Placenta 31 (2010) 1043–1050

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Expression and Localisation of FoxO3 and FoxO4 in Human Placenta and Fetal Membranes

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ARTICLE INFO

Article history:
Accepted 15 September 2010

Keywords:
Placenta
Fetal membranes
FoxO3
FoxO4
Fetal membrane rupture

ABSTRACT

Forkhead box O (FoxO) proteins regulate inflammation, extracellular matrix (ECM) remodelling and apoptosis. We have previously identified FoxO1 proteins in human gestational tissues, and demonstrated a link between FoxO1 and rupture of fetal membranes. There is, however, no data available on the expression and localisation of FoxO3 and FoxO4 in human intrauterine tissues. Thus the aim of this study was to characterise the localisation and expression of FoxO3 and FoxO4 in (i) human placenta and fetal membranes before term spontaneous labour onset, and (ii) supracervical site (SCS) and distal site (DS) fetal membranes from non-labouring women. Immunohistochemistry, Western blotting and quantitative RT-PCR (qRT-PCR) was used to localise and quantitate FoxO3 and FoxO4 protein and mRNA expressions. Cytoplasmic and nuclear FoxO3 was localised in the syncytiotrophoblast layer, chorionic trophoblasts, amnion epithelium and decidua. Cytoplasmic FoxO4 was localised in the syncytiotrophoblasts and chorionic trophoblasts. No or very little FoxO4 protein and mRNA was present in amnion epithelium. The intensity and extent of staining of FoxO3 and FoxO4 was greater in fetal membranes obtained from the SCS compared to DS. Presence of FoxO3 and FoxO4 are expected to contribute to apoptosis and/or cell cycle regulation associated with fetal membrane rupture.

1. Introduction

In humans, the FoxO subfamily of Forkhead transcription factors consists of FoxO1a (FKHR), FoxO3a (FKHRL1), FoxO4 (AFX) and FoxO6. FoxO1, FoxO3, and FoxO4 are three functionally related members, which are orthologs of the *Caenorhabditis elegans* transcription factor DAF-16 [1]. These transcription factors are important physiological targets of phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) signalling. Activation of PI3K/PKB phosphorylates FoxO4 at three different Ser/Thr residues [2–5], which results in nuclear export and cytoplasmic sequestration effectively inhibiting its transcriptional function. Nuclear localisation of FoxO proteins suspends cell cycle progression [6], promotes apoptosis [7], and negatively regulates angiogenesis [8].

FoxO3 has been causally linked to multiple cellular processes, which are activated during human parturition [9–11]. FoxO3 induces matrix metalloproteinase (MMP)- 3, 9 and 13 expression and activity in cancer cells [12] and decreases tissue inhibitor of MMP (TIMP)-1 in human umbilical vein endothelial cells [13]. It has also been shown to increase resistance to oxidative stress by upregulation of antioxidants including mitochondrial superoxide dismutase (MnSOD) [14], peroxisomal catalase [15], and peroxiredoxins [16].

Although there is no data available on FoxO4 in human parturition, in non-gestational tissues, FoxO4 is regulated by and regulates pro-labour mediators. Pro-inflammatory tumour necrosis factor (TNF)α has been shown to activate the c-Jun N-terminal kinases (JNK) stress signalling pathway and promote nuclear import and activation of FoxO4 [17–19] resulting in enhancement of MMP-9 gene transcription and enzymatic activity [18]. In podocytes, exposure to advanced glycation endproducts (AGEs) is associated with FoxO4 transcriptional activation, leading to increased expression of effector proteins of apoptosis [20]. Inflammatory cytokines and the transcriptional activity of nuclear factor kappa B (NF-κB) are up-regulated in FoxO4 knockout mice [21].

We have previously profiled the expression of FoxO1 proteins in human gestational tissues [22], and shown increased expression of acetylated (ac)-FoxO1 in fetal membranes overlying the cervix [23],

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doi:10.1016/j.placenta.2010.09.009

which may be involved in the increased apoptosis and extracellular matrix (ECM) degradation associated with this site [11,24–28]. There is however, no data available on the expression of FoxO3 and FoxO4 in human placenta and fetal membranes. Thus, the aims of this study are to (i) characterise the localisation, gene and protein expression of FoxO3 and FoxO4 in human placenta, amnion and choriodecidua from normal term pregnancies; and (ii) elucidate the effect of supracervical (SC) apposition on FoxO3 and FoxO4 expression in human fetal membranes in the absence of labour.

2. Materials and methods

2.1. Sample collection

The Research Ethics Committee of Mercy Health and Aged Care approved this study. Written, informed consent was obtained from participating women. Human placenta and attached fetal membranes were obtained (with Institutional Research and Ethics Committee approval) from women who delivered healthy, singleton infants at term (≥37 weeks gestation) before labour undergoing elective Caesarean section (indications for Caesarean section were breech presentation and/or previous Caesarean section). 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. In samples from non-labouring women, amnion and choriodecidua were obtained from the SC site (SCS) and a distal site (DS). Identification of the SCS was performed as we have previously detailed [23,27,29]. Briefly, prior to commencement of the Caesarean section, a swab soaked in Bonney's Blue dye (1:1 mixture of brilliant green (0.5%) and crystal violet (0.5%) dissolved in 90% ethanol) is introduced through the cervix onto the chorion lying above the internal os of the cervix. Upon delivery of the placenta, a blue mark is obvious on the membrane where the dye had been applied. The fetal membranes were washed briefly in PBS and three membrane samples were taken from the identified SCS (Bonney's Blue staining) and three membrane samples from a distal site (DS, no Bonney's Blue staining). The placenta and fetal membranes were collected within 30 min of application of Bonney's Blue and transferred within 10 min of delivery to the laboratory within the hospital. Those patients with a low lying placenta were excluded from the study. Tissue samples were fixed and paraffin embedded for immunohistochemical analysis, or snap frozen in liquid nitrogen and immediately stored at −80 °C for RNA and protein analysis.

2.2. RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from approximately 100 mg of tissue using Tri Reagent according to manufacturer's instructions (Sigma–Aldrich, Saint LouisMissouri). 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. One μg of RNA was converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The cDNA was diluted ten-fold, and 2 μl of cDNA was used to perform RT-PCR using Sensimix Plus SYBR green (Quantace, Alexandria, NSW, Australia) and 100 nM of QuantiTect Primer Assays (Qiagen, Germantown, Maryland, USA). The FOXO3 (catalogue number QT00031941) gene ID is 2309 and FOXO4 (catalogue number QT00029141) gene ID is 4303. Average gene C_T values were normalised to the average actin C_T values of the same cDNA sample. There was equal efficiency of PCR amplification of target (FoxO3 and FoxO4) and reference (actin) mRNA (average between 98 and 103%). There was no significant difference in the mRNA expression of actin between any of the sample groups. 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. Fold differences were determined using the $2^{-\Delta\Delta Ct}$ method [30].

2.3. Immunohistochemistry

Tissues were placed in embedding cassettes (Techno-Plas, SA, Australia) fixed in buffered formaldehyde solution (4%) for 48 h and embedded in paraffin. Serial sections (4 μm thick), were cut and mounted on sections onto superfrost plus slides. Four slides were prepared consecutively for each sample. Each site was immunolabelled with each of the antibodies and 2 were used as a negative control slide. Sections were deparaffinised followed by an antigen retrieval step (boiled in 10 mM Tris and 1 mM EDTA, pH 9.0 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 TBS, and incubated in a humidity chamber for 1 h in antibody diluted in 1% BSA in TBS. Rabbit polyclonal anti-FoxO3 (H-144): sc-11351 and goat polyclonal anti-FoxO4 (N-19): sc-5221, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used at 2 μg/ml FoxO3 recognises the product of the human FOXO3 (FKHRL1) gene (ID: 2309, Chromosome 6q21), whereas FoxO4 recognises the product of the human FOXO4 gene (ID: 4303, Chromosome Xq13.1). 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 cover slipped 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 or goat IgG serum were also included. Sections were assessed microscopically for both intensity and extent of staining. The evaluation of all immunohistochemical staining was done as a blind assessment and independently scored by an experienced pathologist (CR). The entire tissue section was scored and the extent of staining was determined on a scale of 0–5 according to the estimated percentage of cells stained: 0 < 10%; 1 = 11–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–90%; 5 > 90%. Staining intensity was assessed on a scale of 0–3: 0 = no staining, negative; 1 = pale brown, weak; 2 = brown, moderate; 3 = dark brown, strong [31]. The average of the extent and intensity score was taken for each case and subsequently used in the final analysis.

2.4. Western blotting

Cytosplamic and nuclear protein extracts were preformed as we have previously described [32]. Assessment of FoxO3 and FoxO4 cytoplasmic and nuclear protein expression was analysed by Western blotting. Rabbit polyclonal anti-FoxO3 and goat polyclonal anti-FoxO4 (N-19) (as detailed above) were used at 1 μg/ml for 24 h. Forty micrograms of protein was separated on polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to PVDF. Protein expression was identified by comparison with the mobility of protein standard. Membranes were viewed and analysed using the Chemi-Doc system (Bio-Rad). Quantitative analysis of the relative density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad). Data were corrected for background, and expressed as optical density (OD/mm^2).

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). Two sample comparisons were analysed by paired sample comparison, Student's *t*-test or Mann–Whitney (Wilcoxon) test. For all other comparisons, analysis was performed using a one-way ANOVA (using Tukey HSD correction to discriminate among the means); homogeneity of data was assessed by Bartlett's test, and when significant, data were logarithmically transformed before further analysis. Statistical significance was ascribed to P value <0.05. Data was expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Expression and localisation of FoxO3 in human gestational tissues

3.1.1. FoxO3 mRNA expression in term placenta and fetal membranes

FoxO3 mRNA expression in human gestational tissues at term was analysed using qRT-PCR. Actin mRNA was used for normalisation of the data. FoxO3 mRNA was detected in all placenta, amnion and choriodecidua samples, with the data presented in Fig. 1A. FoxO3 mRNA expression was significantly higher in placenta and amnion compared to choriodecidua ($p = 0.004$). The gene expression of FoxO3 was similar between placenta and amnion.

3.1.2. Immunohistochemical localisation of FoxO3 expression in term placenta and fetal membranes

Placenta exhibited FoxO3 staining only in the syncytiotrophoblast layer, that was both cytoplasmic and nuclear (Fig. 2A). FoxO3 was not present within the cytotrophoblasts, villous structure or endothelial cells. Amnion and chorion exhibited, both cytoplasmic and nuclear FoxO3 (Fig. 2B). FoxO3 was present in the amnion epithelium, chorion trophoblast and fibroblast cells of the spongy layer. Decidua, also exhibited cytoplasmic FoxO3. No staining was present in the negative controls for FoxO3 in placenta (Fig. 2C) and fetal membranes (Fig. 2D).