

THEMATIC REVIEW

The diversity of sex steroid action: the role of micro-RNAs and FOXO transcription factors in cycling endometrium and cancer

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Abstract

The rise and fall in ovarian oestrogen and progesterone production orchestrates a series of events that are indispensable for reproduction, including ovulation, implantation, decidualisation and menstruation. In the uterus, these events involve extensive tissue remodelling, characterised by waves of endometrial cell proliferation, differentiation, recruitment of inflammatory cells, apoptosis, tissue breakdown, menstruation and regeneration. The ability of ovarian hormones to trigger such diverse physiological responses is foremost dependent upon interaction of activated steroid receptors with specific transcription factors, such as Forkhead box class O (FOXO) proteins, involved in cell fate decisions. Furthermore,

micro-RNAs (miRNAs), small non-coding RNAs that function as posttranscriptional regulators of gene expression, have emerged as a major regulator system of steroid hormone responses in the female reproductive tract. Consequently, increasing evidence shows that deregulated uterine miRNA expression underpins a spectrum of common reproductive disorders, ranging from implantation failure to endometriosis. Furthermore, by targeting FOXO transcription factors and other key regulators of tissue homeostasis, oncogenic endometrial miRNAs promote tumourigenesis and cancer progression.

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Introduction

Micro-RNAs (miRNAs) are small non-coding RNAs, ~22 nucleotides in length, which function as posttranscriptional regulators of gene expression. To date, almost 1000 human miRNAs have been identified and validated (Griffiths-Jones 2010). However, *in silico* studies suggest that there might be as many as tens of thousands potential miRNA species in mammalian cells (Miranda *et al.* 2006), and it has been predicted that at least 60% of all transcripts are targeted by miRNAs (Friedman *et al.* 2009). MiRNAs are essential for normal mammalian development, determining cell identity and fate and regulating diverse biological processes including cell proliferation, metabolism, differentiation and apoptosis. Conversely, miRNAs are also increasingly implicated in the pathogenesis of numerous benign and malignant diseases, including hormone-dependent endometrial, ovarian and breast cancers. In fact, different cancers have distinct

miRNA expression signatures, referred to as oncomiRs, that contribute towards cancer initiation and progression by enhancing or inhibiting the expression of oncogenes and tumour suppressors respectively.

The magnitude of hormone-dependent tissue remodelling in the uterus is unsurpassed in the human body, characterised by ~400 cycles of endometrial cell proliferation, differentiation, menstrual shedding and regeneration. Although sex steroid hormones and activation of their cognate nuclear receptors are the key regulators in cyclic tissue remodelling of the endometrium, a host of interacting transcriptional partners, including Forkhead box class O1 (FOXO1; Christian *et al.* 2002c), signal transducers and activators of transcription 5 (STAT5) and CCAAT/enhancer-binding protein β (C/EBP β) (Christian *et al.* 2002a,b), also play a vital role in determining and fine-tuning the transcriptional responses in target cells. Furthermore, certain factors have emerged as important for safeguarding genomic integrity and tissue homeostasis during these rapid cycles of intense tissue remodelling. Among these factors are members of the FOXO family of transcription factors. FOXO proteins, functioning downstream of the phosphoinositol-3-kinase (PI3K)/AKT

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oncogenic signalling pathway, are central to a diversity of cellular functions, including cell proliferation, apoptosis, differentiation and resistance to oxidative stress and DNA damage (Lam *et al.* 2006, Reedquist *et al.* 2006, Huang & Tindall 2007, Myatt & Lam 2007, Arden 2008, Burgering 2008, Calnan & Brunet 2008, Fu & Tindall 2008, Gomes *et al.* 2008, Ho *et al.* 2008, Maiese *et al.* 2008b).

The human FOXO family consists of four members: FOXO1 (also known as FKHR), FOXO3 (FKHRL1), FOXO4 (AFX or Mllt7) and FOXO6. Their expression is highly regulated depending on the tissue type and developmental stage (Maiese *et al.* 2008a). In the human uterus, for example, FOXO1 is expressed in the endometrial stroma but only following differentiation of stromal fibroblasts into decidual cells during the mid-secretory phase of the cycle, whereas in the epithelial compartment, it is expressed constitutively throughout the menstrual cycle (Christian *et al.* 2002c, Goto *et al.* 2008). FOXO3a expression, by contrast, is downregulated upon decidualisation of endometrial stromal cells (Kajihara *et al.* 2006). Although FOXO4 transcripts are detectable in human endometrium, evidence of expression at protein level is yet lacking (Kajihara *et al.* 2006). FOXO6 expression is thought to be restricted to adult brain tissues (Jacobs *et al.* 2003). It is the ability of FOXOs to control opposing gene networks which are implicated in cell survival, differentiation and cell death responses, which render them key regulators of endometrial homeostasis. Consequently, loss of FOXO disables the ability of cells to arrest at key checkpoints, thereby facilitating tumour development. The notion that FOXO proteins are indeed *bona fide* tumour suppressors is supported by animal studies demonstrating that loss of FOXO activity leads to the development of pre-cancer conditions, including thymic lymphomas and haemangiomas (Paik *et al.* 2007), as well as accelerating Myc-driven lymphomagenesis (Bouchard *et al.* 2007). In this review, we summarise the mechanism of miRNA biogenesis and highlight the role of miRNA and FOXO transcription factors in modulating the hormonal responses in the endometrium. We also discuss how deregulated miRNA expression impacts on FOXO function, disrupts endometrial tissue homeostasis and contributes to the malignant transformation of the endometrium.

miRNA biosynthesis and mechanism of action

The biogenesis of miRNAs is a multi-stage process. It commences in the nucleus where the miRNA genes are first transcribed by RNA polymerase II, or sometimes by RNA polymerase III, into long primary miRNA transcripts (pri-miRNAs; Schwarz *et al.* 2003, Du & Zamore 2005, 2007). These capped and polyadenylated pri-miRNAs contain a hairpin stem of 33 bp, a terminal loop and two single-stranded flanking regions (Cai *et al.* 2004). Many miRNA gene loci are clustered and are, therefore, transcribed together and encoded in polycistronic transcripts

(Tanzer & Stadler 2004). The pri-miRNAs are then processed in the nucleus by a complex consisting of the RNase III enzyme, DROSHA, as well as the DiGeorge syndrome critical region 8 protein (DGCR8; Han *et al.* 2004). The DROSHA/DGCR8 containing the complex truncates the flanking segments and a <11 bp stem region, converting the pri-miRNAs into precursor miRNAs (pre-miRNAs; Fig. 1), which are about 70 bp long hairpin RNAs with 2 bp overhanging at the 3'-end. Pre-miRNAs can also be generated by a DROSHA/DGCR8-independent processing pathway, whereas pre-miRNAs are derived directly from introns through the action of mRNA splicing mechanisms (Lin *et al.* 2005, Ying & Lin 2006). Either way, pre-miRNAs are then transported by the exportin-5/RanGTP complex from the nucleus to the cytoplasm (Fig. 1), where they are processed by RNase III enzyme DICER (Fig. 1; Filipowicz *et al.* 2008, Chekulaeva & Filipowicz 2009). Dicer then cleaves pre-miRNAs into 21–25 bp long miRNA/complementary miRNA (miRNA*) duplexes. The miRNA/miRNA* duplex is essential for the assembly of miRNA-induced silencing complexes (RISC), which is made up primarily of Argonaute (Ago) proteins as the core catalytic components. During the miRISC assembly, the miRNA strand of the duplex is loaded into the (RISC) complex, whereas the other miRNA* strand is discarded. With the mature miRNA as a guide, the miRNA-RISC complex hybridises to complementary nucleotide sequences on target mRNAs. MiRNAs bind to target sites frequently located in the 3'-untranslated region (UTR) of the target mRNA but can also sometimes be found within the 5'-UTR or coding region. There has been much debate about how exactly miRNAs affect protein expression levels. Early studies in *Caenorhabditis elegans* concluded that miRNAs decrease translational output with little effect on target mRNA levels. Other studies involving microarray analysis and overexpression of particular miRNAs indicated that mRNA levels of predicted targets were reduced by miRNA activity. A recent study provided further evidence that miRNAs influence protein output mainly by destabilising target transcripts rather than just repressing their translation (Guo *et al.* 2010). Furthermore, emerging evidence suggests that primary and pre-miRNAs can also potentially interact with target mRNAs and repress gene expression (Trujillo *et al.* 2010). In particular, miRNA biosynthesis is influenced by steroid hormones. For example, DROSHA has been shown to interact with the activated oestrogen receptor (ER). This inhibited the maturation of a subset of pri-miRNAs and, consequently, the mRNA targets of these miRNAs were more abundantly expressed (Yamagata *et al.* 2009).

miRNAs and endometrial function

The endometrial remodelling in response to the rise and fall in ovarian oestradiol and progesterone production is exquisitely regulated by coordinated activation of the ER

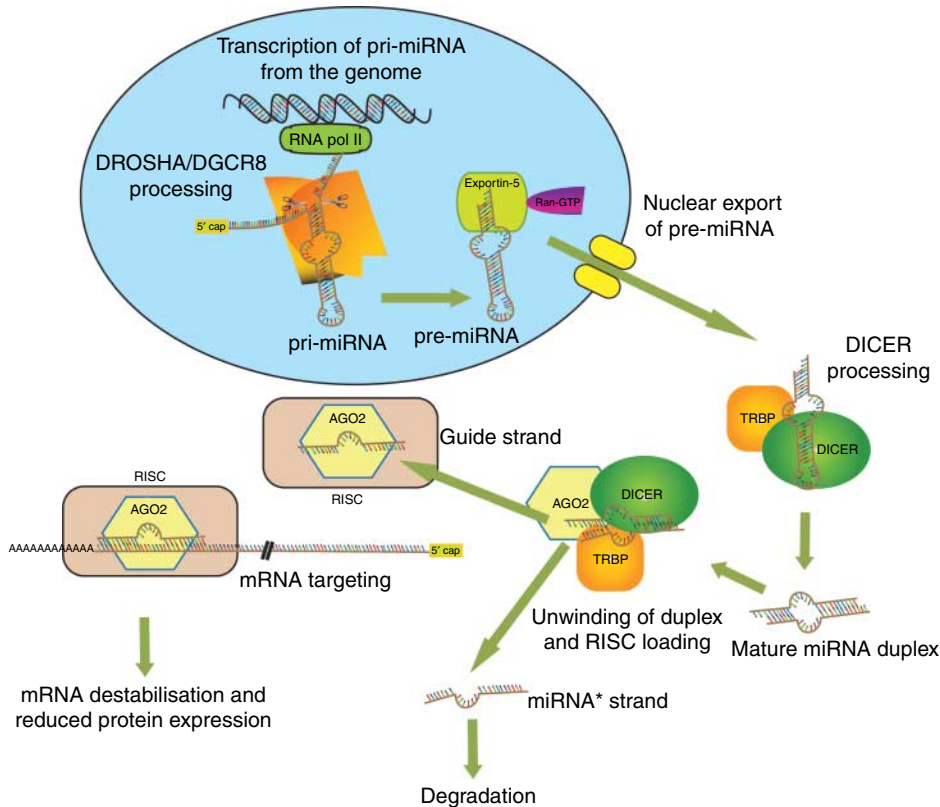


Figure 1 Biogenesis of miRNAs. Micro-RNA genes are transcribed from the genome by RNA polymerase II to generate primary transcripts (pri-miRNAs) that are > 1 kb in length. The microprocessor complex consisting of DROSHA and DGCR8 then processes the pri-miRNAs to release ~70 nucleotide stem-loop precursor miRNAs (pre-miRNAs). The re-miRNA molecules are exported from the nucleus to the cytoplasm by exportin-5 and Ran-GTP. To generate functional miRNAs, pre-miRNAs are further processed in the cytoplasm by Dicer, which dimerises with TRBP. DICER removes the loop region to generate a mature miRNA/miRNA* duplex. Unwinding of the duplex coincides with loading of one strand (the guide strand) onto the RNA-induced silencing complex (RISC), whereas the other (passenger) strand is discarded. The miRNA-loaded RISC also contains Argonaute (AGO) proteins, of which AGO2 has ribonuclease activity, and specific mRNA molecules can be targeted by RISC due to the ability of the guide strand to bind complementary sites in the 3'-UTR of mRNAs. This targeting has a negative impact on mRNA translation mediated mainly by destabilisation of the mRNA.

and progesterone receptor (PR) respectively, members of the superfamily of ligand-dependent nuclear transcription factors. Although ER and PR are expressed in both the epithelial and the stromal compartments, it should be observed that stromal cells also abundantly express androgen and glucocorticoid receptors (AR and GR respectively). AR expression resembles that of PR, with the highest levels occurring during the proliferative phase, followed by declining receptor levels throughout the secretory phase of the cycle (Cloke *et al.* 2010). In contrast, GR expression in the stromal compartment has been reported to be fairly constant across the cycle (McDonald *et al.* 2006).

Ovarian oestradiol induces the ordered growth of the endometrium, whereas the postovulatory rise in progesterone levels controls differentiation of this tissue in preparation for embryo implantation. This differentiation process, termed 'decidualisation', is characterised first by the secretory transformation of the endometrial glands, followed by an influx of immune cells, including macrophages and uterine

natural killer cells, angiogenesis and transformation of endometrial stromal cells into specialised epitheloid decidual cells (Brosens *et al.* 2002). In the absence of pregnancy, declining progesterone levels trigger a switch in the secretory repertoire of decidual stromal cells, now characterised by the expression of pro-inflammatory cytokines, chemokines and matrix metalloproteinases, which activate a sequence of events leading to tissue breakdown of the superficial endometrial layer, focal bleeding and menstrual shedding (Brosens & Gellersen 2006).

Compelling evidence has emerged to indicate that the ability of progesterone to counteract the proliferative effects of oestrogen involves expression of miRNAs that repress genes implicated in cell cycle progression. Profiling studies of the endometrium revealed 12 distinct miRNAs (miR-29b, miR-29c, miR-30b, miR-30d, miR-31, miR-193a-3p, miR-203, miR-204, miR-200c, miR-210, miR-582-5p and miR-345), whose expression was significantly upregulated in the secretory compared with the proliferative phase of

the cycle, when progesterone exerts its function. These miRNAs are predicted to repress the expression of many cell cycle regulators, including cyclins, cyclin-dependent kinases and the transcription factor E2F3, a validated target of miR-210 (Kuokkanen *et al.* 2010). Another microarray study identified 32 miRNAs differentially expressed between isolated human endometrial stromal and glandular epithelial cells (Pan *et al.* 2007). Furthermore, treatment of these primary cultures with synthetic steroids 17 β -oestradiol and medroxyprogesterone acetate differentially regulated the expression of miR-20a, miR-21 and miR-26a, which was partly reversed following co-treatment with the pure oestrogen ICI 182 780 (Pan *et al.* 2007). These observations highlight that miRNA expression in both the stromal and the epithelial compartments of the endometrium is under hormonal control.

Expression of DICER and the AGO proteins, which make up the RISC complex, is maintained at high levels in the pregnant uterus, at least in the mouse (Chakrabarty *et al.* 2007, Hu *et al.* 2008). Furthermore, 32 miRNA species were found to be upregulated during early pregnancy in the mouse (Chakrabarty *et al.* 2007). The expression of miR-101 and miR-199*, induced by oestradiol, was found to enhance the expression of cyclooxygenase-2, an enzyme indispensable for implantation in mice (Chakrabarty *et al.* 2007). Additional analysis identified 13 miRNAs upregulated at implantation sites compared with inter-implantation sites (Hu *et al.* 2008), indicating that the spatiotemporal expression of uterine miRNA is regulated by embryonic signals (Chakrabarty *et al.* 2007). Interestingly, placenta-specific miRNAs have been detected in the maternal circulation during pregnancy (Luo *et al.* 2009). Furthermore, the BeWo trophoblast cell line was shown to secrete miRNA in exosomes, raising the possibility that placental miRNAs are taken up at the foetal-maternal interface by decidual or immune cells with functional consequences on gene expression. The expression of miRNAs has also been studied in human endometrial stromal cells that were decidualised in culture for 4 days. Interestingly, out of 49 miRNAs regulated twofold or more, two-thirds were reported to be downregulated in decidualising cells (Qian *et al.* 2009). However, these results should be interpreted with caution as decidualising endometrial cells acquire a highly secretory phenotype, suggesting that miRNAs may be produced primarily for export. It should also be observed that several miRNA species can regulate ER and PR expression (Pandey & Picard 2010), and the experimentally validated miRNAs that can control ER expression include miR-18ab, miR-19, miR-22, miR-26ab/1297, miR-181, miR-206 and miR-222/221 (Adam *et al.* 2009, Castellano *et al.* 2009, Liu *et al.* 2009, Pandey & Picard 2009, Di Leva *et al.* 2010). PR expression has also been found to be modulated by miR-181 and miR-26ab/1297 (Castellano *et al.* 2009). However, the expression of these species and their function in the endometrium has not been examined systematically.

Role of miRs in ovarian hormone production

Endometrial function is also indirectly regulated by miRNA involved in modulating ovarian steroidogenesis. In the ovary, the granulosa and thecal cells support the growth and maturation of the oocytes as well as secreting hormones, including progesterone, testosterone and oestradiol to regulate the development of endometrium. Thus, miRNAs also regulate endometrial proliferation and development indirectly through modulating the ovarian development and hormone production. A number of ovarian-specific miRNAs have already been identified by miRNA profiling studies in mice (Choi *et al.* 2007, Ro *et al.* 2007, Mishima *et al.* 2008, Ahn *et al.* 2010). Of these, the miRNAs let-7a, miR-125b and miR-143 have also been confirmed to be negatively regulated by FSH in mouse granulosa cells of primary, secondary and antral follicles (Yao *et al.* 2009). In a similar human study, 212 specific miRNA species have been identified in the granulosa cells isolated from peri-ovulatory follicles, 13 of which are regulated by the LH (Fiedler *et al.* 2008). A genome-wide screen has identified both potential inhibitory and stimulatory miRNAs affecting the release of the major sex steroid hormones progesterone, testosterone and oestradiol in human ovarian cells, with 36, 51 and 57 specific miRNAs decreasing and 10, 0 and 1 miRNAs increasing progesterone, testosterone and oestradiol production respectively (Sirotkin *et al.* 2009).

Role of miRNAs in endometriosis

As the expression of endometrial miRNAs is under hormonal control, and in turn modulates the cellular responses to steroid signalling, several investigators have focussed on their role in pelvic endometriosis, a common oestrogen-dependent reproductive disorder characterised by the presence of endometrial tissues outside the uterine cavity (Eskenazi & Warner 1997). Endometriosis affects as many as 10% of all women of reproductive age and is a major cause of pelvic pain and conception delay. Microarray analyses have been employed to identify differentially expressed miRNAs in paired eutopic endometrium and ectopic lesions (Filigheddu *et al.* 2010, Teague *et al.* 2010). Although one study yielded 22 differentially expressed miRNAs and another 50 species, the validity of this approach is somewhat questionable, as endometriotic implants differ profoundly in architecture and cellular composition when compared with eutopic endometrium.

In recent years, abundant evidence has emerged to indicate that steroid hormone responses in eutopic endometrium are grossly perturbed in patients suffering from endometriosis. Microarray studies have been particularly informative to delineate the nature and magnitude of impaired endometrial gene expression in patients with endometriosis. For example, Kao *et al.* (2003) identified in excess of 200 dysregulated genes

in mid-secretory biopsies from women with minimal or mild endometriosis compared with disease-free controls. A subsequent study showed that impaired gene expression in eutopic endometrium of patients with endometriosis encompasses the entire cycle, including the proliferative phase, although the most extensive perturbations were found in early secretory endometrium (Burney *et al.* 2007). Many of the dysregulated genes identified during this phase of the cycle are not only *bona fide* progesterone targets but the overall signature suggests a persistent proliferative phenotype of the endometrium. Interestingly, the perturbed gene expression in eutopic secretory endometrium of affected patients coincides with differential expression of several miRNA species (Burney *et al.* 2009). In particular, members of the miR-9 and miR-34 families (i.e. miR-9, miR-34b* and miR-34c-5p) are downregulated in the endometrium from women with endometriosis compared with healthy individuals. The predicted targets of the miR-9 and miR-34 families include the cell-cycle regulator genes *cyclin E1*, *cyclin E2*, *CDK4*, *CDK6* and *CDC25A* and the anti-apoptotic gene, *BCL-2*, which are also differentially expressed in endometrium from women with and without endometriosis.

FOXO proteins: regulators of hormone-dependent cell fate

FOXO transcription factors are at the centre of an emerging paradigm that links longevity, cell fate and tumour development. Like members of the p53 family of tumour suppressors, FOXO proteins have the innate ability to control distinct and opposing gene programs, leading to cell death or survival and differentiation. Although only partially understood, this contrasting behaviour of FOXO proteins is largely determined by specific and interrelated posttranslational modifications that dynamically respond to changes in growth factor, hormonal and environmental cues. Perhaps, the best characterised posttranslational modification of FOXO proteins involves inactivating phosphorylation by the PI3K/AKT pathway in response to insulin and growth factor signalling. PI3K/AKT-dependent phosphorylation of FOXO proteins triggers their export from the nucleus in a CRM1 transporter-dependent manner and promotes binding of these transcription factors to the 14-3-3 chaperone proteins in the cytosol. PI3K/AKT signalling further antagonises FOXO-dependent transcription by earmarking the phosphorylated proteins for proteasomal degradation (Plas & Thompson 2003). Other kinases, such as serum- and glucocorticoid-inducible kinase 1 (SGK1), casein kinase 1 (CK1) and dual-specificity tyrosine-phosphorylated and regulated 1A (DYRK1A), have also been implicated in FOXO phosphorylation and nuclear export (Brunet *et al.* 2001, Woods *et al.* 2001, Rena *et al.* 2002, Feroze-Zaidi *et al.* 2007). In addition, stress signalling and activation of mitogen-activated protein kinase pathways, including JNK, ERK and p38, also converge on FOXO proteins, often enhancing their

transcriptional activity (Essers *et al.* 2004, Asada *et al.* 2007, Yang *et al.* 2008, Clavel *et al.* 2010).

In addition to phosphorylation and ubiquitination, FOXO proteins are also subject to methylation, O-GlcNAcylation and acetylation. The sum of these posttranslational modifications determines the transcriptional output of FOXO proteins. A case in point is the functional consequences of altered FOXO1 acetylation. Protein acetylation is essentially controlled in a substrate-specific manner by a balance between histone acetyl transferase and histone deacetylase (HDAC) activity. In case of FOXO1, acetylation is mediated by p300/CBP, whereas deacetylation depends on binding to the class III HDAC SIRT1 (Matsuzaki *et al.* 2005, Calnan & Brunet 2008). Intriguingly, SIRT1 promotes the pro-survival function of FOXO1, insofar that it directs FOXO activity away from pro-apoptotic genes (e.g. *FASL* and *BIM*) towards target genes involved in stress resistance (e.g. *GADD45A*) and cell cycle arrest (e.g. *CDKN1B*) genes (Vogt *et al.* 2005).

FOXO proteins are of particular relevance for cell fate decisions in hormone-responsive reproductive tissues, such as prostate, breast, ovary and uterus, because of their complex interactions with nuclear steroid receptors (Fig. 2). For example, FOXO1 binds PR, an interaction essential for endometrial homeostasis (Christian *et al.* 2002c, Takano *et al.* 2007), as outlined in more detail below. In breast cancer cells, FOXO3a engages in cross talk with the ER and inhibits its transcriptional activity of this nuclear receptor (Zou *et al.* 2008, Morelli *et al.* 2010). Accordingly, ectopic expression of FOXO3a in a breast cancer mouse model suppresses oestradiol-dependent tumourigenesis (Zou *et al.* 2008). In the normal prostate and early prostate cancers, the liganded AR induces the expression of genes involved in cell proliferation and survival (Li *et al.* 2008). FOXO proteins, including FOXO1 and FOXO3a, interact with the activated AR, thereby attenuating its DNA binding activity and inhibiting its ability to upregulate pro-apoptotic genes, such as *FASL* (Li *et al.* 2003). In addition, interaction with FOXO proteins may further attenuate AR activity by preventing the recruitment of co-activators (Ma *et al.* 2009). Therefore, the loss of FOXO3a expression, or its inactivation by inhibitory phosphorylation and other posttranslational modifications, is a hallmark androgen-independent progression of human prostate cancer (Lynch *et al.* 2005, Cornforth *et al.* 2008, Yang & Hung 2009).

FOXO transcription factors in cycling endometrium

As mentioned, FOXO1 is the most prominent and best characterised family member in human endometrium. It was first identified in this tissue as a transcriptional regulator of prolactin (PRL), a defining differentiation marker gene of decidualising endometrial stromal cells (Christian *et al.* 2002a,b). Subsequent siRNA depletion studies revealed that the transcription factor FOXO1 is also central to the

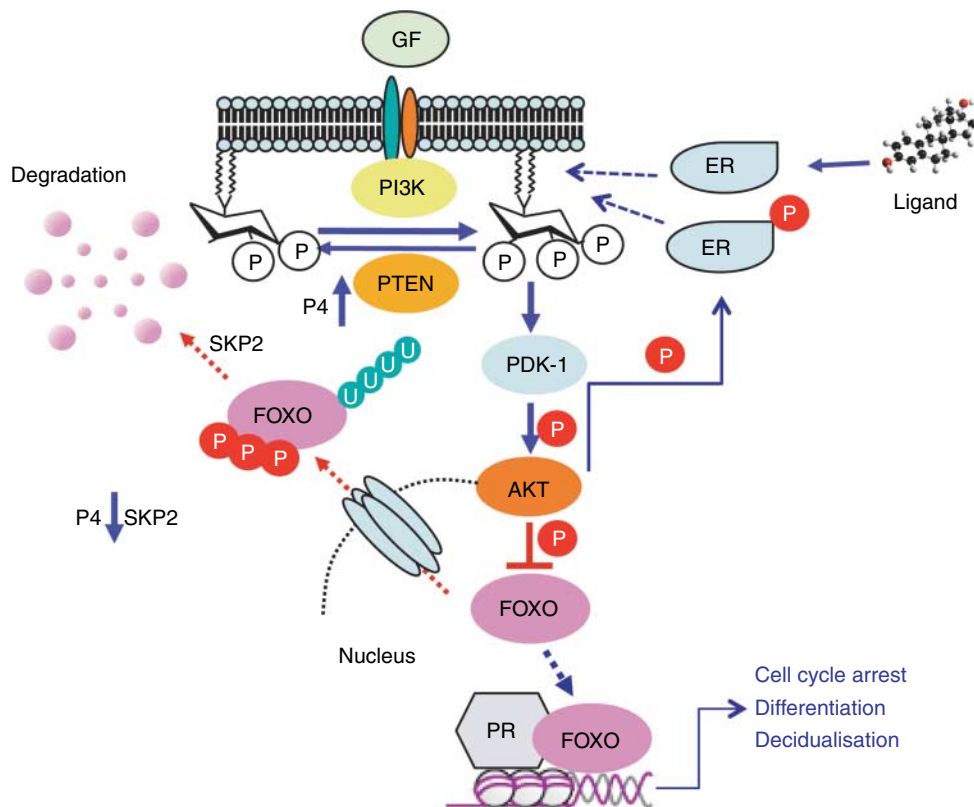


Figure 2 The effects of steroid hormone signalling on the PI3K/AKT/FOXO pathway. Progesterone signalling in normal endometrium promotes the transcriptional activity in FOXO1, which is necessary for decidualisation. Progesterone increases PTEN expression, which leads to PIP3 turnover and a consequent reduction in AKT signalling. Some FOXO1 remains unphosphorylated and nuclear, which enables FOXO1/PR target genes to be expressed. Progesterone also leads to activation of SGK1 and phosphorylation of FOXO1. Some FOXO1 is, therefore, translocated to the cytoplasm; however, progesterone also decreases the expression of SKP2, the FOXO1 ubiquitin ligase and inactive FOXO1 accumulates in the cytoplasm instead of being degraded. Loss of PTEN, which is observed in many cancers, promotes the accumulation of phosphatidylinositol-3,4,5-triphosphate (PIP3), which results in hyperactivity of AKT and FOXO phosphorylation. In the phosphorylated state, FOXO is excluded from the nucleus and degraded in the cytoplasm in a ubiquitin-dependent manner. FOXO target genes are, therefore, not expressed due to loss of FOXO. Oestradiol-bound ER can activate PI3K by binding to the p85 regulatory subunit and promote the generation of PIP3. AKT also phosphorylates ER, which further promotes the production of PIP3 and consequent AKT signalling.

induction of the decidual programme (Grinius *et al.* 2006). The expression of FOXO1 in mid-secretory endometrium coincides with that of several other transcriptional regulators of the decidual process, including p53, STAT5 and C/EBP β (Christian *et al.* 2002a,b,c, Mak *et al.* 2002, Pohnke *et al.* 2004). Moreover, many of these factors are capable of physically interacting with the activated PR and regulate PRL expression by binding to a discrete region in the proximal decidual-specific promoter that contains overlapping FOXO- and C/EBP β -DNA binding sites as well as PR response element half-sites. Furthermore, a recent study demonstrated that recruitment of FOXO1 to the decidual PRL promoter converts HOXA11, a transcription factor essential for development and function of the female reproductive tract, from a repressor to a strong activator of gene expression (Lynch *et al.* 2009). These observations suggest that the activated PR serves as a platform for binding cAMP-induced transcription factors, including FOXO1, and that these

multimeric complexes regulate the expression of decidual gene networks (Jones *et al.* 2006, Gellersen *et al.* 2007).

Although the primary signal for induction and nuclear accumulation of FOXO1 in decidualising endometrial cells is cAMP, its transcriptional output is tightly regulated by progesterone signalling through multiple mechanisms. First, progesterone triggers a reciprocal inhibition of AKT activity and enhanced SGK1 expression and phosphorylation, which in turn causes a partial translocation of FOXO1 to the cytoplasm (Feroze-Zaidi *et al.* 2007). Secondly, progesterone inhibits the expression of Skp2, the oncogenic subunit of the Skp1/Cul1/F-box protein complex that directs FOXO1 ubiquitination and proteasomal degradation (Huang *et al.* 2005). Consequently, decidual cells also accumulate a pool of inactive FOXO1 in the cytoplasm, in addition to nuclear FOXO1, in response to continuous progesterone signalling. Withdrawal of progesterone from differentiated cultures leads to re-accumulation of nuclear FOXO1, enhances BIM

expression and induces cell death, a response that may initiate menstrual shedding of the endometrium upon falling circulating progesterone levels at the end of the cycle (Labied *et al.* 2006).

By combining small interfering RNA technology and microarray screening, Takano *et al.* (2007) found that ~15% of all regulated upon differentiation of human endometrial fibroblasts into decidual cells are under direct or indirect control of FOXO1. Not surprisingly, several FOXO1-dependent genes encode for major differentiation markers (e.g. *IGFBP1*, *PRL* and *LEFTY2*) as well as for numerous proteins involved in cell cycle regulation. For example, FOXO1 regulates the induction of *CDKN1C* (p57^{Kip2}), a cyclin-dependent inhibitor involved in G1 arrest, while simultaneously repressing several genes important for DNA replication/S phase (e.g. *MCM5*), G2/M transition (e.g. *CCNB1*, *CCNB2*, *CDC2*, *BIRC5* and *BRIP1*) or mitosis (e.g. *PRC1*, *NUSAP1*, *CENPE*, *SPBC25* and *ASPM*). Decidualisation of endometrial stromal cells is further associated with silencing of the JNK signalling pathway and its downstream target FOXO3a, which in turn protects these cells against oxidative cell death in pregnancy (Kajihara *et al.* 2006, Leitao *et al.* 2010).

The ability of FOXO proteins to regulate cellular homeostasis, i.e. to activate or repress the appropriate gene networks in response to hormonal, growth factor and environmental cues reflects the fact that a wide range of signal transduction pathways converge on these transcription factors and, conversely, that FOXO protein can regulate the activity of upstream pathways. For example, FOXO transcription factors bind to SMAD3 and SMAD4 in response to transforming growth factor- β (TGF- β) signalling and to β -catenin upon activation of the canonical WNT pathway (Seoane *et al.* 2004, Essers *et al.* 2005). On the other hand, FOXO1 induces the expression of *INSR* in endometrial cells, encoding the insulin receptor as well as *WNT4* and BMP and activin membrane-bound inhibitor (*BAMBI*; Takano *et al.* 2007). *BAMBI* is a transmembrane glycoprotein related to TGF- β family type I receptors. However, it lacks an intracellular kinase domain and serves as a potent inhibitor of BMP, activin and TGF- β signalling (Sekiya *et al.* 2004). This ability of FOXO proteins to engage in complex feedback mechanisms that fine-tune the activity of upstream regulatory pathways is not confined to endometrial cells. For example, in chemoresistant cancer cells, FOXO3a has been shown to induce *PIK3CA* expression, a gene that encodes for the Class 1A PI3K catalytic subunit p110 α . Importantly, p110 α is a rate-limiting factor of PI3K/AKT activation in response to growth factor signalling (Hui *et al.* 2008). In *Drosophila*, the dFOXO orthologue also induces the expression of the insulin receptor (Puig *et al.* 2003), resulting in increased PI3K activity and cell growth under low-nutrient conditions. Another example involves hepatocytes where FOXO1 represses the expression of tribble 3 (*Trb3*), a pseudokinase that antagonises AKT (Matsumoto *et al.* 2006). Thus, depending on the cellular and environmental context, FOXO proteins seem to

be capable of enhancing as well as inhibiting the activity of the upstream inhibitory PI3K/AKT pathway.

FOXO1 is also expressed in the epithelial compartment of the endometrium, but little is known of its function in these cells during the menstrual cycle. Based on the cell line work, however, there is evidence that expression of *FOXO1* in epithelial endometrial cells is under direct control of that activated PR and essential to mediate the anti-proliferative effects of progesterone and synthetic progestins (Shimizu *et al.* 2010).

The miRNA-FOXO axis in endometrial carcinogenesis

Endometrial cancer is the commonest malignancy of the female genital tract in Europe and North America with an incidence of ~22 per 100 000 women per year (Bray *et al.* 2005, Parkin *et al.* 2005). Worldwide, ~50 000 patients die from this cancer each year. Endometrial carcinoma can be divided into two groups: type I (endometrioid) and type II (non-endometrioid). Type I, which accounts for 80% of all endometrial carcinomas, is oestrogen related, low grade, affects pre- and peri-menopausal women and is often preceded by complex atypical endometrial hyperplasia. In contrast, type II tumours are not oestrogen driven and mostly develop in atrophic endometrium.

The *PTEN* tumour suppressor gene encodes a protein/lipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate, the second messenger of the PI3K signalling pathway. Thus, *PTEN* antagonises PI3K activity, inhibits AKT and thus enhances FOXO activity (Fig. 2). It has been estimated that *PTEN* is either completely lost or mutated in 37–51% of primary endometrioid endometrial cancers (EEC), the highest incidence of mutation in this tumour suppressor gene in any cancer analysed to date (Kong *et al.* 1997). *PTEN* is already mutated in 20% of endometrial hyperplasia (Maxwell *et al.* 1998a,b, Risinger *et al.* 1998), indicating that is a very early genetic event in the multi-step process leading to EEC. Furthermore, *SKP2*, the FOXO1 ubiquitin ligase, is overexpressed in endometrial cancer (Kau *et al.* 2003, Lahav-Baratz *et al.* 2004). Thus, based on the loss of *PTEN*, leading to PI3K/AKT hyperactivity, and increased *SKP2* expression, it was predicted that FOXO proteins would be rapidly degraded in EEC. Analysis of 60 surgical specimens revealed indeed that FOXO1 immunoreactivity was undetectable in ~75–80% of EEC, irrespective of the grade of the tumours (Goto *et al.* 2008). However, additional analysis demonstrated that loss of FOXO1 expression in EEC was not primarily a consequence of increased protein turnover. Unexpectedly, FOXO1 transcripts were sixfold less abundant in EEC than in cycling endometrium, whereas the expression of FOXO3a and FOXO4 mRNAs did not differ.

Methylation-specific PCR analysis revealed that the FOXO1 promoter becomes methylated in EEC, but only partially and inconsistently (Goto *et al.* 2008). At the same

Table 1 Differentially expressed miRNAs in endometrioid endometrial cancers (EEC)

Study (reference)	miRNAs differentially expressed in endometrioid endometrial cancers (EEC)
Cohn <i>et al.</i> (2010)	Stage I/IIA grade I EEC versus benign tissue from control patients ↑: miR-9, miR-19b, miR-146, miR-181c, miR-183, miR-200c, miR-205, miR-223, miR-423, miR-425 ↓: let-7a, miR-32, miR-33b, miR-369, miR-409, miR-424, miR-431, miR-451, miR-496, miR-503, miR-516
Ratner <i>et al.</i> (2010)	EEC carcinoma versus benign tissue from control patients ↑: miR-34a, miR-182, miR-183, miR-200a, miR-205, miR-572, miR-622, miR-650 ↓: miR-411, miR-487b
Chung <i>et al.</i> (2009)	EEC versus benign tissue from control patients ↑: miR-10a, miR-23a, miR-25, miR-28, miR-34a, miR-95, miR-103, miR-106a, miR-107, miR-130b, miR-141, miR-151, miR-155, miR-17-5p, miR-182, miR-183, miR-184, miR-191, miR-194, miR-200a, miR-200c, miR-203, miR-205, miR-210, miR-215, miR-223, miR-301, miR-325, miR-326, miR-330 ↓: miR-125b, miR-296, miR-323
Wu <i>et al.</i> (2009)	EEC adenocarcinoma versus adjacent benign tissue from EEC patients ↑: miR-10a, miR-31, miR-96, miR-133b, miR-141, miR-142-5p, miR-155, miR-182, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-210, miR-363, miR-429, miR-432, miR-449 ↓: miR-99b, miR-133, miR-193a, miR-193b, miR-204, miR-368
Boren <i>et al.</i> (2008)	EEC versus atypical hyperplasia versus normal endometrium ↑: let-7c, miR-103, miR-106a, miR-107, miR-181a, miR-185, miR-210, miR-423 ↓: let-7i, miR-30c, miR-152, miR-193, miR-221

time, it became increasingly apparent that deregulated miRNA expression was a hallmark of endometrial carcinogenesis (Table 1). Initial profiling analysis of miRNAs in normal endometrium, atypical hyperplasia and endometrial cancer uncovered 13 miRNA species to be differentially expressed. Within this group, five miRNAs (miR-let 7i, miR-30c, miR-221, miR-193 and miR-152) were decreased and eight (miR-let 7c, miR-185, miR-106a, miR-181a, miR-210, miR-423, miR-103 and miR-107) increased upon transition from normal endometrium to atypical hyperplasia to cancer (Boren *et al.* 2008). Among the most differentially expressed miRNAs, miR-205, miR-182 and miR-200a are most upregulated in endometrioid samples, whereas miR-411 was most downregulated in cancer samples compared with benign tissue (Ratner *et al.* 2010). Another small-scale screening analysis revealed 30 miRNAs that were significantly dysregulated in EEC (Chung *et al.* 2009). Together, these miRNA profiling studies demonstrated that miRNA expression is commonly dysregulated in human endometrial cancer and that there are putative miRNA signatures for endometrial cancer (Takano *et al.* 2007, Boren *et al.* 2008, Chung *et al.* 2009, Wu *et al.* 2009, Cohn *et al.* 2010, Ratner *et al.* 2010). For example, the miRNAs miR-182, miR-183 and miR-205 are consistently upregulated in endometrial cancer samples compared with benign tissues. However, despite the strong correlations between miRNA expression patterns and endometrial cancer, little is known about the role of these differentially expressed miRNAs and their targets in endometrial cancer tumourigenesis and progression (Table 2; Huang *et al.* 2009, Myatt *et al.* 2010).

A recent expression profiling study of miRNAs in normal endometrium and EEC has identified an inverse correlation between FOXO1 expression and the abundance of several of the *in silico*-predicted miRNAs (i.e. miR-9, miR-27, miR-96, miR-153, miR-182, miR-183 and miR-186) that

potentially bind the 3'-UTR of FOXO1 transcripts (Myatt *et al.* 2010). This finding suggests that loss of FOXO1 expression in endometrial cancer may be mediated by miRNAs. Consistently, these differentially expressed miRNAs are also overexpressed in the endometrial cancer cell line Ishikawa, which expresses low levels of FOXO1 but not in HEC-1B cells, another endometrial cancer cell line characterised by high FOXO1 expression. Importantly, overexpression of any of the differentially expressed miRNAs in HEC-1B cells was sufficient to significantly reduce FOXO1. Conversely, FOXO1 expression could be efficiently restored in the Ishikawa cell line upon simultaneous depletion of miR-9, miR-27, miR-96, miR-153, miR-183 and miR-186. 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. These findings suggest that a group of miRNAs act coordinately to repress FOXO1 expression, which in turn deregulates cell cycle control and apoptotic responses in endometrial cancer. A similar scenario may contribute to breast cancer development

Table 2 Validated functional consequences for miRNA in endometrioid endometrial cancers**Functional miRNA studies in endometrial cancer**

Study (reference)	Main functional findings for miRNA
Cohn <i>et al.</i> (2010)	Repression of miR-129-2 expression by epigenetic mechanisms leads to SOX4 overexpression in endometrial cancer
Myatt <i>et al.</i> (2010)	miR-9, miR-27, miR-96, miR-153, miR-182, miR-183 and miR-186 regulate FOXO1 expression in endometrial cancer cell lines

where FOXO1 transcripts and expression are also down-regulated in tumour samples compared with normal tissue. Silencing of the miRNA processing enzymes, DROSHA and DICER, in breast cancer cell lines results in enhanced FOXO1 expression (Guttilla & White 2009). Indeed, three miRNAs (miR-27a, miR-96 and miR-182) that limit FOXO1 expression in EEC are also highly expressed in breast cancer cells with low FOXO1 levels. Antisense inhibitors to each of these miRNAs significantly increase endogenous FOXO1 expression and decrease cell proliferation and viability (Guttilla & White 2009). Thus, in terms of disabling the tumor suppressor function of FOXO1 in hormone-responsive endometrial and breast cancer, miRNAs play an important, if not central, role.

Future directions: miRNAs as cancer biomarkers and therapeutic targets

MiRNA represents a new class of biomarkers that can complement existing conventional markers, including metabolites, antigens and mRNA transcripts. The fact that miRNAs remain largely intact in formalin-fixed, paraffin-embedded clinical samples highlights their potential in molecular phenotyping of benign and malignant reproductive disorders as well as for monitoring treatment efficacy (Bitossi *et al.* 2008, Szafranska *et al.* 2008, Tam 2008, Jorgensen *et al.* 2010, Nuovo 2010, Schuster *et al.* 2010). Furthermore, recent evidence suggests that some neoplastic processes, for example, ovarian cancer, generate mRNA profiles in blood cells characteristic of the tumours, suggesting that whole blood miRNA profiling could be used for diagnostic purposes (Balch *et al.* 2009, Lodes *et al.* 2009, Resnick *et al.* 2009, Hausler *et al.* 2010). Evidence for miRNA secretion by the trophoblast has already been established, but it is not yet known if secretion is also a feature of decidualising endometrium. Nevertheless, the clear potential exists to use circulating miRNAs for the prediction of pregnancy complications associated with impaired placentation, such as miscarriage, pre-eclampsia and foetal growth restriction.

In the context of proliferative disorders of the endometrium, such as endometriosis and EEC, it is striking, but perhaps not entirely surprising, that many deregulated miRNAs potentially target cell cycle regulators, oncogenes and intermediates in the PI3K/AKT/FOXO as well as the steroid hormone signalling pathways. This observation emphasises the importance of hormone-dependent coordinated miRNA expression in ensuring tissue homeostasis during the process of cyclic cell proliferation, differentiation, apoptosis and tissue breakdown. Besides targeting oncomiRs in the context of endometrial hyperplasia or cancer, it is conceivable that miRNAs or components of the processing machinery could also be targeted for fertility control. Therapeutic interventions, however, will require a much more in-depth understanding of the role of miRNA in modulating the endometrial responses to ovarian hormones.

Thus, miRNAs represent an important class of diagnostic and prognostic biomarkers and therapeutic targets that warrant further investigation before their true potential can be realised.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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