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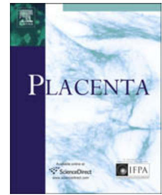

Localisation and Expression of FoxO1 Proteins in Human Gestational Tissues

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ABSTRACT

In non-gestational tissues, emerging data indicate that the FoxO1 family of Forkhead transcription factors play diverse roles in many cellular processes coordinating programs of gene expression that regulate apoptosis, oxidative stress resistance, and immune cell homeostasis. Successful outcome of human parturition rely on many of these processes, however there is no data available on FoxO1 proteins in human intrauterine tissues, nor their role in pregnancy complications such as pre-eclampsia. Thus the aim of this study was (i) to characterises the localisation and expression of FoxO1, acetylated (ac)-FoxO1 and phosphorylated (p)-FoxO1 in human placenta and fetal membranes obtained from term Caesarean sections ($n = 5$); and (ii) to compare the expression of FoxO1 proteins in term placental samples from normal and pre-eclamptic pregnancies ($n = 5$ per group). In placenta, weak FoxO1 staining was localised to the syncytiotrophoblast layer, whereas ac-FoxO1 and p-FoxO1 staining was mainly localised in the syncytiotrophoblasts and cytotrophoblasts. In fetal membranes, FoxO1, ac-FoxO1 and p-FoxO1 were localised to the trophoblast layer of the chorion, amnion epithelium and decidual cells. Quantitative RT-PCR (qRT-PCR) analysis showed a 6-fold and 12-fold higher mRNA expression in the choriodecidua compared to placenta and amnion, respectively. In both amnion and choriodecidua, FoxO1 protein expression was higher in the cytoplasmic fractions than in the nuclear fractions. On the otherhand, ac-FoxO1 and p-FoxO1 protein expression was higher in the nuclear fractions for all three tissues. There was no difference in the mRNA or protein expression of FoxO1 proteins in placental samples from normal and pre-eclamptic term pregnancies. The exact role of FoxO1 proteins in human pregnancy are unknown, however the finding that they are expressed in human gestational tissues warrants further research into their function in these tissues.

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1. Introduction

The forkhead superfamily of transcription factors which comprises more than 100 members in humans are classified from FoxA to FoxS on the basis of sequence similarity, and are characterised by a conserved 100 amino-acid DNA-binding domain designated the 'Forkhead box' [1]. FoxO proteins (also known as Fox 'Other' proteins) contain a unique insert of five amino acids within the region of the DNA-binding domain (α-helix 3) that is directly involved in sequence-specific interaction with DNA binding sites [1,2]. In mammals, there are four FoxO genes, FoxO1, 3, 4 and 6. FoxO1 is the most widely studied of all FoxO proteins, and as such is expressed in a variety of tissues [3], including being the predominant isoform in normal cycling human endometrium [4–6]. There is however no data available about the expression of FoxO1 proteins in human gestational tissues.

In vitro and *in vivo* studies have shown that FoxO1 proteins control the regulation of many genes involved in fundamental cellular processes, including cell cycle regulation, cell death, modulation of inflammation, metabolism, protection from oxidative stress and survival [7–12]. In addition, several genetic models have been also been used to explore the function of FoxO1 proteins *in vivo* [13,14]. Mouse models have demonstrated that in the developing embryo, FoxO1 plays a critical role in establishing normal vasculature. The FoxO1-null (FoxO$^{-/-}$) state results in an embryonic lethal phenotype, whereby death takes place on embryonic day 10.5 as a consequence of incomplete vascular development in the yolk sacs. In addition, FoxO1$^{-/-}$ embryos are approximately 50% the size of their FoxO1$^{+/+}$ littermates.

FoxO1 proteins are tightly regulated, with the multiple levels of FoxO1 control including phosphorylation, acetylation, deacytylation and proteolytic degradation. These post-translational modifications

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control the translocation between the nucleus-cytoplasm, transcriptional activity and protein–protein interactions with other transcription factors and co-activator or co-repressor proteins [12–18]. Depending on the type of stimuli, phosphorylation of Thr-24, Ser-256 and Ser-319 results in nuclear/cytoplasmic shuttling of FoxO1. For example, in response to growth and survival factors, through phosphorylation by a variety of kinases, FoxO1 translocates from the nucleus to the cytosol, resulting in the inactivation of its transcriptional activity [19,20]. In contrast, in response to stress stimuli, FoxO1 is phosphorylated by the mitogen activated protein kinase (MAPK) family member, jun N-terminal kinase (JNK), resulting in nuclear translocation and thus activation of its function as a transcription factor resulting in increased cellular protection against oxidative stress [21]. Nuclear import also results in acetylation of FoxO1 proteins with histone acetyltransferase (HAT) activity containing proteins, such as calcium response element-binding (CREB)-binding protein (CBP) and/or p300 [22]. Acetylation of FoxO1 exerts inhibitory effects on its transactivation activity, possibly by reducing its DNA binding activity and/or via enhancement of its phosphorylation [17]. The end result is a shift in the function of FoxO1 from cell-cycle arrest and protection against oxidative stress towards cell death.

To date, there is nothing known about the expression, localisation and function of FoxO1 proteins in human gestational tissues. This study describes the use of immunohistochemistry, quantitative real-time PCR (qRT-PCR) and Western blotting to determine the cellular distribution, mRNA and protein expression of FoxO1, acetylated (ac)-FoxO1, and phosphorylated (p)-FoxO1 in human placenta, amnion and choriodecidua from normal term pregnancies. Further, in order to ascertain whether FoxO1 proteins play a role in pregnancy complications, the expression of FoxO1 was also studied in placentas obtained from pre-eclamptic patients.

2. Materials and methods

2.1. Tissue collection and preparation

Human placentae and attached fetal membranes ($n = 5$) were obtained (with Institutional Research and Ethics Committee approval) from women who delivered healthy, singleton infants at term ($\geq$37 weeks gestation) undergoing elective caesarean section (indications for caesarean section were breech presentation and/or previous caesarean section). Tissues were obtained within ten minutes of delivery and dissected fragments were placed in ice-cold PBS. A placental lobule (cotyledon) was removed from the central region of the placenta. The basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross-section. Placental tissue was blunt dissected to remove visible connective tissue and calcium deposits. Amnion and choriodecidua was obtained 2 cm away from the peri-placental edge. Tissue was collected and stored at -80 °C until assayed for FoxO1, ac-FoxO1 and p-FoxO expression (i) by Western blotting, and (ii) quantitative RT-PCR (qRT-PCR) as detailed below. Tissues (placenta and fetal membrane tissue rolls with amnion innermost) were also placed in embedding cassettes (Techno-Plas, SA, Australia) and fixed in buffered formaldehyde solution (4%) and embedded in paraffin for FoxO1, ac-FoxO1 and p-FoxO analysis by immunohistochemistry.

Pre-eclamptic patients were selected for study after demonstrating symptoms of pre-eclampsia as defined by the Council of the Australasian Society for the Study of Hypertension in Pregnancy (blood pressure greater than 140 mm Hg systolic or 90 mm Hg diastolic; or a rise of $>$30 mm Hg or $>$15 mm Hg above initial systolic and diastolic pressures, respectively and proteinuria: defined as $>$0.3 g of protein secreted/24 h). All pre-eclamptic patients delivered vaginally at term ($\geq$37 weeks gestation) ($n = 5$). Gestationally age-matched placental samples were also collected from non-hypertensive pregnancies who also delivered vaginally at term ($n = 5$) and had no significant past medical history, current illness or used regular medication. Placental samples were prepared as detailed above and snap frozen in liquid nitrogen, and stored at -80 °C until analysis.

2.2. Immunohistochemistry

Localisation of FoxO1, ac-FoxO1 and p-FoxO1 in human gestational tissues was performed by immunohistochemistry. Serial sections of placenta, amnion and choriodecidua (4 μm thick), were cut and mounted on sections onto superfrost plus slides. Sections were deparaffinised followed by an antigen retrieval step (boiled in 10 mM citrate buffer, pH 6.0 for 10 min followed by 20 min incubation). Endogenous peroxidase activity was removed using 3% H_2O_2 in methanol for 10 min. Sections were transferred to tris-buffered saline (TBS; 20 mM Tris pH 7.6, 150 mM NaCl). The sections were incubated in a humidity chamber for 1 h in antibody diluted in 1% bovine serum albumin (BSA) in TBS. The primary antibodies, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), were rabbit polyclonal anti-FoxO1 (sc-11350) at 1/50 dilution, rabbit polyclonal anti-ac-FoxO1 (sc-49437) at 1/100 dilution, and rabbit polyclonal anti-p-FoxO1 ser 256 (sc-101681) at 1/100. After incubation the binding sites were labelled with Dako Envision + polymer linked secondary reagent and visualized using Dako DAB+ (Dakocytomation). Nuclei were counterstained with Mayer's haematoxylin and the sections were dehydrated and coverslipped using a resinous mounting agent. Positive controls, which were composite slides with tonsil, breast tumour and ovarian tumour, were included in each run. Negative control slides, where primary antibody was replaced with normal rabbit IgG serum were also included.

2.3. Western blotting

Assessment of FoxO1, ac-FoxO1 and p-FoxO1 protein expression was analysed by Western blotting. Rabbit polyclonal anti-FoxO1 (sc-11350), rabbit polyclonal anti-ac-FoxO1 (sc-49437), rabbit polyclonal anti-p-FoxO1 ser 256 (sc-101681) and horseradish peroxidase conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Forty micrograms of cytoplasmic and nuclear protein [prepared as detailed in [23]] was separated on 10.5–14% Criterion polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene fluoride (PVDF) as previously described [24]. The membrane was blocked with blocking buffer (5% BSA in TBST) for 1 h at room temperature and then probed overnight at 4 °C with primary antibody at 1/250 dilution (0.8 μg/ml) in blocking buffer. After incubation with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1/5000 (20 ng/ml) at room temperature for 30 min, chemiluminescent signal was detected and captured using a ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA). To normalise target protein levels against a loading control, all membranes were stripped and re-probed with rabbit polyclonal anti-β-actin antibody. Protein expression was identified by comparison with the mobility of protein standard. Quantitative analysis of the peak optical density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad Laboratories, Hercules, CA).

2.4. RNA extraction and real-time PCR

Total RNA was extracted from approximately 100 mg of tissue. Extraction was performed using Tri Reagent according to manufacturer's instructions (Sigma–Aldrich, Saint Louis, Missouri). RNA concentrations were quantified using a spectrophotometer (Smart Spec, Bio-Rad). RNA quality and integrity was determined via the A_{260}/A_{280} ratio and agarose gels electrophoresis. 1 μg of RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacture's instructions. 1 μl of cDNA was used to perform RT-PCR using Sensimix Plus SYBR green (Quantace, Alexandria, NSW) and 100 nM of FoxO1 primer (Hs_FOXO1_1_SG QuantiTect Primer Assay (QT000442347); Qiagen, Germantown, Maryland, USA). The specificity of the product was assessed from melting curve analysis. RNA without reverse transcriptase during cDNA synthesis as well as PCR reactions using water instead of template showed no amplification. A positive control sample was also used in each run on each plate.

Following baseline correction, the fluorescence threshold level was set during the geometric (exponential) phase of PCR amplification to generate the threshold cycle (C_T) value for each amplification curve. Average gene C_T values were normalised to the average 18S rRNA C_T values of the same cDNA sample. Variations in gene expression were calculated by the comparative C_T method that compares test samples to a calibrator sample. This method uses results obtained for a uniformly expressed control gene to correct for differences in the amount of RNA present in the two samples being compared in order to generate a ΔC_T value. The 18S gene was used as the control gene, the placental tissues were the test samples and the amnion and choriodecidua tissues were the calibrator. Fold differences were determined using the $2^{-\Delta\Delta C_T}$ method [25].

2.5. Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, Maryland, USA). Differences between the groups were analysed by a one-way ANOVA. Tukey-HSD correction was used to discriminate among the means between the groups. Statistical significance was ascribed to p-value <0.05. Data was expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Immunohistochemical localisation of FoxO1, ac-FoxO1 and p-FoxO1 expression in term placenta

Immunohistochemistry was used to determine the cellular locatisation of FoxO1, ac-FoxO1 and p-FoxO1 in human placenta,

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