Interplay between steroid signalling and microRNAs: implications for hormone-dependent cancers

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Abstract

Hormones are key drivers of cancer development. To date, interest has largely been focussed on the classical model of hormonal gene regulation, but there is increasing evidence for a role of hormone signalling pathways in post-translational regulation of gene expression. In particular, a complex and dynamic network of bi-directional interactions with microRNAs (miRs) at all stages of biogenesis and during target gene repression is emerging. miRs, which act mainly by negatively regulating gene expression through association with 3' -UTRs of mRNA species, are increasingly understood to be important in development, normal physiology and pathogenesis. Given recent demonstrations of altered miR profiles in a diverse range of cancers, their ability to function as oncogenes or tumour suppressors, and hormonal regulation of miRs, understanding mechanisms by which miRs are generated and regulated is vitally important. miRs are transcribed by RNA polymerase II and then processed in the nucleus by the Drosha-containing Microprocessor complex and in the cytoplasm by Dicer, before mature miRs are incorporated into the RNA-induced silencing complex. It is increasingly evident that multiple cellular signalling pathways converge upon the miR biogenesis cascade, adding further layers of regulatory complexity to modulate miR maturation. This review summarises recent advances in identification of novel components and regulators of the Microprocessor and Dicer complexes, with particular emphasis on the role of hormone signalling pathways in regulating their activity. Understanding hormone regulation of miR production and how this is perturbed in cancer are critical for the development of miR-based therapeutics and biomarkers.

An overview of microRNA biogenesis

MicroRNAs (miRs) were first discovered in 1993, with two independent studies describing the Caenorhabditis elegans lin-4 gene product as a small non-coding RNA (ncRNA; Lee et al. 1993, Wightman et al. 1993). Later it was shown that the C. elegans let7 gene product, also an ncRNA, could associate with the 3'-UTR of target genes to initiate a cascade of regulatory processes acting on heterochronic genes (Reinhart et al. 2000). The functionality of miRs in other cell systems was first demonstrated by three groups in 2001 (Lagos-Quintana et al. 2001, Lau et al. 2001, Lee & Ambros 2001). It is now understood that miRs act as gene regulators in almost all plant and animal species.

Mammalian miRs are transcribed by RNA polymerase II, generating a primary miR (pri-miR) transcript containing one or more hairpin structures, comprising a stem and...
terminal loop. Following transcription, pri-miRs are 5' capped and adenylated. The majority are polycistronic and generate several functional mature miRs. In the nucleus, the pri-miR is cleaved into one or more ~70 nt precursor miRs (pre-miRs), which have a hairpin structure; this is performed by the Microprocessor complex, which contains the RNase III enzyme, Drosha, and the double-stranded RNA-binding domain protein DiGeorge Critical Region 8 (DGCR8)/Partner of Drosha (Pasha). Purified Drosha can cleave pri-miRs in vitro (Gregory et al. 2004), but requires cofactor proteins for increased efficiency and specificity; therefore the Drosha Microprocessor is very large multi-protein complex (>650 kDa in human cells (Gregory et al. 2002, Lee et al. 2003)). Containing at least 20 different polypeptides, thought to promote fidelity, specificity and/or Drosha cleavage activity and to act as scaffolding proteins. Some general cofactors modulate biogenesis of the majority of miRs, e.g. DGCR8, whilst others promote or inhibit biogenesis of a defined miR subgroup, e.g. KH-type splicing regulatory protein 1 (KHSRP1), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), and the DEAD box RNA helicases, p68 (DDX5) and p72 (DDX17) (Fukuda et al. 2007). The primary function of DGCR8 is to bind to the junction between single-stranded and double-stranded regions of the pri-miR stem via its two RNA-binding domains, leading Drosha to cleave ~11 bp from the junction. DGCR8 ensures correct orientation of Drosha on the miR hairpin (Landthaler et al. 2004). Only about 25–100 nucleotides surrounding both ends of the stem–loop are required for Drosha cleavage and pri-miR processing (Zeng et al. 2002, Lee et al. 2003).

Drosha cleavage results in a product with a 2 nt 3' overhang, which is vital both for recognition by Exportin 5, which facilitates Ran-GTP-dependent export of the pre-miR to the cytoplasm, and for Dicer-mediated pre-miR cleavage, because processing occurs at a defined distance from the free RNA end produced by Drosha. In the cytoplasm, pre-miRs are further processed by another RNase III enzyme, Dicer (enhanced by the accessory dsRBD protein TAR RNA-binding protein (TRBP)/PACT), which cleaves the stem loop to produce a ~22 nt miR duplex. The two strands subsequently separate and one strand associates with Argonaute 2 (AGO2), a protein component of the RNA-induced silencing complex (RISC). Unwinding of the duplex to generate the guide strand and the passenger strand is achieved by a combination of helicases associated with the RISC complex (exactly which remains to be determined, although helicases associated with RISC formation and/or activity include p68, p72, RNA helicase A, TNRC6B and Gemin3/4 (Meister et al. 2005, Robb & Rana 2007, Salzman et al. 2007)). The mature miR guides RISC to complementary sequences within the 3'-UTR of target mRNAs, resulting in translational repression and/or transcript degradation. For a subset of intronic miRs (mirtrons), following transcription, pre-miR hairpins are released from host introns by splicing and debranching to bypass Drosha-mediated cleavage (Okamura et al. 2007, Ruby et al. 2007). These pre-miRs re-enter the conventional processing pathway in the cytoplasm, where they are further processed by Dicer. An overview of miR biogenesis is shown in Fig. 1.

Currently, little is known about the factors determining which strand of the miR duplex is incorporated into RISC. One important parameter may be thermodynamic asymmetry of the miR duplex, because the strand with the less stably base-paired 5’ end is more frequently incorporated into RISC (Kawamata et al. 2009, Pratt & MacRae 2009, Yang et al. 2011). The other strand (passenger strand or miR* strand) was thought to be degraded (Yang et al. 2011). However, recent deep-sequencing experiments in Drosophila have revealed that frequently miR*s are not degraded but associated with AGO1 or AGO2, hence both strands of the miR duplex are functional (Okamura et al. 2008, Mah et al. 2010, Yang et al. 2011), and 3’-UTR-repressive activities have been assigned to several human miR*s (Kim et al. 2011, Yang et al. 2011). Humans express four different AGO proteins demonstrating similar preferences for miR duplex structures, in that central mismatches promote RISC association (Murphy et al. 2008). However, it is likely that many other factors beyond the scope of this review could impinge upon strand selection.

**Regulation of miR maturation**

miR biogenesis is emerging as a stringently controlled and remarkably complex pathway; coordinated regulation likely serves to prevent mis-expression of miRs, both spatially and temporally. miR levels are thought to be particularly important in development and tumourigenesis. For example, in early development many pri-miRs are expressed but not efficiently converted into their mature forms, while reduced processing has been shown to contribute to widespread downregulation of many miRs in human cancers. It is widely accepted that miRs can function as oncogenes and tumour suppressors (Shenouda & Alahari 2009). Global miR downregulation has been identified as a general characteristic of a number of human cancers (Kumar et al. 2007, Ozen et al. 2008), suggesting abrogation of miR biogenesis may induce or maintain tumourigenesis. Indeed, downregulation of Drosha and Dicer and mutations in genes encoding TRBP and Exportin...
Figure 1
Regulators of microRNA biogenesis. Schematic illustration of proteins that modulate miR maturation through multiple mechanisms. miRs regulated by a particular factor are displayed beside that protein. miR, microRNA; O-methyl, O-methylation; AI, adenosine to inosine; ADAR, deaminase acting on RNA; pri-miR, primary microRNA; pre-miR, precursor microRNA; ERα, estrogen receptor alpha; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; KHSRP1, KH-type splicing regulatory protein 1; ARS2, arsenite-resistance protein 2; AR, androgen receptor; BRCA1, breast cancer type 1 susceptibility protein; TDP43, TAR DNA-binding protein 43; DGCR8, DiGeorge critical region 8; TRIM32, tripartite motif-containing protein 32; NHL2, NDR1/HIN1-like protein 2; XRNX2, 5’-3’ exoribonuclease 2; GLD2, defective in germ line development 2; AGO2, Argonaute 2; TUT4, terminal uridylyltransferase 4; TRBP, HIV 1 TAR RNA-binding protein; BCDIN3D, BCDIN3 domain-containing protein; MCPIP1, monocyte chemotactic protein-induced protein 1.
miR-specific regulation by Microprocessor The Microprocessor complex contains many proteins and there is evidence that certain of these may be important for processing distinct subsets of miRs, suggesting these proteins can act as specificity factors; for instance, depletion of Microprocessor cofactors p68 or p72 in knockout mice results in reduced levels of a subset of mature miRs, whilst others remain unchanged (Fukuda et al. 2007). In addition, the tumour-suppressor protein p53 and TGFβ/BMP4-stimulated SMAD are known to enhance the processing of distinct subsets of miRs through interaction with p68 and Drosha to increase pri-miR cleavage (shown in Fig. 1; Davis et al. 2008, Suzuki et al. 2009). One miR whose biogenesis is enhanced by SMAD is miR-21, which targets several tumour-suppressor genes including PTEN, therefore may act as an oncomiR (Kumaraswamy et al. 2011). Also BRCA1, which is vital for maintenance of genomic stability, increased the levels of mature cancer-associated miRs including let7a-1, miR-16-1 and miR-34a via a mechanism involving direct association of BRCA1 with Drosha and pri-miRs through recognition of branched secondary structures (Kawai & Amano 2012). Processing of the BRCA1-regulated miR subset is likely to be lost in BRCA1-mutant cells, perhaps a mechanism by which BRCA1 mutation enhances tumourigenesis.

Self-regulation of the Microprocessor Drosha and DGCR8 can mutually regulate each other’s function post-translationally: DGCR8 interacts with the central domain of Drosha to stabilise Drosha protein (Yeom et al. 2006), while the Drosha–DGCR8 complex cleaves hairpin structures in the 5′-UTR and coding region of the DGCR8 transcript, leading to the degradation of DGCR8 mRNA, generating a negative feedback loop to regulate DGCR8 levels if Microprocessor activity is sufficient (Han et al. 2009). These data imply collaborative and dynamic mutual regulation of pathways that regulate both mRNA stability and miR maturation.

Regulation of miR maturation by RNA processing modulators miRNA splicing regulators have been shown to preferentially induce maturation and accumulation of one or more miRs from a polycistronic pri-miR transcript (Michlewski et al. 2008). hnRNPA1 is a multifunctional RNA-binding protein involved in many aspects of RNA processing, including alternative splicing. Its direct association with its consensus binding site, UAGGGA/U, in pri-miR stem–loops can either increase Drosha processing by inducing a stem–loop conformational shift, in the case of pri-miR-18a (Guil & Caceres 2007, Newman & Hammond 2010), or repress maturation by inhibiting Drosha association, as is the case for let7a (Michlewski & Caceres 2010). TAR DNA-binding protein 43 shares significant sequence homology with hnRNPs and promotes miR biogenesis as a component of both the Microprocessor, where it binds pri-miRs and Drosha, and the cytoplasmic Dicer complex, where it associates with the terminal loop regions of a subset of pre-miRs to enhance their maturation (Kawahara & Mieda-Sato 2012).

The RNA-binding protein KHSRP1 (also known as FBP2) also regulates miR biogenesis as a component of both the Microprocessor and Dicer complexes (Trabucchi et al. 2009). KHSRP1 (previously shown to interact with single-stranded, AU-rich mRNAs to mediate mRNA decay (Gherzi et al. 2004)) promotes maturation of a miR subset through association of its KH3 domain with a specific 5′ GGG triplet within terminal loops of target pre-miRs, such as pre-let7a-1 and pre-miR-155 (Ruggiero et al. 2009, Trabucchi et al. 2009). Furthermore, KHSRP is itself a target of AR signalling hence could mediate androgen regulation of this miR subset (Massie et al. 2007; C E Fletcher, D A Dart and C L Bevan, unpublished observations).

TRBP is a key component of the Dicer-containing complex (Melo et al. 2009). Truncating TRBP mutations have been identified in colorectal and endometrial carcinoma cell lines with microsatellite instability, leading to decreased TRBP protein, Dicer destabilisation and defective pre-miR processing. Reintroduction of TRBP into TRBP-deficient colon cancer cells restored processing of known tumour-suppressive miRs such as miR-26a (Sander et al. 2008) and miR-125a (Scott et al. 2007), and interestingly, repressed the cells’ ability to form tumours in nude mice (Melo et al. 2009), suggesting that TRBP may
function as a tumour suppressor by maintaining Dicer-mediated biogenesis of tumour-suppressive miRs.

Other factors regulating miR maturation  Enzymes that catalyse RNA/DNA modifications can strongly influence miR biogenesis. For instance, BCDIN3D, an RNA-methyltransferase, O-methylates 5’ monophosphates of target pre-miRs, resulting in loss of negative charge and consequently of Dicer binding and miR maturation (Xhemalce et al. 2012). Microprocessor-mediated pri-miR cleavage is regulated by RNA-editing adenosine deaminase acting on RNA (ADARs). ADAR conversion of adenosine (A) to inosine (I) inhibits effective pri-miR processing by Drosha and can alter sequence and structural properties, because base pairing of inosine is similar to that of guanosine (O’Connell & Keegan 2006, Yang et al. 2006). This editing can target the pri-miR for degradation, produce modified mature miRs that regulate a different set of targets, modify extent of target regulation or alter pre-miR processing by DGCR8 (O’Connell & Keegan 2006, Yang et al. 2006).

In addition, an immune regulator ribonuclease, monocyte chemoattractant protein 1-induced protein 1 (MCPIP1), suppresses biogenesis of a subset of miRs through pre-miR terminal loop cleavage, antagonising Dicer activity (Suzuki et al. 2011). Commensurate with a physiological role for this mode of regulation, an inverse correlation was identified between Dicer and MCPIP1 expression in lung cancer, and high MCPIP1 expression was associated with poor prognosis in lung adenocarcinoma patients (Karube et al. 2005, Shedden et al. 2008).

Together, these observations highlight the potential for signalling cascades to regulate the biogenesis of individual miRs or miR subsets, and suggest that interaction of Microprocessor cofactors with accessory proteins such as p53, hnrNPA1, KHSRP1 or SMAD can alter the specificity and/or activity of Drosha processing. It may be speculated that this is mediated through conformational changes in the Microprocessor and/or pri-miR, or through displacement of repressive proteins bound to RNA.

Hormonal regulation of miR biogenesis

There are clear indications in the literature to support direct and indirect hormonal regulation of miR biogenesis, with hormone signalling altering miR biogenesis at all stages of maturation, from transcription to control of mature miR stability. Such regulatory mechanisms provide numerous opportunities to impart subtle but stringent control over gene expression in order to maintain appropriate levels of key protein activity during normal cellular homeostasis. Aberrations in these subtle mechanisms of hormone-modulated miR biogenesis may promote disease pathogenesis, particularly in hormone-related cancers.

Estrogen modulation of miR biogenesis

Estrogens have been described as key regulators of miR biogenesis in breast cancer cells, endometrial stromal cells and myometrial smooth muscle cells; these effects are achieved through liganded ERα directly regulating miR transcription, ERα modulation of miR maturation, or via direct or indirect autoregulatory feedback loops, both positive and negative (Castellano et al. 2009, Klinge 2009).

High throughput profiling identifies estrogen-regulated miRs  As for protein-coding genes, hormone stimulation alters transcription of numerous miR-coding genes, and ERα has been demonstrated to directly associate with response elements in the promoters of such to initiate gene expression (Bhat-Nakshatri et al. 2009). miRNAome-wide profiling arrays, such as microarrays and qPCR arrays, have helped to define a signature of estrogen-regulated miRs. The first examples of such arrays are limited in their applicability to ERα signalling in humans, being performed in zebrafish, rat breast tissue and mouse spleen (Kovalchuk et al. 2007, Cohen et al. 2008, Dai et al. 2008), and to date only around 15 studies have examined estrogen regulation of miRs in human cells (Iorio et al. 2005, Bhat-Nakshatri et al. 2009, Castellano et al. 2009, Klinge 2009, Maillot et al. 2009, Wickramasinghe et al. 2009, Cicatiello et al. 2010, Cochrane et al. 2010, Di Leva et al. 2010, 2013, Hah et al. 2011, Ferraro et al. 2012, Masuda et al. 2012, Zhao et al. 2013). Despite the majority of these being performed in MCF7 breast cancer cells, inconsistencies are evident, presumably attributable to differences in ligand, treatment duration, array platform, statistical processing and threshold setting as well as lab-specific cell line differences. As such, very few estrogen-regulated miRs have been consistently identified and validated to date (Fig. 2). In contrast, considerable efforts have been focussed on identification of miRs that show differential expression in breast cancer, resulting in upwards of 50 publications. Such studies are vital in the identification of novel diagnostic/prognostic biomarkers and therapeutic targets to improve clinical management and treatment of a disease that is a very considerable cause of morbidity and
mortality. However, the relationship between miRs that are disregulated in breast cancer and those that are estrogen-regulated is complex and the degree of overlap is currently unclear.

A summary of the most commonly identified estrogen-regulated miRs is shown in Table 1 and Figure 2. In contrast to the identification of numerous estrogen-regulated miRs in MCF7 cells, no miRs demonstrated...
estrogen regulation following 24 h treatment of T47D breast cancer cells with 10 nM estradiol (E2; Katchy et al. 2012). In one of the most widely cited studies, Bhat-Nakshatri et al. (2009) demonstrated estrogen upregulation of 21 miRs (several of which target ERz coactivators in vitro) and downregulation of seven in MCF7 cells through microarray analysis. Several of the identified miRs were located within intragenic regions of estrogen-regulated genes. Of the identified estrogen-upregulated miRs, let7f, miR-98 and miR-21 were shown to reduce levels of c-MYC and E2F2 proteins (required for optimal ERz activity and secondary estrogen response respectively), providing evidence for a negative feedback loop to repress ERz activity. Another microarray analysis identified 17 miRs as significantly being upregulated by estrogen treatment in MCF7 cells. Interestingly, however, only miR-7 (which targets EGFR in ERz-positive but not ERz-negative breast cancer cells) was subsequently found to be suppressed by treatment with an anti-estrogen, Faslodex (Masuda et al. 2012). Reasons for the lack of converse effects of anti-estrogens on estrogen-regulated miRs in this case remain to be identified, but this approach is one means of validating ER-dependent regulation.

As a more direct, and arguably more physiologically relevant method for identification of transcriptionally estrogen-regulated miRs, Hah et al. (2011) used global nuclear run-on and sequencing (GRO-seq) to map the position and orientation of all RNA polymerases across the MCF7 genome following treatment with 100 nM E2 for 10, 60 or 160 min. 119 miR-containing transcripts that were regulated by E2 at one or more time points were identified. Interestingly, MCF7 cells were enriched for 2700 predicted targets of these E2-regulated miRs compared with other cells, indicating the presence of a complex regulatory network of miRs and their mRNA targets (Hah et al. 2011). Other studies have combined microarray expression profiling of miRs with global ERz-binding site mapping through ChIP-seq to provide mechanistic insights: Bhat-Nakshatri et al. examined functional ERz-binding sites within 20 kb regions of miR genes following estrogen stimulation that resulted in increased pri-miR transcription. One estrogen-responsive ER was found in the miR-21 promoter, with five and four ERs identified in the miR-23b~27b~24-1 and miR-23a~27a~24-2 promoters respectively, although different cluster members showed differing extents of regulation (Bhat-Nakshatri et al. 2008, 2009). Ferraro et al. (2012) used this approach to compare responses in estrogen-treated MCF7 and ZR-75.1 cells. They demonstrated that miR-135a-2, miR-181c, miR-26b and the clustered miR-23a, miR-27a and miR-24-2 were estrogen-regulated in both cell lines and located within 10 kb of an ERz-binding site, while estrogen-regulated miR-25, miR-26a, miR-424, miR-618, miR-760 and miR-942 were located in the intragenic region of CYP1B1, cytochrome P450 family 1B1; E2, estradiol; Sp, specificity protein; ZBTB10, zinc finger and BTB domain containing 10; RIP-140, receptor interacting protein 140; SRC1, steroid receptor coactivator 1; ER, estrogen receptor alpha; AIB1, amplified in breast cancer 1; PR, progesterone receptor; ATP1B1, ATPase Na+ K+ transporting beta 1; AR, androgen receptor; ERBB2, v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2; TGFBR2, transforming growth factor beta receptor interacting protein 140; SRC1, steroid receptor coactivator 1; ERz, estrogen receptor alpha; AIB1, amplified in breast cancer 1; PR, progesterone receptor; ATP1B1, ATPase Na+ K+ transporting beta 1; AR, androgen receptor; ERBB2, v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2; TGFBR2, transforming growth factor beta receptor 2; PTEN, phosphatase and tensin homologue; PHB, prohibitin.

Figure 2
Mutual regulation by steroid hormone receptors and microRNAs (miRs). A schematic representation of the mechanisms by which steroid hormone receptors (SHRs) and miRs mutually regulate each other's activity through feedback loops, permitting a myriad of complex and subtle responses to stimuli. (1) ERz represses miR-206, miR-221 and miR-222, which directly target ERz, thereby reducing the levels of ERz inhibitory miRs. (2) ERz represses miR-26a, miR-26b, miR-181a, miR-181b, miR-23a and miR-23b, which target the ERz effector and growth-stimulatory protein, PR, to inhibit cell growth and proliferation. (3) A negative-feedback loop whereby ERs upregulates c-MYC, a transcription factor required for the expression of the miR-17 ~ 92 cluster, whose component miRs directly targets ERz. This estrogen-upregulated cluster also targets the ERz coactivator AIB1. (4) miR-18a, miR-18b, miR-193b and miR-302c represses ERz protein translation through direct association with its 3'-UTR. (5) ERs has been demonstrated to directly upregulate miR-20a, miR-19a/b, miR-92a-1, miR-21, let7a, let7g, miR-203, miR-200a, miR-30b and miR-182, amongst others. (6) ERs represses Drosha cleavage of a subset of pri-miRs through association with p800/p72 to promote Drosha dissociation from the Microprocessor. (7) miR-140-3p and miR-22 indirectly repress ERz transcriptional activity by targeting the ERz coactivators, RIP140 and SRC1 respectively. (8) miR-27a targets ZBTB10, an inhibitor of Sp transcription factors that function to enhance ERs transcriptional activity. By repressing an inhibitor of ERs, miR-27a indirectly increases ERz activity, (9) miR-27b targets CYP1B1, an enzyme responsible for estrogen synthesis, thereby preventing ERz activation. (10) PR inhibits miR-633, miR-29c, miR-29*, miR-193b and miR-29, which targets the PR effector protein, ATP1B1. (11) PR upregulates miR-625*, miR-21, miR-142-5p, miR-146b-5p and miR-513a-5p, which targets PR in a negative-feedback loop. (12) AR transcriptionally upregulates miR-141, miR-17-5p, miR-22, miR-27a/miR-29a, miR-29b, miR-32, miR-101, miR-125b, miR-148a, miR-594 and others. (13) miR-331-3p indirectly reduces AR activity by targeting ERBB2, which can activate transcription of AR target genes in the absence of androgens. (14) AR indirectly maintains its own activity in positive feedback loop whereby AR induces transcription of miR-21, which targets the 3'-UTR of PTEN (known to inhibit AR nuclear translocation and promotes its degradation). Androgen-upregulated miR-21 also targets the tumour suppressor, TGFB2R, to stimulate growth. (15) AR directly represses transcription of miR-221 and miR-126*. (16) miR-205, miR-185, miR-34a/b, miR-135b, miR-297, miR-299-3p, miR-371-3p, miR-421, miR-449/a/b, miR-634, miR-654-5p and miR-9 directly repress AR through binding to its 3'-UTR. (17) AR indirectly increases its own activity by enhancing transcription of miR-27a, which targets the AR corepressor, PHB. AR also inhibits PHB transcription. (18) Let7 indirectly inhibits AR by targeting the AR transcription factor, c-Myc, CYP1B1, cytochrome P450 family 1B1; ERs, estrogen receptor alpha; AIB1, amplified in breast cancer 1; PR, progesterone receptor; ATP1B1, ATPase Na+ K+ transporting beta 1; AR, androgen receptor; ERBB2, v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2; TGFBR2, transforming growth factor beta receptor 2; PTEN, phosphatase and tensin homologue; PHB, prohibitin.
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estrogen-regulated genes. Furthermore, Di Leva et al. (2013) demonstrated that binding of ERα to the promoter of the miR-191-425-DALRD3 unit leads to increased levels of miR-191 and -425 and loss of DALRD3 levels, with subsequent loss of miR-191 and miR-425 targets that include the tumour suppressor EGR1. Both these studies provide evidence for mutual estrogen-regulation of protein-coding genes and their intragenic miRs.

In a further study, Maillot et al. (2009) identified 23 miRs as repressed by estrogen in MCF7 breast cancer cells, with at least some of the inhibitory effects attributable to ERα repression of miR cluster transcription. Most interesting was the observation that a number of estrogen-downregulated miRs reduce DNA synthesis and proliferation of breast cancer cells upon their transient transfection (Maillot et al. 2009). Analysis of predicted mRNA targets of these estrogen-downregulated, growth inhibitory miRs demonstrated enrichment for genes with roles in cell growth and proliferation. Of particular interest was the finding that six of seven miRs predicted to target the progesterone receptor (PR) 3’-UTR (PR is an ERα-upregulated target gene and a vital mediator of ER signalling) are downregulated by estrogen (Maillot et al. 2009). These observations provide further evidence for a positive feedback loop, whereby ERα downregulates miRs that repress mediators of estrogen signalling in order to amplify its own activity. Hence, estrogen signalling has two ways to increase activity of a vital mediator of its own activity (PR) – direct and indirect feedback loops (Fig. 2).

In addition, hormone treatment can induce recruitment of other transcription factors to miR promoter regions. For example, estrogen-inducible c-MYC is recruited to the promoters of the paralogous miR-17-92 and miR-106a-363 clusters and increases mature levels of encoded miRs (Castellano et al. 2009). This generates a feedback loop whereby ERα self-regulates through increasing levels of ERα pathway-targeting miRs, since miRs 18a, 19b and 20b directly target and downregulate ERα, whilst miRs 20a, 20b, 17-5p and 106a inhibit translation of the ERα coactivator AIB1 (Castellano et al. 2009). These constitute a complex and highly sensitive network of interactions for subtle manipulation of ERα signalling in breast cancer cells, and highlight that negative as well as positive feedback loops contribute to this.

It is clear that greater research effort is required to isolate the effects of estrogen on pri-miR transcription from the post-transcriptional effects of liganded ERα to the latter stages of miR biogenesis. As an example, let7g demonstrated direct regulation by ERα, but not at the levels of transcription, because estrogen treatment reduced levels of mature let7g, but not pri-let7g in a MAPK-dependent manner in MCF7 cells (Qian et al. 2011). Although the precise mechanism of such regulation remains unclear, reduction in mature let7g may be attributable to p68/p72-mediated ERα inhibition of Drosha (discussed below).

**ERα modulation of Microprocessor activity**

A seminal study by Yamagata et al. (2009) provided the first in vivo evidence for ERα regulation of miR expression. It was also the first to demonstrate ERα modulation of Microprocessor function. Expression of a set of mature miRs was upregulated in female mouse embryos deficient in ERα, whilst estrogen treatment of ovariectomised female mice inhibited the production of a subset of mature miRs in the uterus, a target organ of estrogen signalling (Yamagata et al. 2009). Upon further examination, it was found that the levels of the relevant pri-miRs were unchanged following estrogen treatment, but levels of the cognate mature miRs and pre-miRs were downregulated, suggesting that estrogen-mediated modulation of miR levels occurs at the level of pri-miR to pre-miR processing. The authors hypothesised that estrogen signalling via ERα may modulate Drosha activity through physical association of ERα with the RNA helicases p68 (DDX5) and/or p72 (DDX17), which are important components of the Drosha Microprocessor complex and vital to miR maturation. Indeed, it was found that mouse ERα was associated with mouse Drosha, but only in the presence of E2 and that this requires p72 and p68, as confirmed by immunoprecipitation assays (Yamagata et al. 2009).

In another study, RNA-ChIP experiments showed dissociation of pri-miRs from Drosha and p72 in the presence of estrogen. Interestingly, the N-terminal of ERα associates with the C-terminal of Drosha, which contains the RNaseIII- and dsRNA-binding motifs (Macias et al. 2009). This could explain the observations of Castellano et al., who found that the extent of estrogen regulation of pri-miRs was greater than that of their encoded mature miRs (Castellano et al. 2009). It is possible that these miRs are also subject to ERα-mediated inhibition of pri-miR processing through repression of Drosha activity in an E2- and p68/p72-dependent manner, yielding the observed biphasic regulatory pattern.

These data indicate a mechanism whereby ERα inhibits Drosha-mediated processing of a subset of miRs through association with Drosha in a p68/p72- and E2-dependent manner, resulting in the dissociation of the Microprocessor complex from the pri-miR. However, it is likely that further auxiliary factors dictate the specificity of
estrogen-mediated miR regulation and that liganded ERα acts at other steps of miR biogenesis, for example, pre-miR processing or mature miR stability.

**Regulation of miR biogenesis pathway proteins by estrogen** Further evidence for estrogen regulation of miR maturation comes from the findings of Nothnick *et al.* (2010), who demonstrated that estrogen and progesterone significantly upregulate the levels of Exportin 5 mRNA in mouse uterine tissue, an effect abrogated by the addition of the anti-estrogen ICI 182 780. They found that the levels of miR-451 were concurrently increased, but did not demonstrate that these effects are specifically attributable to the effects of these steroid hormones on Exportin 5 expression, failing to exclude the possibility of estrogen-enhanced transcription of pri-miR-451 independent of Exportin 5. The authors also showed that P4, but not E2, increased Dicer expression (Nothnick *et al.* 2010). However, evidence for estrogen regulation of Dicer in human cells comes from Lin *et al.* (2007) and Bhat-Nakshatri *et al.* (2009) who demonstrated that Dicer protein levels are upregulated by estrogen in MCF7 breast cancer cells, with ERα binding to the enhancer region of the Dicer promoter.

In a further study, Cheng *et al.* evaluated the expression of miR biogenesis-associated genes in ER-positive and ER-negative cell lines. They found that ER-positive cell lines had increased levels of TRBP and Dicer transcripts and decreased levels of AGO1 and AGO2 compared with ER-negative cell lines (Cheng *et al.* 2009). This is supported by Adams *et al.* (2009) who showed reduced AGO2 expression in ER-positive breast cancer cell lines. It has also been demonstrated that Dicer is regulated by MAPK, and that phosphorylation of TRBP by this kinase pathway increases the expression of the growth-promoting miRs 17 and 20 and inhibits maturation of the let7 tumour suppressor in HeLa cells (Paroo *et al.* 2009). As it is well established that ERα activates MAPK in several cell lines (Levin 2011), together these data highlight a putative indirect mechanism for estrogen regulation of Dicer activity through activation of hormone-stimulated MAPK pathway. Furthermore, the Dicer-targeting tumour-suppressive let7, which reduces Dicer protein levels in several cell lines (Tokumaru *et al.* 2008), is downregulated by estrogen treatment (Qian *et al.* 2011), constituting a mechanism for indirect upregulation of Dicer activity by estrogen modulation of miRs. Further, Dicer mRNA is targeted by estrogen-regulated miR-29a, miR-125, let7 and miR-21, and TRBP by let7 (Tokumaru *et al.* 2008, Cochrane *et al.* 2010, Shu *et al.* 2011). From these studies (summarised in Table 2), it is clear that a pattern of regulation is increasingly observed whereby feedback loops involving steroid signalling permit the maintenance of hormone-regulated miR biogenesis at appropriate levels.

**A potential novel role for estrogen in modulating miR:3′-UTR interaction** Finally, a novel mechanism has been proposed whereby, complementary to its role in the modulation of miR levels, ERα may modulate miR activity by altering the availability of miR-binding sites within target 3′-UTRs. One report has demonstrated that ERα signalling can induce alternative polyadenylation and 3′-UTR shortening of CDC6, which led to loss of miR-binding sites and increased CDC6 protein levels (Akman *et al.* 2012). This represents a possible additional mechanism for estrogen regulation of gene expression through modulating the potential for repressive miR–3′-UTR interactions; however, additional independent studies are required to corroborate these observations, and a defined mechanism for this phenomenon remains to be elucidated. If confirmed, such a mechanism would allow ERα not only to regulate its target genes by altering miR-mediated repression, but also to regulate cellular availability of some miRs in a transcription-independent manner.

### Table 2 Steroid hormone regulation of microRNA biogenesis pathway components

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<th>miR biogenesis protein</th>
<th>Regulating SHRs</th>
<th>Direction of regulation</th>
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<td>Exportin-5, Dicer</td>
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<td>Nothnick <em>et al.</em> (2010)</td>
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<td>Cheng <em>et al.</em> (2009)</td>
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<td>ERα</td>
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### Targeting of ER pathway by miRs: feedback circuits involving hormone-regulated miRs modulate ER expression and activity

**miRs targeting ER** miRs known to target ER itself are summarised in Table 3. One of the first to be identified was miR-206. Adams *et al.* (2007) demonstrated that miR-206 targets ERα (ESR1) mRNA and decreases ERα protein levels to reduce the growth of breast cancer cells. The tumour-suppressive let7 family of miRs has also been demonstrated to repress ERα expression in MCF7 cells following...
their transient overexpression (Zhao et al. 2011). Leivonen et al. employed protein lysate microarray technology to identify ERα-targeting miRs following transfection of 319 pre-miRs into MCF7 and BT-474 cells. Twenty-one miRs were confirmed to downregulate ERα as validated by western blotting and qPCR analyses, and miRs 18a, 18b, 193b, 206 and 302c were confirmed to target ERα 3'UTR in reporter assays (Leivonen et al. 2009). Notably, clustering analysis of mRNA targets of these miRs demonstrated enrichment for estrogen-induced genes.

Other miRs identified as directly targeting ERα are miR-221 and -222 (Leivonen et al. 2009). Indeed, knockdown of either miR in ERα-negative MDA-MB-468 cells restores ERα expression and initiates estrogen responsiveness and anti-estrogen sensitivity (Zhao et al. 2008). Again, the authors propose that miR-221/-222 also act by additionally targeting downstream components of the ERα signalling pathway. Interestingly, although miR-221/-222 decrease ERα protein levels, proliferation of the transfected cells was accelerated through decreased expression of tumour-suppressor proteins, thereby highlighting the importance of examining all miR targets and utilising pathway analysis tools to predict the effects of miR manipulation (Di Leva et al. 2010, Rao et al. 2011). In the context of miR-221/-222 targeting of ERα, it is interesting that several studies have demonstrated dramatic downregulation of these miRs upon estrogen treatment (Cochrane et al. 2010), and that ERα directly inhibits transcription of these miRs by recruiting the transcriptional corepressors NCoR and SMRT to the defined miR-221/-222 promoter (Di Leva et al. 2010). Together, these data provide further strong evidence for autoregulation of ERα activity through direct downregulation of miRs that directly target its 3'UTR. Similarly, the ERα-targeting miRs 18a and 206 are estrogen-regulated, suggestive of a potential feedback loop to modulate ERα activity. Interestingly, whilst miR-18a is upregulated through estrogen stimulation of MCF7 cells (Castellano et al. 2009), miR-206, which also targets the ERα coactivators SRC1 and AIB1, is downregulated via inhibition of Drosha-mediated maturation (Adams et al. 2007, 2009). The opposite direction of regulation of these ERα-targeting miRs suggests a complex regulatory mechanism that permits subtle yet stringent control of ERα activity, allowing cells to adjust their transcriptional output to varied challenges.

**Modulation of ERα activity regulators and synthesis of proteins by miRs**

ERα-mediated gene transcription requires a plethora of accessory factors and coactivators in order to modify chromatin structure such that it is compatible with RNA polymerase binding, and there is increasing evidence that miRs can modulate ERα activity through targeting coregulators. For example, miR-17-5p reduced translation of AIB1 and reduced ER activity on transfected and endogenous target genes by up to 50% in CHO-K1 cells (Hossain et al. 2006). In addition, miR-206 was demonstrated to bind to the 3'UTRs of both SRC1 and AIB1 and the transcription factor GATA3 in MCF7 cells (Adams et al. 2009). This led to a significant reduction in ERE luciferase reporter activity and inhibition of ERα target gene transcripts, for example, PR (Adams et al. 2009). Furthermore, levels of the ERα target gene and effector protein, cyclin D1, were reduced upon infection of MCF7 cells with a retroviral vector containing the miR-17-20 cluster, although effects of cluster expression on ERα activity were not examined (Yu et al. 2008). Additional studies have confirmed miR-22 repression of SRC1 through 3'UTR binding in Huh7 hepatocarcinoma cells, whilst miR-140-3p directly inhibits RIP-140 translation through association with 3'UTR seed region (Takata et al. 2011).

Corepressors of ERα also display miR-mediated regulation. miRs 10a/b and 284 have been shown to directly suppress SMRT expression through 3'UTR binding and translational inhibition in neuroblastoma and HeLa cells respectively (Foley et al. 2011, Wu et al. 2011), although the impact of these miRs on ERα activity was not assessed. Steroid-regulated miR-27a provides a further example of a miR targeting downstream regulators of
steroid signalling: miR-27a represses both the steroid receptor corepressor Prohibitin and also ZBTB10, a zinc finger gene which functions to repress Sp transcription factors that have pro-proliferative and angiogenic effects in breast cancer cells (Mertens-Talcott et al. 2007, He et al. 2008, Fletcher et al. 2012). Thus miR-27a is an indirect oncomiR by repressing transcription factor repressors. miR27a also indirectly activates Erzα, as Erz expression and E2 response are dependent on Sp1, which is derepressed by miR-27a (Li et al. 2010). Interestingly, and in keeping with these findings, a single-nucleotide polymorphism in the terminal loop of miR-27a that is predicted to inhibit pre-miR-27a processing is associated with reduced risk of familial premenopausal breast cancer (Yang et al. 2010). However, the effect of this mutation on the ability of miR-27a to indirectly increase ERz activity through repression of ZBTB10 remains to be evaluated.

It has also been postulated that miRs may modulate estrogen synthesis and release from cells, and there is some limited evidence to support this: transfection of a miR precursor library into primary human granulosa cells (the principal site of pre-menopausal estrogen production) identified 51 miRs that suppress the release of estrogen, including miR-15a, miR-24, miR-125b, and members of the let7 family. Interestingly, no miRs were found to increase estrogen release in this study (Sirotkin et al. 2009). Local production of estrogen in breast and other tissues is mediated by aromatase (encoded by CYP1B1) and 17β-hydroxysteroid dehydrogenase. It has been shown that the CYP1B1 3’-UTR contains putative miR-27b-binding sites, and that exogenous miR-27b reduced CYP1B1 levels and enzymatic activity, thus presumably decreasing production of local estrogen (Tsuchiya et al. 2006, Nakajima & Yokoi 2011). However, the effect of this mutation on the ability of miR-27a to indirectly increase ERz activity through repression of ZBTB10 remains to be evaluated.

A number of studies have described the roles of miRs as vital mediators and enhancers of AR signalling for both breast and prostate cancer. It will be of major interest to determine whether these are disregulated in both tumour types and whether they are regulated by steroid signalling themselves.

### Androgen regulation of miR biogenesis

Androgens have been demonstrated to alter miR biogenesis in a range of cell lines and in vivo models. ChIP-seq data have provided evidence for the association of AR with the promoters of a number of miR genes, and AR, like ER, demonstrates autoregulation through miRs (Fig. 2). A number of studies have described the roles of miRs both as mediators or effectors of AR action and regulators of AR activity, discussed below.

### Androgen regulation of miR expression

Androgen-responsive tissues express distinct sets of miRs, as demonstrated by castration of Sprague–Dawley rats, which resulted in a dramatic reduction in a large subset of miRs in the prostate compared with intact animals, whilst treatment of castrated animals with androgen increased miR expression in a tissue-specific manner (Narayanan et al. 2010). The miR profile was not altered in the liver upon androgen treatment, but the levator ani muscle (which has an anabolic response to androgens) demonstrated an upregulated miR profile that differed significantly from that of the prostate, preferentially targeting components of the Raf/MEK/ERK signalling pathway (Narayanan et al. 2010). Interestingly, Dicer siRNA completely inhibited dihydrotestosterone (DHT) induction of the well-characterised androgen-responsive gene PSA in LNCaP cells, and Dicer−/− mice developed androgen-insensitivity syndrome, indicating that androgen-regulated miRs are vital mediators of AR action in vivo (Narayanan et al. 2010).

It was also hypothesised that reduced AR function upon loss of androgen-stimulated miR synthesis may be a result of androgen-regulated miR targeting of AR corepressors. Consistent with this, 75% of predicted NCoR or SMRT-targeting miRs were upregulated in the prostate by DHT, and Dicer siRNA treatment increased transcription of both NCoR and SMRT (Narayanan et al. 2010). Taken together, these innovative data identify androgen-responsive tissue-specific miR profiles, and confirm androgen-upregulated miRs as vital mediators and enhancers of AR signalling through corepressor targeting (discussed also below).

Takayama et al. were amongst the first to describe the association of AR with miR promoters in prostate cancer cells through integration of 5’ cap analysis of gene
expression (CAGE), to identify androgen-regulated transcription start sites, with ChIP on array (ChIP-chip) to identify AR-binding sites throughout the human genome. In addition to protein-coding genes, miR-222, miR-221, miR-21, miR-125b, miR-218-1, miR-218-2, let7c and miR-100 were found to be located adjacent to AR-binding sites (Takayama et al. 2011). In corroboration, Ribas et al. (2009) demonstrated androgen-induced AR binding to the miR-21 cluster promoter, which resulted in increased levels of oncomiR-21. This increased androgen-dependent growth and induced androgen-independent growth of LNCaP and LAPC-4 cells. It should be noted, however, that the CAGE-with-ChIP-chip approach favoured by Takayama et al. may not identify all miRs regulated by AR binding to ARE promoters, because the authors only assessed one time point of androgen treatment, thus possibly overlooking earlier or later androgen binding events.

In one of the first miR microarray studies to examine androgen regulation of miRs, Waltering et al. (2011) showed that 17 miRs were significantly modulated by treating VCaP cells with DHT, at least half of which were confirmed as androgen-regulated in subsequent studies (given in Table 4). Later, Mo et al. (2013) combined time course microarrays with bioinformatic methods to identify androgen-regulated miRs and their targets in androgen-stimulated LNCaP cells, focussing on those that showed an early and enduring response to androgen, a subgroup they term ‘primary targets’. Amongst 137 miRs primary targets, 22 miRs had existing well-documented roles in prostate cancer (Bonci et al. 2008, Ozen et al. 2008, Rokhlin et al. 2008, Tong et al. 2008). Of these, miR-101, miR-145, miR-34a, miR-182, miR-375, miR-181a, miR-92b and miR-125 were upregulated by androgen, whilst miR-16, miR-126*, miR-23b, miR-100, miR-222, miR-133a, miR-499 and miR-340 were downregulated by androgen, corroborating previous demonstrations of androgen regulation by miRs and altered expression in prostate cancer (Amb et al. 2008, Bonci et al. 2008, Tong et al. 2008). Two additional primary targets, miR-19a and miR-27a, showed significant enrichment for roles in key cellular processes upon pathway analysis. Concordant with this, ChIP analysis confirmed AR binding to putative AREs within the promoter regions of these miRs (Mo et al. 2013). These data are consistent with our earlier findings, in which we also demonstrated AR binding to the miR-27a promoter after androgen stimulation (Fletcher et al. 2012). Similar extents of miR-27a upregulation were observed in the two studies, thereby supporting AR association with the miR-27a promoter as a bona fide physiological event. Pathway analysis of predicted target sets of these miRs showed enrichment for proteins with previously described roles in prostate cancer. For example ABCA1, targeted by miR-27a, inhibits proliferation of prostate cancer cells (Fukuchi et al. 2004, Yang et al. 2012) whilst RAB13, a GTPase that positively regulates epithelial cell tight junction formation (Kanda et al. 2007), is a target of miR-19a.

Shi et al. (2007) have demonstrated that miR-125b is significantly transcriptionally upregulated by androgen treatment in LNCaP cells, and its transient transfection increases prostate cancer cell growth whilst reducing Bax1 expression and 3’-UTR activity. Given that a subset of estrogen-upregulated miRs with putative oncogenic function was demonstrated to target this tumour suppressor in estrogen-treated endometrial carcinoma cells to positively reinforce ERα activity (Zhang et al. 2012), it is very interesting that the same phenomenon and mechanism of action were observed for an androgen-regulated miR in prostate cancer (Shi et al. 2007).

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Positive feedback regulation of AR through androgen-upregulated miRs Several studies have now identified mechanisms whereby AR exploits miRs to maintain high levels of its own expression and activity. Mishra et al. (2013) demonstrated that AR inhibition reduced the levels of miR-21 in AR-positive prostate cancer cells, whilst overexpression of AR increases it. Interestingly, miR-21 mimic increased AR mRNA levels, restored AR protein to AR-negative PC3 cells and enhanced PSA promoter reporter activity. This therefore represents a positive feedback loop whereby androgen-regulated miR-21 increases AR levels to further increase miR-21, which targets several tumour suppressors. The mechanism whereby miR-21 increases AR activity was not addressed, although it was hypothesised this may involve miR-21 targeting PTEN, which inhibits AR nuclear translocation and promotes degradation of AR in several cancers (Lin et al. 2004, Mulholland et al. 2006). Our own studies demonstrated a similar positive feedback loop: androgen-upregulated miR-27a increases AR activity by targeting the AR corepressor and tumour suppressor, PHB, in prostate cancer cells (Fletcher et al. 2012). These data combined suggest that androgen upregulation of miRs that target tumour suppressors and corepressors of AR transcriptional activity to positively reinforce AR signalling (Fig. 2) may be commonplace in androgen-responsive tissues. Such mechanisms could potentially play a vital role in increasing AR activity in the absence of circulating androgens in the case of advanced, castrate-resistant prostate cancer (CRPC), particularly if such miRs are overexpressed in cancerous tissue, as has been demonstrated for miR-21 (Krichevsky & Gabriely 2009, Dart et al. 2011).

Androgen regulation of protein components of the miR biogenesis pathway Regulation of one or more components of the miR processing complexes is a possible mechanism for androgen regulation of miR biogenesis. For example, Dicer and DGCR8 are upregulated by DHT treatment of LNCaP cells (Mo et al. 2013), consistent with the observed increase in Dicer protein levels in prostate cancer (Chiosea et al. 2006). In addition, AR was shown to accelerate miR biogenesis through increasing Drosha cleavage of pri-miR species, although the mechanism of androgen-increased Drosha activity remains to be described (Fletcher et al. 2012). However this effect is opposite to the inhibition of Drosha cleavage by ERα. It may be that both ERα and AR can act to regulate Drosha and Dicer activities, exerting distinct and opposing effects in the nucleus and the cytoplasm to control hormone-stimulated miR biogenesis in different tissues and developmental stages. It may be hypothesised that these regulatory mechanisms are perturbed in cancer, because altered expression of Drosha or Dicer has been reported in a number of cancers (Chiosea et al. 2006, 2007, Merritt et al. 2008, Faber et al. 2011, Passon et al. 2012).

There is also circumstantial evidence for links between androgen signalling and miR processing pathways. The Microprocessor component P68 was identified as a novel AR-interacting protein in a yeast two-hybrid screen (Clark et al. 2008) and demonstrated to be recruited to AREs upon ligand treatment and to act as an AR coactivator in LNCaP cells. Interestingly, mutation of Y593 (a putative phosphorylation site) in p68 abolished AR coactivation, indicating that post-translational modifications of p68 may be vital for this role (Clark et al. 2008), although effects of this mutation on RNA helicase activity were not investigated. The above study also found increased P68 immunohistochemical staining in a cohort of prostate cancer patients compared with benign hyperplasia patients (Clark et al. 2008). These data potentially support a link between miR processing and AR-mediated transcription, and also suggest that P68-dependent miR processing could be altered in prostate cancer.

miR regulation of androgen signalling miRs targeting the AR It is well established that miRs can directly regulate AR signalling through targeting the AR 3’-UTR (Table 5). In one study, Östling et al. (2011) coupled protein lysate microarrays in LNCaP, LAPC-4, CWR-R1, 22Rv1 and MDA-PCa-2b cells with miR microarray expression analysis in 13 prostate cell lines to quantify miR-mediated changes in AR protein content in prostate cancer. Seventy-one miRs that altered AR protein levels in all five cell lines were selected for validation, with 52 decreasing and 19 increasing AR protein levels, and 21 AR-targeting miRs were shown to reduce both AR protein levels and androgen-stimulated growth upon transient transfection of the miR mimics into LNCaP and 22Rv-1 cells, and MDA-PCa-2b cells respectively. Thirteen of these were then further validated as significantly reducing AR 3’-UTR activity: miR-135b, miR-185, miR-297, miR-299-3p, miR-34a, miR-34c, miR-371-3p, miR-421, miR-449a, miR-449b, miR-634, miR-654-5p and miR-9 (Östling et al. 2011).
Recent studies have reported shortening of 3′-UTRs in cancer cells, leading to enhanced oncogene activation. Indeed, mRNAs with shorter 3′-UTRs produce more protein, in part due to loss of miR-mediated repression (Mayr & Bartel 2009). In this context, it is interesting that the AR 3′-UTR length in several PCA cell lines was found to be ~6680 bp; far greater than the sequence length of 436 bp specified by the AR RefSeq file (NM_0000444) and used by target prediction algorithms. This dramatically increases the potential for direct miR regulation of the AR transcript, although it should be noted that there is evidence to suggest that the extent of regulation of a transcript by a miR is generally greater closer to the 5′ end of the 3′-UTR (Grimson et al. 2007, Bartel 2009). It has been proposed that the AR 3′-UTR can function as dynamic entity, and active 3′-UTR shortening in response to environmental stimuli may represent a further mechanism of PCA progression (Sikand et al. 2011b), permitting AR to evade regulation by tumour-suppressive miRs, such as long AR 3′-UTR-binding miR-34a and miR-34c (Östling et al. 2011), which are proposed potent mediators of p53 tumour suppression. However, very limited information is currently available on miR targeting of the relatively poorly characterised longer AR 3′-UTR isoforms. In fact, there remains no definitive evidence for reduced miR binding and consequently higher AR protein levels following AR 3′-UTR shortening. Further studies are required to compare AR 3′-UTR length in malignant vs benign prostate tissue, and in hormone-naïve PCa vs CRPC, to assess its potential role in cancer progression.

**Inhibition of AR activity by tumour-suppressive miRs**

LET7 expression is frequently decreased, or the gene is deleted, in human cancers (Calin et al. 2004, Jiang et al. 2005, Ozen et al. 2008). Its targets include the oncogenes RAS and MYC (Johnson et al. 2005, Kumar et al. 2007) and it has thus been proposed as a tumour-suppressor miR. This tumour suppressor capacity may be achieved in prostate cancer through decreased AR activity, since let7c was demonstrated to target c-MYC, which is required for transcription of AR (Nadiminty et al. 2012). It was found that let7c reduced AR activity and decreased growth of C4-2B cells, attributable to let7c association with c-MYC 3′-UTR leading to a loss of c-MYC-mediated AR transcription (Nadiminty et al. 2012).

Epis et al. (2009) investigated the regulation of AR activity by miR-331-3p. It was found that miR-331-3p reduces AR activity in LNCaP cells by targeting the oncogene ERBB2, because AR 3′-UTR lacks miR-331-3p-binding sites and ERBB2 can activate transcription of

<table>
<thead>
<tr>
<th>miR</th>
<th>Direction of AR activity</th>
<th>References</th>
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<tbody>
<tr>
<td>205</td>
<td>‡/‡</td>
<td>Hagman et al. (2013)</td>
</tr>
<tr>
<td>185</td>
<td>‡/‡</td>
<td>Östling et al. (2011) and Qu et al. (2013)</td>
</tr>
<tr>
<td>34</td>
<td>‡/‡</td>
<td>Östling et al. (2011) and Kashat et al. (2013)</td>
</tr>
<tr>
<td>135b</td>
<td>‡</td>
<td>Östling et al. (2011)</td>
</tr>
<tr>
<td>297</td>
<td>‡</td>
<td>Östling et al. (2011)</td>
</tr>
<tr>
<td>299-3p</td>
<td>‡</td>
<td>Östling et al. (2011)</td>
</tr>
<tr>
<td>371-3p</td>
<td>‡</td>
<td>Östling et al. (2011)</td>
</tr>
<tr>
<td>421</td>
<td>‡</td>
<td>Östling et al. (2011)</td>
</tr>
<tr>
<td>449a/b</td>
<td>‡</td>
<td>Östling et al. (2011)</td>
</tr>
<tr>
<td>634</td>
<td>‡</td>
<td>Östling et al. (2011)</td>
</tr>
<tr>
<td>654-5p</td>
<td>‡</td>
<td>Östling et al. (2011)</td>
</tr>
<tr>
<td>124</td>
<td>‡</td>
<td>Shi et al. (2011)</td>
</tr>
<tr>
<td>488*</td>
<td>‡ (indirect via targeting Shp)</td>
<td>Xiao et al. (2012)</td>
</tr>
<tr>
<td>141</td>
<td>‡ (indirect via targeting Myc)</td>
<td>Nadiminty et al. (2012)</td>
</tr>
<tr>
<td>17-5p</td>
<td>‡ (indirect by targeting PCAF)</td>
<td>Gong et al. (2012)</td>
</tr>
<tr>
<td>27a</td>
<td>‡ (indirect via targeting AR corepressor PhB)</td>
<td>Fletcher et al. (2012)</td>
</tr>
<tr>
<td>331-3p</td>
<td>‡ (indirect via targeting ERBB2)</td>
<td>Epis et al. (2009)</td>
</tr>
</tbody>
</table>

(Fig. 2). Targeting of AR by miR-185 is consistent with the findings of Qu et al. (2013), who demonstrated that miR-185 significantly reduces the levels of AR protein, but not mRNA levels, in LNCaP cells, with concomitant decreases in LNCaP cell proliferation and xenograft tumourigenicity upon miR-185 overexpression. Furthermore, targeting of AR by miR-34a is in keeping with data from Kashat et al. (2013): in an observational study showing that miR-34a reduced protein and mRNA levels of AR in C4-2B and CWR22Rv-1 cells.

Other miRs are also reported to target AR directly. Hagman et al. (2013) demonstrated that ectopic miR-205 overexpression significantly decreased AR protein levels and activity of an AR 3′-UTR reporter in VCaP cells, although this was not recapitulated in LNCaP cells. However, as levels of miR-205 were low in all seven but one in prostate cancer cell lines, the physiological relevance of miR-205 targeting of AR remains to be ascertained. Similarly, AR levels were decreased both at the RNA and protein level following ectopic expression of miR-488* in androgen-dependent and -independent prostate cancer cells, and both AR transcriptional activity and AR 3′-UTR reporter activity were also reduced (Sikand et al. 2011a).

A potential novel role and mechanism for regulation of AR involving miRs involves shortening of the 3′-UTR.
AR-target genes in the absence of androgen (Craft et al. 1999, Mellinghoff et al. 2004). ERBB2 was demonstrated to be a direct target of miR-331-3p through 3′-UTR reporter assays and protein expression studies in multiple prostate cancer cell lines. The above theory of indirect miR targeting of AR via ERBB2 is supported by a decrease in PSA promoter activity upon miR-331-3p ectopic expression (Epis et al. 2009).

Such tumour-suppressive, AR-inhibitory miRs could be exploited as potential therapeutics for prostate cancer, either alone or in combination with anti-androgens.

Nuclear RISC components as coactivators of ERα and AR

Further evidence for cooperation and functional interaction between the miR biogenesis cascade and steroid signalling pathways has been reported from the recent study by Redfern et al. which demonstrated that components of the RISC complex, such as Dicer, PACT and TRBP, bind to an RNA transcript that functions as a coactivator of nuclear receptor (NR) activity, called steroid receptor RNA activator (SRA) (Lanz et al. 2002, Hube et al. 2006, Redfern et al. 2013). PACT, TRBP and Dicer increased SRA-enhanced ERα and AR reporter activities, and were recruited to AR-target gene promoters, whilst siRNA-mediated depletion of PACT and TRBP reduced the expression of AR and ERα-target genes (Redfern et al. 2013). Interestingly, RISC protein-bound pre-miRs were identified in the nucleus as well as in the cytoplasm, suggesting the existence of a nuclear RISC complex in which SRA functions as a scaffold for PACT, TRBP and Dicer (Redfern et al. 2013), raising the possibility of coupling pre-miR processing and NR coregulation of transcription within a single-nuclear complex. It is also possible that SRA could sequester RISC components away from their pre-miR substrates to regulate RISC activity. It is feasible that other proteins may fulfill similar roles to SRA in coupling NR transcriptional activity with Drosha Microprocessor-mediated pri-miR cleavage, since both FUS, a Microprocessor component that stimulates pri-miR processing (Morlando et al. 2012), and p68, known to promote cleavage of a subset of pri-miRs (Fukuda et al. 2007, Salzman et al. 2007, Kawai & Amano 2012, Samaan et al. 2013), are known AR cofactors (Clark et al. 2008, Brooke et al. 2011, Haile et al. 2011).

Conclusions and summary

The multiple steps of the miR biogenesis cascade provide a plethora of opportunities for hormone regulation of miR synthesis, adding further complexity to allow the most stringent regulation and fine-tuning of a pathway with vital roles in almost all physiological processes, and the dysregulation of which has undesirable consequences for the pathogenesis of human cancers. It is clear that distinct subsets of miRs are regulated by estrogen and androgen signalling in responsive tissues, but that these hormones share common mechanisms for regulating the activity of miR biogenesis pathway components, altering transcription of miRs and exploiting target miRs to reinforce their own activity. From in vivo studies, we evidenced that miRs are vital effectors of AR and ERα signalling pathways, and conversely, it is increasingly apparent that multiple miRs can directly target the 3′-UTRs of ERα and AR to regulate hormone signalling. Furthermore, NRs preferentially regulate miRs that target their own effectors and cofactors, resulting in feedback that may amplify or restrict their signalling. In addition, several fascinating emerging theories highlighted herein may yet have sizeable implications for the bi-directional regulation of miR biogenesis and hormone signalling. We should also remember that miRs themselves have been postulated to function similarly to hormones, influencing gene expression, microenvironment and disease pathogenesis at distant sites, and potentially functioning as readily assayable biomarkers. The interplay between hormone signalling and miR biogenesis, and further characterisation of its components, provides significant opportunity for increased understanding of steroid-related cancer progression and the potential for exploitation of such interactions for designing novel cancer therapeutics.

Declaration of interest

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

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