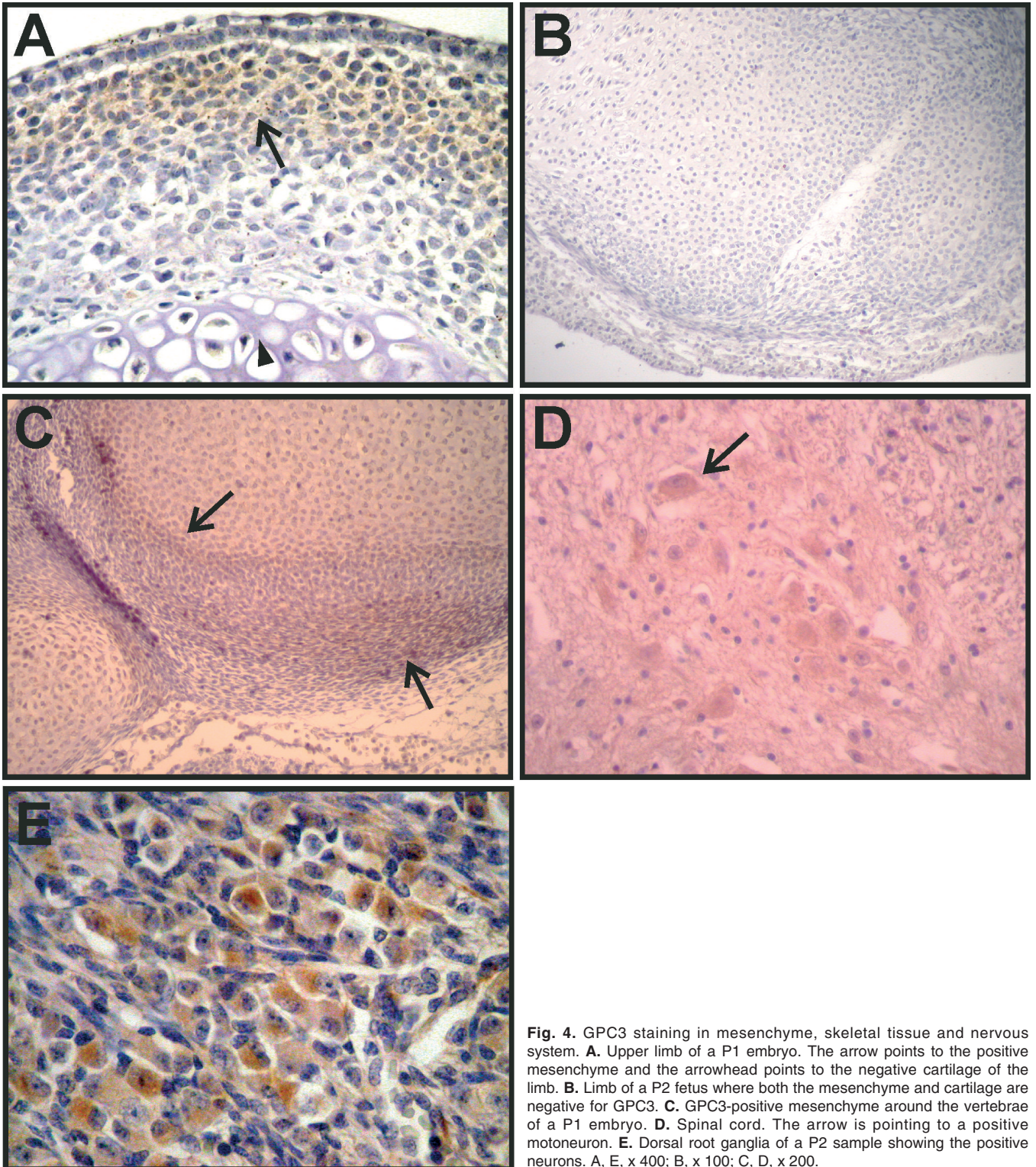


Fig. 4. GPC3 staining in mesenchyme, skeletal tissue and nervous system. **A.** Upper limb of a P1 embryo. The arrow points to the positive mesenchyme and the arrowhead points to the negative cartilage of the limb. **B.** Limb of a P2 fetus where both the mesenchyme and cartilage are negative for GPC3. **C.** GPC3-positive mesenchyme around the vertebrae of a P1 embryo. **D.** Spinal cord. The arrow is pointing to a positive motoneuron. **E.** Dorsal root ganglia of a P2 sample showing the positive neurons. A, E, x 400; B, x 100; C, D, x 200.

ganglia, not detected in mouse embryos.

Discussion

Mice carrying mutant alleles of GPC3 created by either targeted gene disruption or gene trapping (Cano-Gauci et al., 1999; Chiao et al., 2002) display many, but not all, of the abnormalities associated with SGBS, including renal cystic dysplasia, ventral wall defects, and some skeletal abnormalities. These findings are consistent with the pattern of GPC3 expression in the mouse embryo, detected in the kidney, gonads, mesenchymal cells, cartilage and intervertebral disks among other tissues (Pellegrini et al., 1998). However, studies of human embryos and fetuses to show the developmental differences in the expression of this proteoglycan respect to mouse model organisms are missing. Moreover, although some authors have studied GPC3 expression at mRNA level in some adult and fetal samples, there are almost no studies available that assess GPC3 expression at protein level in human tissues.

Our immunohistochemistry results in developing humans show a remarkable pattern of cell type as well as stage-specific GPC3 expression from the 5th to the 28th week of development. In addition, we observed that human placenta at term, in coincidence with Khan et al. (2001), displays an intense GPC3 staining of the syncytiotrophoblast cell layer and negative mesenchymal elements.

Interestingly we have found that several organs, such as brain, thymus, thyroid, salivary glands and spleen, do not express GPC3 during the embryonic and fetal periods studied. However, it has to be considered that GPC3 could be expressed in these tissues before the 5th week of gestation or after the 28th week.

We found that hepatocytes are stained for GPC3 at the cytoplasmic and membrane levels from P1 through P4 in all the cases examined. It should be noted, however, that GPC3 is absent in the adult liver (Pellegrini et al., 1998). Interestingly, GPC3 is expressed in hepatocellular carcinoma (HCC) cells, suggesting that it could be considered an oncofetal antigen in this pathology (Capurro et al., 2003; Kandil et al., 2007).

The endocrine pancreas, represented by the islets of Langerhans which were reported to be hyperplastic and increased in number in SGBS patients (DeBaun et al., 2001), was negative in the period analysed in this study. On the contrary the exocrine pancreas, which has not been reported to be affected in SGBS patients, presented a rather strong GPC3 staining during the first and second periods that gradually disappeared, being absent by the fourth period, concurrent with the fact that adult human pancreas does not express GPC3 mRNA (Pellegrini et al., 1998).

In contrast to the detection of GPC3 in endoderm and mesenchyme-derived cells from developing rat intestine (Li et al., 1997), in the human intestine we only found high positive staining in the visceral smooth muscle in all samples along the studied period. It

remains to be investigated whether this finding is related to some of the abdominal wall defects, like omphalocele and hernias, displayed by the SGBS patients (DeBaun et al., 2001). Interestingly, with the exception of glypican-2, all the other glypicans are also expressed by smooth muscle cells during development (Song and Filmus, 2002).

A very high expression of GPC3 was found in all tissues forming the tongue in the studied cases. Interestingly, macroglossia is one of the most common clinical features of SGBS patients (DeBaun et al., 2001). While several other craniofacial dysmorphisms are frequently associated with SGBS, in general we did not find specific GPC3 staining in any other craniofacial tissues, except the nose, lips and palate in one P1 embryo.

Regarding the stomach mucosa, we could observe staining of parietal cells in P2, P3 and P4. While other authors have reported that adult gastric mucous cells are positive for GPC3, we could not detect its expression in those cells during the studied period. Interestingly, and in contrast with the previously mentioned high expression of GPC3 in HCC, GPC3 expression is not detected in most gastric cancer samples (Zhu et al., 2002).

The kidney is an organ affected both in SGBS patients and in GPC3 null mice (Grisaru and Rosenblum, 2001; Chiao et al., 2002). It is known that the expression of GPC3 is critical for normal branching morphogenesis of the ureteric bud and normal organization of the mesenchymal-derived glomeruli and tubules (Grisaru et al., 2001; Chiao et al., 2002). We found that several mesenchymal-derived structures of the renal cortex expressed GPC3 in the P2 and P3 fetal periods but that this expression is gradually downregulated and only some proximal convoluted tubules remained positive in P4 fetuses. In agreement with our results, Pellegrini et al. (1998) have found GPC3 mRNA expression in the mouse metanephric mesenchyme (Pellegrini et al., 1998).

On the contrary, while several abnormalities in ureteric bud branching have been described in GPC3 null mice (Grisaru et al., 2001) and, as mentioned before, SGBS patients present dysplastic and cystic kidneys (Grisaru and Rosenblum, 2001), neither the ureteric bud nor its derivatives were stained for GPC3 in any of the samples analysed in the present study. These findings may contribute to the discussion regarding whether GPC3 functions in a non-cell autonomous manner, exerting its effects on distant tissues and cells as a secreted molecule (Grisaru and Rosenblum, 2001).

Interestingly, in addition to the fact that SGBS patients have a high risk for Wilms' kidney embryonal tumor, Saikali and Sinnett (2000) have identified GPC3 as a highly differentially expressed gene in all tested cases of Wilms' cell lines and tumors.

Regarding the genital system, we have found a stage and sex dependent expression of GPC3. For example, while the sexual cords were poorly positive during P1

and P2, P3 male fetuses presented staining of the seminiferous epithelium, Leydig cells, epididymus, vas deferens and urethral epithelium. Interestingly SGBS patients also appear to have an increased risk for cryptorchidism, hypospadias and small penis (DeBaun et al., 2001). In contrast, the ovary of the female fetus of the same group was negative.

Contrarily to observations made in mice (Pellegrini et al., 1998), we found a strong GPC3 signal in the cytoplasm of neurons and neuropilum of the spinal cord and the neurons of the sensory dorsal root ganglia in the second and third periods. This finding is relevant since SGBS patients may present with hypotony and have a high risk for developing neuroblastomas and medulloblastomas (Pellegrini et al., 1998).

While in general, the GPC3 expression profile during human development was similar to that described in mice, several specific differences in the nervous tissue, cartilage and skin were observed. Although we are comparing the expression pattern of GPC3 at the protein level in human tissues with the expression at mRNA level during mouse development (Pellegrini et al., 1998), some of the differences observed between our results shown here and the ones published by Pellegrini et al. (1998) may be due to differences in the techniques employed; others may be due to the differential expression of GPC3 between human and mouse and it is possible that these differences could explain some of the dissimilar characteristics between SGBS patients and GPC3 null mice (Cano-Gauci et al., 1999).

While the expression of GPC3 during human development suggests a possible role in the formation of the organs affected in SGBS, the precise mechanism of how GPC3 may mediate SGBS abnormalities remains unknown. Further, it would be interesting to compare the ontogeny of GPC3 with human tissues from SGBS patients.

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