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LEFTY2 expression and localization in rat oviduct during early pregnancy

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Summary

In mammals, fertilization and preimplantation embryo development occurs in the oviduct. Cross-talk between the developing embryos and the maternal reproductive tract has been described in such a way as to show that the embryos modulate the physiology and gene expression of the oviduct. Different studies have indicated that transforming growth factor beta (TGF-β) can modulate the oviductal microenvironment and act as an autocrine/paracrine factor on embryo development. LEFTY2, a novel member of the TGF-β superfamily is involved in the negative regulation of other cytokines in this family such as nodal, activin, BMPs, TGF-β1 and Vg1. In previous studies, we have reported that LEFTY2 is differentially expressed in the rat oviduct during pregnancy. In this study, we describe the temporal pattern of LEFTY2 in pregnant and non-pregnant rat oviduct by western blotting, which showed higher levels of LEFTY2 on day 4 of pregnancy, a time at which the embryos are ending their journey along the oviduct. The cellular location of LEFTY2 was assessed by immunohistochemistry, which showed immunolabelling in the cytoplasm and at the apical surface of the oviductal epithelial cells. The oviductal fluid also presented a 26 kDa band, which corresponds to the biologically active form of this protein, at the preimplantation period of pregnancy, indicating LEFTY2 secretion to the lumen. As LEFTY2 is expressed at a high level just before the embryos pass to the uterus, its biological effect might be relevant and significant for the preimplantation stage of embryo development in the oviduct. The fact that embryos do not express LEFTY2 at this stage of development supports this hypothesis.

Keywords: Embryo development, *Lefty2*, Oviduct, Pregnancy, TGF-β

Introduction

The oviduct provides an optimal environment for crucial events that occur during the early reproductive stage, leading to the establishment of pregnancy. These occurrences include transportation of gametes, fertilization and embryo early development. In fact, the development of preimplantation embryo occurs almost entirely inside the oviduct, where a wide range of growth factors, cytokines and their receptors are synthesized (Buhi *et al.*, 2000; Hardy & Spanos, 2002). Early embryos also express growth factors and their receptors, therefore the developing embryos find themselves in a rich growth-factors milieu (Hardy & Spanos, 2002; Osterlund & Fried, 2000). Transforming growth factor beta (TGF-β) superfamily members are closely associated with tissue remodelling and reproductive processes and are involved in maternal–embryo dialogue and in embryo development (Chang *et al.*, 2002; Wolf *et al.*, 2003). A novel member of the TGF-β family, LEFTY2, has been detected in uteri in humans and mice, and is involved in endometrial tissue remodelation during the sexual

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cycle and the embryo implantation process (Cornet *et al.*, 2002; Tang *et al.*, 2005a, b). Valdecantos *et al.* (2004) and Argañaraz *et al.* (2007) reported, for the first time, *lefty2* expression in the rat oviduct. We have also shown that *lefty2* transcripts were more abundant at 4 days after mating, when the embryos are completing their transit along the oviduct (Valdecantos *et al.*, 2004; Argañaraz *et al.*, 2007). *In silico* analyses of the rat *lefty2* sequence (AY758558) revealed that this gene encodes a 366 amino acid preproprotein with a predicted mass of 40.91 kDa and with two RXXR putative cleavage sites, which could lead to the secretion of two cleavage products: a 26 kDa (short processed) form and a 32 kDa (long processed) form (Argañaraz *et al.*, 2007). These data are in agreement with other studies that reported that the shorter processed form is biologically active and induces MAPK activation, while the 32 kDa form is inactive (Ulloa *et al.*, 2001). At present, it is known that the LEFTY2 biologically active forms require processing of their preproteins by members of the proprotein convertase (pc) family. The proprotein convertases are secretory proteolytic enzymes that activate precursor proteins into biologically active forms by limited proteolysis at one or multiple internal sites (Scamuffa *et al.*, 2006). In addition, there is evidence that the LEFTY2 preproprotein can be differentially processed depending on the cell of synthesis (Meno *et al.*, 1996). However, several questions related to *lefty2* remain unknown: i.e., how its transcriptional, posttranscriptional and posttranslational modifications affect protein secretion, maturation and movement within the extracellular milieu. Furthermore, no mechanism of action in other organs, besides the uterus, has been described. The abundance or scarcity of LEFTY2 protein in the oviduct during the early pregnancy and estrous cycle would be significant for understanding its role in early pregnancy or in embryo development. *lefty2* expression in the rat embryo at the morula stage was also studied to assess new evidence of the cross-talk between embryos and the maternal tract.

Material and methods

Animals

Wistar virgin 3-month-old adult female rats, weighing 200–300 g were used. Animals were housed individually under standard environmental conditions (12 h light:12 h dark cycle) with chow and water *ad libitum*.

Each phase of the estrous cycle was determined by vaginal smears (Marcondes *et al.*, 2002). Vaginal smears were checked daily and only rats that showed at least two consecutive 4-day estrous cycles were included.

To achieve pregnancies, the animals in proestrus were caged with adult males. The first day of pregnancy was designed by the presence of spermatozoa in the vaginal smears. Animals were sacrificed by carbon dioxide inhalation and oviductal samples were collected between 10 a.m. and 12 a.m. on days 2, 4 and 6 of pregnancy and during proestrus (PE), estrus (E) and diestrus (DE). In each case, oviducts were immediately removed, flushed with phosphate-buffered saline (PBS) and processed for SDS-PAGE and western blot analysis or immunohistochemistry. All assays were performed on single oviducts, and samples were never pooled.

Oviductal fluid collection and preparation

Oviductal fluids of rats on the fourth day of pregnancy were recovered by flushing both oviducts with a 32G blunted syringe and 100 μl of PBS. The fluid was centrifuged to eliminate cells and debris. The supernatants from the six oviducts were pooled and precipitated by addition of 20% cold trichloroacetic acid (TCA), pellets were dissolved and the protein contents of the samples were quantified using the Quant-iT protein assay kit (Invitrogen).

Oviductal protein isolation

Total proteins from flushed oviducts were isolated using a lysis buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P40, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. Both oviducts were homogenized in 250 μl ice-cold buffer by using an Ultraturrax homogenizer (1:10 w/vol.); the homogenates were centrifuged at 10,000 *g* for 10 min at 4°C and supernatants were collected for assays. Total proteins were quantified using the Quant-iT protein assay kit (Invitrogen).

Western blot analysis

The pooled oviductal fluid or 100 μg of oviductal protein, obtained as indicated before, was subjected to 12.5% SDS-PAGE. Then, proteins were transferred onto nitrocellulose membranes (Sigma-Aldrich) and run at 30 volts for 10 h at 4°C. The membranes were blocked in 2% albumin in PBS-T (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 0.1% Tween 20) for 8 h, then incubated overnight at 4°C with a goat polyclonal antibody against the LEFTY carboxyl terminal peptide, M-20 (1:100, goat anti-mouse, Santa Cruz Biotechnology) or rabbit anti-γ-tubulin (1:750, T3320, Sigma-Aldrich), followed by incubation with the secondary antibody for 1 h at 37°C (1:1000, biotinylated rabbit anti-goat

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IgG or biotinylated goat anti-rabbit IgG; Sigma-Aldrich). Finally, blots were incubated for 15 min with alkaline phosphatase-linked ExtrAvidin (1:50,000, Sigma-Aldrich) and protein bands were developed using the Sigma Fast kit (Sigma-Aldrich). Membrane digital images were obtained with an Olympus C5060 Wide Zoom colour digital camera (Olympus) and densitometry of band intensities was performed using ImageJ software (http://rsbweb.nih.gov/ij/), then the ratios of LEFTY2/γ-TUBULIN band intensities were calculated. Prestained molecular weight marker proteins were run on a separate lane to determine the molecular weights of the immunostained bands. Primary antibody or secondary antibody omitted from the staining reaction was used as the control.

Immunohistochemistry

Oviducts were fixed in 4% paraformaldehyde, dehydrated in ethanol and embedded in paraffin. Sections of 7-μm thickness were collected on Frosted HiFix slides (Inprot). Paraffin sections were deparaffinized and rehydrated. Non-specific sites were blocked for 6 h with 2% albumin in PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4) and then incubated with goat polyclonal antibody against the Lefty peptide, M-20 (1:50), overnight at 4°C. Afterwards, the slides were incubated with biotinylated anti-goat (1:1000) for 1 h at room temperature and detected with alkaline phosphatase conjugated ExtrAvidin (1:1000). Tissue sections were developed for 30 min in the dark using the Sigma Fast kit (Sigma-Aldrich). A minimum of five sections was examined for each oviduct sample. Representative tissue sections were photographed with an Olympus C5060 Wide Zoom colour digital camera (Olympus). Two types of assay controls were performed: (i) the primary antibody was omitted; and (ii) the antibody was neutralized with the immunogenic peptide, according to the manufacturer's recommendation. Briefly, the antibody was incubated with a five-fold excess of the peptide (20 ng/ml) in PBS overnight at 4°C. Sections were viewed, analysed and photographed under a light microscope without a counterstain.

Embryo collection and RT-PCR assays

Embryos at the morula stage were obtained by oviductal flushing from day-4 pregnant rats. The embryos were pooled and immediately processed for total RNA extraction with SV Total RNA Isolation System (Promega) and reverse transcribed using oligo-dT primers (Invitrogen) and M-MLV Reverse Transcriptase (Promega). *β-actin* expression was used as an internal RNA loading control for each sample. PCR was performed with specific primer pairs for *lefty2* (forward 5′-ACCATCGAGTGGCTGAGAG-3′ and reverse 5′-GATGGTCAGAGACTCTGGCA-3′), and primer pairs for the *β-actin* (forward 5′-CGTGGGCCGCCCTAGGCACCA-3′ and reverse 5′-TTGGCCTTAGGGTTCAGGGGGG-3′). The PCR conditions were: an initial denaturation time of 2 min at 94°C, followed by 30 cycles for 15 s at 94°C; for 35 s at 58°C; for 1 min at 72°C and an extension step of 7 min at 72°C. *lefty2* and *β-actin* RT-PCR products were run by 1.5% agarose gel electrophoresis, stained with SYBR Green, and then visualized by using a Gel Doc 1000 image analyzer (BioRad).

Statistical analysis

All experiments were performed with four different samples and repeated at least twice to confirm reproducibility of data. Density of bands and ratios of LEFTY2/γTUBULIN were pooled and analyzed by ANOVA followed by Student's *t*-test. Every experiment showed the same trend. *p*-values of less than 0.05 were considered to be significant.

Results

LEFTY2 protein expression in rat oviducts during estrous cycle and pregnancy

To ascertain that LEFTY2 is translated in the oviductal tissues, western blotting was carried out on extracted proteins from rat oviducts during proestrus, estrus, diestrus and early pregnancy (days 2, 4 and 6). LEFTY2 was identified by its reactivity with M-20, a commercially available polyclonal goat antibody raised against a synthetic LEFTY peptide. The antibody detects the LEFTY2 different isoforms: the preproprotein of 41 kDa, as well as the long processed form of 32 kDa and the short processed form of 26 kDa. Only two immunoreactive bands were detected in all oviductal samples: the preproprotein of 41 kDa and the 26 kDa short processed form, showing different intensities according to the physiological stage.

During the estrous cycle, the 41 kDa LEFTY2 preproprotein level remained unchanged, whereas the 26 kDa LEFTY2 short processed form decreased at proestrous, in relation to estrous and diestrous stages. In addition, the immunoreactivity of the 41 kDa band was markedly greater than 26 kDa bands in all stages of the estrous cycle (Fig. 1).

During early pregnancy, the 41 kDa LEFTY2 preproprotein level remained unchanged. The 26 kDa LEFTY2 short processed form intensity was higher at day 4 of pregnancy, increasing 3.86-fold respect on day 2 of gestation ($p = 0.0001$) and 1.94-fold respect on day 6 of gestation ($p = 0.0125$) (Fig. 2). Remarkably, on the second day of gestation the 41 kDa

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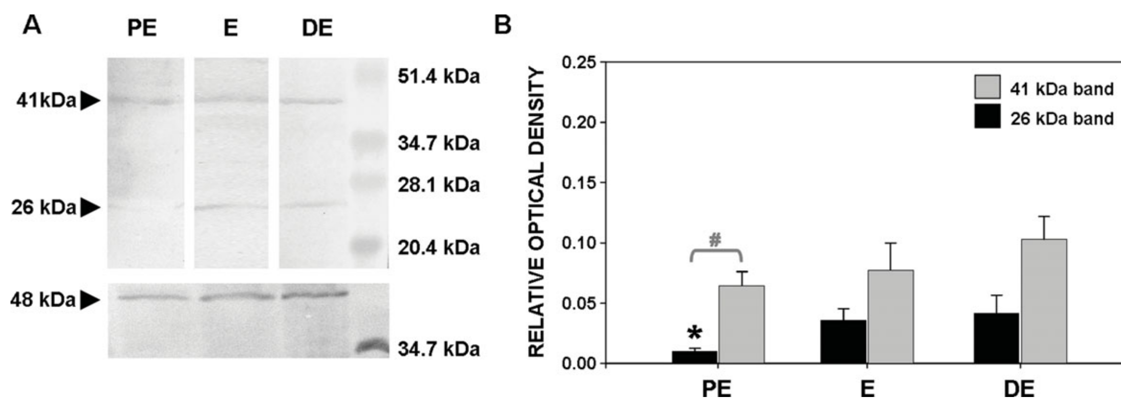

Figure 1 (*A*) Western blot analysis of LEFTY2 at estrous (E), diestrous (DE) and proestrous (PE) in oviductal homogenates by using polyclonal anti-human *lefty* M-20 antibody. Each sample was reprobed with antibody to γ-tubulin to ensure equal loading. (*B*) Plot of LEFTY2/γ-TUBULIN optical relative densities (arbitrary units) of the mature LEFTY2 (26 kDa band) and immature LEFTY2 (41 kDa band) during the estrous cycle. *Short processed form significant difference among the estrous cycle, $p \leq 0.02$. #Significant difference between the precursor and the short processed form, $p \leq 0.0005$.

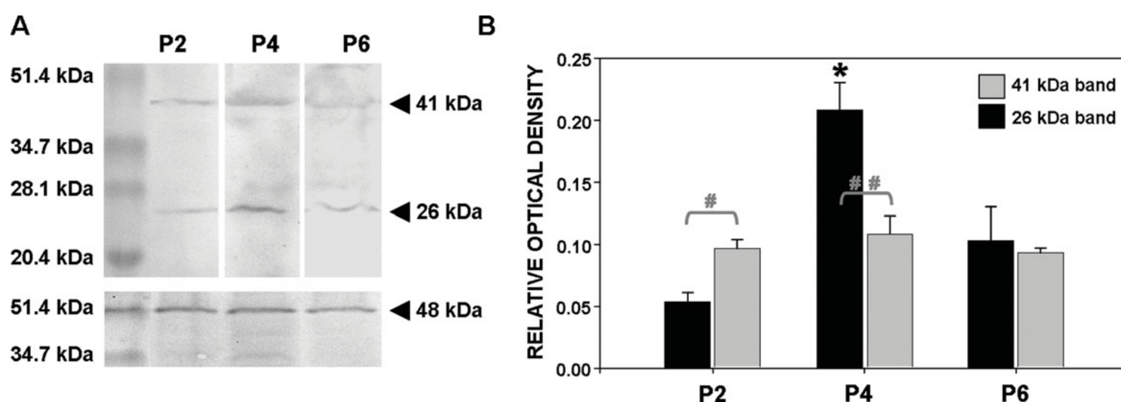

Figure 2 (*A*) Western blotting of LEFTY2 of pregnant rat oviduct 2, 4 and 6 days after mating by using polyclonal anti-human lefty M-20. Each sample was reprobed with antibody to γ-tubulin to ensure equal loading. (*B*) Plot of LEFTY2/γ-TUBULIN optical relative densities (arbitrary units) of the mature LEFTY2 (26 kDa band) and immature LEFTY2 (41 kDa) during pregnancy. *Short processed form significant difference among pregnancy, $p \leq 0.01$. Significant difference between the precursor and the short processed form, #$p \leq 0.025$; ##$p \leq 0.01$.

band intensity is higher than that of the 26 kDa band ($p = 0.024$); in contrast at day 4 of pregnancy, the 26 kDa short processed form was more abundant than the 41 kDa form ($p = 0.011$) (Fig. 2).

Immunolocalization of LEFTY2 in rat oviduct

Sections of rat oviductal tubes from the fourth day of pregnancy, when LEFTY2 protein levels were higher, were subjected to immunohistochemical staining. Sections incubated with the primary antibody showed positive staining in several different oviductal compartments (Fig. 3*B*). In the oviductal mucosa, staining was observed at the apical surface and in cytoplasm of epithelial cells. In this area, both ciliated and non-ciliated cells of the ampulla and isthmus showed no difference in intensity of immunostaining (data not shown) (Fig. 3*C*). Connective tissue located under the mucosa also exhibited positive immunoreactivity for LEFTY2, staining was observed in the cytoplasm of fibroblasts but not in their nuclei.

Sections either without lefty antibody or with lefty antibody absorbed with the immunogenic peptide showed no staining or showed slight staining in the apical surface of oviductal mucosa (Fig. 3*A*).

LEFTY2 in the oviductal fluid

To determine whether LEFTY2 produced in the oviduct was secreted into the lumen, the oviductal fluids of day-4 pregnant rats were subjected to western blot analysis. Only the 26 kDa immunoreactive band, corresponding to the short processed form of LEFTY2, was detected. Neither the 41 kDa precursor nor the 33 kDa long processed form was observed (Fig. 4).

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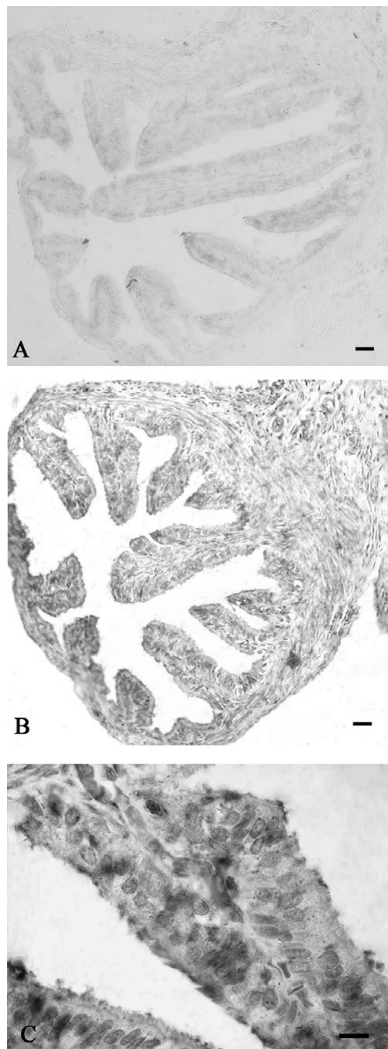

Figure 3 Immunohistochemical detection of LEFTY2 in pregnant rat oviduct on day 4 after mating revealed with alkaline phosphatase (see Material and methods for details). A representative section of ampulla is shown. (*A*) Negative control, sections were incubated without the polyclonal anti-human lefty M-20. Bar 100 µm. (*B*) Sections incubated with polyclonal anti-human lefty M-20. Bar 100 µm. (*C*) Positive staining in epithelial cells and mucosa apical surface. Bar 10 µm.

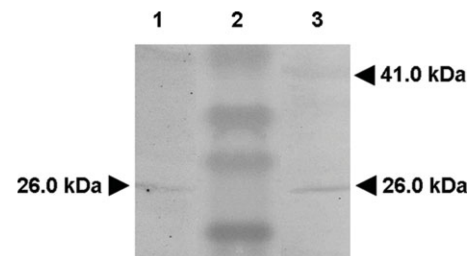

Figure 4 Western blot analysis of LEFTY2 in oviductal fluid on the fourth day of pregnancy using polyclonal anti-human lefty M-20. Lane 1, pooled oviductal fluid from pregnant rats 4 days after mating, lane 2, prestained protein marker (51.4, 34.7, 28.1 and 20.4 kDa), lane 3, positive control, day-4 pregnant oviduct.

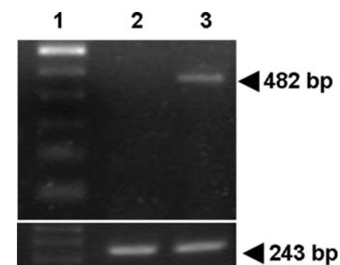

Figure 5 Lack of *lefty2* gene expression in preimplantation embryos at morula stage. Lane 1, 100 bp molecular weight marker, gene *β-actin* in the oviduct of pregnant rats (fourth day); lane 2, *lefty2* in preimplantation embryos; lane 3, positive control of *lefty2* in the oviduct of pregnant rats on the fourth day after mating. *β-actin* was amplified as loading control.

lefty2 expression in preimplantation embryos

The expression of *lefty2* in the Wistar rat embryo at the morula stage was examined by RT-PCR. *lefty2* expression was not detected at the fourth day of pregnancy, during the preimplantation period. Figure 5 shows a unique band corresponding to the predicted size of *β-actin*, the 484-bp product corresponding to *lefty2* was absent. As a positive control, a sample of oviductal RNA from day-4 pregnant rats was used; in this sample, both genes were amplified. These results showed that rat embryos did not express *lefty2* on the fourth day of pregnancy (Fig. 5).

Discussion

Understanding the molecules and mechanisms that are involved in the reproductive process has been of great interest for enhancement of *in vitro* production of embryos. The development of optimal human embryo culture conditions is critical for assisted reproduction programmes (Jones *et al.*, 1998), and also beneficial to the derivation of human embryonic stem cells (Mercader *et al.*, 2006). *In vitro* experiments suggest that oviductal cells secrete a variety of components that enhance embryo development (Yeung *et al.*, 2002) by influencing embryo gene expression and metabolism (Lee *et al.*, 2003, 2004; Knijn *et al.*, 2005). Different studies have found that TGF-βs signalling derived from the oviduct may act as an autocrine/paracrine factor for embryo development and for modulation of the microenvironment where this development takes place (Hardy & Spanos, 2002; Armant, 2005). Cornet *et al.* (2002) and Tang *et al.* (2005) demonstrated that LEFTY2 is expressed in the uteri of humans and mice, where it has been exclusively related to the endometrial remodelling and embryo implantation. For

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functional studies of LEFTY2 in the oviduct during the preimplantation embryonic stages, we have evaluated LEFTY2 temporal expression and localization in the oviductal cells. In this way, if LEFTY2 is secreted to the oviductal lumen, participation in embryonic development would be a possibility.

As for other members of the TGF-β superfamily, LEFTY2 is synthesized as a preproprotein and then cleaved at RXXR sites to release the mature form of the protein (Drummond & Findlay, 2006). By carrying out *in silico* analyses, we predicted that LEFTY2 preproprotein (40.91 kDa) exhibits at least two such RXXR sites, which are located at amino acid residues 74–77 and 132–135. If one of these sites was the cleavage site, a mature protein of 32.58 kDa (long processed form) or 25.81 kDa (short processed form) should be produced (Argañaraz *et al.*, 2007). Therefore, there must be endoproteolytic processing in order to release the LEFTY2 bioactive polypeptide.

In this study, western blot analysis of rat oviduct during estrous cycle and pregnancy revealed the presence of two bands; one of 41 kDa, corresponding to the preproprotein, and the mature 26 kDa form (short-processed form), whereas the 33 kDa (long processed form) mature protein was not detected. There is evidence that the preproprotein can be differentially processed depending on the cell of synthesis and that proteolytic cleavage may regulate not only the activity of LEFTY2 but also its specificity (Meno *et al.*, 1996). Recently, it was demonstrated that a soluble protein convertase, PC5A, is localized in the extracellular matrix and exerts its proteolytic action on the cell surface or in the extracellular matrix (Nour *et al.*, 2005). PC5A cleaves the preproprotein at the 74–77 amino acid residue site yielding the long processed form (33 kDa); meanwhile, cleavage at the 132–135 amino acid residue site may require an unknown PC that is expressed only in certain cell types (Sakuma *et al.*, 2002). Therefore, our results suggest that this unknown PC should exist in the rat oviduct and that it might be more abundant or/and active during early pregnancy.

During the estrous cycle, LEFTY2 protein was mainly present as an inactive preproprotein, whilst the short processed form was detected at low levels – suggesting that LEFTY has a low biological activity in the oviduct of non-pregnant rats.

We observed that LEFTY2 expression pattern changes throughout pregnancy. Interestingly, the LEFTY2 biologically active form (short processed form) increased 3.8-fold from day 2 to day 4 of pregnancy, a fact that coincides with the embryo final steps in the oviduct. When the embryos have reached the uterus at 6 days after mating, the biologically active form of LEFTY2 in the oviduct began to decrease (1.94-fold). These protein levels were almost consistent with our previous observations in which *lefty2* transcripts increased five-fold from day 2 to day 4 of pregnancy (Argañaraz *et al.*, 2007). These data might indicate that the function of LEFTY2 is restricted to the preimplantation stage, consistent with the final steps of the embryo in the oviduct on its way into the uterus. In agreement with this hypothesis, we found LEFTY2 biologically active polypeptide (26 kDa band) in the oviductal fluid, consistent with it being produced and secreted by oviductal cells to the embryo milieu. LEFTY secretion was previously reported by Meno *et al.* (1996), who detected LEFTY processed forms of 32 and 26 kDa in BALB/3T3 cell conditioned medium. Therefore they concluded that these cells could secrete these processed forms. Also, LEFTY2 has been described in endometrial fluid during the secretory phase, this finding would suggest a role in human reproduction (Tabibzadeh *et al.*, 2000).

Immunoreactive staining shows that LEFTY2 synthesis mostly takes place in the oviductal epithelial cells, mainly on the luminal surface. Human endometrial glands and adjacent stromal cells could synthesize LEFTY as was suggested by *in situ* hybridization assays. LEFTY2 plays a role in human reproduction, as it has been described in endometrial fluid during the secretory phase (Tabibzadeh *et al.*, 1998; 2000).

LEFTY2 participates in the negative modulation of TGF-β1 and BMP4 signalling by inhibition of R-Smad receptor phosphorylation and by providing a repressed state of responsive genes, such as metalloproteinases, collagen and the connective tissue growth factor (CTGF). However, the mechanism of action remains unknown (Ulloa *et al.*, 2001). Also, LEFTY blocks the effects of NODAL, another TGF-β, by binding to NODAL itself or to its co-receptor CRIPTO. In this way, LEFTY prevents NODAL binding to its receptor (Chen & Shen, 2002; Hamada *et al.*, 2003; Cheng *et al.*, 2004). In this way, it is possible that a LEFTY2 negative feedback response is likely to be involved during TGF-β and BMP signalling in preimplantation embryo development. Interestingly with LEFTY2 expression, one of its targets, TGF-β1, was detected in embryos and in non-pregnant and pregnant oviductal tissues (Zhao *et al.*, 1994; Fitzpatrick *et al.*, 2002; Strandell *et al.*, 2004; Refaat *et al.*, 2008). In fact, *in vitro* studies have demonstrated that growth factors, including TGF-β1, play an important role in oocyte maturation, embryogenesis, and early embryo development (Marquant-Le Guienne *et al.*, 1989, Larson *et al.*, 1992; Osterlund & Fried, 2000). In support of the concept that LEFTY regulates other TGF-β members, it had been demonstrated that TGF-β1, TGF-β2, TGF-β3 and their receptors were present in oviductal epithelial cells, and in human and mouse oviductal fluid (Zhao *et al.*, 1994; Chow *et al.*, 2001; Strandell *et al.*, 2004), as well as in preimplantation

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in the embryo (Hardy & Spanos, 2002; Chow *et al.*, 2001).

During the fourth day of the embryo journey through the oviductal tube, LEFTY2 secretion would be restricted to the oviduct. Our results demonstrate that preimplantation embryos at in the morula stage (fourth day after mating) did not express the lefty2 gene. In mice, the expression of *lefty2* is first detectable in 6-day embryos in the visceral endoderm of the distal region (Shen, 2007). In this context, the particular timing and location of LEFTY2 in the oviduct, four days after mating, might be important for the preimplantation period of pregnancy in rats.

To summarize, we demonstrated that LEFTY2 is present in the rat oviduct. The epithelial cells synthesize and secret LEFTY2 to the lumen. The preproprotein is more abundant during the estrous cycle, while the short processed form is higher in early pregnancy, specifically when the embryos are still in the oviduct. In addition, the day-4 embryo expressed *lefty2*. Taken together, our results, and the fact that LEFTY2 regulates TGF-β family members, suggest that Lefty2 signalling has a role during normal *in vivo* rat embryo preimplantation development in the oviduct. These findings extend the understanding of the LEFTY2 pathway in the reproductive process, demonstrating its participation in the oviduct of mammals throughout early pregnancy. Nevertheless, further studies are needed to determine Lefty2 function in the oviduct.

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References

Argañaraz, M.E., Valdecantos, P.A., Abate, C.M. & Miceli, D.C. (2007). Rat *Lefty2/EBAF* gene, isolation and expression analysis in the oviduct during the early pregnancy stage. *Genes Genet. Syst*. **82**, 171–5.

Armant, D.R. (2005). Blastocysts don't go it alone. Extrinsic signals fine-tune the intrinsic developmental program of trophoblast cells. *Dev. Biol*. **280**, 260–80.

Buhi, W.C., Alvarez, I.M. & Kouba, A.J. (2000). Secreted proteins of the oviduct. *Cells Tissues Organs* **166**, 165–79.

Chang, H., Brown, C.W. & Matzuk, M.M. (2002). Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr. Rev*. **23**, 787–823.

Chen, C. & Shen, M.M. (2002). Two modes by which Lefty proteins inhibit nodal signaling. *Curr. Biol*. **14**, 618–24.

Cheng, S.K., Olale, F., Brivanlou, A.H. & Schier, A.F. (2004). Lefty blocks a subset of TGFbeta signals by antagonizing EGF-CFC coreceptors. *PLoS Biol*. **2**, 215–26.

Chow, J.F., Lee, K.F., Chan, S.T. & Yeung, W.S. (2001). Quantification of transforming growth factor beta1 (TGFbeta1) mRNA expression in mouse preimplantation embryos and determination of TGFbeta receptor (type I and type II) expression in mouse embryos and reproductive tract. *Mol. Hum. Reprod*. **7**, 1047–56.

Cornet, P.B., Picquet, C., Lemoine, P., Osteen, K.G., Bruner-Tran, K.L., Tabibzadeh, S., Courtoy, P.J., Eeckhout, Y., Marbaix, E. & Henriet, P. (2002). Regulation and function of LEFTY-A/EBAF in the human endometrium. mRNA expression during the menstrual cycle, control by progesterone, and effect on matrix metalloproteinases. *J. Biol. Chem*. **277**, 42496–504.

Drummond, A. & Findlay, J. (2006). Focus on TGF-beta signalling. *Reproduction* **132**, 177–8.

Fitzpatrick, R., Casey, O.M., Morris, D., Smith, T., Powell, R. & Sreenan, J.M. (2002). Postmortem stability of RNA isolated from bovine reproductive tissues. *Biochim. Biophys. Acta* **1574**, 10–4.

Hamada, H., Meno, C., Watanabe, D. & Saijoh, Y. (2003). Establishment of vertebrate left–right asymmetry. *Nat. Rev. Genet.* **3**, 103–13.

Hardy, K. & Spanos, S. (2002). Growth factor expression and function in the human and mouse preimplantation embryo. *J. Endocrinol*. **17**, 221–36.

Jones, G.M., Trounson, A.O., Lolatgis, N. & Wood, C. (1998). Factors affecting the success of human blastocyst development and pregnancy following *in vitro* fertilization and embryo transfer. *Fertil. Steril*. **70**, 1022–9.

Knijn, H.M., Wrenzycki, C., Hendriksen, P.J., Vos, PL., Zeinstra, E.C., Van Der Weijden, G.C., Niemann, H. & Dieleman, S.J. (2005). *In vitro* and *in vivo* culture effects on mRNA expression of genes involved in metabolism and apoptosis in bovine embryos. *Reprod. Fertil. Dev*. **17**, 775–84.

Larson, R.C., Ignotz, G.G. & Currie, W.B. (1992). Transforming growth factor beta and basic fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle. *Mol. Reprod. Dev.* **33**, 432–5.

Lee, Y.L., Lee, K.F., Xu, J.S., He, Q.Y., Chiu, J.F., Lee, W.M., Luk, J.M. & Yeung, W.S.B. (2004). The embryotrophic activity of oviductal cell derived complement C3b and IC3b-a novel function of complement protein in reproduction. *J. Biol. Chem*. **279**, 12763–8.

Lee, Y.L., Lee, K.F., Xu, J.S., Kwok, K.L., Luk, J.M., Lee, W.M. & Yeung, W.S. (2003). Embryotrophic factor-3 from human oviductal cells affects the messenger RNA expression of mouse blastocyst. *Biol. Reprod*. **68**, 375–82.

Marcondes, F.K., Bianchi, F.J., Tanno, A.P. (2002). Determination of the estrous cycle phases of rats: some helpful considerations. *Braz. J. Biol.* **62**, 609–14.

Marquant-Le Guienne, B., Gérard, M., Solari, A. & Thibault, C. (1989). *In vitro* culture of bovine egg fertilized either *in vivo* or *in vitro*. *Reprod. Nutr. Dev*. **29**, 559–68.

Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y. & Hamada, H. (1996). Left–right asymmetric expression of the TGFβ family member lefty in mouse embryos. *Nature* **381**, 151–5.

https://doi.org/10.1017/S0967199410000602 Published online by Cambridge University Press

Mercader, A., Valbuena, D. & Simon, C. (2006). Human embryo culture. *Methods Enzymol*. **420**, 3–18.
Nour, N., Mayer, G., Mort, J.S., Salvas, A., Mbikay, M., Morrison, C.J., Overall, C.M. & Seidah, N.G. (2005). The cysteine-rich domain of the secreted proprotein convertases PC5A and PACE4 functions as a cell surface anchor and interacts with tissue inhibitors of metalloproteinases. *Mol. Biol. Cell* **16**, 5215–26
Osterlund, C. & Fried, G. (2000). TGFbeta receptor types I and II and the substrate proteins Smad 2 and 3 are present in human oocytes. *Mol. Hum. Reprod*. **6**, 498–503.
Refaat, B., Amer, S., Ola, B., Chapman, N. & Ledger, W. (2008). The expression of activin-betaA and -betaB subunits, follistatin, and activin type II receptors in fallopian tubes bearing an ectopic pregnancy. *J. Clin. Endocrinol. Metab*. **93**, 293–9.
Sakuma, R., Ohnishi, Yi.Y., Meno, C., Fujii, H., Juan, H., Takeuchi, J., Ogura, T., Li, E., Miyazono, K. & Hamada, H. (2002). Inhibition of Nodal signalling by Lefty mediated through interaction with common receptors and efficient diffusion.*Genes Cells* **7**, 401–12.
Scamuffa, N., Calvo, F., Chrétien, M., Seidah, N.G. & Khatib, A.M. (2006). *FASEB J.* **20**, 1954–63.
Shen, M.M. (2007). Nodal signaling, developmental roles and regulation. *Development* **134**, 1023–34.
Strandell, A., Thorburn, J. & Wallin, A. (2004). The presence of cytokines and growth factors in hydrosalpingeal fluid. *J. Assist. Reprod. Genet*. **21**, 241–7.
Tabibzadeh, S., Lessey, B. & Satyaswaroop, P.G. (1998). Temporal and site-specific expression of transforming growth factor-beta4 in human endometrium. *Mol. Hum. Reprod*. **4**, 595–602.
Tabibzadeh, S., Mason, J.M., Shea, W., Cai, Y., Murray, M.J. & Lessey, B. (2000). Dysregulated expression of EBAF a novel molecular defect in the endometria of patients with infertility. *J. Clin. Endocrinol. Metab*. **85**, 2526–36.
Tang, M., Mikhailik, A., Pauli, I., Giudice, L.C., Fazleabas, AT., Tulac, S., Carson, D.D., Kaufman, D.G., Barbier, C., Creemers, J.W. & Tabibzadeh, S. (2005a). Decidual differentiation of stromal cells promotes pc5/6 expression and lefty processing. *Endocrinology* **146**, 5313–20.
Tang, M., Xu, Y., Julian, J., Carson, D. & Tabibzadeh, S. (2005b. Lefty is expressed in mouse endometrium in estrous cycle and preimplantation period. *Hum. Reprod*. **20**, 872–80.
Ulloa, L., Creemers, J.W., Roy, S., Liu, S., Mason, J. & Tabibzadeh, S. (2001). Lefty proteins exhibit unique processing and activate the MAPK pathway. *J. Biol. Chem.* **276**, 21387–96.
Valdecantos, P.A., Argañaraz, M.E., Abate, C.M. & Miceli, DC. (2004). RNA fingerprinting using RAP-PCR identifies an EBAF homologue mRNA differentially expressed in rat oviduct. *Biocell* **28**, 287–97.
Wolf, E., Arnold, G.J., Bauersachs, S., Beier, H.M., Blum, H., Einspanier, R., Frohlich, T., Herrler, A., Hiendleder, S., Kölle, S., Prelle, K., Reichenbach, H.D., Stojkovic, M., Wenigerkind, H. & Sinowatz, F. (2003). Embryo–maternal communication in bovine-strategies for deciphering a complex cross-talk. *Reprod. Domest. Anim*. **38**, 276–89.
Yeung, W.S.B., Lee, K.F. & Xu, J.S. (2002). The oviduct and development of preimplantation embryo. *Reprod. Med. Rev.* **10**, 21–44.
Zhao, Y., Chegini, N. & Flanders, KC. (1994). Human fallopian tube expresses transforming growth factor (TGF beta) isoforms, TGF beta type I-III receptor messenger ribonucleic acid and protein, and contains [125I]TGF beta-binding sites. *J. Clin. Endocrinol. Metab*. **79**, 1177–84.

https://doi.org/10.1017/S0967199410000602 Published online by Cambridge University Press