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Molecular and morphologic analyses of expression of ESX1L in different stages of human placental development

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Abstract

The mRNA expression of the ESX1L gene was analyzed by RT-PCR and *in situ* hybridization in human normal cytogenetically placentas, of different gestational ages. Our RT-PCR analysis showed that ESX1L mRNA is expressed from 5 weeks of gestation until term, suggesting a role not only in trophoblast differentiation but also in the maintenance of the villi and microvasculature. We also observed, by *in situ* hybridization, that ESX1L mRNA is expressed by cytotrophoblast from chorionic plate, syncytiotrophoblast and stromal cells of all terminal, intermediate and stem villi of term placentas. ESX1L mRNA expression was more pronounced in trophoblast cells of terminal villi than in intermediate and stem villi. In conclusion, ESX1L is expressed during all stages of placental development and is localized to sparse areas of trophoblast in terminal villi in association with cytotrophoblastic cells.

Keywords: placenta • ESX1L • transcriptional factor • *in situ* hybridization

Introduction

The placenta is a feto-maternal organ that possesses two components; the larger foetal portion formed by the chorionic villi, and a smaller maternal portion, derived from the endometrium or decidua. The chorionic villi are composed of both foetal and maternal structures. The chorion is derived from the cyto and syncytiotrophoblast initially, as the pregnancy advances, the secondary villi are formed with the presence of a core of mesoderm, and at the end of the third week, the tertiary villi are formed, composed of an external layer of syncytiotrophoblast, the adjacent, cytotrophoblast, and a conjunctive tissue with foetal vessels. At the 16th week vasculosyncytial membranes are seen in the villi [1].

The regulation of gene expression is fundamental for normal morphogenesis of the placenta in mammals [2]. Currently, various genes which are important for placental morphogenesis are known. In mice, two of such genes are Esx1, a homeobox-type transcription factor that during embryogenesis has its expression restricted to extra embryonic tis-

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sues, including the endoderm of the visceral yolk sac, the ectoderm of the chorion, and subsequently the labyrinthine trophoblast of the chorioallantoic placenta [3] and the Id-2 (inhibitor of DNA binding 2), expressed by villous cytotrophoblasts [4]. Esx1 was identified as an essential X-chromosome-imprinted regulator of placental development that influences foetal growth [5]. The Esx1 protein contains several notable features that are not often associated with homeoproteins, including an atypical homeodomain of the paired-like class, a proline-rich region that contains an SH3 binding motif, and a novel repeat region consisting of prolines alternating with phenylalanines or asparagines - PF/PN motif. Moreover, the Esx1 protein is expressed in the labyrinth layer of the mouse placenta *in vivo*, where its subcellular localization is primarily in the cytoplasm [6]. In humans, the ESX1L gene that is orthologous to Esx1, presents 4 exons and 3 introns, is located on chromosome X (Xq21.33-q22) and encodes a transcription factor that presents a DNA binding homeodomain. The expression of ESX1L is restricted to the human placenta and testicle [7]. Recently, ESX1L was found to be equally expressed in intrauterine growth restriction and normal placentas in the presence of both random and skewed X chromosome inactivation [8]

In order to provide evidence that ESX1L is expressed during all stages of human placental tissue we analyzed the mRNA expression by reverse transcriptase polymerase chain reaction (RT-PCR) and confirmed that it is localized in the syncytiotrophoblast and also in the villi stroma by *in situ* hybridization of term placentas.

Materials and Methods

The fragments of 39 placentas were collected at two counselling service at Rio de Janeiro, Brazil (Vivicel and Clinisul) from different foetal stages. Gestational age of the patients was estimated based on the date of the last normal menstrual period (LNMP). Placental tissue samples, (> 31 weeks), were obtained from a total of five normal pregnancies at the Military Police Central Hospital. The study protocol (859) was approved by the UERJ Ethics Committee and informed consent was obtained from the mothers.

RNA extraction

A fragment of approximately 1cm^3 was removed from the each placenta (n=44) and placed in TRIZOL (Invitrogen, Carlsbad, CA, USA) for molecular analysis. Total RNA was extracted following the manufacturer's recommendations and quantified on a spectrophotometer. The integrity of the RNA was checked by electrophoresis on a formaldehyde agarose gel.

ESX1L Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Complementary DNA (cDNA) was synthesized from total RNA (2µg) by reverse transcription (RT) using random hexamers and MMLV-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 1 hour at 37º C. Following the RT reaction, 5 µL of the cDNA was used for PCR with an *in situ* hot start procedure [9]. The oligonucleotide primers used for the amplification of the *ESX1L* gene were: 5' GCG TTC ACG CAG TTT CAG 3' (Exon2) and 5' CTG ATT TCG TTT CCA CTT 3'(Exon4). The reactions were performed on a thermal cycler using the following program: (95ºC for 5 min, 35 cycles of 95ºC for 30 s, 55ºC for 1 min, 72ºC 1 min). The 162 bp amplified products, were visualized in a 2.5% agarose gel stained with ethidium bromide, and compared to a molecular weight marker.

ESX1L mRNA *in situ* hybridization

The normal term placentas were fixed in 4% paraformaldehyde for 14 hours, paraplast embedded, and sections of 4 µm were used for *in situ* hybridization and for routine hematoxylin-eosin staining. For in situ hybridization, a 710 bp fragment of the ESXlL gene provided by Dr. Behringer (Department of Molecular Genetic, University of Texas, M.D. Anderson Cancer Center) was used. *In situ* hybridization was performed on the sections following the removal of the paraplast using standard methods [10]. The digoxigenin labelled probe had 710 bp and corresponded to the 3' portion of the ESX1L gene. Both antisense and sense -specific digoxigenin-labelled riboprobes were made by *in vitro* transcription and diluted in hybridization buffer. Hybridization took place overnight at 65ºC and, afterwords, the sections were washed with 50% formamide/ 1X SSC/ 0.1% Tween 20 for 15 min at 65º C and with

1X MABT (MAB- maleic acid 0,05 M/ 0,5M NaCl- pH 7,5 and 1%Tween 20) at room temperature. The slides were blocked with MABT/ 10% goat serum and incubated at room temperature for 1.5 hours. The detection was performed with an anti-digoxigenin antibody (1:2000) at room temperature overnight, after which the slides were washed 3 times with MABT for 30 min each. The slides were washed with NTMT (0.1M NaCl/ 0.06M Tris pH 9.5/ 0.03M $MgCl_2$/ 1% Tween 20) for 5 min. Visualization was carried out using Fast Red Naphtol (Sigma) for 72 hours after which the slides were counterstained with Light green and Mayer's hematoxylin and mounted with aqueous medium. As a negative control the sense probe was used with the same *in situ* hybridization protocol.

ESX1L mRNA expression morphometry in placental villi

Twenty five fields (of 1 slide for sample) were randomly selected and digitalized using a microscope (model BX40, Olympus) attached with a camera (Optronics, USA). Each image was projected on a screen monitor and the following parameters were evaluated using point count: number of positive trophoblastic cells, number of positive stromal cells, number of villi (terminal intermediate and stem), and a number of signal [11]. The terminal villi were characterized by presenting a continuous layer of syncytiotrophoblast, a discontinuous layer of cytotrophoblast, dilated capillaries and vasculosyncycial membranes [1]. All evaluations for semi-quantitative analysis were performed by blinded observers.

Statistic evaluation

The data are presented as mean +/- standard error. The Mann-Whitney non-parametric test for different samples was used to comparison of means, and chi-square was used to compare results from semi-quantitative analysis [12].

Results

RT-PCR analysis showed that ESX1L mRNA was expressed in different gestational stages of normal placentas, from 5 weeks up until term (Fig. 1). ESX1L mRNA expression was detected, by non radioactive *in situ* hybridization, in term placentas. Trophoblastic cells, both cytotrophoblast and syncytiotrophoblast, expressing ESX1L mRNA were sparsely distributed in villous and in the extra-villous tissue (Fig. 2C). The syncytiotrophoblast expressing cells were close to cytotrophoblast cells. In these locations we also detected the expression by endothelial cells and in cells from syncytiotrophoblast of the syncytial knots (Fig. 2A and D). Moreover, other villi cells exhibiting their nuclei with a fusiform shape or with irregular nuclei, seeming to be fibroblasts or macrophage cells, respectively, were less stained (Fig. 2B).

The quantification of the expression of ESX1L mRNA in terminal, intermediate and stem villi showed that trophoblastic cells showed the highest expression. Both syncytiotrophoblast and cytotrophoblast cells had a similar level of expression and differences were detected among terminal and intermediate or stem villi (Table 1).

Discussion

In mice, heterozygous females with mutated Esx1 inherited from their mothers or hemyzigous mutant males were born 20% smaller and the labyrinthine layer was defective with oedematous, larger and heavier placentas as early as 13.5 dpc [5]. A decreased expression of the X-chromosomal Esx1 gene was detected in abnormally enlarged placentas of mouse interspecif hybrids [13]. Moreover, further investigation of gene expression with interspecies hybridization, cloning by nuclear transfer, and mutation of the Esx1 gene revealed that genes whose expression is altered in abnormal placental growth are likely to be important in the occurrence of placentopathies [14]. On the other hand, ESX1L was equally expressed in 13 intra uterine growth restriction and normal placentas, and shows the same methylation pattern in the presence of both random and skewed X chromosome inactivation. Although it has been demonstrated that ESX1L was not imprinted in human third-trimester placentas and there was no parent-of-origin effect of chromosome X associated with placental insufficiency [8], our findings suggest that the ESX1L gene may be an important tran-

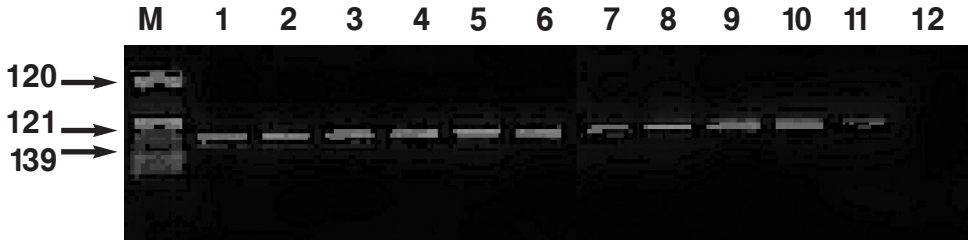

Fig. 1 RT-PCR analysis of ESX1L mRNA expression with 162pb. M- molecular weight markers, 1-placenta at 5 weeks, 2- placenta at 6 weeks, 3- placenta at 7 weeks, 4- placenta at 8 weeks, 5- placenta at 9 weeks, 6- placenta 10 weeks, 7- placenta at 12 weeks, 8- placenta at 16 weeks, 9- placenta t 20 weeks, 10- placenta at 24 weeks, 11- placenta at 39 weeks, C negative control.

scriptional factor to the placental vascular bed during normal development.

During pregnancy, several and complex structural transformations occur in the placenta. However, the entire process requires a coordinated control of gene expression [2, 15, 16]. This work shows that ESX1L mRNA is expressed in different stages of placental development. Our results are in accordance with previous results that show ESX1L mRNA expression in human term placentas [7] but, by RT-PCR, we observed that human placentas expressed this gene from five weeks to term. In comparison to some genes expressed only in certain stages of the development of the trophoblastic cells, such as HAND1 [17], we suggest that this gene could be important for the differentiation and maintenance of the syncytiotrophoblast phenotype, since it is detected both in cyto and syncytiotrophoblast cells in term placentas. Moreover, our results confirmed the detection of ESXL1 in normal term placentas by [8].The spotty distribution of ESX1L mRNA expression in cyto and syncytiotrophoblast cells in all kinds of villi could be related to the incorporation of new cells to the syncytiotrophoblast. If we compare with the percent of

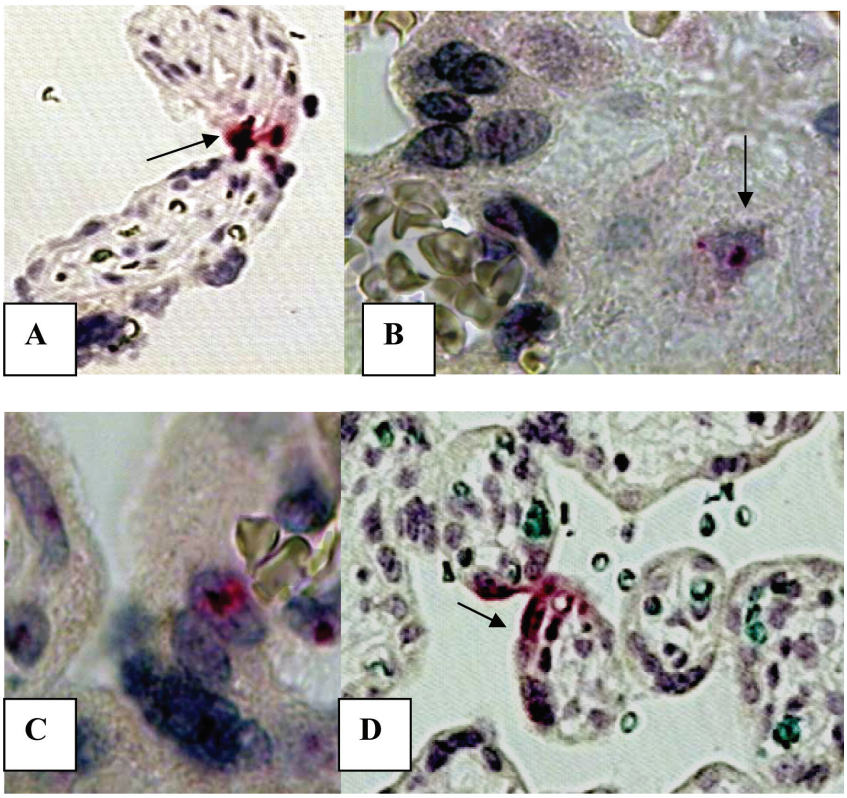

Fig. 2 *In situ* hybridization to localize ESX1L mRNA expression. **A.**Syncytial knot with labelling to ESX1L m RNA. (arrow) **B**. Nucleus of a stromal cell (arrow) showing a positive labelling. 20x. **C.** A syncytiotrophoblast cell with a positive labelling **D**.Terminal villi with labelling in syncytiotrophoblastic cells

Table 1 Percent volume of ESX1L mRNA expression in placental villi.

	Terminal a (n=23)	Intermediate (n=53)	Stem (n=49)	Total
Syncyciotrophoblast	0.122 ± 0.015	0.052 ± 0.005	0.065 ± 0.007	0.071
Cytotrophoblast	0.108 ± 0.017	0.042 ± 0.001	0.063 ± 0.004	0.080
Stroma	0.009 ± 0.002	0.023 ± 0.004	0.025 ± 0.004	0.019

a - Non parametric Tukey Test. Difference significant between terminal and intermediate and between terminal and stem villi ($p < 0.01$). Values area mean ± standart error. Number of points of ESX1L mRNA expression / Total number of points in non superposable fields of 5 normal term placentas.

total villous volume [20], the percent of trophoblast cells expressing ESX1L represents less than 50% of the total trophoblast cell population. The decreased ESX1L mRNA expression in stem and intermediate villi compared to terminal villi may be related to the formation of new villi or to specific structures in the villous. ESX1L mRNA expression in endothelial cells in vessels in the proximity of the ESX1L expressing trophoblast was also noted. Perhaps, the role of this gene in vasculogenesis should be further investigated. In the placenta of mice, Esx1 knock-out caused a significant modification in blood vessel organization with irregular laminin deposition [5]. We also observed by *in situ* hybridization that stromal cells express ESX1L mRNA. As important interactions between the endothelial, perivascular and trophoblastic cells are necessary for the normal development of the vasculogenesis in the placenta both in the villous and extra-villous tissue [17-20], the differential expression of ESXL1 in these cells could be related to the regulation of other proteins found during angiogenesis and vasculogenesis in the placental villi. In conclusion, ESX1L is expressed during all stages of placental development and sparse areas of trophoblast in terminal villi in association with cytotrophoblastic cells exhibited ESX1L mRNA expression detected by *in situ* hybridization.

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