Androgen-regulated processing of the oncomir MiR-27a, which targets Prohibitin in prostate cancer

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MicroRNAs (miRs) play an important role in the development of many complex human diseases and may have tumour suppressor or oncogenic (oncomir) properties. Prostate cancer is initially an androgen-driven disease, and androgen receptor (AR) remains a key driver of growth even in castration-resistant tumours. However, AR-mediated oncomiR pathways remain to be elucidated. We demonstrate that miR-27a is an androgen-regulated oncomir in prostate cancer, acting via targeting the tumour suppressor and AR corepressor, Prohibitin (PHB). Increasing miR-27a expression results in reduced PHB mRNA and protein levels, and increased expression of AR target genes and prostate cancer cell growth. This involves a novel mechanism for androgen-mediated miR regulation, whereby AR induces a transient increase in miR-23a27a24-2 transcription, but more significantly accelerates processing of the primiR-23a27a24-2 cluster. Androgens therefore regulate miR-27a expression both transcriptionally (via AR binding to the cluster promoter) and post-transcriptionally (accelerating primiR processing to the mature form). We further show that a miR-27a antisense oligonucleotide, by opposing the effects of mir-27a, has therapeutic potential in prostate cancer.

INTRODUCTION

Prostate cancer (PCa) is the most prevalent non-cutaneous malignancy in western males (1). Initially, growth of prostate tumours is dependent on circulating androgens, which exert their growth-stimulatory effects through association with the androgen receptor (AR) (2). AR is a ligand-dependent transcription factor, activation of which by androgens initiates target gene transactivation resulting in PCa cell proliferation. This involves dimerization and recruitment to androgen response elements (AREs) of coactivators and/or corepressors (3–7), whose roles in PCa remain poorly understood. MicroRNAs (miRs), which have been shown to function as oncogenes and tumour suppressors in multiple cancers (8–11), may play an important role in PCa through targeting coregulators of AR and targets involved in growth response. Further, several miRs demonstrate androgen regulation, although the mechanisms surrounding such regulation remain to be elucidated. In this study, we demonstrate inhibition of the AR corepressor, Prohibitin (PHB), by hsa-miR-27a (henceforth referred to as miR-27a) in PCa, and identify a mechanism by which AR mediates post-transcriptional miR-27a maturation. Importantly, as well as being an AR corepressor, PHB is an AR target protein, downregulation of which promotes cell growth, implying a tumour suppressor function (12–14). How much of this relates to its corepressor role is not clear. Thus, miRs that inhibit AR targets and/or coregulators to modulate androgen response may represent novel targets for PCa therapeutics.

PHB is an evolutionarily conserved protein with multiple cellular functions (15–22) that negatively regulate AR activity and androgen-stimulated growth of PCa cells (13). A reduction in PHB either by androgens or RNAi-mediated knockdown resulted in increased AR activity and enhanced growth of prostate xenografts (LNCaP) (14). Our initial observation that AR downregulates PHB was followed by evidence that this occurs at least partly at the level of transcription. Indeed, PHB regulation may involve several mechanisms, such as genomic effects at the PHB promoter or post-translational modifications. A more recent hypothesis suggests a role for miR association with PHB-3’UTR in regulation of PHB (23). However, the precise mechanism of androgen or

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AR-mediated downregulation of genes such as PHB is unknown. Interestingly, we found that the optimum RNAi oligo for PHB knockdown shared considerable sequence similarity to a previously published oncomiR-targeting PHB-3′UTR—namely miR-27a (23). PHB has a large, highly conserved 3′UTR, with several putative miR-binding sites, indicating that miRs may be heavily involved in its regulation.

MiRs are 19–25 nt non-coding RNAs that regulate target gene expression through translational inhibition or transcript degradation (24), regulating a wealth of biological processes including tumourigenesis (25). Further, 30% of miR genes are in cancer-associated genomic regions or in fragile sites (26). It is, therefore, conceivable that miRs have a significant effect on cell physiology and that common single-nucleotide polymorphisms within key miR target sites could contribute significantly to an individual’s risk of developing complex diseases. Functional studies have also established that individual miRs can act as oncogenes (oncomiRs) or tumour suppressors (8), and although targets of many miRs remain to be elucidated, some have been shown to repress well-known oncogenes or tumour suppressors. For example, miR-15/16 inhibit the anti-apoptotic factor BCL2, an anti-apoptotic factor (27), while the let-7 family of miRs target the Ras oncogene (28). Thus, miR aberrations may be involved in prostate oncogenesis and development of castration-resistant PCa (10,29,30).

Several studies have revealed distinct miR signatures in prostate tumours compared with benign samples (11,31,32). MiR-27a is encoded in an intergenic cluster with miR-23a and miR-24-2 located on chromosome 19p13.1. The ~2159 nt primary transcript is transcribed from a promoter region located between −603 and +36 nt (33). The −530 to −410 promoter portion contains two GC-rich domains and is thought to be the location for transcription factor binding (34,35). Expression of the miR23a27a24-2 cluster is altered in many cancers, including leukaemia (36,37), breast cancer (38), colorectal cancer (39), hepatocellular carcinoma (40), ovarian cancer (41) and PCa (11,32). Upregulation of this cluster has been shown to reduce tumour suppressive effects of TGFβ in human hepatocellular carcinoma cells (40); conversely, it induced apoptosis in human embryonic kidney cells (42). The diverse effects of this cluster suggest context-dependent activities, warranting investigation into the individual molecular functions of the cluster members and identification of targets, especially given evidence of differential regulation of miR-23a27a24-2 cluster members. For example, downregulation of miR-27a has been observed independently of miR-23a and miR-24-2 (43), and it is likely that both transcriptional and post-transcriptional mechanisms may be involved in cluster regulation.

As miR-27a has been proposed to function as an oncomiR in gastric cancer through downregulation of PHB (23) and given the androgen-mediated downregulation of PHB and the role of PHB in AR repression, we hypothesized that the AR itself may modulate levels of miR-27a thus downregulating its own repressor (PHB) in a ‘positive feedback loop’. MiR-27a targeting of PHB could play an important role in PCa progression in several ways; through reducing AR repression and by downregulating other repressive roles of PHB, resulting in maintenance of oncogenic phenotype.

RESULTS

Androgen regulation of PHB occurs largely at the 3′UTR

In the LNCaP PCa cell line, androgen treatment reduces PHB levels by ~50% (13). However, PHB promoter activity shows a maximum reduction of only 25% following AR activation (Supplementary Material, Fig. S1), therefore we hypothesized that androgen regulation of PHB may occur at the 3′UTR. PHB-3′UTR sequence was fused to a luciferase reporter (Supplementary Material, Fig. S1B and C) and HeLa cells were transiently transfected with this reporter and AR expression vector. Luciferase activity was reduced by ~25% upon androgen treatment (Fig. 1A), supporting the hypothesis that androgens downregulate PHB via the 3′UTR. This is mediated by AR, since AR silencing (using a doxycycline-inducible system (44)), resulted in increased PHB-3′UTR levels, which was not observed using scrambled shRNA (Fig. 1B). In further support of a role for the 3′UTR in androgen regulation of PHB, when another PCa cell line, PC3wtAR cells, was treated with androgen and primer-specific quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) performed for either the PHB coding region or PHB-3′UTR, reduction in PHB 3′UTR was 85% compared with 45% for the coding region (Fig. 1C). Finally, anti-androgen treatment abrogated the effects of Mibolerone (MB) at the endogenous PHB-3′UTR: while PHB-3′UTR PCR product levels were drastically reduced following androgen treatment of LNCaP cells (Fig. 1D), addition of Bicalutamide (Bic) reversed this effect. These data suggest that androgen exerts inhibitory effects on PHB via the 3′UTR.

PHB is a target of MiR-27a in PCa cells

Putative miR-binding sites in the PHB-3′UTR were mapped (microRNA.org, last accessed on 11 April 2012) (Supplementary Material, Fig. S2) and showed eight groupings within the 3′UTR, including a binding site for miR-27a, of interest due to its reported role as an oncomiR-targeting PHB in gastric cancer (23). High sequence complementarity exists between PHB 3′UTR and the miR-27a seed region (Fig. 2A). To confirm that PHB-3′UTR contains bona fide miR-27a target sequences, the PHB 3′UTR reporter vector was transfected into HeLa cells with expression vectors for AR and miR-27a mimic [p2TetR-miR-27a: since HeLa cells lack Tet repressor, doxycycline (dox) was not required to derepress miR-27a mimic expression]. A mutant form of the PHB-3′UTR was also tested, carrying two nucleotide changes in the putative miR-27a seed region (Fig. 2A). While addition of miR-27a mimic resulted in 60% reduction in PHB-3′UTR activity, it did not significantly alter activity of the mutated reporter (Fig. 2B), demonstrating that PHB-3′UTR is a direct target of miR-27a via this site. To assess whether miR-27a can reduce levels of endogenous PHB in PCa cells, LNCaP cells expressing miR-27a under dox control were created (LNCaP/TR2/miR-27a). Induction of exogenous miR-27a mimic resulted in a considerable reduction in PHB protein levels 24 h post-dox treatment compared with non-induced cells, with optimal 90% reduction in the PHB protein observed following 0.1 μM dox treatment (Fig. 2C). To address the physiological relationship between endogenous miR-27a and
PHB in PCa, PHB protein and miR-27a levels were assessed by western blotting and qRT–PCR, respectively, in a panel of representative human cell line models. A significant inverse correlation between PHB protein levels and miR-27a levels was found, with a Pearson correlation coefficient of 0.881. This suggests that levels of PHB and miR-27a are closely linked in PCa, supporting PHB as an endogenous target of miR-27a in this disease.

To investigate whether repression of PHB-3′UTR is via translational repression or transcript degradation, levels of PHB-3′UTR were assessed by qRT–PCR in LNCaP/TR2/miR-27a cells induced with dox. Increasing miR-27a levels resulted in up to 70% reduction in PHB-3′UTR levels (representing the complete mRNA) compared with untreated cells (Fig. 2E). This suggests that miR-27a induces degradation of the PHB transcript following binding to the PHB-3′UTR. To confirm whether these effects on PHB are specifically attributable to miR-27a, miR-27a anti-sense oligonucleotide (ASO) was transfected into LNCaP cells in the presence or absence of miR-27a. In agreement with Figure 2C, miR-27a significantly reduced PHB protein levels by up to 70% (Fig. 2F). Addition of ASO rescued miR-27a-mediated PHB loss, while transfection of ASO alone increased PHB protein levels ~2.6-fold. Next, the impact of ASO introduction on PHB-3′UTR activity was assessed in HeLa cells. MiR-27a reduced PHB-3′UTR activity by ~60% and this was restored to empty vector-transfected control levels upon addition of 1–10 μg ASO, in a dose-dependent manner (Fig. 2G). Further, transfection of the ASO into LNCaP cells increased PHB-3′UTR transcript levels compared with untransfected or scrambled control-transfected cells, presumably by acting on endogenous miR-27a (Fig. 2H). Taken together, these data confirm that miR-27a targets PHB-3′UTR in PCa cells through seed region binding, leading to transcript disruption.

Figure 1. Androgen signalling downregulates PHB-3′UTR activity. (A) Luciferase activity in extracts of HeLa cells cotransfected with wild-type AR expression construct pSVAR and 1 μg SV40-GL4-PHB3’UTR prior to 24 h treatment with 10 nM Mibolerone (MB) vehicle (EtOH). Luciferase was normalized for transfection efficiency (β-galactosidase activity) and mean ± SEM of three independent experiments is shown. (B–D) qRT–PCR analysis of PHB-3′UTR, AR and L19 levels in LNCaP/Scram and LNCaP/AR shRNA cells treated with doxycycline (dox) for 48 h to induce expression of scrambled AR shRNA or AR shRNA. Western blotting was performed on protein lysates to confirm silencing of AR, for which β-actin was used as a loading control. (C) qRT–PCR analysis of PHB coding region and PHB-3′UTR from PC3wtAR cells treated with MB (10 nM) for 24 h prior to RNA extraction. Reverse transcription and qPCR were performed using primers as indicated. Columns: mean relative gene expression from three independent experiments ± SEM. (D) Semi-quantitative RT–PCR analysis of LNCaP cells treated with MB (1 nM) + Bic (10 μM) for 24 h. P ≤ 0.05, **P ≤ 0.005. See also Supplementary Material, Figure S1.
and/or translational inhibition and loss of PHB protein, which can be abrogated through addition of miR-27a ASO.

MiR-27a overexpression results in increased expression of androgen-regulated genes and increases PCa cell growth

Since PHB is an AR corepressor, the impact of PHB reduction on expression of PSA and TMPRSS2 (well-characterized AR-responsive genes) was examined by qRT–PCR after dox induction of LNCaP/2TetR-p2TetR-miR-27a cells. PSA and TMPRSS2 mRNA levels were increased up to 3.2-fold following induction of exogenous miR-27a expression (Fig. 3A). To investigate effects of miR-27a on LNCaP PCa cell growth, the same cells were treated with 10 nM MB or dox, and cell number assayed at intervals up to 6 days. Androgen treatment increased cell growth 1.5-fold compared with vehicle-treated control cells at 6 days (Fig. 3B). Exogenous expression of miR-27a mimic (Dox + MB) resulted in a significantly greater, 1.7-fold increase in cell growth compared with vehicle-treated control at 6 days. Induction of miR-27a mimic in the androgen-depleted medium did not increase cell growth, suggesting that miR-27a plays a role in androgen-dependent growth. To investigate whether the growth-stimulatory effects of miR-27a could be specifically attributed to targeting of PHB, a plasmid expressing tagged PHB (pcDNA4/TO-GFP-PHB) was introduced into the above system. It was found that increasing PHB resulted in decreased cell growth even in the presence of exogenous miR-27a (Fig. 3B; note that the non-normalized data are shown on the right to illustrate the effect of miR-27a on growth in the presence of androgen), i.e. PHB can indeed suppress miR-27a-mediated growth stimulation. Induction of miR-27a expression and GFP-PHB transfection did not alter cell growth in the absence of MB (Supplementary Material, Fig. S3). In a reciprocal experiment, stable LNCaP/GFP-PHB cells were transfected with miR-27a or a negative control oligo and treated with MB + dox to induce GFP-PHB expression (Fig. 3D). In agreement with Figure 3B, it was found that miR-27a overexpression in the absence of exogenous PHB led to a significant 80% increase in androgen-induced cell growth. In contrast, upon induction of GFP-PHB expression, no significant difference was observed between miR-27a-transfected and non-transfected cells, suggesting that overexpression of PHB is able to compensate for the growth increase induced by miR-27a of PHB-3′UTR. It should be noted that the exogenous PHB lacks the 3′-UTR hence would not be targeted by miR-27a. These data suggest that the growth-stimulatory effects of miR-27a in PCa cells are largely attributable to miR-27a targeting of PHB, without ruling out a contribution of other miR-27a-regulated transcripts.

As miR-27a increased PCa cell growth, we hypothesized that miR-27a ASO may inhibit proliferation. In support of this, while androgen treatment of LNCaP cells transfected with scrambled ASO gave a 3-fold increase in cell number after 6 days, the increase was reduced to 1.5-fold in cells transfected with miR-27a ASO in the presence of androgen (Fig. 3E). Together, these data indicate that miR-27a targeting of PHB increases AR activity in PCa cells through loss of PHB corepressive activity, and that manipulation of miR-27a alters PCa cell growth.

The MiR-23a27a24-2 cluster is regulated by androgen and AR in PCa cells

Since both androgen signalling and miR-27a downregulate PHB, we investigated whether miR-27a is androgen regulated. LNCaP cells were treated with 10 nM MB or ethanol for 0–24 h and endogenous mature miR-27a assayed by qRT–PCR. MiR-27a expression increased by >2-fold after 4 h and by 2.9-fold after 24 h in LNCaP cells treated with MB, compared with time zero (P ≤ 0.05, Fig. 4A). This is consistent with the magnitude of fold-increases reported in microarray analysis of AR-regulated miRs in PCa (11).

As miR-27a is polycistronically transcribed (Fig. 4B), we next examined whether miR-27a cluster partners, miR-23a and miR-24-2, demonstrate androgen regulation. qRT–PCR was performed for mature miRs 27a, 23a and 24-2 following treatment of LNCaP cells with androgen and an anti-androgen (Bic). All three miRs were significantly increased following MB treatment, but decreased to near-vehicle control levels upon addition of anti-androgen (Fig. 4C). The extent of regulation varied for the three cluster members, with miR-27a demonstrating a greater response to MB (5-fold increased expression) compared with miR-23a and miR-24 (3.8-fold and
Figure 3. MiR-27a manipulation alters expression of AR target genes and prostate cancer cell growth. (A) qRT–PCR analysis of PSA and TMPRSS2 mRNA levels from LNCaP/TR2/p2TetR-miR-27a cells treated with dox (0–0.2 μM) for 24 h. L19 expression was used for normalization of mRNA levels. Columns: mean relative mRNA levels from two independent experiments performed in triplicate ± SEM. *, *P ≤ 0.05; **P ≤ 0.05. (B and C) Sulphorhodamine B growth assays of LNCaP/TR2/p2TetR-miR-27a cells treated with 10 nM MB + 1 μM dox to induce miR-27a expression (B) or with additional transfection of GFP-PHB (pcDNA4/TO-GFP-PHB) or empty pcDNA4/TO using Lipofectamine LTX (C). See also Supplementary Material, Figure S3. (D) Sulphorhodamine B growth assays of LNCaP/TR2/pcDNA4/TO-GFP-PHB cells treated with 10 nM MB + 1 μM dox to induce GFP-PHB expression, with additional transfection of miR-27a mimic or negative control oligo at a final concentration of 200 nM. (E) Sulphorhodamine B growth assays of LNCaP cells transfected with miR-27a ASO or a scrambled miR-27a ASO at a final concentration of 50 ± 10 nM MB. (B–E) SRB assays were performed at 0, 2, 4 and 6 days post-treatment (B and E) or at 0, 1, 3, 5 and 7 days post-transfection/treatment (C and D). Points: mean relative absorbance at 492 nm, where absorbance at day 0 was set at 1 for all treatment conditions (B and E), absorbance at day 5 in the presence of MB and absence of PHB was set at 1 (C), or absorbance at day 5 in the absence of any treatment was set at 1 (D). Points represent mean of three independent experiments performed in six replicate wells per experiment ± SEM. *, *P ≤ 0.05.
These changes in mature miR levels are confirmed as AR-specific as they can be negated by anti-androgen treatment. To confirm that androgen regulation of miR-27a is not a cell line-specific effect, PC3wtAR cells (45) were treated with MB + Bic for 24 h followed by northern blotting of total RNA (Fig. 4D). Again, androgen treatment increased miR-27a levels in a dose-dependent manner, and this was abrogated by anti-androgen treatment.

AR associates with the proximal MiR23a27a24-2 promoter in PCa cells in response to androgen treatment to enhance transcription and also acts to enhance Drosha-mediated PrimIR-23a27a24-2 processing

The observation that all three members of the miR-23a27a24-2 cluster demonstrate androgen regulation suggested this may be mediated through association of liganded AR with AREs.
Figure 5. AR associates with the proximal Mir23a27a24-2 promoter in prostate cancer cells in response to androgen, transiently increasing primary transcript levels and enhancing Drosha-mediated PrimiR cleavage. (A) Luciferase activity assay of Cos-1 cells transfected with 100 ng AR expression vector pSVAR and 1 μg of either proximal or distal miR-23a27a24-2 promoter reporter vector for 24 h, followed by 24 h treatment with MB (10 nM) or an equal volume of ethanol. β-Galactosidase activity was used as a control for cell number. Data represent mean relative luciferase activity for three independent experiments with duplicate analyses of each RNA sample ± SEM. (B) ChIP analysis of AR recruitment to the miR-23a27a24-2 cluster promoter in LNCaP cells treated with MB (10 nm) for 0–2 h. The purified DNA fraction was probed for the presence of proximal miR-23a27a24-2 promoter by qPCR. AR relative enrichment is displayed as a
within the cluster promoter. We used the ALGGEN algorithm (http://alggen.lsi.upc.es/) to predict putative AREs or half-sites within the miR-23a27a24-2 promoter. Two predicted AREs were identified within 300 bp 5' of the miR-23a27a24-2 transcription start site (Supplementary Material, Fig. S3A). To assess whether the proximal miR promoter can confer androgen responsiveness upon a luciferase reporter gene, indicative of primiR transcription, an 800 bp proximal portion (−726 to −23) and a 1.1 kb distal portion (−2982 to −1942) of the miR cluster promoter were inserted upstream of the luc2 gene in the pGL4.18 vector and transacted into Cos-1 cells alongside AR expression vector. Activity of the proximal fragment was increased 5-fold following androgen treatment compared with vehicle-treated cells (Fig. 5A), however miR distal promoter activity was unchanged, suggesting that this region is not androgen responsive. To assess whether liganded AR can directly associate with the proximal miR cluster promoter, ChIP analysis was performed on LNCaP cells following treatment with MB or vehicle for 0–2 h, using primers to amplify a 352 bp portion of the proximal miR cluster promoter (Supplementary Material, Fig. S4A). AR was significantly enriched by 2.5-fold on the proximal miR cluster promoter in LNCaP cells after 30 min of androgen treatment compared with vehicle-treated cells (Fig. 5B). However, association appears to be transient, as enrichment was reduced to basal levels after 60 and 120 min of androgen treatment. The well-defined androgen-regulated PSA gene promoter was used as a positive control throughout ChIP experiments (Supplementary Material, Fig. S4B–E). The above data suggest that AR is able to associate with the miR-23a27a24-2 promoter in response to androgen to initiate transcription of the cluster, albeit in a transient manner.

As miR-27a and its cluster partners are polycistrionically transcribed and all demonstrate androgen regulation, we wished to confirm whether AR association with the miR-23a27a24-2 promoter leads to androgen-dependent transcription of the entire endogenous miR-23a27a24-2 cluster. LNCaP cells were treated with MB for 0–24 h and qRT−PCR was performed using primers designed to amplify miR-23a27a24-2 primary transcript (primiR-23a27a24-2). PrimiR levels were increased 7-fold 1 h post-androgen treatment, and thereafter primiR levels declined until, after 24 h of androgen treatment, primiR levels were similar to vehicle-treated cells (Fig. 5C). MB-mediated AR activation across this time frame was confirmed by increased PSA expression (Supplementary Material, Fig. S5). Together with Figure 4A, Figure 5C suggests that an initial peak in primiR levels is translated into later increase in mature miR levels, with the possibility that androgen treatment acts not only at the miR cluster promoter, but also to enhance pri-mir or pre-mir turnover. In corroboration of this, examination of primiR-23a27a24-2 levels following AR silencing revealed primiR accumulation, rather than loss, in PCa cells following induction of AR shRNA, whereas induction of scrambled shRNA had no effect on primiR levels (Fig. 5D). These data indicate a further role for liganded AR in enhancing primiR/premiR processing, as AR-mediated miR promoter activation could not explain the observed decrease in primiR transcript levels following AR silencing. To investigate the ability of AR to enhance Drosha-mediated primiR-23a27a24-2 processing, we generated a reporter vector in which the genomic primiR-23a27a24-2 sequence is located 5' of the luc2 gene, under the control of a CMV promoter. Transcription yields primiR-23a27a24-2 transcript linked to the luc2 transcript. Drosha-mediated cleavage of the primiR disrupts the transcript, resulting in loss of luciferase activity—thus Drosha activity is inversely correlated with luciferase activity. This system has been shown to be a sensitive assay for Drosha-mediated cleavage of a specific primiR species in previous studies (46–48). The primiR-23a27a24-2 Drosha cleavage reporter was transfected into Cos-1 cells alongside AR and increasing doses of MB and Bic. Luciferase activity was significantly reduced in the presence of MB with reference to vehicle-treated cells, suggesting that androgen treatment increased Drosha cleavage of primiR-23a27a24-2. Addition of Bic to this system was able to abrogate the effect of androgen treatment on Drosha activity, confirming that liganded AR is able to specifically enhance Drosha processing of primiR-23a27a24-2. These effects were not observed in the absence of AR (Supplementary Material, Fig. S6A) or upon transfection of the empty CMV-GL4 plasmid (Supplementary Material, Fig. S6B).

AR regulation of miR-27a occurs during post-transcriptional processing of PrimiR to PremiR

To confirm that androgen regulation of miR-27a occurs post-transcriptionally, we investigated the ability of miR-27a and its cluster partners to accumulate in the absence of active transcription through treatment of LNCaP cells with the transcription-blocking agent, Actinomycin D (ActD), + androgen. We observed that after 6 h treatment of cells with ActD and androgen, primiR levels were increased 6-fold due to the presence of MB prior to degradation of transcripts (Fig. 6A). However, at later time points, primiR levels were drastically reduced due to transcriptional blocking and transcript degradation (Fig. 6A). Of note, transcription of another androgen-regulated gene, KLK2, showed a similar decrease at 24 and 48 h post-actinomycin treatment but without the initial peak at 6 h, presumably due to different dynamics of...
Figure 6. Androgen regulation of the PrimIR-23a27a24-2 cluster occurs post-transcriptionally. (A) qRT–PCR analysis of primiR-23a27a24-2 and mature miR-27a levels (i,ii), or L19 levels (iii) from LNCaP cells grown in stripped medium and treated with Actinomycin D (1 μM) and MB (1 nM), ethanol or ethanol or MB (1 nM) for 0–48 h. Reverse transcription was performed using oligo d(T) primers (primiR-23a27a24-2 and L19) or miR-specific primers (miR-27a). (B–D) qRT–PCR analysis of miR-23a (B), miR-24 (C) and miR-182 (D) from LNCaP cells grown in stripped medium and treated with Actinomycin D (1 μM) + MB (1 nM) for 0–48 h. Reverse transcription was performed using miR-specific primers. (A–D) Data represent mean relative gene expression for three independent experiments ± SEM. See also Supplementary Material, Figure S7. (E and F) qRT–PCR analysis of primiR-23a27a24-2 and miR-27a levels from PC3wtAR cells treated with 10 nM MB + 10 μM Bic for 24 h. L19 and U18 expression levels were used as normalization controls for primiR-23a27a24-2 and miR-27a levels, respectively. Data represent mean ± SEM for three independent experiments. (G) qRT–PCR analysis of miR-27a levels from LNCaP/TR2/scram and LNCaP/TR2/AR shRNA cells grown in stripped medium and treated with MB (10 nM) + dox for 48 h. U18 expression was used as a normalization control and data represent mean relative miR-27a expression for three independent experiments ± SEM. *P ≤ 0.05, **P ≤ 0.001.
AR regulation of this gene (Supplementary Material, Fig. S7). In contrast to these primary transcripts, mature miR-27a levels were increased 6-fold at 48 h post-treatment (Fig. 6Ai), showing a similar pattern of expression to that observed following androgen treatment in the absence of ActD (Fig. 4A). As expected, miR-27a levels were unchanged in the presence of ActD and ethanol (Fig. 6Aii), likely due to the lack of activated transcription and miR stability. L19 mRNA levels were significantly reduced by ActD treatment in the presence or absence of androgen, confirming transcriptional inactivation by ActD (Fig. 6Aiii). These data suggest that active transcription is not required to increase miR-27a levels following androgen treatment, and in combination with results shown in Figure 5E support the hypothesis that active androgen regulation of the miR cluster occurs during primiR to premiR processing. In confirmation of this, miR23a and miR24-2 are also increased in the presence of androgen, but not ethanol, following transcriptional inactivation (Fig. 6B and C, respectively). In contrast, miR-182, which we previously found to be unresponsive to androgen (data not shown), shows no difference in expression between vehicle- and androgen-treated cells following addition of ActD (Fig. 6D). In addition, Figure 6E and F shows that, while primiR-23a27a24-2 levels are reduced following 24 h MB treatment (Fig. 6E), mature miR-27a levels show the opposite, demonstrating a near 1.8-fold increase (Fig. 6F). Treatment with the antiandrogen Bic abrogated both MB-induced primiR decrease and miR-27a increase. Addition of Bic alone did not alter miR-27a levels but led to a small increase in primiR levels, presumably due to stabilization of the primiR. These data demonstrate that androgen treatment alters the equilibrium between primiR-23a27a24-2 and mature miR-27a, favouring accelerated miR-27a production by enhancing Drosha-mediated primiR cleavage. To confirm that androgen-enhanced post-transcriptional processing of primiR-23a27a24-2 requires AR, levels of mature miR-27a were assessed in LNCaP cells following dox-mediated induction of AR shRNA. Levels of miR-27a were 50% lower in PCa cells following AR silencing in the presence of androgen with reference to androgen-only-treated cells (Fig. 6F). This indicates that AR is required for androgen-induced primiR-23a27a24-2 to premiR-23a27a24-2 processing.

Together, these data indicate that androgen regulation of the miR-23a27a24-2 cluster occurs via two distinct processes. Initial regulation is at the level of transcription through trans-ient association of AR with the miR cluster promoter, resulting in increased levels of the primiR. Additionally, androgens then promote Drosha-mediated primiR to premiR processing resulting in increased mature mir-27a (and other cluster partners). Data presented here also suggest that there may exist a mechanism of androgen-mediated regulation that is specific to mir-27a, as mir-27a shows a greater androgen response than either miR-23a or miR-24-2.

DISCUSSION

Previous studies suggested that downregulation of PHB by AR is a key step in cell cycle initiation in androgen-dependent cells, and PHB may thus play an important role in PCa (14). We found that PHB regulation by androgens is largely mediated by miRs, specifically miR-27a. MiRs can act as oncogenes and tumour suppressors in a multitude of cancers. It is our hypothesis that oncomiRs can act by targeting important components of the androgen signalling pathway in PCa, and such oncomiRs may themselves be androgen regulated. This would result in a complex regulatory feedback loop that permits fine-tuning of AR activity. In gastric adenocarcinoma cells, inhibiting miR-27a was shown to inhibit growth, suggesting that it can act as an oncogene (23). We show here that miR-27a is subject to androgen regulation and demonstrate the direct consequences of miR-27a activity, and suppression thereof, on PHB expression, PCa cell growth and AR target gene expression. Further, we identify a novel mechanism for post-transcriptional modulation of miR biogenesis by AR.

The PHB-3′UTR contains one predicted miR-27a-binding site ~100 bp 3′ of the end of the coding region, which is highly conserved. This UTR conferred susceptibility for androgen-mediated downregulation to a luciferase reporter gene, suggesting that an androgen-regulated factor associates with PHB-3′UTR to reduce its activity (Fig. 1). AR silencing/inhibition increased levels of PHB transcript, confirming the requirement for AR in regulation of PHB-3′UTR. A candidate intermediary factor was miR-27a, which we confirmed reduced PHB levels via specific binding to the 3′UTR (Fig. 2). The dominant mechanism appears to be transcript degradation, rather than translational inhibition. Interestingly, PHB-3′UTR mutations have been reported in a number of breast cancer cell lines, including BT-20, SK-BR-3 and MCF7 (49), raising the possibility that mutations may occur in miR-binding sites. Indeed, Sato et al. (50) demonstrated mutation of PHB in sporadic breast cancer and Jakubowska et al. (51) have identified a C>T polymorphism in the 3′UTR of PHB as a breast cancer risk modifier in a Polish cohort carrying a BRCA1 mutation. In silico analysis performed on PHB-3′UTR mutations identified in breast cancer cell lines (49) has shown mutations in putative-binding sites for several miRs. However, it is as yet unknown whether these miRs target PHB in vivo and whether they have roles in cancer; no mutations have yet been identified in the miR-27a-binding sequence.

Functional consequences of miR-27a manipulation in PCa cells include increased expression of androgen-regulated genes, PSA and TMPRSS2, and increased androgen-dependent growth. Conversely, the miR-27a ASO significantly reduced growth in the presence of androgen (Fig. 3). In this context, it is noteworthy that WEE-1 and MYT-1 (important regulators of cyclin B and cdc2, which, like PHB, have roles in cell cycle progression) both contain potential miR-27a complementary sites in their 3′UTRs and have been demonstrated to be bone fide targets (38). Further, it has been demonstrated in breast cancer that miR-27a targets ZBTB10, a repressor of the Sp family of transcription factors that have been implicated in carcinogenesis in breast cancer (38), and this may indirectly affect ERα levels (52). The observed increase in PARP cleavage upon miR-27a ASO transfection and decrease in anti-apoptotic survivin and anti-angiogenic VEGF and VEGFR1 (38) demonstrates the potential of miR-27a to function as an oncomiR in vivo by targeting several tumour
suppressor proteins. An additional miR-27a target is the tumour suppressor FOXO1, which has important roles in cell cycle regulation and initiation of apoptosis (53). Similarly to PHB, it has been shown to be hormonally regulated and is downregulated in several cancers, including endometrial carcinoma and ovarian cancer (54). These observations suggest that miR-27a targets proteins with important cell cycle regulatory roles, and, at least in PCa, androgen-mediated upregulation of miR-27a may be required to downregulate cell cycle inhibitors for cell cycle initiation and cell growth. This highlights the potential ‘oncomiR’ function of miR-27a.

The multitude of potential miR-27a targets, several of which are also tumour suppressors, make it difficult to ascertain the relative contribution of miR-27a-targetting of a particular mRNA to observed phenotypic changes. An additional complicating factor is that miRs often act as ‘tuning agents’. In this model, miRs can function as rheostats to dampen protein levels to a more desirable level while still permitting the target protein to carry out essential functions in the cells. The combinatorial effects of low-level downregulation of multiple miR targets likely account for observed physiological effects following altered miR expression, rather than high efficiency targeting of a small number of mRNAs. Thus, it cannot be discounted that the effects of miR-27a suppression outlined here are due to low-level repression of several mRNA species. An argument for the key role of PHB is that we demonstrated that adding back PHB to cells with increased miR-27a resulted in inhibition of miR-27a-stimulated growth (Fig. 3), indicating that here miR-27a is exerting its effects largely via PHB. However, the suppression was not total. This could be a dosage effect but another likely explanation is that miR-27a suppression of other targets also contributes to the observed growth stimulation.

We found that androgen regulation of miR-27a occurs via several mechanisms. Levels of the cluster primary transcript, primiR-23a27a24-2, initially increased with androgen treatment but soon after decreased, suggesting that androgen acts both to rapidly and directly increase primiR levels and also increase the rate of primiR or premiR processing thus depleting the primiR (Figs 5 and 6). This hypothesis is supported by the steady increase in mature miR-27a following androgen addition. The mechanism of androgen regulation, therefore, appears not to be primarily stabilization of the mature miR. As no evidence exists for independent primiR expression within the miR-23a27a24-2 cluster (unlike the miR-23b27b24-1 cluster) and no internal poly(A)-binding sites have been identified (43), and given the observation that both cluster partners, miR-23a and miR-24-2, demonstrate androgen regulation, we initially hypothesized that such regulation occurs at the level of transcription from the miR promoter and/or processing of the primary transcript. However, since the three miRs of the cluster show differing degrees of androgen regulation (Fig. 4), it is possible that other unique regulation mechanisms exist, acting after primiR cleavage.

When assessing miR cluster promoter activity, we found that the proximal 700 bp conferred androgen responsiveness to a reporter, and within this saw androgen-dependent AR association with the proximal −366 to −24 region (containing two predicted AREs) (Fig. 5). However, these moderate effects may not entirely explain the rapid accumulation of primary transcript 1 h after MB treatment. AR may associate with additional regions or may require other factor(s) to associate efficiently with the miR promoter. Taken together, these data results indicate that transient association of AR with the miR-23a27a24-2 promoter results in a pulse of transcription of the miR cluster shortly after androgen treatment. However, primiR levels were increased following AR silencing (Fig. 5D) or anti-androgen treatment (Fig. 6E), an apparently paradoxical result. We showed using a Drosha cleavage reporter vector specific for the miR-23a27a24-2 cluster that androgens promote the cleavage of the primiR, which explains the increase in primiR upon AR silencing/inhibition, since blocking this processing would result in accumulation of the primiR. Thus, it seems that AR also acts post-transcriptionally during miR biogenesis to increase miR-27a levels, supported by the fact that miR-27a, 23a and 24-2 accumulate in the absence of transcription following addition of androgens (Fig. 6). While we have demonstrated that AR acts during Drosha-mediated primiR processing, the possibility that AR also acts to enhance Exportin 5-mediated primiR export, or to accelerate primiR processing by Dicer, cannot be entirely discounted. Work is on-going to elucidate exactly where AR exerts its effects on miR-23a27a24-2 processing and if this mechanism is unique to this cluster.

This report proposes a novel mechanism for androgen regulation of oncomiR-27a and its growth suppressor target, PHB, in PCa, summarized in Figure 7. Androgen signalling results in a reduction in transcription of the PHB gene as shown previously (12). Additionally, androgen treatment leads to upregulation of miR-27a, through enhanced cluster transcription and increased primiR processing. MiR-27a then associates with the PHB-3′UTR, resulting in transcript disruption and translational inhibition. These effects may be combinatorial and result in loss of PHB activity with a subsequent increase in AR activity and cell growth. As miR-27a ASO has been shown to increase PHB protein levels and reduce androgen-dependent PCA cell growth, miR-27a ASO may represent a novel therapeutic for the treatment of PCA.

In conclusion, we have identified complex, multi-level regulation of PHB by AR, mediated by several interconnected pathways. Regulation occurs at the levels of transcription, translation and post-translation in a spatial as well as temporal manner. Such intricate regulation is vital for such a ubiquitous protein with many fundamental cellular roles and proven tumour suppressor capacity. The deregulation of PHB control through increased miR27a may promote carcinogenesis or maintenance of the oncogenic phenotype in PCa.

MATERIALS AND METHODS

Mammalian cell culture

Cells were maintained at 37°C in 5% CO₂. HeLa and Cos-1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma), and LNCaP, DuCaP, VCaP, C42, DU145, PC3 and PC3wtAR cells were maintained in RPMI-1640 (Sigma). All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM 1-glutamine (Sigma). Twenty-four
to 48 h prior to ligand treatment, media were replaced with phenol red-free DMEM or RPMI-1640 as appropriate, supplemented with 5% charcoal-stripped FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. RWPE-1 cells were maintained in Keratinocyte-SFM (Life Technologies) supplemented with epidermal growth factor 1–53 (5 ng/ml) and bovine pituitary extract (50 μg/ml).

Stably transfected LNCaP cell variants, LNCaP/AR-shRNA, LNCaP/scrambled-shRNA (44), were maintained as above with 2.5 μg/ml blasticidin and 1 μg/ml puromycin. LNCaP/TR2/p2TetR-miR-27a and LNCaP/TR2/pcDNA4/TO-GFP-PHB were generated by stable transfection of LNCaP/TR2 cells (14) with p2TetR-miR-27a and pcDNA4/TO-GFP-PHB, respectively, and were maintained as above with 12 μg/ml blasticidin and 300 μg/ml zeocin.

**Cell lysis and protein extraction**

Cells were lysed and protein extracted as described (14). Lysates were stored at −20°C. See Supplementary Material, Experimental Procedures.

**Western blot analysis**

Proteins were resolved by 8–12% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membrane (Bio-Rad). After blocking (5% non-fat dried milk powder in 0.05% Tween-20 in 1× TBS) for 40 min, membrane was incubated with mouse anti-AR mAb (Dako, 1/1000), mouse anti-PHB mAb (Neomarkers, 1/1000) or mouse anti-β-actin mAb (Abcam, 1/10000) for 1 h and visualized using goat anti-mouse IgG-HRP. Detection was by ECL reagents (Amersham).

**RNA extraction and non-radioactive MiR northern blotting**

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Northern blotting was performed on 5–20 μg total RNA using double digoxigenin-labelled, LNA-modified probe (Exiqon) complementary to the sequence of mature miR-27a, as described previously (55) with the following amendments: RNA was electrotransferred to positive nylon membrane (Roche) at 300 mA for 40 min and hybridization was performed at 37°C using UltraHyb hybridization buffer (Ambion), followed by washing in 2× SSC, 0.1% SDS and then 0.1× SSC, 0.1% SDS at 37°C. Ethidium bromide staining of rRNAs was used as a loading control. Blots were analysed by addition of CDP-Star (Roche) and exposure of film to membrane or by membrane staining with BCIP/NBT.
Semi-quantitative RT–PCR

Five hundred nanograms of total RNA was reverse transcribed using oligo d(T) primers and the qScript Reverse Transcription kit (PrimerDesign). PCR was performed using 12.5 μl Taq-Reddy Mix (Abgene), 0–2 μg cDNA and 25 pmol forward and reverse primers per reaction and made to 25 μl with ddH2O. Primer sequences for amplification of PHB 3′UTR, AR and L19 are shown (Supplementary Material, Experimental Procedures and Table S1). Samples were thermocycled as follows: 94°C for 4 min, 30–40 cycles at (94°C for 30 s, 52–65°C for 30–60 s, 72°C–30 for 120 s), 72°C for 10 min. PCR products were analysed by agarose gel electrophoresis.

MiR and mRNA quantification by quantitative real-time RT–PCR

Mature miR expression was quantified by quantitative real-time RT–PCR using the TaqMan microRNA assay for hsa-miR-27a, hsa-miR-23a, hsa-miR-24, hsa-U18 and hsa-miR-182 (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. Five to 10 ng of total RNA was reverse transcribed per reaction. PCR was performed using 1.5 μl of the obtained cDNA in an ABI 900HT Real-Time PCR System under the 9600 Emulation mode. Cycling conditions were: 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 60 s. For detection of PHB, primiRs, AR and AR target genes, cDNA was prepared from 500 ng total RNA using Pre-mix. Oligo d(T) primer. cDNAs were amplified using 2× Fast SYBR Green Master Mix (Applied Biosystems) and 250 nm forward and reverse primers (Supplementary Material, Experimental Procedures and Table S2). All data were analysed using the ΔΔCt method, with U18 and L19 as endogenous references for miR and mRNA levels, respectively, using ethanol-treated samples as calibrators.

Oligonucleotide and plasmid transfection

MiR-27a anti-sense (ASO), scrambled ASO and miR-27a mimic were synthesized by MWG Eurofins (see Supplementary Material, Experimental Procedures and Table S3). Transfection of miR-27a/ASO was performed using Dharmafect 2 (Dharmacon) on LNCaP cells at 50% confluence. Transfection complexes were added to cells at a final concentration of 0–500 nm. After 24 h, RNA was extracted. Whole cell lysates were prepared 48 h after transfection. For transfection of LNCaP/GFP-PHB with miR-27a for SRB assays, miR-27a and negative control mimics were purchased from Dharmacon. Mimic transfection was performed using Lipofectamine RNAiMAX (Invitrogen) to give a final mimic concentration of 100 nm. Lipofectamine LTX (Invitrogen) was used for transfection of LNCaP/miR-27a cells with pcDNA4/TO-GFP-PHB to yield final plasmid concentrations of 0.3 or 1.5 ng/μl.

PHB-3′UTR analysis

The 200 bp SV40 promoter mRNA sequence was inserted into the MCS of pGL4.18 vector (Promega) using BglII and HindIII restriction sites to generate pSV40-GL4.18. A 900 bp DNA fragment corresponding to the PHB-3′UTR was then inserted 3′ of the luc2 gene using XbaI and SalI to generate the pSV40-GL4.18-PHB-3′UTR vector. Subsequently, miR-27a-binding site residues of the 3′UTR were mutated using the QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene) to produce pSV40-GL4.18-PHB-3′UTR mutant (Fig. 2A). pSV40-GL4.18-PHB-3′UTR or the mutant derivative vector (0–8 μg) was cotransfected with pSVAR and pdmLacZ into HeLa cells using the calcium phosphate method (56). For inhibition of miR-27a activity, miR-27a antisense (0–10 μg) was transfected into HeLa cells alongside expression and reporter vectors. Twenty-four hours post-transfection, cells were treated with MB or vehicle (EtOH) and harvested after a further 24 h.

PrimiR-23a27a24-2-specific Drosha cleavage activity assay

The 602 bp CMV promoter mRNA sequence was inserted into the MCS of pGL4.18 vector (Promega) using NheI and KpnI restriction sites to generate pCMV-GL4.18. The 390 bp miR-23a23a27a24-2 genomic sequence was then inserted 5′ of the luc2p gene and 3′ of the CMV promoter in the pCMVGL4.18 using BglII and HindIII restriction sites to generate the pCMV-primiR-23a27a24-2-GL4.18. This vector was cotransfected into Cos-1 cells alongside pSVAR and pdmLacZ using the calcium phosphate method (56). Twenty-four hours post-transfection, cells were treated with MB ± Bic, or vehicle (EtOH) and harvested after a further 24 h.

Reporter assays

Luciferase assays were performed using the Luclite assay (Packard, USA) and activity normalized for transfection efficiency using the Galacton kit (Tropix) as previously described (12) (See Supplementary Material, Experimental Procedures).

Chromatin immunoprecipitations (ChIP)

Cells grown in the starvation medium for 3 days were treated with 10 nm MB for 0–2 h. ChIP was performed on formaldehyde cross-linked cell samples as described (57). The presence of miR-23a27a24-2 promoter in immunoprecipitates was quantified by qRT–PCR using 5 μl DNA and Fast SYBR Green qPCR mix (Applied Biosystems). Primers shown in Supplementary Material, Experimental Procedures and Table S4.

Sulphorhodamine B (SRB) assay

Cell number was estimated using the SRB assay as previously described (13) (See Supplementary Material, Experimental Procedures).
Statistical analyses

Normally distributed continuous variables were assessed by student’s t-test. Strength of correlation between two normally distributed continuous variables was assessed by Pearson’s correlation coefficient (r). P ≤ 0.05 was interpreted to denote statistical significance.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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