

Forkhead transcription factor FOXO1A is critical for induction of human decidualization

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Abstract

Experiments utilizing RNA interference technology were performed to determine whether the forkhead transcription factor FOXO1A, a member of the FOXO family of proteins, plays a critical role in the induction of human uterine decidualization. Human decidual fibroblast cells were decidualized *in vitro* for 6 days with medroxyprogesterone, estradiol, and dibutyryl cAMP in the presence or absence of a highly specific FOXO1A small interfering RNA (siRNA) that inhibits FOXO1A mRNA and protein expression by more than 80%. RNA and proteins were extracted from the cells at 0, 2, 4, and 6 days. FOXO1A and IGFBP-1 proteins were determined by immunoblotting; and intracellular mRNA levels for several decidualization marker genes were determined by real-time PCR. Exposure of the cells to FOXO1A siRNA in five separate experiments resulted in a 40–75%

inhibition of prolactin, IGFBP-1, tissue inhibitor of metalloproteinase 3 (TIMP3), somatostatin and endometrial bleeding-associated factor (EBAF) mRNAs, all of which are markedly induced during the decidualization process. In contrast, actin and GAPDH mRNA levels did not change during decidualization. The inhibition of mRNA levels was first noted at day 2 and persisted for the remainder of each experiment. Western blot analysis indicated that the FOXO1A siRNA inhibited IGFBP-1 protein expression by 60–80%. Decidual fibroblast cells exposed in an identical manner to a control RNA that had no effect on FOXO1A expression caused only a 0–15% inhibition of the marker genes and IGFBP-1 protein. Taken together, these findings strongly suggest a critical role for FOXO1A in the induction of human decidualization.

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Introduction

Transcription factors of the forkhead (FOXO) family of DNA-binding proteins are involved in regulation of diverse cellular functions, such as differentiation, metabolism, proliferation, and survival (for review see Accili & Arden 2004). Several lines of evidence suggest that one of the family members, FOXO1A (forkhead box O1A (rhabdomyosarcoma), also known as FKHD and FKH1), is involved in the regulation of human uterine decidualization. DNA microarray studies have shown that FOXO1A is targeted to the nucleus of human decidual cells (Christian *et al.* 2002) and is one of the earliest and most induced genes during human decidualization (Brar *et al.* 2001). In addition, transfection studies in human decidual cells have shown that overexpression of FOXO1A induces expression of the prolactin (Christian *et al.* 2002) and insulin-like growth factor-binding protein 1 (IGFBP-1; Lane *et al.* 1994, Tseng *et al.* 1997, Kim *et al.* 2005) genes, both of which are markedly induced during human decidualization. In a recent study, Kim and

co-workers (2005) also reported that a FOXO1A small interfering RNA (siRNA) blocked IGFBP-1 expression in human uterine fibroblasts and HEC-1B cells, but they did not examine whether silencing of FOXO1A expression affects other markers of decidualization and is important in the induction of the decidualization process.

In this study, we have used RNA interference (RNAi) technology to investigate the role of FOXO1A in the induction of decidualization using an *in vitro* model in which human decidual fibroblasts exposed to progesterone, estradiol, and dibutyryl cAMP differentiate to a phenotype that expresses FOXO1A, prolactin, IGFBP-1, and other genes induced during decidualization of human endometrial stromal cells.

Materials and Methods

Preparation and culture of decidual fibroblast cells

Approval to obtain human placentas for this study was obtained by the Institutional Review Boards at the

Table 1 FOXO1A mRNA sequences targeted by FOXO1A siRNAs. The FOXO1A mRNA (accession number NM_002015) contains 1969 bases

mRNA sequence	Target sequence (5' → 3')	Positions of bases targeted
siFOXO1A-A	AAGCCGAGCTGCCAAGAAGAA	801–821
siFOXO1A-B	AAGAGCTGCATCCATGGACAA	756–776
siFOXO1A-C	AAGCCCTGGCTCTCACAGCAA	891–911
siFOX91A-D	AAGTTCATTCGTGTCAGAAT	664–684

University of Cincinnati, College of Medicine and the Children's Hospital Medical Center, Cincinnati, OH, USA; permission to use the placentas for these investigations was given in each instance. Term decidual tissue was dissected from the chorionic layer, and fibroblasts were isolated and purified to >95% purity by differential plating on plastic as previously described (Brar *et al.* 2001, Richards *et al.* 1995). Flow cytometry and immunocytochemistry studies performed earlier by us (Richards *et al.* 1995) demonstrated that fibroblast cells purified by this technique are free of cells expressing bone marrow-derived cell-surface antigens and the epithelial cell marker, cytokeratin. No trophoblast or endometrial stromal cells were observed. The cells tested positive for the cytoskeletal protein vimentin, establishing that they are mesenchymal cells. Approximately 2×10^6 cells were plated per well in six-well tissue-culture plates (Falcon Plastics, Becton–Dickinson, Franklin Lakes, NJ, USA) in RPMI 1640 medium (Gibco/Invitrogen) supplemented with 10% fetal bovine serum (Gibco/Invitrogen) and cultured in an atmosphere of 5% CO₂ at 37 °C. After reaching 80–90% confluence, the cells were transfected with siRNA or scrambled RNA (Qiagen) and decidualized as described below.

Design and transfection of siRNAs

Sequences of the siRNAs used to attenuate FOXO1A expression are shown in Table 1. The target sequences within the FOXO1A mRNA were selected and complementary siRNAs were synthesized by Qiagen as a 4-for-silencing kit. A control scrambled RNA against a target sequence 5'-AATTCTCCGAACGTGTCACGT-3', which does not share homology with the human genome, was also provided by Qiagen. Adherent fibroblast cells were transfected with siRNAs and control RNA using the RNAiFect transfection reagent from Qiagen according to the vendor's specifications. Briefly, siRNA or scrambled RNA solutions were prepared 15–25 min before the cell transfection. The ratio of siRNA to the RNAiFect reagent was 1 µg siRNA to 6 µl transfection reagent. The siRNA–RNAiFect complexes were

allowed to form for 15 min at room temperature. After replacing the FBS-containing medium with 1 ml RPMI 1640 medium, the siRNA–RNAiFect suspension was added drop-wise to a final concentration of 40–60 nM siRNA. The cells with adherent complexes were incubated 4 h at 37 °C. Decidualization was induced by replacing 1 ml RPMI 1640 medium with 4 ml aliquots of fresh RPMI 1640 medium supplemented with 2% FBS, 1 µM medroxyprogesterone, 10 nM estradiol, and 150 µM dibutyryl cAMP (all from Invitrogen). Aliquots of medium and cell extracts were collected for 6 days at the time intervals indicated in the figures and tables. The efficiency of the transfection was determined in three preliminary experiments in which a fluorescent control RNA–RNAiFect complex (Qiagen) was transfected into the cells instead of the siRNA–RNAiFect complex. The uptake of the fluorescent RNA into the decidual fibroblast cells in the three experiments, as determined by fluorescence microscopy of 150–200 cells per experiment, was in the range of 75–85%.

Protein and RNA extraction

For the preparation of protein extracts, the medium in the wells was removed by aspiration; and the cells were washed with 1 ml cold PBS. The cells were then extracted with 0.1 ml extraction buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.1 M potassium fluoride, 50 mM potassium chloride, 10 mM sodium pyrophosphate, 1 mM EDTA, 0.2 mM sodium orthovanadate, 20 mM dithiothreitol and 5 mM PMSF). The protein concentration in each cell extract was determined using the Bradford assay (BioRad). RNA was extracted from the fibroblasts with Trizol reagent (Invitrogen) according to the vendor's specifications. The protein extracts and RNA samples were kept at –70 °C.

SDS/PAGE and Western blotting

Relative FOXO1 and IGFBP-1 protein levels were determined in total cell extracts and medium samples by Western blot analysis. Equal amounts of proteins (15 µg) in the extracts and medium samples were separated on a NuPAGE 4–12% Bis–Tris gel (Invitrogen) before electrotransfer at 32 V onto a nitrocellulose membrane (Protran, 0.2 µm; Schleicher and Schuell, Keene, NH, USA). Transfer efficiency was 90–100% as judged by the transfer of stained protein markers (Invitrogen). Nonspecific binding sites on the blots were blocked by incubation for 1 h at room temperature with 5% dried non-fat milk in Tris-buffered saline (TBST; 130 mM NaCl, 20 mM Tris/HCl, pH 7.6, and 0.1% Tween 20). Blots were exposed to a mixture of primary rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA) against phosphorylated (Ser-256) and non-phosphorylated forms of FOXO1A. Both antibodies were diluted 1:2000 in TBST

Table 2 List of decidualization markers and reference genes

Gene	Primer sequence (5' → 3')	Forward (F) or reverse (R), position on cDNA (bp)	Efficiency (%)	Amplicon size (bp)
Real-time PCR				
Actin	CTGGACTTCGAGCAAGAGAT GATGTCACCGTCACACTCA	F (661–680) R (848–867)	111·6	208
FOXO1A	TCATGTCAACCTATGGCAG CATGGTGCTTACCGTGTG	F (1500–1526) R (1621–1638)	96·6	140
Prolactin	GCTGTAGAGATTGAGGAGCA CATTTCCTTTGGTTTCAGGA	F (431–449) R (498–517)	110	86
EBAF	CCTGAGAGGGTGCTAAGAG GGTAGGTAGGGGCTGTCT	F (37–55) R (99–116)	85·9	81
GAPDH	CCATGGAGAAGGCTGGGGCTCATTGC GGACCGGTTCCAGTAGGTAAGTGAAC	F (6–23) R (80–99)	95·6	95
IGFBP-1	CTATGATGGCTCGAAGGCTCCTCATGTCAC CCCTTCCTAGGGACCCAGAGGTCTCTAG	F (610–628) R (679–696)	107·4	88
Somatostatin	AGACTCCGTCAGTTTCTGC CTGGTTGGGTTTCAGACAG	F (88–105) R (169–186)	90·7	100
TIMP3	GACGCCTCTGCAACTCCGACA GATGACGTCGACCATGGCTCCT	F (283–304) R (756–777)	91·4	49
RT-PCR				
FOXO1A	AGAGCGTGCCTACTTCAA ATACTGGCTTGTCTACTA	F (575–593) R (993–1011)	NA	437
Actin	GTGGATCAGCAAGCAGGAGTATGA TTTAGGATGGCAAGGGACTTCTCTG	F (300–1323) R (1637–1660)	NA	362

NA, not applicable; RT, reverse transcriptase; TIMP3, tissue inhibitor of metalloproteinase 3.

with 5% dried non-fat milk. The blots were incubated with antibodies overnight at 4 °C, and then incubated for 1 h at room temperature with secondary peroxidase-conjugated goat anti-rabbit IgGs (Chemicon, Temecula, CA, USA; 1:5000 dilution in TBST). To control for loading efficiency, actin bands were identified using mouse anti-human actin monoclonal antibody (1:50 000 dilution in TBST) provided by Dr J. Lessard (Children's Hospital Medical Center, Cincinnati, OH, USA). Peroxidase-conjugated goat anti-mouse IgGs were used as secondary antibodies (Chemicon; 1:10 000 dilution in TBST). IGFBP-1 was identified on the blots with a goat anti-IGFBP-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:2000 dilution in TBST) and a peroxidase-conjugated donkey anti-goat IgG (Chemicon; 1:5000 dilution in TBST) as a secondary antibody. Protein bands were visualized by enhanced chemiluminescence (SuperSignal West Femo Maximum Sensitivity Substrate for FOXO1A detection and SuperSignal West Pico Chemiluminescent Substrate for IGFBP-1 detection, both from Pierce, Rockford, IL, USA).

mRNA analysis

Total RNA was isolated from decidual fibroblasts using Trizol reagent according to the vendor's specifications. One to two micrograms of total RNA were reverse

transcribed and PCR amplification performed. Decidualization was monitored in one set of experiments by determination of FOXO1A, prolactin, IGFBP-1, endometrial bleeding-associated factor (EBAF), and somatostatin mRNA levels by semi-quantitative reverse transcription PCR using GAPDH, and actin mRNA levels for normalization. The sequences of the prolactin, IGFBP-1, EBAF, and GAPDH primers are listed elsewhere (Kessler *et al.* 2005). The sequences of the FOXO1A, somatostatin, and actin primers are shown in Table 2. The reactions were performed for 25 cycles, which was in the linear range of the amplification curve for each set of primers. The PCR products were separated by electrophoresis on 6% polyacrylamide gels at 200 V for 3 h. The gels were transferred to 3 M paper, dried, and quantified using a phosphorimager and Imagequant 1·2 software (Molecular Dynamics, Sunnyvale, CA, USA). The primer pairs used for the PCR reactions were specific for each DNA, and the products of the PCR reactions were identical to the predicted sizes (Table 2).

Changes in mRNA levels in most experiments were quantified in these templates by using the Stratagene MX3000P robotic system (Stratagene, LaJolla, CA, USA) designed for the real-time PCR. Quantitative PCR amplifications were performed using the HotMasterMix (Brinkman, Westbury, NY, USA) supplemented with Sybr Green (Invitrogen) and Rox (Stratagene). The mix

was used according to the manufacturer's instructions using a 20 μ l final volume. The PCR reaction was performed after a 2 min incubation at 95 °C followed by 40 cycles at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s. Dissociation/association curves for each reaction were determined after the fortieth cycle. Preliminary experiments determined that the conditions used for each primer pair were optimized for these conditions. Each dissociation curve yielded a single peak at the expected melting point, and the amplicon was of the expected molecular size. Human term decidua cDNA (25 ng) was used as a positive control, and the reaction mixture without cDNA template was used as a negative control in each PCR run. The intra- and interassay variation of the real-time PCR reactions for each primer pair was within \pm 10%. The primers used for quantitative PCR, the size of each amplicon, and the efficiency of the PCR reactions are shown in Table 2. The same somatostatin probes were used in the semi-quantitative and real-time PCR reaction.

Statistical analysis

The effects of the FOXO1A siRNAs on the decidualization markers and other RNAs (GAPDH and actin) were calculated as the percent change in the mRNA levels relative to the mRNA levels in cells treated with the scrambled RNA. Each difference was expressed as the mean \pm S.E.M. from triplicate cultures. The statistical difference between sample means was determined by analysis of variance followed by Duncan's multiple comparison test or the Tukey *post hoc* test. *P* values of <0.05 were considered significant.

Results

Effects of FOXO1A siRNAs on induction of FOXO1A mRNA and protein during decidualization

In preliminary experiments to determine whether attenuation of FOXO1A expression inhibits decidualization, four siRNAs targeting different regions of FOXO1A were added to cultures of human decidual fibroblasts undergoing decidualization in response to progesterone, estradiol, and dibutyryl cAMP. The siRNAs were added 4 h prior to the induction of decidualization, and FOXO1A mRNA and protein levels were determined after 2, 4, and 6 days of exposure. The amounts of FOXO1A mRNA and protein in the four groups of siRNA-treated cells were compared with the amounts in cells cultured with a scrambled control RNA that was not targeted against FOXO1A and to the amounts in cells decidualized in the absence of added RNA.

As shown in Fig. 1, FOXO1A mRNA levels in fibroblast cells cultured in control medium alone (no scrambled RNA or siRNA) increased markedly from day 0

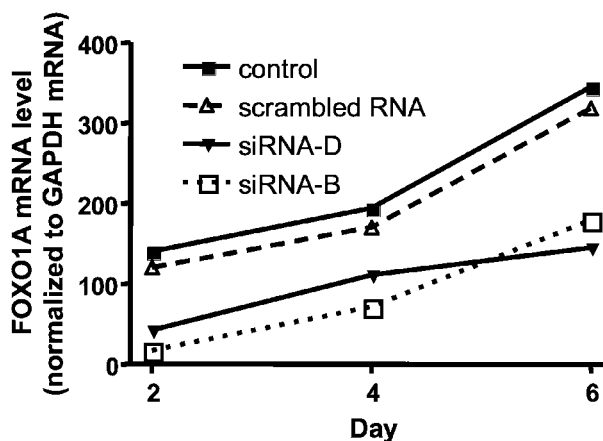


Figure 1 FOXO1A siRNAs inhibit FOXO1A mRNA levels during decidualization. Human decidual fibroblast cells were incubated for 4 h in control medium or medium containing a scrambled RNA oligomer or two RNA oligomers (siRNA-D and siRNA-B). The cells were then exposed to medroxyprogesterone, estradiol, and dibutyryl cAMP as described in Materials and Methods. FOXO1A mRNA levels were determined after 2, 4, and 6 days by real-time PCR. The amounts of FOXO1A mRNA were quantitated by densitometry and then normalized to the amounts of GAPDH mRNA in each sample. Each point represents the mean value for the mRNA in duplicate culture wells. Two RNA oligomers targeted to FOXO1A (siRNA-A and siRNA-C) had no effects on FOXO1A mRNA levels (data not shown).

(just prior to the addition of steroids and dibutyryl cAMP when FOXO1A mRNA levels were not detectable) to day 2 and remained at high levels over the remainder of the 6-day culture period. FOXO1A mRNA levels in the cells that had been exposed to the scrambled RNA had a pattern during decidualization that was nearly identical to those of the fibroblast cells that were decidualized in the absence of added RNA. In contrast, cells decidualized after exposure to siRNA oligomers B and D expressed significantly less FOXO1A mRNA than cells exposed to the scrambled RNA oligomer and to cells that were not exposed to added RNA. After 2 days of decidualization, FOXO1A mRNA levels in the cells exposed to siRNA-B and siRNA-D were 85.9 and 44.2% less, respectively, than those of cells exposed to the scrambled RNA. After 6 days, FOXO1A mRNA levels were 59.0 and 25.5% less, respectively. The cells that had been exposed to RNA oligomers A and C expressed the same amounts of FOXO1A mRNA during decidualization as the scrambled RNA-exposed cells or the cells exposed to control medium alone (data not shown).

Exposure of decidual fibroblasts to the scrambled RNA immediately prior to decidualization had no effect on the levels of decidualization marker mRNAs following exposure of the cells to progesterone, estradiol, and dibutyryl cAMP. As shown in Fig. 2, decidual cells prior to decidualization contained undetectable amounts of FOXO1A, prolactin, IGFBP-1, EBAF, and somatostatin

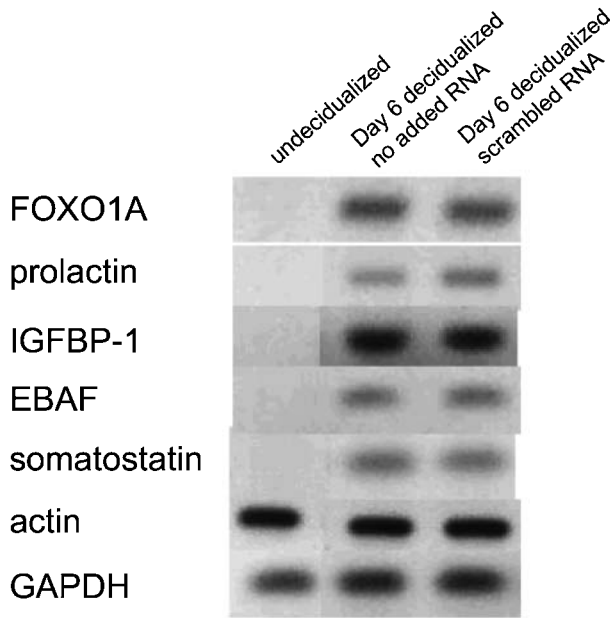


Figure 2 The effects of the scrambled RNA on marker mRNA levels during decidualization. Human decidual fibroblasts were exposed to a scrambled RNA oligomer and to control medium without added RNA as described in Fig. 1. After 6 days of decidualization, lysates of the cells were analyzed for FOXO1A, prolactin, IGFBP-1, EBAF, somatostatin, actin, and GAPDH mRNA levels by RT-PCR. The mRNA levels for FOXO1A, prolactin, IGFBP-1, EBAF, and somatostatin have been normalized to GAPDH mRNA levels in the same cell lysates. The GAPDH and actin mRNA levels were not normalized. Normalization of the GAPDH mRNA levels to actin mRNA and normalization of the actin mRNA levels to GAPDH mRNA also revealed that the normalized GAPDH and actin mRNA levels did not change during *in vitro* decidualization.

mRNA levels. The decidual cells that were exposed to the scrambled RNA prior to decidualization showed a marked increase in the mRNA level for each of the marker mRNAs after 6 days of decidualization that was identical or nearly identical to the increase observed in cells decidualized following exposure to control medium containing no added RNA. Actin and GAPDH mRNA levels did not change during decidualization, and the mRNA levels in the two groups of cells after 6 days of decidualization were nearly identical.

The attenuation of FOXO1A mRNA levels during decidualization by FOXO1A siRNA-B and FOXO1A siRNA-D was associated with decreased FOXO1A protein expression (Fig. 3). The upper portion of Fig. 3 shows the results of a single experiment in which FOXO1A and phosphorylated FOXO1A protein levels were determined in control cells and cells exposed to scrambled RNA, FOXO1A RNAi-B, and FOXO1A RNAi-D prior to decidualization. FOXO1A protein levels in the control cells not exposed to exogenous RNA increased markedly

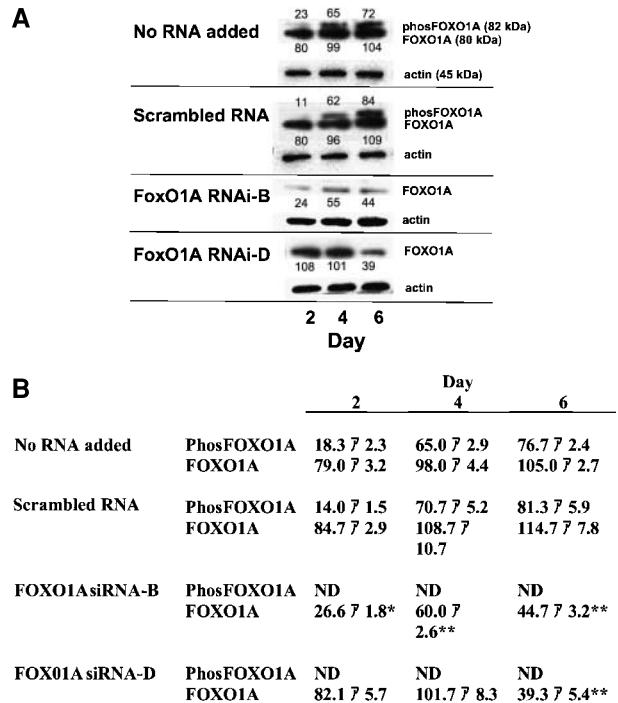


Figure 3 The effects of FOXO1A siRNAs on FOXO1A protein levels during decidualization. Human decidual fibroblasts were exposed to no exogenous RNA, a scrambled RNA oligomer, and FOXO1A siRNA-B and FOXO1A siRNA-D as described in Fig. 1. After 2, 4, and 6 days of decidualization, cell extracts from triplicate wells were pooled and analyzed for FOXO1A by Western blot analyses using highly specific antisera to the phosphorylated (A, upper band) and non-phosphorylated (A, lower band) forms of the protein. Actin protein levels were determined to be certain that equal amounts of protein were loaded for each sample. (A) shows the protein bands from a single experiment. The numbers above the phosphorylated FOXO1A protein bands indicate the relative densitometric values for the phosphorylated FOXO1A proteins normalized to the actin values. The numbers below the non-phosphorylated FOXO1A bands indicate the relative values for the non-phosphorylated FOXO1A proteins. (B) shows the densitometric results (mean ± S.E.M.) from three separate experiments, including the experiment depicted in the upper portion of the figure. ND, not detected; * $P < 0.01$; ** $P < 0.001$.

during the first 2 days of decidualization. At the end of day 2, both phosphorylated and non-phosphorylated forms of FOXO1A protein were detected. Both FOXO1A forms then increased progressively over the next 4 days. FOXO1A siRNA-B and siRNA-D attenuated the expression of FOXO1A protein, while the scrambled RNA oligomer had no significant effect. At the end of day 2 of decidualization, cells transfected with FOXO1A siRNA-B expressed approximately 80% less of the non-phosphorylated form of FOXO1A-B than cells transfected with the scrambled RNA; and no phosphorylated form of FOXO1A protein was detected at any time during the 6 day culture. FOXO1A siRNA-D also attenuated

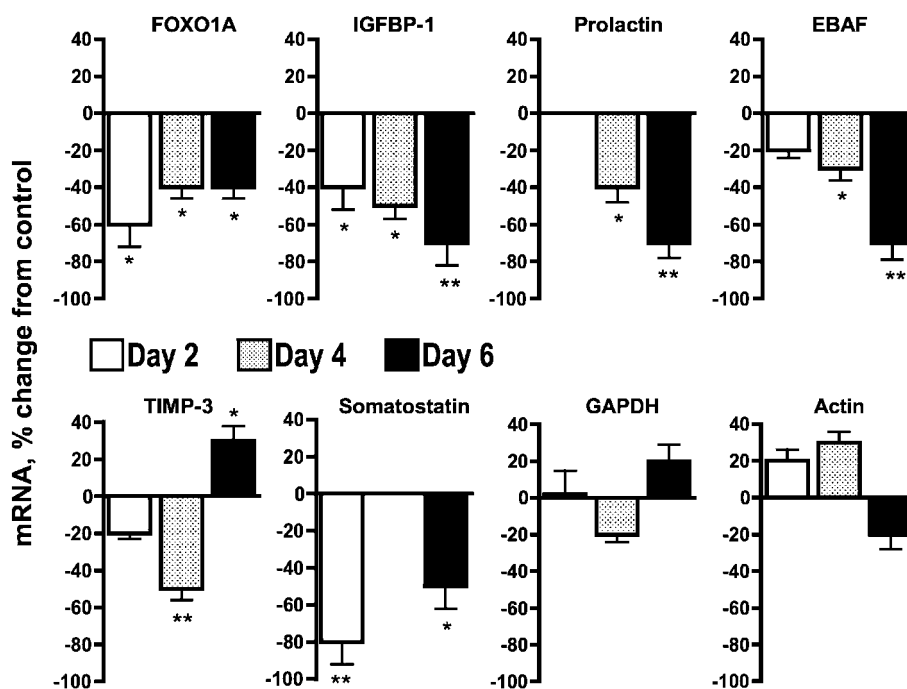


Figure 4 Attenuation of FOXO1A expression inhibits expression of decidualization-specific marker genes. Decidual fibroblast cells were treated with scrambled RNA or FOXO1A siRNA-B for 6 days as described in Fig. 1. Levels of FOXO1A, IGFBP-1, prolactin, EBAF, TIMP-3, somatostatin, GAPDH, and actin mRNAs were determined by real-time PCR at the indicated times. Results are expressed as percent change from the corresponding mRNA levels in cells treated with scrambled RNA. Each bar represents the mean \pm S.E.M. from triplicate wells. * $P < 0.05$; ** $P < 0.01$.

FOXO1A expression but the decrease in expression was less than that by FOXO1A siRNA-B.

The table in the lower half of Fig. 3 shows the relative densitometric values for FOXO1A and phosphorylated FOXO1A proteins in three separate experiments, including the experiment depicted in the upper half of the figure. The changes in the FOXO1A proteins in response to the two siRNAs in the two experiments not depicted in the upper panel were nearly identical to that of the depicted experiment. Since FOXO1A siRNA-B markedly attenuated the induction of FOXO1A mRNA and protein levels and the attenuation by siRNA-B was greater than that of siRNA-D, subsequent experiments were performed using FOXO1A siRNA-B.

Effect of FOXO1A siRNA on mRNA levels of decidualization markers

To determine whether inhibition of FOXO1A expression during decidualization attenuates decidualization, experiments were performed to examine whether fibroblast cells exposed to progesterone, estradiol, and dibutyryl cAMP in the presence of FOXO1A siRNA-B express less decidualization marker mRNAs than cells exposed under identical conditions to scrambled RNA. As

in the preliminary experiments, the cells were exposed for 4 h to control medium or medium containing scrambled RNA or FOXO1A siRNA-B. The medium of all three groups of cells was then changed to medium containing progesterone, estradiol, and dibutyryl cAMP and cultured for an additional 6 days. RNA was isolated from the cells after 2, 4, and 6 days and analyzed by real-time PCR for FOXO1A, prolactin, IGFBP-1, EBAF, tissue inhibitor of metalloproteinase 3 (TIMP3), and somatostatin mRNAs, all of which are known to be induced during decidualization (Brar *et al.* 2001). Actin and GAPDH mRNAs, which do not change during decidualization of decidual fibroblasts, were measured to control for sample loading.

As anticipated from earlier experiments (Brar *et al.* 2001), prolactin, IGFBP-1, EBAF, TIMP3, and somatostatin mRNAs were undetectable in the decidual fibroblast cells prior to decidualization. Following exposure to progesterone, estradiol, and dibutyryl cAMP, mRNA levels for each of the marker genes in the cells treated with the scrambled RNA were markedly induced, reaching levels identical or nearly identical to cells decidualized in the absence of the scrambled RNA (data not shown). As expected, FOXO1A mRNA levels in the siRNA-treated cells were significantly less than those of the scrambled RNA-exposed cells at each time interval ($P < 0.05$ at each

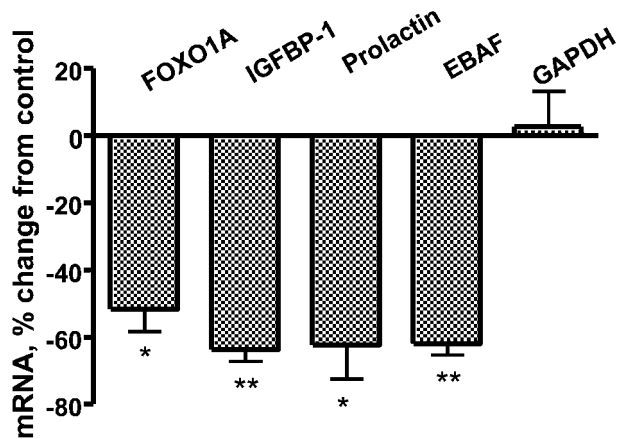


Figure 5 The effects of a FOXO1A siRNA on selected decidualization-specific marker genes after 6 days of decidualization. Human decidual fibroblasts were exposed to FOXO1A siRNA-B and decidualized for 6 days as described in Fig. 1. FOXO1A, IGFBP-1, prolactin, EBAF, and GAPDH mRNA levels were determined at the end of day 6. The mRNA levels for each of the marker genes in the scrambled mRNA-treated cells in each of the experiments showed the anticipated increases during the 6 days of *in vitro* decidualization (data not shown). The effect of the FOXO1A siRNA on each of the mRNAs is expressed as the percent change from the corresponding mRNA level in the control cells treated with scrambled RNA. Each bar represents the mean \pm S.E.M. from four separate experiments; triplicate wells were analyzed in each experiment. The analysis of variance was blocked for replicates. * $P < 0.05$; ** $P < 0.01$.

time; Fig. 4). At the end of day 2, FOXO1A mRNA levels in the FOXO1A siRNA-exposed cells were $60 \pm 11\%$ (mean \pm S.E.M.) less than that of the scrambled RNA-exposed cells ($P < 0.01$). At the end of day 6, prolactin, IGFBP-1, EBAF, TIMP3, and somatostatin levels were 60–81% less ($P < 0.05$ or 0.01) in each instance. In contrast, GAPDH and actin mRNA levels were essentially unchanged by FOXO1A siRNA. Attenuation of FOXO1A, prolactin, IGFBP-1, and EBAF mRNA levels was also observed in four other experiments (Fig. 5). After 6 days, the mean levels of FOXO1A mRNA in the FOXO1A siRNA-exposed cells were $62 \pm 3\%$ less than that of the scrambled RNA-exposed cells, while the levels of prolactin, IGFBP-1, and EBAF were 60–70% less ($P < 0.05$ or 0.01) in all instances. GAPDH mRNA levels were unchanged.

The attenuation of IGFBP-1 mRNA levels was accompanied by a marked decrease in the release of IGFBP-1 protein. As shown in Fig. 6, decidual fibroblast cells exposed to FOXO1A siRNA-B during decidualization released approximately 75–80% less IGFBP-1 protein on day 6 of culture than cells exposed to scrambled RNA.

Discussion

In an earlier study, we demonstrated that FOXO1A mRNA levels increase markedly during human decidualization

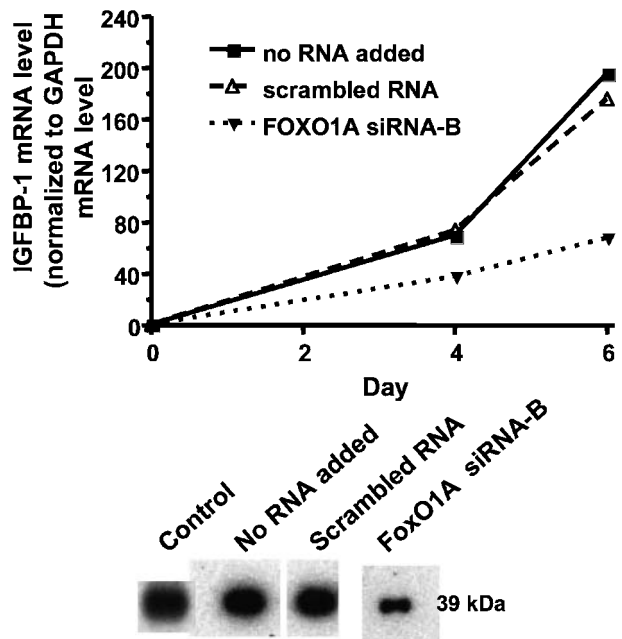


Figure 6 The effect of FOXO1A siRNA on IGFBP-1 mRNA and protein levels. Decidual fibroblast cells were decidualized for 6 days following exposure to FOXO1A siRNA-B, a scrambled RNA or no added RNA as described in Fig. 1. The graph shows the relative IGFBP-1 mRNA levels, normalized to GAPDH mRNA levels, in the three groups of cells prior to decidualization (time 0) and after 4 and 6 days of decidualization. The lower panel shows the relative amounts of IGFBP-1 protein (determined by Western blot analysis) released into the medium during the sixth day of decidualization. A control lane containing a cell extract of human endometrial stromal cells showed a single protein with the same apparent molecular mass as the protein band detected in the cultured decidual fibroblasts. Each lane contained an identical amount of total protein.

in vitro (Brar *et al.* 2001). In the present study, we have shown that FOXO1A protein levels, which are undetectable in untreated decidual fibroblast cells, are markedly induced during the first 2 days after exposure to progesterone, estradiol, and dibutyryl cAMP and then increase by about 3-fold over the next 4 days of decidualization. At 2 days after the initiation of decidualization, almost all of the FOXO1A protein is non-phosphorylated. By 4 and 6 days, most of the transcription factor has been phosphorylated.

Numerous studies have shown that the FOXO proteins are phosphorylated *in vivo* on multiple threonine and serine residues, three of which lie within a consensus sequence for phosphorylation by protein kinase B (PKB) and that PKB-induced phosphorylation inhibits transcriptional activity of the FOXO proteins (Kops *et al.* 1999, Rena *et al.* 1999). In the absence of PKB activity, the FOXO proteins are localized predominantly to the cell nucleus. Subsequent PKB-mediated phosphorylation

induces relocalization of the transcription factor from the nucleus to the cytoplasm (Biggs *et al.* 1999, Brownawell *et al.* 2001, Brunet *et al.* 1999). The phosphorylation-induced relocation appears to protect the cells against elevated levels of FOXO1A (Brunet *et al.* 1999, Nakae *et al.* 1999, 2000, 2001, Paradis & Ruvkun 1998, Rena *et al.* 1999) that may promote cellular senescence and atrophy (Accili & Arden 2004). Our data therefore suggest that the FOXO1A phosphorylation may occur when a critical level of intranuclear FOXO1A is reached. The subsequent relocalization of FOXO1A may prevent over-accumulation of the active form of FOXO1A and the induction of cellular atrophy (Accili & Arden 2004).

In initial experiments to elucidate the role of FOXO1A in decidualization, we used four siRNAs that were designed to target different region of FOXO1A mRNA. Control experiments with a scrambled RNA that does not target sequences in the human genome indicated lack of silencing activity against FOXO1A, while siRNAs targeted to different regions of FOXO1A RNA caused different amounts of inhibition. Preliminary experiments permitted us to select the most active siRNA that reduced FOXO1A mRNA and protein levels.

Fibroblast cells decidualized in the presence of FOXO1A siRNA-B expressed significantly less amounts of IGFBP-1, prolactin, EBAF, TIMP3, and somatostatin than cells decidualized in the presence of scrambled RNA. Decreased levels of the decidualization-specific markers were noted at 2 days of exposure and the decreased expression persisted over the 6 day observation period. The mRNA levels of two housekeeping genes, GAPDH and actin, were not affected by transfection with FOXO1A siRNA. The observation that the attenuation of FOXO1A expression during decidualization results in a marked decrease in the expression of decidualization-specific marker genes strongly suggests a critical role for FOXO1A in the induction of human decidualization. In a recent study, Kim and co-workers (2005) reported that a FOXO1A siRNA blocked IGFBP-1 expression in human uterine fibroblasts and HEC-1B cells, but they did not examine whether silencing of FOXO1A expression affects other markers of decidualization and is important in the induction of the decidualization process.

Microarray studies have identified three classes of FOXO1A-regulated transcripts (Ramaswamy *et al.* 2002) based on the binding to insulin-response sequences IRSA and IRSB (Guo *et al.* 1999, Kim & Fazleabas 2004). Class I genes, including IGFBP-1, are activated by FOXO1A and require IRS binding activity. Class II genes, including *CB LB*, *SOD-2*, and several DNA-damage response genes, are induced by FOXO1A without a strict requirement for interaction with an IRS element. Class III genes, responsible for cell-cycle inhibition, soft agar growth suppression, and tumor suppression, are transcriptionally regulated by FOXO1A. Analysis of the promoter regions of the set of human decidualization markers used in this

study revealed the presence of IRS response elements in the promoters of FOXO1A, prolactin, and IGFBP-1, indicating that these genes belong to Class I. The presence of an IRS in the FOXO1A promoter suggests that there may be self-regulation of FOXO1A expression. The promoter regions of EBAF and somatostatin, as well as the genes encoding for GAPDH and actin, do not have IRS elements, indicating that these genes are Class II genes. The housekeeping genes GAPDH and actin, which do not have IRS elements, were not affected by the silencing of FOXO1A.

Although the exact mechanisms by which FOXO1A affects the induction of decidualization is unknown, several lines of evidence suggest that FOXO1A action is mediated via the cAMP/protein kinase A signal transduction pathway. During decidualization there is a marked, sustained increase in intracellular cAMP levels, and agents that induce decidualization, such as progesterone and relaxin, lead to a sustained elevation of cAMP levels (Christian *et al.* 2002). In addition, inhibition of protein kinase A (PKA) action by a competitive inhibitor has been shown to block the decidualization process (Brar *et al.* 1997). Furthermore, FOXO1A augments the effects of PKA-dependent activation of the prolactin receptor in human endometrial stromal cells through functional cooperation with CCAAT/enhancer binding protein- β (C/EP β) and binding to a composite FOXO1/C/EP β response element in the proximal promoter region (Christian *et al.* 2002). FOXD2, another member of the FOX family, also has been shown to interact with the cAMP signal transduction pathway (Johansson *et al.* 2003). In Jurkat T cells, over-expression of FOXD2 increases endogenous levels of RI α , a regulatory subunit of PKA, through inhibition of RI α 1b promoter activity and increase the sensitivity of the promoter to cAMP (Johansson *et al.* 2003). Co-expression experiments also demonstrated that PKB α /Akt1 works together with FOXD2 to induce the RI α 1b promoter and increase endogenous RI α protein levels further (Johansson *et al.* 2003), suggesting that FOXD2 sets the threshold for cAMP-mediated negative modulation of T-cell activation.

In summary, relatively little is known about the molecular mechanisms involved in the induction of decidualization. The results of this study suggest that FOXO1A expression is critical for induction of the decidualization process.

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