Definition of microRNAs That Repress Expression of the Tumor Suppressor Gene FOXO1 in Endometrial Cancer

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Abstract

Endometrial cancer is the most common malignancy of the lower female reproductive tract. The tumor suppressor FOXO1 is downregulated in endometrial cancer compared with normal endometrium but the underlying mechanisms are not well understood. Using microRNA (miR) target prediction algorithms, we identified several miRs that potentially bind the 3′-untranslated region of FOXO1 transcripts. Expression profiling of normal and malignant endometrial samples by quantitative real-time PCR and Northern blot analysis revealed an inverse correlation between the levels of FOXO1 protein and the abundance of several of the in silico–predicted miRs, suggesting that loss of FOXO1 expression in endometrial cancer may be mediated by miRs. To determine the role of candidate miRs, we used the endometrial cancer cell lines HEC-1B and Ishikawa, which express FOXO1 at high and low levels, respectively. Expression of miR-9, miR-27, miR-96, miR-153, miR-182, miR-183, or miR-186, but not miR-29a, miR-128, miR-152, or miR-486 mimetics in HEC-1B cells was sufficient to significantly reduce the abundance of FOXO1. Conversely, FOXO1 expression was efficiently restored in the Ishikawa cell line upon simultaneous inhibition of miR-9, miR-27, miR-96, miR-153, miR-183, and miR-186. Moreover, induction of FOXO1 in Ishikawa cells by miR inhibitors was accompanied by G1 cell cycle arrest and cell death, and was attenuated by the small interfering RNA–mediated downregulation of FOXO1 expression. Our findings identify several miRs overexpressed in endometrial cancer that function in concert to repress FOXO1 expression. Further, aberrant miR expression results in deregulated cell cycle control and impaired apoptotic responses, and thus, may be central to endometrial tumorigenesis.

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Introduction

Endometrial cancer is the most common uterine cancer, accounting for 6% of all cancers in women in the United States. Endometrioid adenocarcinoma (type I) or endometrioid endometrial cancer (EEC) is the most common histologic subtype that represents 75% to 80% of all endometrial cancers (1–4). The remaining endometrial cancers consist predominantly of highly aggressive type II endometrial cancers, including uterine papillary serous (<10%) and clear cell carcinomas (4%; ref. 2). The etiology of endometrial cancer remains unclear, although unopposed estrogen exposure, complex hyperplasia with atypia, and treatment with tamoxifen during breast cancer therapy are recognized risk factors for endometrial cancer. Hysterectomy and bilateral salpingo-oophorectomy is still the primary and most effective treatment for patients with localized disease. Although adjuvant radiation therapy may reduce locoregional recurrence in patients with stage I disease, the associated toxicity and morbidity is significant. Patients with inoperable disease that are not candidates for radiation therapy may be treated with progesterational agents (5). A combination of cisplatin or paclitaxel with doxorubicin has been reported to improve overall survival in a subset of patients with stage III or IV disease compared with radiation therapy (5). However, prognosis remains poor for the 15% of patients that develop persistent or recurrent tumors, or those patients that present with advanced disease. Thus, there is an urgent need for new therapeutic targets and strategies, both of which may be realized through an increased understanding of the molecular mechanisms governing endometrial tumorigenesis.

Deregulation of the PTEN/phosphoinositide 3-kinase (PI3K)/PKB (also called Akt) signaling pathway is a hallmark of endometrial cancer (6). PTEN is inactivated in >80% of endometrioid carcinomas, leading to unrestrained PKB signaling. Loss of PTEN is likely to be an early event in endometrial tumorigenesis as it is found in 55% of precancerous endometrial lesions (3, 7, 8). In addition, 40% to 80% of type II endometrial cancers display HER2 amplification, and 36% of endometrioid carcinomas have mutations in the PI3KCA gene, both of which enhance the activity of the PI3K/PKB...
signaling pathway (6, 8). Key effectors of PI3K deregulation are the FOXO family of transcription factors, consisting of FOXO1, FOXO3a, and FOXO4, which are direct downstream phosphorylation targets of the protein kinase PKB and the related kinase SGK1 (9–11). PKB-dependent phosphorylation of FOXO proteins results in their nuclear exclusion, leading to loss of trans-activation of FOXO target genes, many of which are important for cell cycle regulation, apoptosis, and differentiation. Chemosensitization of endometrial cancer cells in response to PKB inhibition has been shown to require FOXO1 activity (12). FOXO1 has also been shown to be a crucial regulator of progesterone-dependent differentiation of the normal human endometrium, a process termed decidualization, and the subsequent menstrual shedding (refs. 13, 14). The central role of FOXO1 in endometrial differentiation was highlighted by a recent microarray study demonstrating that FOXO1 knockdown in primary endometrial cells using small interfering RNA (siRNA) perturbs the expression of 15% of all transcripts regulated during decidualization (14). Importantly, FOXO1 was shown to simultaneously regulate the induction of CDKN1C, which encodes for the cyclin-dependent inhibitor p57Kip2 involved in G1 cell cycle arrest, and the repression of several genes involved in DNA replication (e.g., MCM5), G2-M transition (e.g., CCNB1, CCNB2, CDC2, BRIC5, and BRIPI), and mitosis (e.g., PRC1, NUSAP1, CENPF, SPBC25, and ASPM).

MicroRNAs (miRNAs or miRs) are a class of small non-coding RNAs that regulate gene expression by facilitating mRNA degradation or translational inhibition. They play important roles in development, cellular differentiation, proliferation, cell cycle control, and cell death (15, 16) and have been implicated in a variety of human diseases, including cancer (16, 17). Recent evidence suggests that the FOXO family of transcription factors are targets for regulation by miRs. For example, aberrant miR-182 expression has been implicated in melanoma metastasis through downregulation of FOXO3a (18). In addition, a number of studies reported altered patterns of miR expression in EEC and identified a subset of miRs that correlated with disease stage and recurrence (19, 20). Using an in silico approach, we identified several cross-species conserved miRs predicted to target the 3′-untranslated region (UTR) of FOXO1. In both clinical samples and endometrial cancer cell lines, we investigated the role of miRs in the deregulation of FOXO1 expression and activity in endometrial cancer.

Materials and Methods

miRNA selection. In silico prediction of FOXO1 targeting miRNAs was performed using the PicTar, TargetScan, miRanda, and miRNA targets (Memorial Sloan-Kettering Computational Biology Center), miRBase, and RNAReg algorithms (21–24). Venn diagram analysis was then performed and used to identify miRNAs which displayed both interspecies conservation and were identified by multiple algorithms.

Cell culture and medium. Human endometrial cancer cell lines HEC-1B and Ishikawa originated from the American Type Culture Collection and were acquired from Cancer Research UK, where they were tested and authenticated. These procedures include cross-species checks, DNA authentication, and quarantine. Cell lines used in the present study were in culture for <6 mo and maintained in DMEM/F12 medium (Sigma) containing 10% fetal calf serum and 2 mmol/L of glutamine (10% CO2 at 37°C).

Clinical specimens. The participating Local Research and Ethics Committees approved the study and patient consent was obtained before tissue collection. Snap-frozen endometrial cancer samples were obtained from patients undergoing hysterectomy without preoperative chemotherapy or radiation and histologically validated for type and grade. Normal endometrial samples were obtained from premenopausal women awaiting in vitro fertilization treatment. All of the samples were frozen in liquid nitrogen immediately after resection and stored at −80°C until use.

Immunohistochemistry. Paraffin-embedded, formalin-fixed endometrial specimens were immunostained for FOXO1 using the Universal LSAB Plus Kits (DAKO) as described previously (25) using primary antibodies against FOXO1 (1:50 dilution; Cell Signaling Technology, Inc.). Histologic and immunohistochemical assessments were performed by two independent pathologists. Every tumor was given a score reflecting the average intensity of the staining (no staining, 0; low staining, 1; medium staining, 2; strong staining, 3).

Western blot analysis. Protein was extracted and SDS-PAGE performed as previously described (26). FOXO1 (C20), GAPDH (6C5), p27Kip1 (SX18F7), and β-tubulin (H-40) antibodies were from Santa Cruz Biotechnology (Autogen Bioclear). Western blot quantification was performed using ImageJ software (Image Processing and Analysis in Java). All Western blot experiments were performed in triplicate.

Quantitative real-time PCR. Total RNA was extracted from cell lines using TRI reagent (Ambion) as previously described (27) and used for quantitative real-time PCR (qRT-PCR) analysis without enrichment for miRs (28). cDNA synthesis and qRT-PCR was performed using miRVana qRT-PCR miRNA detection kit and primer sets (Ambion) according to the instructions of the manufacturer. Samples were normalized to 5S (Ambion) and quantified accordingly using standard curves. For FOXO1 mRNA quantification, the following gene-specific primer pairs were used: L19 sense (5′-GCCGAAGGTACAGCCAT-3′) and L19 antisense (5′-GCAGCGCGGCABAA-3′); FOXO1 sense (5′-TGCACTAGCCCAATC-3′) and FOXO1 antisense (5′-TTGGTCAAGCGGTCTC-3′); L19, a nonregulated ribosomal housekeeping gene was used as an internal control to normalize input cDNA. qRT-PCR was performed using the Applied Biosystems HT-7900 using SYBR Green Master Mix (Applied Biosystems). Each analysis was performed in three experimental replicates with three technical replicates within each experiment and SEM determined.

Northern blot analysis. Northern blot analysis was performed essentially on pooled RNA extracted from 7 normal and 10 malignant endometrium samples using Trizol reagent (Invitrogen). 5′-DIG–labeled LNA-modified probes against
hsa-miR-182, hsa-miR-96, and hsa-miR-194 (which was predicted by qRT-PCR not to change) were used for hybridization (Exiqon). Probe sequences were hsa-miR-96 5′-AGCAAAAATGTGCTAGTGCCAAA-3′, hsa-miR-182 5′-AGTGAGATTCATTACAGGCCAAA-3′, and hsa-miR-194 5′-TCCACATGGAGTGTTACA-3′. Northern blotting was performed essentially as described (29). Perfect HybPlus was used for hybridization (68°C; Sigma-Aldrich). Membranes were then developed using the Block and Wash Buffer Set (Roche) and CDP-Star Reagent (Roche). RNA loading was determined by staining of the tRNAs with ethidium bromide.

Transfection. Cells were seeded on six-well plates 24 h prior to transfection (Invitrogen). miR mimetics and inhibitors were transfected into cells at a final concentration of 60 nmol/L using OligofectAMINE (Invitrogen) in serum-free conditions. miRNA transfection conditions were optimized to allow for comparative mimetic levels in HEC-1B compared with miRNA levels in Ishikawa cells. Cells were incubated with miRNA inhibitors, synthetic pre/anti-miRNA mimetics, or appropriate scramble controls (all from Ambion) for 4 h in Opti-MEM media before the addition of normal growth medium. The cells were then assayed for 48 h after transfection. Propidium iodide staining was performed as previously described (30, 31).

Statistical analysis. All values are presented as mean ± SEM where appropriate. Statistical significance between the two groups was determined by use of a two-tailed t test, and values of P < 0.05 were considered significant (*), values of P < 0.001 were very significant (**). To test for differences between tissue types and FOXO1 staining, one-way ANOVA was performed followed by Dunnett’s two-tailed t test, and the mean difference considered significant at the P < 0.05 level (Supplementary Fig. S1). All statistical analyses were performed with SPSS v.16.

Results

Discordance between FOXO1 mRNA and protein levels in endometrioid endometrial carcinomas. The levels of FOXO1 protein expression were first investigated in 9 normal endometrial tissues, 73 hyperplasia cases, and 52 endometrial carcino

Expression of putative FOXO1-targeting miRs in endometrial cancer. By combining the results of several miRNA target prediction programs, we first identified a panel of highly conserved miRs with the potential to target the 3′-UTR of FOXO1 transcripts (Fig. 3A and B), and then examined their expression levels in the same 10 malignant and 7 normal endometrial samples by qRT-PCR analysis. As shown in Fig. 3C, the levels of several of these in silico–predicted miRs, including miR-9, miR-27, miR-96, miR-128, miR-153, miR-182, miR-183, and miR-186, were significantly upregulated in endometrial cancer compared with normal endometrium. Notably, the abundance of some miR species, such as miR-27, miR-128, and miR-186 was >10-fold higher in the cancer samples. To further confirm the difference in miR expression between normal endometrium and endometrial cancer, we used Northern blot analysis to examine miR expression levels (Fig. 3C). In agreement with the qRT-PCR data, both miR-96 and miR-182 showed a higher level of expression in the endometrial cancer samples. As an internal control, we examined the expression of miR-194, which showed no significant difference in expression by qRT-PCR; miR-192 was detected at a similar level in both normal and malignant tissue by Northern blot analysis (Fig. 3C). These data suggest that the loss of FOXO1 expression upon malignant transformation of human endometrium coincides with a strong induction of several miRs, suggesting a causal link between these phenomena.

Validation of putative FOXO1-targeting miRs in endometrial carcinoma cell lines. To explore the mechanisms that perturbed FOXO1 expression in endometrial cancer, we made use of two well-characterized endometrial cancer cell lines, HEC-1B and Ishikawa. Western blot analysis confirmed that HEC-1B, but not Ishikawa cells, abundantly express FOXO1 (Fig. 4A) as previously reported (33, 34). In agreement, Ishikawa cells also express much lower levels of p27kip1, a FOXO1 target in endometrial cells. As was the case for normal and malignant endometrial biopsies, qRT-PCR analysis showed lower FOXO1 transcript levels in Ishikawa cells (Fig. 4B), albeit much less so than would have been inferred by Western blot analyses. The discrepancy between FOXO1 mRNA and protein levels prompted an analysis of putative FOXO1 miRs in HEC-1B and Ishikawa cells. Strikingly,
qRT-PCR analysis showed that several microRNAs, including miR-27, miR-96, miR-128, miR-153, miR-182, miR-183, and miR-186, were expressed at much higher levels in Ishikawa cells compared with HEC-1B cells (Fig. 4). Others, including miR-135, miR-142-3p, and miR-194, were expressed at comparable levels in both cell lines whereas miR-486 and miR-9 were more abundant in HEC-1B cells (Fig. 4C). Thus, loss of FOXO1 expression in endometrial cancer in vivo and in Ishikawa cells correlated with increased expression of a number of the same miRs that included miR-9, miR-27a, miR-96, miR-183, and miR-186.

To test the repressive potential of these miRs, synthetic mimetics were transfected individually into HEC-1B cells and endogenous FOXO1 expression levels monitored by Western blot analysis. In addition, cells were also transfected with a scramble mimic and a mimetic of miR-29a, which was not predicted to target FOXO1. Overexpression of several miRs (miR-9, miR-27a, miR-96, miR-153, and miR-186) effectively downregulated FOXO1 expression (Fig. 5A; Supplementary Fig. S2). In contrast, miR-128 (despite its strong induction in endometrial cancer), miR-152, miR-486, and the miR-29a control had little effect on FOXO1 expression.

In the reverse experiment, we used synthetic hairpin miRNA inhibitors to silence the activity of specific miRs in Ishikawa cells, and monitored the expression of FOXO1 and its target p27Kip1. Transfection of some individual miR inhibitors, targeting miR-9, miR-27a, miR-96, miR-153, miR-183, and miR-186, elicited a small but reproducible induction in FOXO1 and p27Kip1 levels (Fig. 5B and C; Supplementary Fig. S2). In contrast, FOXO1 levels were effectively unchanged.
upon transfection of anti-miR scramble control or a miRNA not predicted to target FOXO1, miR-29a. These observations indicate that targeting of individual miRs is insufficient to fully restore FOXO1 expression in endometrial cancer cells. However, transfection of individual inhibitors did not restore FOXO1 to the levels observed in HEC-1B cells. To further examine this effect 2× (anti–miR-153 and anti–miR-183), 6× (anti–miR-9, anti–miR-27, anti–miR-96, anti–miR-153, anti–miR-183, and anti–miR-186), or 8× (anti–miR-9, anti–miR-27, anti–miR-96, anti–miR-128, anti–miR-153, anti–miR-182, anti–miR-183, and anti–miR-186) were cotransfected into Ishikawa cells. Cotransfection of miR inhibitors dramatically enhanced FOXO1 levels in Ishikawa cells, and was accompanied by the induction of the FOXO1 target p27kip1 (Fig. 6A).

Repression of miR expression induces cell cycle arrest and cell death in Ishikawa cells in a FOXO1-dependent manner. During the development of endometrial cancer, the repression of FOXO1 by miR may be hypothesized to confer a proliferative advantage, or to allow cells to escape apoptosis. As such, we next determined the effect of repressing the miR expression in Ishikawa cells. Cell cycle analysis of propidium iodide–stained cells by flow cytometry showed that transfection of the 6× anti-miRs was effective in inducing cell cycle arrest and apoptosis in Ishikawa cells (Fig. 6B). To show the requirement of FOXO1 induction for anti-miR–induced cell death, the 6× anti-miR panel was cotransfected with a siRNA targeting the coding domain of FOXO1, causing a repression in FOXO1 levels (Fig. 6C). Under these circumstances, a reduction in anti-miR–induced cell death was observed, consistent with FOXO1 induction being critical for this effect (Fig. 6C). Although HEC-1B cells already express high levels of FOXO1, we next determined whether the transfection of the 6× anti-miR pool would result in cell death (Fig. 6D). In fact, only a moderate effect was observed on FOXO1 expression level, consistent with low endogenous levels of the miRs observed in the qRT-PCR analysis, and moreover, no significant change in the cell cycle distribution was observed (Fig. 6D). Together, these observations suggest that a number of miRs may repress endometrial FOXO1 expression, thereby promoting proliferation and survival of endometrial cancer cells.

Discussion

In this study, we identified and characterized a defined set of FOXO1-targeting miRs that may have a role in endometrial tumorigenesis. The expression of the tumor suppressor FOXO1 is strongly repressed in endometrial cancer samples compared with normal cycling endometrium.

Figure 2. Downregulation of FOXO1 expression in type I endometrial cancer (EEC). A, Western blot analysis of FOXO1 expression in 7 snap-frozen normal endometrial samples and 10 snap-frozen EECs. β-Tubulin staining was included as a loading control. B, statistical analysis of FOXO1 expression after Western blot quantification using ImageJ software, demonstrating significantly lower FOXO1 protein levels in EECs compared with normal endometrium. C, qRT-PCR analysis of FOXO1 transcripts in the same 7 snap-frozen normal endometrial samples and 10 snap-frozen EECs. Columns, mean; bars, SD (n.s., not significant).
Figure 3. Profile of FOXO1-targeting microRNA levels in type I endometrial cancer (EEC) and normal endometrium. A, Venn diagram analysis was used to select miRNAs that displayed interspecies conservation and were identified by multiple algorithms. B, schematic diagram illustrating the location of predicted miRNA binding sites identified through Venn diagram analysis. C, qRT-PCR analysis of miR expression in the same 7 snap-frozen normal endometrial samples and 10 snap-frozen EECs. Columns, mean; bars, SD. D, Northern blot analysis of miRNA expression in normal (N) versus endometrial cancer (EC). Membranes were hybridized with hsa-miR-182, hsa-miR-96, or hsa-miR-194–specific DIG-labeled probes; miR-194 served as a control. RNA loading was confirmed by staining of rRNA with ethidium bromide. Result is representative of three individual experiments.
Using a combination of miR target prediction programs, we identified a panel of miRs that may theoretically target the 3′-UTR of FOXO1 transcripts. Comparative analysis of these miRs and FOXO1 in normal endometrium and EEC revealed that loss of FOXO1 expression inversely correlated with significant upregulation of several of the in silico–predicted targeting miRs, including miR-9, miR-27, miR-96, miR-128, miR-153, miR-182, miR-183, and miR-186. However, the comparative expression of miRs in endometrial cancer versus normal, and HEC-1B versus Ishikawa cells were not always in agreement. In particular, miR-9 was increased in endometrial cancer tissue, but lower in the HEC-1B compared with Ishikawa cells, whereas miR-486 showed no change in tissue samples, but was higher in HEC-1B cells compared with Ishikawa. This is likely a reflection of the limitations of comparing cancer tissues and cancer cell lines, respectively. In addition, whereas HEC-1B and Ishikawa cells display different FOXO1 expression levels, both are still transformed cell lines and thus not exact models of normal and malignant endometrium. However, HEC-1B did display a miR profile more akin to normal endometrium than cancer, and this cell line was used to examine the ability of miRs to repress endogenous FOXO1 expression.

The relatively higher levels of FOXO1 expression in HEC-1B cells is consistent with the fact that HEC-1B proliferates slower compared with other EEC cell lines (e.g., Ishikawa and

Figure 4. Differential expression of FOXO1 and targeting miRs in HEC-1B and Ishikawa cells. A, Western blot analysis of FOXO1 and p27Kip1 expression in HEC-1B and Ishikawa cells. β-Tubulin was used as a loading control. B, qRT-PCR analysis showed lower FOXO1 transcript levels in Ishikawa cells compared with HEC-1B cells. C, qRT-PCR analysis of miR expression in HEC-1B and Ishikawa cells. Columns, mean of triplicate measurements; bars, SEM.
ECC1; ref. 33). Interestingly, not all predicted miRs inhibited endogenous FOXO1 protein expression in HEC-1B cells. In particular, miR-128, which was expressed at a much higher level in Ishikawa cells, had no significant effect on the expression level of FOXO1, suggesting that reintroduction of miR-128 alone is not sufficient to inhibit FOXO1 expression, or that FOXO1 is not a direct target of miR-128 in this instance. However, these data further narrow the set of putative FOXO1-targeting miR to miR-9, miR-27, miR-96, miR-153, miR-182, miR-183, and miR-186, all of which were also overexpressed in endometrial cancer.

FOXO1 expression is substantially lower in Ishikawa compared with HEC-1B cells (34) and, as is the case in vivo, coincided with increased expression of several putative FOXO1 targeting miRs. Yet, knockdown of individual miRs has only marginal effects on FOXO1 expression in Ishikawa...
Figure 6. Effects of expression of pooled anti-miRs on FOXO1 expression and cell cycle status in Ishikawa cells. A, Ishikawa cells seeded for 24 h were transfected with either mock conditions, scrambled anti-miRs, or anti-miRs as indicated for 48 h. The anti-miRs used for transfection were 2× (anti–miR-153 and anti–miR-183), 6× (anti–miR-9, anti–miR-27, anti–miR-96, anti–miR-153, anti–miR-183, and anti–miR-186), or 8× (anti–miR-9, anti–miR-27, anti–miR-96, anti–miR-128, anti–miR-153, anti–miR-182, anti–miR-183, and anti–miR-186). Western blot analysis of FOXO1, p27Kip1, and β-tubulin expression was performed after anti-miR pool transfection; HEC-1B cell lysate was included for comparison. Quantitative analysis of FOXO1 protein expression after miRNA transfection is shown. Columns, mean results of at least three independent anti-miR transfection experiments after quantification with ImageJ software and normalization with β-tubulin; bars, SD. Statistical analysis was performed using Student’s t test.

B, Ishikawa cells were transfected with 6× anti-miRs, and cell cycle phase distribution analyzed by flow cytometry after propidium iodide staining. Percentage of cells in each phase of the cell cycle (sub-G1, G1, S, and G2-M) is indicated. Representative data from three independent experiments are shown. Analysis of cell death as judged by sub-G1 cells. Statistical analysis was performed on three independent sets of transfections using Student’s t test.

C, Ishikawa cells were transfected with 6× anti-miRs or 6× anti-miR scramble, as described in Fig. 3B, with or without FOXO1-specific siRNA or scramble siRNA for 48 h and cycle phase distribution determined by propidium iodide staining, or analyzed by Western blot for FOXO1 expression. siNS, siRNA nonspecific; miR-NS, anti-miR nonspecific; siFOXO1, siRNA targeting FOXO1; miR-6×, 6× anti-miR. D, HEC-1B cells were transfected with mock, 6× anti-miR scramble, or 6× anti-miR. Forty-eight hours later, cells were either fixed with ethanol and cell cycle phase distribution determined by propidium iodide staining, or analyzed by Western blot for FOXO1 expression.
cells in contrast to the overexpression experiments in HEC-1B cells. This is, however, predictable as elevated levels of other FOXO1-targeting miRs will compensate for the loss of one miR species. Similarly, in vivo, some but not all FOXO1-targeting miRs may be critical to endometrial tumorigenesis depending on their relative expression levels in the endometrial cancer cells. Co-overexpression of these miRNAs with overlapping function in endometrial cancer may have significant implications in vivo, and may result in the loss of cell cycle and cell death control. The fact that some but not all FOXO1-targeting miRNAs have a predominant role in repressing FOXO1 expression could be related to their relative expression levels in the endometrial cancer cells. Consistent with this postulation, the 6x miRs (miR-9, miR-27, miR-96, miR-128, miR-153, miR-183, and miR-186) which cooperated in FOXO1 repression in Ishikawa cells were also among the most upregulated species in EEC compared with normal endometrium. Interestingly, the 3'-UTR seed sites of a number of the miRs implicated in FOXO1 regulation, in particular, miR-27 and miR-128, and miR-182 and miR-96, were proximally located, possibly resulting in cooperative repression. Several of the miRs that repressed FOXO1 most effectively were located in the 5' region of the 3'-UTR, which has previously been reported to be indicative of higher site activity. It is also intriguing to note that miR-183, miR-96, and miR-182 are located proximally in genome (chr7:129201768–129201845, chr7:129201981–129202000, and chr7:129197459–129197568, respectively). Moreover, a previous study identifying the proximal promoters of a large number of miRNAs (35) showed that miR-96, miR-183, and miR-182 share the same transcription start site (chr7: 129207158), suggesting that these miRNA may be coordinately deregulated, and therefore, be of particular importance during endometrial tumorigenesis. A recent report also supports our findings by describing the targeting of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells (36). However, this article does not determine whether the deregulation of these miRNA observed in breast cancer cell lines was also observed in breast cancer tissue. As such, our findings represent the first demonstration of FOXO1 targeting miRNA in vivo.

In addition to the miR-mediated repression, other mechanisms may contribute to the loss of FOXO1 expression in endometrial cancer. Previously, we reported that the FOXO1 promoter is methylated in some but not all endometrial cancers (33). Furthermore, a recent report proposed that the low levels of FOXO1 protein observed in Ishikawa cells are due to the upregulation of Skp2, an oncogenic subunit of the Skp1/Cul1/F-box protein ubiquitin complex that promotes ubiquitination and degradation of phosphorylated FOXO1 (37). Consistent with this, Skp2 is overexpressed in EEC (34, 38). However, treatment with the proteasomal inhibitor is insufficient to restore FOXO1 expression in Ishikawa cells (33), indicating that enhanced proteasomal degradation is not the primary pathway that accounts for the loss of this transcription factor in endometrial cancer.

FOXO1 has emerged as a major regulator of progesterone-dependent differentiation of the human endometrium and subsequent apoptosis associated with menstrual shedding (14). In addition, FOXO1 has been implicated in safeguarding genomic stability of the endometrium during the rapid waves of intense tissue remodeling by regulating the expression of DNA repair genes, such as GADD45α. Consequently, several studies have functionally linked the decrease or complete loss of FOXO1 expression in EEC to uncontrolled cell proliferation, impaired apoptosis, and increased susceptibility to genotoxic stress (33, 34, 39). Our results showed that reexpression of FOXO1 in Ishikawa cells upon knockdown of six miRs was accompanied by cell cycle arrest and cell death, emphasizing the cardinal role of FOXO1 in cell cycle control and cellular death response in endometrial cells. The extent to which deregulation of the miR machinery could be implicated in benign proliferative disorders of the endometrium, such as endometriosis, is currently under investigation. The corollary of these observations suggests that miR-based gene therapy may provide a novel approach for the treatment of endometrial hyperplasia and cancer (40–42). In summary, we identify a group of FOXO1-targeting miRNAs that are upregulated in endometrial cancer. The ability of this group of miRs to promote FOXO1 repression may precipitate in a key role in endometrial tumorigenesis by bypassing cell cycle and cell death control.

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