Rat 

Lefty2/EBAF Gene: Isolation and Expression Analysis in the Oviduct During the Early Pregnancy Stage

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It has been demonstrated, by RNA Arbitrarily Primed Polymerase Chain Reaction (RAP-PCR), that the endometrial bleeding associated factor (ebaf or lefty2) is expressed in rat oviduct. In this work we isolated and sequenced the full-length lefty2 cDNA from Rattus norvegicus oviducts and described its expression level in this organ during the estrous cycle and early pregnancy stage. The coding deduced sequence (CDS) codifies a 40.91 kDa protein with a highly conserved TGF-β functional domain. RT-PCR semiquantitative analysis indicated that oviduct cells transcribe lefty2 among different stages of the estrous cycle with the maximum expression at diestrus phase. The highest expression of lefty2 was at the 4th day after mating (five folds respect to day one), just when the embryos have completed their transit through the oviduct. The lefty2 expression declined rapidly thereafter and the levels of their transcripts in the oviduct remained low until 7th days after mating.

Key words: Lefty2/Ebaf, oviduct, early pregnancy, expression

During early embryo development, the embryo undergoes cell divisions, apoptosis and differentiation, influenced by the components of the oviductal luminal secretion (Buhi et al., 2000). Different studies have shown that a wide range of cytokines, growth factors and their receptors are expressed in the reproductive tract during preimplantation development, providing evidence of endocrine, paracrine and autocrine pathways of regulation (Hardy and Spanos, 2002; Chow et al., 2001; Zhao et al., 1994). In a previous work we have isolated a differentially expressed cDNA fragment (Pr14) from pregnant R. norvegicus oviduct (Genbank accession N°: AF202268) which expression is restricted to the oviduct and uterus. The comparison of Pr14 sequence with the rat genomic database showed 100% of identity to the R. norvegicus chromosome 13 (Accession No. NW_047400). This fragment was 100% identical to a rat DNA sequence downstream terminal exon of a mRNA gene similar to lefty2. The fragment comprised the nucleotides 4,476 and 5,091 of the 3' untranslated region (UTR) (Valdecantos et al., 2004). Lefty2 is a transforming growth factor-beta (TGF-β) family member, cloned by Meno et al. (1996) from mouse embryos, that acts in the left-right axis determination. On the other hand, Kothapalli et al., (1997) isolated and cloned Ebaf cDNA from human endometrium and showed by northern blot assays its lack of expression in lung, kidney, ovary, liver, colon, stomach, breast, lymph node and spleen and fallopian tube. The Lefty2 protein has been implicated in the antagonic action over other members of the TGF-β family such as nodal, activin, bone morphogenetic proteins (BMPs), TGF-β1 and Vg1 (Branford and Yost, 2004). It has also been reported that Lefty2 stimulates the production of matrix metalloproteinases (MMPs) in human (Cornet et al., 2002) and mouse uterus (Tang et al., 2005a) during the sexual cycle and pregnancy showing that the progesterone controls both the expression of lefty2 and its effect on these MMPs.

It is known that the oviductal epithelium synthesize growth factors and MMPs (Gabler et al., 2001) and that lefty2 is specifically expressed in rat oviduct and uterus but not in other organs (Valdecantos et al., 2004). The objectives of this work were to isolate, characterize the lefty2 full-length cDNA and study its expression pattern in the rat oviduct during the estrous cycle and early pregnancy stage for a better understanding of its action.

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mechanism during reproduction. Virgin female *Rattus norvegicus* (Wistar) weighing 200-250 g were used. Vaginal smears were checked daily, and only rats that showed at least two consecutive 4-day estrous cycles were included. In order to obtain the pregnant rats, animals in proestrus were mated with fertile males of the same strain. Day 1 of pregnancy was defined as the one in which a vaginal plug and sperm in the vaginal smear were found in paired animals, the following morning. After mating, steroids hormones measurements were made at the time of sample collection (data not shown). Female animals were killed between 10 and 11 am, oviducts were colleted and stored at –80°C.

Total RNA samples from early pregnant rat oviducts were obtained with SV Total RNA Isolation System (Promega, Madison, WI) and reverse transcribed using oligo dT and M-MLV Reverse Transcriptase (Promega). Table 1 shows the primers sequences which were designed according to the *R. norvegicus* chromosome 13 DNA sequence (Genbank accession No. NW_047400) harboring the rat *lefty2* gene CDS and the differentially expressed cDNA fragment (Pr14). Amplification reactions were carried out in an automated thermal cycler (Perkin-Elmer, model 2400). PCR conditions consisted of an initial denaturation time of 2 min at 94°C followed by 30 cycles at 94°C for 30 sec; 60°C for 35 sec; 72°C for 1 min and an extension step of 10 min at 72°C. A single RT-PCR product of 1,384 bp was isolated and sequenced (Fig. 1A). Sequencing reactions were performed in a DNA ABI 373 A Genetic Analyzer. The novel rat cDNA sequence was submitted to the National Center for Biotechnology Information database under the accession number AY758558. The BLAST search program analysis revealed that the RT-PCR amplified sequence is identical to the exons predicted sequences of the rat *lefty2* gene locus and to the selected Pr14 partial sequence (indicated

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<th>Table 1. Primers employed. The NCBI accession No; the primers sequence; the position and the size of the product amplified are shown</th>
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<td><strong>Sequence denomination</strong></td>
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<td><em>Rattus norvegicus</em> chromosome 13 genomic contig</td>
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<td><em>Rattus norvegicus</em> cDNA clone Pr14</td>
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<td><em>Rattus norvegicus</em> EBAF precursor, mRNA</td>
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<td><em>Rattus norvegicus</em> beta actin, mRNA</td>
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Fig. 1. A. Agarose 1% gel electrophoresis: 1) MWM: 400 bp Molecular weight marker (PB-L, Productos Bio-Lógicos) and 2) RT-PCR *lefty2* fragment of pregnant rat oviducts B. Rat *lefty2* mRNA nucleotide sequence, GenBank accession number: AY758558. The gene consists of 4 exons as well as in other mammalian species. The highlighted sequence encodes the conserved functional domains; the initiation and termination codons are boxed.
Isolation and oviductal expression of rat ebaf/lefty2 cDNA during early pregnancy in Table 1). Computer analysis of the nucleotide sequence revealed a 1,101 bp open reading frame flanked by a 64 bp 5' UTR and a 219 bp 3' UTR (Fig. 1B). It has 93% identity with mouse lefty2 (GenBank Accession No: BC066224) and 82% with human lefty2 (GenBank Accession No: BC035718). The rat lefty2 isolated sequence encodes a 366 amino acids protein with a predicted mass of 40.91 kDa that contains a 21 amino acid signal peptide, a 186 amino acid TGF-β propeptide domain and a 90 amino acid TGF-β domain. Two putative proteolytic cleavage sites, characteristics of the TGF-β family, at amino acids 74–77 and 132–135 that could be involved in releasing the mature protein are present. Six conserved cysteines at amino acid residues 263; 293; 297; 316; 350 and 353, known as cysteines knot, are present; they form intrachain disulfide bonds that stabilize the protein, are also essential for ligand binding. Sequence comparisons of rat Lefty2 protein inferred from the isolated cDNA

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Fig. 2. Multiple alignment of lefty2 complete deducted amino acid sequence from rat, mouse, human, dog and cow. Alignment sequences were performed using the Multalin software (http://prodes.toulouse.inra.fr/multalin/multalin.html). Numbers represent the position of the amino acid in the respective peptide sequence. Only different amino acids are indicated. The putative conserved domains are upper-lined: signal peptide as a continuous line; the latency associated peptide (LAP) domain as a dash-line and the TGF-β domain as a dotted line. The conserved cysteines are highlighted; the putative enzymatic processing domains (RXXR) are boxed. Gaps are represented with broken lines.
(GenBank Accession No:AY758558) revealed a 92% identity and 95% similarity to the Mus musculus (GenBank Accession No: AAH66224.1) and 80% and 89% respectively to the Homo sapiens (GenBank Accession No: AAH35718.1) Lefty2 proteins. Sequence comparisons were also performed between lefty2 genomic deduced amino acid sequences of Canis familiaris (GenBank Accession XP_547508) showing an identity of 71% and a similarity of 76% and Bos taurus (GenBank Accession XP_613627) with an identity of 84% and a similarity of 85%. We noticed that the automated computational predicted sequence of C. familiaris Lefty2, deposited in the GenBank database, has 43 aa above a putative signal peptide sequence (Fig. 2). These extra aa were not present in the other sequences analyzed and did not correspond to any conserved domain.

The lefty2 expression pattern has shown that this gene is expressed in the rat oviduct and uterus but not in other organs. Assays to demonstrate that its expression may be regulated by ovarian steroids were carried out by semi-quantitative RT-PCR using total RNA obtained from both oviducts of each rat, during estrous cycle (Proestrus, Estrus, Diestrus) and early pregnancy (the seven consecutively days after mating). Three different animals for each stage were used. 0.5 ug of total RNA were reverse transcribed using oligo dT and M-MLV Reverse Transcriptase (Promega). Based on the rat lefty2 cloned cDNA sequence reported in this study, forward and reverse primers were designed to yield a 484 bp RT-PCR product (see Table 1). β-actin expression was used as an internal RNA loading control for each sample and as a means of detecting contaminating genomic DNA. In these experiments both pairs of primers were designed to span an intron in order to ensure that the amplified products were cDNA rather than contaminating genomic DNA. No amplified genomic DNA product was observed in any of the RT-PCR assayed samples. The PCR conditions were: an initial denaturation time of 2 min at 94°C, followed by 30 cycles at 94°C for 15 sec; 58°C for 35 sec; 72°C for 1 min and an extension step of 7 min at 72°C. The linear ranges of amplification for lefty2 and β-actin products were determined by performing RT-PCR for each one with reaction of 10, 15, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, and 40 cycles. In order to ensure working in the linear amplification range and to avoid individual variability reaction, each sample was amplified separately in 6 individual PCR reactions with the lefty2 specific primers for 21, 22, 23, 24, 25, and 26 cycles, and in 6 individual PCR reactions with the β-actin specific primers for 18, 19, 20, 21, 22, and 23 cycles. Each PCR assay was carried out at least twice. Lefty2 and β-actin RT-PCR products were combined and run in 1.5% agarose gel electrophoresis, stained with ethidium bromide and then visualized using a Gel Doc 1000 image analyzer (BioRad). The lefty2/β-actin ratios were determined for each sample using the band intensities values obtained by the software Molecular Analyst 1.4.1 (BioRad) and compared using t-test; statistically significant differences were considered when p

![Fig. 3. A. Relative levels of lefty2 mRNA in rat oviduct during the estrous cycle. Proestrus: PE; Estrus: E and Diestrus: DE and early pregnancy from day one: P1 to day 7: P7. B. Lefty2 and β-actin products were amplified by semi-quantitative RT-PCR, run in 1.5% (w/v) agarose gel electrophoresis and stained with ethidium bromide. Bands intensities were analyzed by Gel Doc 1000 Image Analyzer (BioRad). Then the ratio lefty2 mRNA/β-actin mRNA was determined for each sample (n = 3). Means and SE are shown. * P < 0.05.](image-url)
The results show the presence of \textit{lefty2} transcripts among the different stages of the estrous cycle: proestrus, estrus and diestrus (second day after ovulation) (Fig 3). The concentration of \textit{lefty2} mRNA in the oviduct throughout the estrous cycle showed an increase on the day of diestrus, before the oocytes began to pass to the uterus, reaching its lowest value on the day of proestrus, about 12 h before ovulation. However these changes did not follow the variations in neither plasmatic progesterone nor estradiol levels. According to Fuentealba et al. (1987), the oviduct may be responding to an increase in estradiol bioavailability not detected by RIA in the peripheral circulation.

The mRNA \textit{lefty2} concentration during the first 7 days of pregnancy showed a clear peak on day 4. This short-lived rise in \textit{lefty2} expression takes place during the period of embryo passage to the uterus. It is known that in pregnant rats, embryos were found in the uterus not earlier than 21:00 h on day 4 (Fuentealba et al., 1988). From days 4–7 of pregnancy, \textit{lefty2} concentrations decreased and showed no statistically significant difference in their expression level. The mechanism by which the pregnancy may up-regulate \textit{lefty2} expression in the oviduct is not known yet. In the endometrium, \textit{Lefty2} is involved in the extracellular matrix reorganization during the menstrual stage and decidualization (Tang M. et al., 2005 b). It is known that \textit{Lefty2} induces the expression of matrix metalloproteinases such as streptomelysin-1 and matrixiysin-1 proteins in human endometrial explants and the collagenolytic activity in transfected cells (Ulloa L. et al., 2001). If \textit{lefty2} is involved in some remodeling process undergoing by the oviduct MMPs activations remains to be studied (Cornet et al., 2002).

Recent studies on oocyte–oviduct and embryo-oviduct interactions suggest their regulation by growth factors (Hardy and Sapos, 2002). TGF-β1, detected in embryos and reproductive tract, is a multifunctional polypeptide cytokine that influences numerous physiological processes. \textit{Lefty2} participate in negative modulation of TGF-β1 and BMP signaling by inhibition of phosphorylation of the receptor of Smad (R-Smad) and by providing a repressed state of responsive genes to TGF-β (Ulloa et al., 2001). In this way it is possible that \textit{Lefty2} negative feedback response could be involved during TGF-β and BMP signaling in the last steps of the development of pre-implantation embryos.

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REFERENCES


