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Regulation of several androgen-induced genes through the repression of the miR-99a/let-7c/miR-125b-2 miRNA cluster in prostate cancer cells

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Abstract

The androgen receptor (AR) stimulates and represses gene expression to promote the initiation and progression of prostate cancer. Here we report that androgen represses the miR-99a/let7c/125b-2 cluster through AR and anti-androgen drugs block the androgen-repression of the miRNA cluster. AR directly binds to the host gene of the miR-99a/let7c/125b-2 cluster, LINC00478. Expression of the cluster is repressed or activated by chromatin remodelers EZH2 or JMJD3 in the presence or absence of androgen, respectively. Bioinformatics analysis reveals a significant enrichment of targets of miR-99a, let-7c and miR-125b in androgen-induced gene sets, suggesting that downregulation of the miR-99a/let7c/125b-2 cluster by androgen protects many of their target mRNAs from degradation and indirectly assists in the gene induction. We validated the hypothesis with twelve potential targets of the miR-99a/let7c/125b-2 cluster induced by androgen: nine out of the twelve mRNAs are downregulated by the microRNA cluster. To ascertain the biological significance of this hypothesis we focused on IGF1R, a known prostate cancer growth factor that is induced by androgen and directly targeted by the miR-99a/let7c/125b-2 cluster. The androgen-induced cell proliferation is ameliorated to a similar extent as anti-androgen drugs by preventing the repression of the microRNAs or induction of IGF1R in androgen-dependent prostate cancer cells. Expression of a microRNA-resistant form of IGF1R protects these cells from inhibition by the miR-99a/let7c/125b-2 cluster. These results indicate that a thorough understanding of how androgen stimulates prostate cancer growth requires not only an understanding of genes directly induced/repressed by AR but also of genes indirectly induced by AR through the repression of key microRNAs.

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Conflict of interests

The authors declare no conflict of interest.

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Keywords

miRNA cluster; androgen regulation; prostate cancer proliferation

Introduction

The androgen receptor (AR) is essential for normal prostate development as well as the progression of prostate cancer. AR is typically activated by ligand-binding and translocates to the nucleus where it binds to the androgen responsive elements (AREs) and transactivates gene expression (1). The canonical ARE consists of an inverted repeat sequence 5'-AGAACAnnnTGTTCT-3' (http://transfac.gbf.de/TRANSFAC/) (2, 3). Genome-wide studies have revealed other non-canonical AREs sequences and ARE half-sites (4–7). Recent studies reported that androgen represses as many genes as it induces, and the majority of androgen-repressed genes bind AR nearby (8, 9). Androgen-activated AR actively represses gene expression, including the AR gene itself (10–13). Androgen-bound AR could repress genes by directly binding to activating transcription factors utilized by the promoter or competing for cofactors and thus interfering with its transcriptional activity (14–16). AR can also recruit corepressor complexes and histone modification enzymes (9–11, 13). AR-repressed genes displayed similar classification and distribution in gene ontology and pathway analyses as androgen-induced genes and the repression contributes to prostate cancer cell growth, survival and invasion (8, 11, 16).

miRNAs belong to a class of small non-coding RNA that regulates gene expression posttranscriptionally. Their aberrant expression has been observed in various diseases, including prostate cancer (17–20). Several groups have reported regulation of miRNAs by androgen with a focus on the miRNAs upregulated by androgen. miR-32 and miR-148a were upregulated by androgen and overexpressed in prostate cancers compared to benign prostate hyperplasia (21). miR-29a, miR-29b and miR-141 were also induced by androgen in prostate cancer cells (22). miR-22 and miR-134 are upregulated and miR-17, miR-18 and miR-20a are downregulated by androgens (23). miRNAs can be regulated at the levels of transcription, biogenesis or degradation (24). miR-21 was transcriptionally induced by AR directly bound to the miR-21 gene (25). On the other hand, androgen not only increased the transcription of the miR-23a/27a/24-2 cluster, but also promoted Drosha-mediated primary miRNA processing (26). Regulation of miRNA expression by androgen results in aberrant expression of miRNA target genes, which in turn may affect the progression of prostate cancer.

In this study, we report the downregulation of the miR-99a/let7c/125b-2 cluster by androgen in prostate cancer cells. The miR-99a/let7c/125b-2 cluster is transcriptionally repressed by androgen-activated AR. There is a significant enrichment of target genes of the miR-99a/let7c/125b-2 cluster among androgen-induced genes, suggesting that gene induction by androgen in prostate cancer is indirectly assisted through repression of specific miRNAs. The importance of this indirect regulation of gene expression by AR is revealed by the abrogation of androgen-stimulated cell proliferation when the decrease of mR-99a/let7c/125b-2 is prevented.

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Results

The miR-99a/let7c/125b-2 cluster of miRNAs is repressed by androgen

miR-99a, miR-100 and miR-125b are downregulated by androgen treatment (19), but the mechanism of downregulation was not known. The two genomic loci of miR-125b, miR-125b-1 on chromosome 11 is encoded in mir-100/let-7a-2 cluster (MIR100HG) while miR-125b-2 on chromosome 21 is encoded with miR-99a and let-7c within an intron of a long non-coding RNA LINC00478 (Table 1 and Fig. 1C), while miR-125b-1 (Table 1). To distinguish between the clusters, we checked whether let-7c or let-7a was repressed by androgen in a manner similar to miR-99a, miR-100 and miR-125b. let-7c, but nor let-7a, was repressed by synthetic androgen R1881 in a dose dependent manner in LNCaP cells (Fig. 1A), suggesting that the miR-99a/let7c/125b-2 cluster is responsive to androgen. To test whether androgen represses the transcription of the cluster, we measured the expression of primary miRNAs of the miR-99a/let7c/125b-2 cluster. Pri-miR-99a, pri-let7c and pri-miR-125b-1 were all decreased by androgen (Fig. 1B).

Since the miR-99a/let7c/125b-2 cluster is located in an intron, we examined whether the host gene, LINC00478, is also repressed by androgen. Two transcripts of LINC00478, long (NR_027790) and short (NR_027791), could encode the miR-99a/let7c/125b-2 cluster (Fig. 1C). Six pairs of primers were used to assess androgen regulation of both transcripts (Fig. 1C). F1R1 and F2R2 detect the mature and precursor forms of the long transcript, respectively. F3R3 detects the mature short transcript, while the other three primer pairs detect both transcripts. Both transcripts of LINC00478 were downregulated by androgen, with the long transcript more responsive than the short transcript (Fig. 1C; F1R1 vs. F3R3).

Downregulation of the miR-99a/let7c/125b-2 cluster by androgen requires the presence of AR

We next determine whether AR is required for the androgen-repression of the miR-99a/let7c/125b-2 cluster. As in the AR-positive LNCaP cells, the expression of the miR-99a/let7c/125b-2 cluster was decreased by androgen in AR-positive C4-2 prostate cancer cells (Fig. 2A and Fig. S1A), but not in AR-negative PC-3 prostate cancer cells (Fig. 2B and Fig. S1B). PC-3 cells have a wild type AR gene but do not express AR due to weak transcriptional activity at the AR promoter (27). Ectopic expression of wild type AR in PC-3 cells (Fig. S2B) restored the repression of miR-99a/let7c/125b-2 by androgen (Fig. 2C and Fig. S1C). Conversely, knocking down AR in LNCaP cells by siRNA (Fig. S2A) abolished the repression of miR-99a/let7c/125b-2 by androgen (Fig. 2D and Fig. S1D). Moreover, treatment of anti-androgen drugs Bicalutamide and Flutamide in LNCaP cells also blocked the androgen-induced repression of miR-99a/let7c/125b-2 (Fig. 2E). These results suggest that androgen represses the expression of miR-99a/let7c/125b-2 specifically through AR in prostate cancer cells.

AR binds to the host genes of the miR-99a/let7c/125b-2 cluster

Next we evaluated whether AR directly binds to the host gene of the miR-99a/let7c/125b-2 cluster, LINC00478. AR ChIP-seq data in LNCaP cells in the presence or absence of R1881 have been published (28). Peaks of AR binding were identified using HPeak, a Hidden

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Markov Model (HMM)-based peak identifying algorithm (http://www.sph.umich.edu/csg/qin/HPeak/). Seven AR binding sites were reported by the ChIP-seq upstream and within the LINC00478 gene: ARBS1–7 (Fig. 3A). ARBS1 and ARBS2 are within 50kb of the transcription start site (TSS) of the long transcript and ARBS3, ARBS4 and ARBS5 are within 50kb of the TSS of the short transcript. Since genome-wide ChIP-seq analyses often have significant false-positive rates, AR ChIP and quantitative real-time PCR was performed to validate whether AR bound to any of these five potential binding sites. Androgen induced AR binding to ARBS1 and ARBS2 (Fig. 3B) to an extent that was similar to the binding to the well-characterized PSA enhancer (AREIII) (10). Significant AR binding or AREs were not seen at the other AR ChIP-seq sites. Interestingly, ARE half-sites were detected at ARBS1 and ARBS2 by ALGGEN-PROMO, a matrix algorithm for predicting transcription factor binding sites based on TRANSFAC (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) (Fig. S2D). ARBS1 contains "TGTCCT", an exact half site of canonical ARE (5), while ARBS2 contains "GGGACA", which resembles the other half of the canonical ARE ("GGTACA" or "GGAACA") (5).

Polycomb protein EZH2 is involved in AR-repression of the miR-99a/let7c/125b-2 cluster

It has previously been shown that androgen-induced gene expression is mediated by forkhead box protein A1 (FOXA1) and active histone modifications including H3K4 me1 and H3K4me2 (29). However, the mechanisms of androgen-mediated gene repression are more complex. Global ChIP-seq analysis revealed EZH2 binding and a significant enrichment of repressive histone mark H3K27me3 around AR-repressed genes, which suggested that EZH2 was involved in AR-repressed gene expression. To test whether EZH2 was responsible for the downregulation of the miR-99a/let7c/125b-2 cluster by androgen treatment, we transfected siRNA against EZH2 in LNCaP cells, and examined the expression of the miR-99a/let7c/125b-2 cluster in the absence or the presence of androgen. Depletion of EZH2 significantly diminished the repression of the miR-99a/let7c/125b-2 cluster by androgen (Fig. 3C).

Conversely, H3K27 demethylase JMJD3 has been known to counteract polycomb protein mediated gene repression (30, 31). JMJD3 erases the H3K27me3 mark and activates gene expression (32, 33). To examine if JMJD3 is involved in elevating the expression of the miR-99a/let7c/125b-2 cluster upon androgen depletion, we knocked down JMJD3 using siRNAs. In the absence of androgen, where the expression of the miR-99a/let7c/125b-2 cluster is normally induced, knocking-down JMJD3 decreased the level of all three primary miRNAs (Fig. 3D). These results suggest that EZH2 or JMJD3 reversibly inhibits or activates the expression of the miR-99a/let7c/125b-2 cluster in the presence or absence of androgen, respectively.

Target genes of the miR-99a/let7c/125b-2 cluster are enriched in androgen-induced genes

Since the miR-99a/let7c/125b-2 cluster was repressed by androgen, we hypothesized that target genes of miR-99a, let-7c and miR-125b may be upregulated upon androgen treatment as a result of reduced targeting by the miR-99a/let7c/125b-2 cluster. To test this hypothesis, we extracted lists of potential targets of miR-99a, let-7c and miR-125b from TargetScan (version 6.0), a widely used target prediction algorithm of miRNAs. Two lists of androgen-

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induced genes were obtained from two independent studies (GSE17044 and GSE22606), in which LNCaP cells were treated with R1881or vehicle and gene expression was measured by microarray analysis. To determine which (and how many) androgen-induced genes were also miRNA targets, we intersected predicted targets of each miRNA with androgen-induced genes from each microarray dataset (Table S1, S2 and S3). We performed a Monte Carlo simulation to determine the expected number of miRNA targets in the androgen-induced gene sets at random. The fold enrichment (with p-value) was calculated of the observed number of androgen-induced miRNA targets relative to the random expectation. Potential targets of miR-99a were 4.92 and 6.63 fold enriched in the two respective androgen-induced gene sets, while targets of let-7c and miR-125b were also significantly enriched in androgen-upregulated genes (Table 2).

Table 3 contains the androgen-induced genes that are targets of the miR-99a/let7c/125b-2 cluster that were common between the two microarray datasets used. We selected four target genes of each miRNA and validated by qRT-PCR that they were induced after androgen treatment. The expression of all of the genes we tested was increased by androgen (Fig. 4A). To test whether the AR-induced genes were also repressed by the miR-99a/let7c/125b-2 cluster, we measured their mRNAs by qRT-PCR after miRNA transfection. At least 75% of the mRNAs tested were significantly decreased by the miR-99a/let7c/125b-2 cluster (Fig. 4B). Since the androgen-repression of the miR-99a/let7c/125b-2 cluster requires AR, we also examined the expression of these mRNAs after knocking-down AR by siRNA. None of the selected genes were stimulated by androgen after AR knock-down (Fig. S3A, B and C). Thus, many genes induced by androgens through AR, are concurrently de-repressed through AR mediated inhibition of the miR-99a/let7c/125b-2 cluster.

IGF1R is a key target of the miR-99a/let7c/125b-2 cluster

Among the androgen-induced target genes of the miR-99a/let7c/125b-2 cluster, factors in the IGF signaling pathway appeared multiple times: insulin-like growth factor 1 receptor (IGF1R) was a target of miR-99a and let-7c, and insulin receptor substrate 2 (IRS2) was a target of let-7c. miR-125b was also predicted to target IGF1R, but at a site that is conserved only among primates. IGF1R is important for the tumorigenesis and progression of prostate cancer, because of its demonstrated roles in angiogenesis, transformation and mitogenesis (34–36). It is frequently overexpressed in prostate tumors and often associated with poor prognosis (37, 38). Several monoclonal antibodies and small molecule inhibitors of IGF1R are currently under investigation in clinical trials for treating metastatic castration-resistant prostate cancer (CRPC) (39, 40). We therefore decided to follow up on IGF1R and first validated that IGF1R was a direct target of the miR-99a/let7c/125b-2 cluster by luciferase reporter assay (Fig. 5A). In the 3’UTR of IGF1R there was one predicted binding site each for miR-99a and miR-125b and three for let-7c (Fig. 5A). It was shown previously that let-7 directly downregulated several genes in the IGF signaling pathway, including IGF1R, but the report did not clarify whether all three target sites were active in IGF1R repression (41). Therefore, we tested the effect of each miRNA target site on IGF1R by dividing the 3’UTR of IGF1R into three fragments and cloning each of them into a luciferase reporter. The first fragment contained one let-7c binding site, the second fragment had the miR-125b binding site and another let-7c binding site, and the third fragment included the miR-99a binding site

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and the third let-7c binding site (Fig. 5A). In the luciferase reporter assay, ectopic expression of miR-99a and let-7c but not miR-125b decreased the luciferase activity, indicating that miR-99a and let-7c directly bound to the 3'UTR of IGF1R and regulated its expression (Fig. 5A). The two 3'-most binding sites of let-7c responded to the microRNA, with the most significant regulation coming from the third binding site. The differential effects of these miRNA binding sites might be due to the secondary structure of the target mRNA or the binding of other proteins to the target mRNA.

We then checked the expression level of IGF1R after transfecting the miR-99a/let7c/125b-2 cluster into cells. Overexpression of the miR-99a/let7c/125b-2 cluster decreased both mRNA and protein level of IGF1R in LNCaP cells (Fig. 5B and C).

Androgen is known to stimulate the growth of prostate cancer cells, and members of the miR-99a/let7c/125b-2 cluster have been shown previously to have growth suppressive functions (19, 42–45). We therefore hypothesized that androgen-repression of the miR-99a/let7c/125b-2 cluster promoted androgen-dependent growth through the derepression of IGF1R. To test this hypothesis, we first performed a growth assay in LNCaP cells. Androgen stimulated proliferation of LNCaP cells (Fig. 5D, CS vs 1nM R1881 si-GL2), and anti-androgen drugs Bicalutamide or Flutamide blocked the growth stimulation by androgen (Fig. 5D). Interestingly, forced expression of miR-99a, let-7c or miR-125b to prevent their downregulation by androgen also blunted the growth stimulation by androgen, to a similar extent as the anti-androgen drugs (Fig. 5D). This growth suppressive effect of the miR-99a/let7c/125b-2 cluster in LNCaP cells was observed after both 4 day and 8 day treatments (Fig. 5D and Fig. S4A). Let-7c and miR-125b inhibited proliferation more than anti-androgen drugs after 8 days of treatment (Fig. S4A). Introduction of the miR-99a/let7c/125b-2 cluster did not significantly inhibit the growth of LNCaP cells in the absence of androgen, suggesting they specifically inhibit androgen-stimulated growth in LNCaP cells (Fig. S4B).

To further verify our hypothesis, we repeated the growth assays in AR-positive CW22RV1 and AR-negative DU145 prostate cancer cells. Similar to the effect in LNCaP cells, ectopic expression of the miR-99a/let7c/125b-2 cluster significantly inhibited the growth of CW22RV1 cells (Fig. S4C). In contrast, the miR-99a/let7c/125b-2 cluster did not suppress the growth of the AR-negative and androgen-independent DU145 cells (Fig. S4D).

We next hypothesized that the inhibitory effect of the miR-99a/let7c/125b-2 cluster on androgen-dependent growth is through downregulating IGF1R. Indeed, siRNA against IGF1R suppressed cell proliferation of LNCaP cells to a similar extent as the microRNAs (Fig. 5D), suggesting that miR-99a and let-7c, the two miRNAs we found to directly repress IGF1R, may inhibit LNCaP cell growth through targeting IGF1R. To test this possibility, we generated a LNCaP cell line that stably expresses IGF1R, which lacks the 3'UTR and so is not repressed by the miRNAs that target the 3'UTR (Fig. S2C). Overexpression of IGF1R without its 3'UTR rescued the growth defect caused by miR-99a, let-7c or siRNA targeting the 3'UTR of IGF1R, indicating that the growth suppression of LNCaP cells by the miR-99a/let7c/125b-2 cluster in the presence of androgen was primarily due to the downregulation of IGF1R (Fig. 5E). The result also suggests that repression of the miR-99a/

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let7c/125b-2 cluster by androgen will contribute to prostate cancer growth by relieving IGF1R from microRNA inhibition (Fig. 5F).

Discussion

miRNAs, like other RNA polymerase II driven transcripts, are known to be regulated by various transcription factors. miR-34 and miR-205 both possess tumor suppressive function in cancer cells and are directly induced by the tumor suppressor p53 (46, 47). The expression of miR-17 oncomiR cluster, on the other hand, is known to be driven by oncogenic transcription factor c-myc (48). Similar to p53 and c-myc, androgen receptor has also been shown to regulate miRNA expression (21, 25, 26, 49). Androgen-regulated miRNAs also contribute to castration-resistant prostate cancer (CRPC) (21, 25). Many androgen-induced miRNAs, such as miR-32, miR-148 and miR-21, are oncogenic and upregulated in CRPC compared to benign prostate tissue, while androgen-repressed miRNAs, such as miR-99a and miR-221, tend to be tumor suppressive and are usually downregulated in CRPC (19, 21, 25, 49).

In this paper, we demonstrate that the miR-99a/let7c/125b-2 cluster is repressed by androgen and this can contribute to changes in the gene expression program and cell proliferation induced by androgen. Interestingly, let-7c decreased AR expression by targeting c-myc, a known transcription factor for AR, suggesting that repression of miR-99a/let7c/125b-2 cluster by androgen may positively feedback and stimulate AR production (50–52). In contrast to our results, S. Inoue's group reported the miR-99a/let7c/125b-2 cluster as an androgen-induced gene in LNCaP cells after 24 hour treatment with R1881 (53). This may be explained by different conditions of androgen treatment and tissue culture, since we harvested cells after 48 hours of androgen-deprivation (charcoal-stripped media and FBS) followed by 48 hours of R1881 treatment. On the other hand, our results are supported by two other independent studies. One study showed that the host gene of the miR-99a/let7c/125b-2 cluster LINC00478 was repressed by androgen in VCaP and VCS2 cells (10). Another study observed androgen-repression of miR-125b by northern blotting in LNCaP cells (25).

According to published ChIP-seq data, there were two AR binding sites (ARBS) within 50kb of the TSS of the long transcript of LINC00478 and three ARBS near the TSS of the short variant. Our AR ChIP-qPCR analysis only confirmed two of these AR binding sites near the promoter of the long transcript (Fig. 3B). However, our RT-qPCR demonstrated that both of the transcripts were repressed by androgen, though the short variant is repressed to a less extent (Fig. 1C; mature transcripts F3R3 vs. F1R1). The modest regulation of the short variant by androgen may be due to its remote location relative to the potential AR enhancers ARBS1 and ARBS2. Other weaker AR enhancers of the short transcript, not captured by the ChIP-seq analysis, may also exist. An interesting twist is that the two functional ARBS have only half-ARE sites. Such half-ARE sites can bind to AR both *in vitro* and *in vivo* (6, 54). Therefore, these two ARE half-sites likely serve as transcriptional enhancers for AR. It is still unknown how AR binds to ARE half-sites. Since AR forms dimer independent of DNA binding, it is possible that only one DNA binding domain (DBD) of the AR dimer binds to the ARE half-site and this binding is stabilized by other

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DNA binding proteins (55). The AR dimer may also bind to two separate ARE half-sites (ARBS1 and ARBS2) through chromatin looping.

EZH2 (enhancer of zeste 2) is part of the Polycomb Repressive Complex 2 (PRC2) and responsible for the trimethylation of H3K27 on target gene promoters. EZH2 is frequently overexpressed in aggressive tumors including prostate cancer, which is often associated with poor prognosis (56, 57). Knock-down of EZH2 relieved its target genes from repression and inhibited proliferation of prostate cancer cells (58). Global gene repression by AR has been suggested to be mainly mediated by EZH2 and its associated repressive histone mark H3K27me3 (9). Our results are consistent with this suggestion, though androgen still repressed the miR-99a/let7c/125b-2 cluster in cells transfected with si-EZH2. siRNAs cannot completely eliminate the target in the transfected cells and this could account for the residual repression by androgen, but we cannot rule out additional mechanisms that contribute to repression of the miR-99a/let7c/125b-2 cluster by androgen. Many genes repressed by AR and EZH2 promoted cell differentiation and were downregulated in CRPC (9), just as seen with the miR-99a/let7c/125b-2 cluster. The reduced expression of the miR-99a/let7c/125b-2 cluster in CRPC is consistent with upregulation of EZH2 in aggressive prostate cancers.

JMJD3 (jumonji domain containing 3) has recently been discovered as a histone H3K27 demethylase (33). It specifically removes the tri-methylation marks from H3K27 and activates gene expression, counteracting the effect of polycomb proteins, including EZH2 (30, 32). Similar to polycomb proteins, JMJD3 is also involved in regulating development and cell differentiation, as well as cancer formation (32, 33, 59–61). In many cases, JMJD3 and EZH2 counter-balance each other to control expression of specific genes, consistent with what we have observed for the miR-99a/let7c/125b-2 cluster in this study (30, 31). The expression of JMJD3 is upregulated in prostate cancer, especially in metastatic prostate cancer (32). However, JMJD3 is also reported to act as a tumor suppressor and inhibit cell proliferation (33, 61). Our work suggests that JMJD3 may function as a tumor suppressor as it induces the expression of the growth suppressive miR-99a/let7c/125b-2 cluster. Further work is needed to understand the exact function of JMJD3 in prostate cancer cells, especially in response to androgen regulation.

Insulin-like growth factor 1 receptor (IGF1R) is the primary receptor for IGF-I that also binds to IGF-II and insulin. Ligand-activated IGF1R activates downstream signaling pathways, including the PI3K/Akt pathway, MAPK pathway and STAT3 pathway, resulting in cell proliferation, inhibition of apoptosis and increased motility (34–36). Increased serum level of IGF-I and elevated expression of IGF-I and IGF1R in malignant prostate tumors are often associated with poor prognosis (37, 38). Several monoclonal antibodies and small molecule inhibitors of IGF1R are currently under investigation in clinical trials for treating metastatic castration-resistant prostate cancer (CRPC) (39, 40). IGF-I enhances the nuclear translocation of AR, while androgen promotes the cellular response to IGF-I treatment (34, 62) thereby producing a pro-proliferation positive feedback loop. This increased response to IGF-I treatment in the presence of androgen is due to increased expression of IGF1R (62). It was suggested that AR indirectly increased the transcription of IGF1R, possibly through activation of ERK pathway (62, 63), or through androgen-induced expression of KLF6

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(Kruppel factor like 6), a transcription factor for IGF1R (64, 65). Here we report another way by which the expression of IGF1R is regulated by androgen. Our results suggest that treatment of androgen decreases the expression of miR-99a and let-7c and in turn derepresses their common target IGF1R at both the mRNA and protein level. This pathway could also account for conditions where androgen increases the protein level of IGF1R without an increase in mRNA (65). Consistent with previous studies, we also confirm the direct regulation of IGF1R by miR-99a and let-7c (41, 66). Furthermore, we determined that the second and third predicted binding sites in the 3'UTR of IGF1R were the primary target sites for let-7c.

While it is generally accepted that androgen-activated AR binds to promoters of many androgen-induced genes, our results suggest that several of these genes are also targets of microRNAs that are repressed by androgen-activated AR. This forms a feed-forward regulatory loop consisting of transcription factor AR, AR-repressed miRNAs and androgen-induced genes that are also targeted by these AR-regulated miRNAs. A similar miRNA-transcription factor regulatory network has been illustrated in glioblastoma multiforme (GBM) where many of the GBM-related genes are regulated both transcriptionally and by miRNAs (67). In this study, we show that AR and AR-repressed miRNAs both contribute to androgen-induced gene expression. Among the 12 androgen-induced genes that we tested in this study (Fig. 4), only three genes (FKBP5, GREB1 and ANKH) are not regulated by the miR-99a/let7c/125b-2 cluster and are known to be directly regulated by AR at the transcriptional level (3, 68, 69). The remaining nine androgen-induced genes are all regulated by the miR-99a/let7c/125b-2 cluster. Some of them are directly activated by AR at promoters, so we expect the post-transcriptional de-repression resulting from inhibition of the miRNAs to be a secondary means of increasing expression of these genes. However, it is noteworthy that of the 32 targets of the miR-99a/let7c/125b-2 cluster that are induced by androgen and listed in Table 3, 12 have no AR binding peaks within 50kb of their promoters from AR ChIP-seq data (28). Among the nine validated androgen-induced miRNA targets, FZD8, FZD5 and IRS2 do not exhibit AR binding peaks within 50kb of their promoters (28). Thus it is also possible that relief of post-transcriptional degradation by miRNAs plays an important role in induction of these genes by androgen. A recent bioinformatics study found that transcription factors regulating a miRNA often also regulate the target genes of this miRNA, consistent with the dual regulation of gene expression at both transcriptional and post-transcriptional levels we propose in this study (70). Although there have been some studies focusing on miRNAs induced by oncogenes and tumor suppressors, this study highlights the importance of following miRNAs that may be repressed by such genes. Also gene expression changes induced by nuclear hormone receptors like AR are usually believed to be due to the direct action of the receptor on the promoter of the target genes. Our results suggest that some of the gene induction may be explained by integration of miRNAs repressed by such nuclear receptors into the network.

Taken together, our results identify a regulatory network involving key players in the development of prostate cancer: the transcription factor AR, the chromatin remodeling factors, EZH2 and JMJD3, growths suppressive microRNAs in the miR-99a/let7c/125b-2 cluster, and signal transduction factor IGF1R. Upon androgen treatment, activated AR translocates to the nucleus, where it binds to AREs (ARE half-sites) at the enhancer of a

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non-coding RNA LINC00478 and recruits the histone methyltransferase EZH2. This results in decreased expression of LINC00478 and the miR-99a/let7c/125b-2 cluster generated from its intron. Downregulation of miR-99a, let-7c and miR-125b leads to derepression of their target genes, including IGF1R, and triggers a series of downstream signaling cascades, which increases proliferation of prostate cancer cells (Fig. 5F). Similarly, in CRPC cells, hyperactivated AR reduces expression of the miR-99a/let7c/125b-2 cluster and increases expression of their targets, which may contribute to prostate cancer progression. This is consistent with the reduced expression of miR-99a, let-7c and miR-125b and increased level of IGF1R seen in prostate cancer cells (19, 21, 37, 38, 43).

Materials and Methods

Cells and Tissues

Human prostate cancer cells LNCaP, C4-2 and PC3 were obtained from ATCC and maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum. For experiments on androgen responsiveness, cells were cultured in phenol red-free RPMI 1640 medium supplemented with charcoal:dextran stripped fetal bovine serum (Hyclone) for 48 hours before the addition of the androgen analog R1881 (Perkin-Elmer).

Transfection of siRNA and cell growth assay

Transfection of siRNA or miRNA duplex was performed with Lipofectamine RNAiMax reagent (Invitrogen) as described (71). siRNA was transfected on Day 0 and Day 2 separately, and cells were counted on Day 4 using Countess automated cell counter (Invitrogen).

Chromatin Immunoprecipitation (ChIP) Assay

LNCaP cells were fixed using formaldehyde at RT for 15min and then lysed. Chromatin was sonicated to an average length of 300–500bp. Anti-AR (H-280, santa cruz) and rabbit ChIP grade IgG (Abcam) were used to precipitate DNA fragments. Quantitative real-time PCR was used to analyze AR binding at seven putative AR binding sites. PSA enhancer (ARE3) was used as a positive control (10). The primers used in q-PCR analysis were described in Table S4.

Western blotting

The antibodies used were as follows: anti-IGF1R β (Cell Signaling), anti-AR (Millipore) and anti-β-actin (Sigma). The western blot image was captured by G:Box iChemi XT gel documentation and analysis system. Signal intensity of western blots was quantified with GeneTools from SynGene.

RNA isolation and quantification of miRNA

Total RNA was extracted using TRIzol (Invitrogen). 1μg total RNA was reverse transcribed using NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen). The expression level of miRNAs was measured by quantitative PCR using NCode SYBR GreenER miRNA qPCR

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kit in triplicate. U6 small nuclear RNA (snU6) was used to normalize the expression data of miRNAs (19).

Luciferase reporter assay

The 3'-UTR fragments of IGF1R containing miR-99, let-7c and miR-125b binding sites were cloned into a modified vector pRL-CMV (19). The mutations were made to the miRNA binding sites in the 3'UTR-MUT clones. The primers used in 3'UTR or 3'UTR-MUT cloning are described in Table S4. The luciferase reporter assay was performed as previously described (71).

HPeak analysis of ChIP-seq data

We analyzed the AR ChIP-seq data in LNCaP cells in the presence or the absence of androgen. AR ChIP-seq data were downloaded from GEO database (GSM353644 and GSM353643). HPeak version 2.1 (72) was used to find genomic regions enriched for androgen-induced AR binding in LNCaP cells.

Enrichment analysis of miRNA targets in androgen-induced gene sets

The lists of predicted targets of miR-99a, let-7c and miR-125b were downloaded from TargetScan (http://www.targetscan.org/, version 6.0). We only considered the targets where the sites were broadly conserved among vertebrates. Two sets of androgen-induced genes in LNCaP cells were generated from two independent microarray analyses (GSE22606 and GSE17044). All genes with ≥1.2 fold induction by androgen were included. To determine if putative targets of miR-99a/let7c/125b-2 cluster were enriched in androgen-induced gene sets, we first generated the "observed number" of androgen-induced genes that are also miRNA targets by intersecting the potential targets of miR-99a/let7c/125b-2 with each androgen-induced gene set. The randomly "expected number" of androgen-induced genes that are also miRNA targets given the number of miRNA targets were determined using 1000 rounds of Monte Carlo simulation, assuming that miRNA targets were uniformly distributed in the total database of genes. The significance of any enrichment of "observed" to "expected" is also given by the Monte Carlo simulation as the proportion of simulation rounds that were more extreme than the observation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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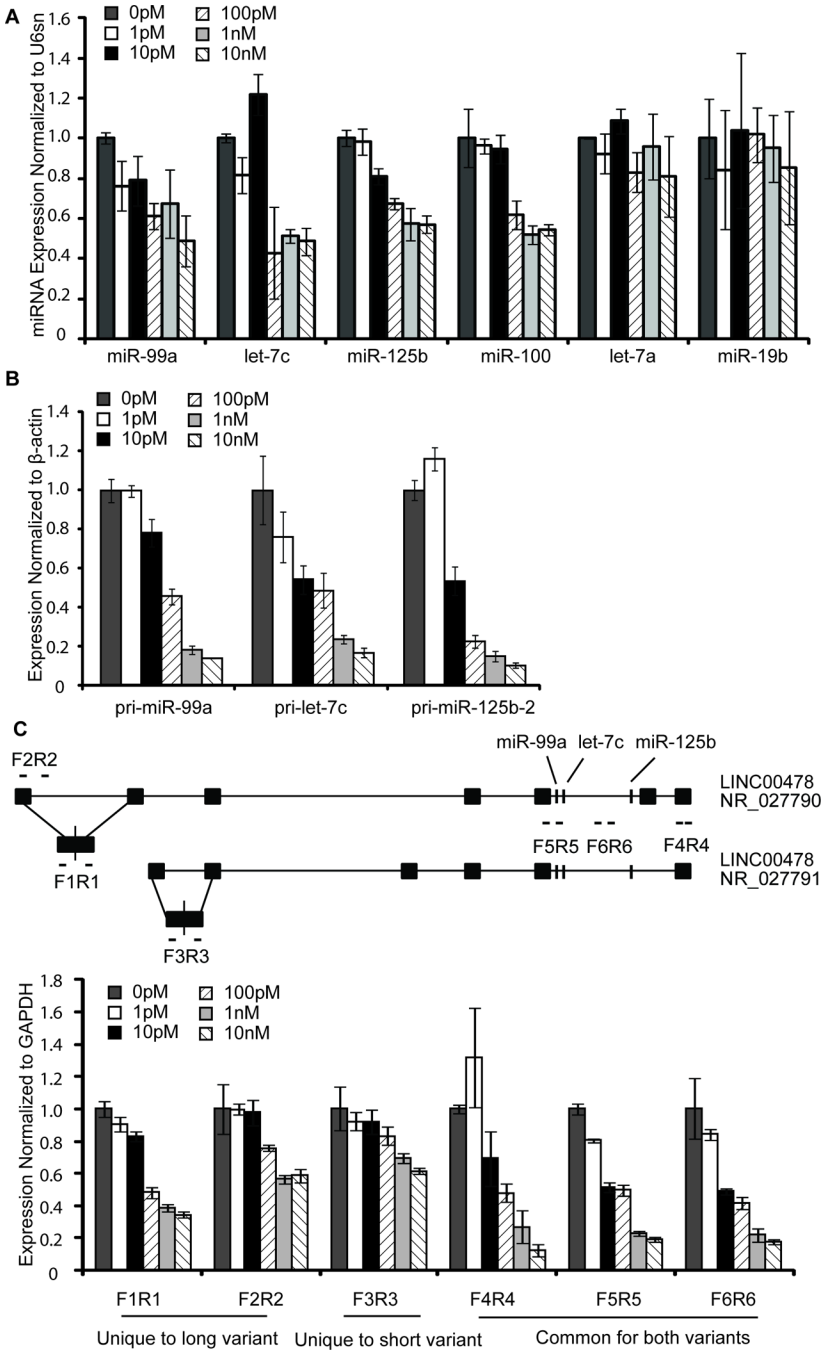

Figure 1. miR-99a/let7c/125b-2 cluster and its host gene LINC00478 are repressed by androgen

A. qPCR was used to measure the expression level of miRNAs. The values were normalized to that of U6sn. Mean±s.d. n=3. The expression level under no androgen treatment is set as 1. LNCaP cells were treated with charcoal-stripped serum for 48 hrs followed by treatment with indicated concentrations of R1881. **B.** The expression of primary miRNAs was measured by qPCR and normalized to β-actin. Rest as in Fig. 1A. **C.** Six pairs of primers were used to measure the expression of the two variant transcripts of LINC00478 at different concentrations of androgen. Schematic at the top shows the two transcripts of

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LINC00478 with the exons indicated by black boxes and the microRNAs shown by vertical lines. Primer pairs are indicated by horizontal lines. F1R1 and F3R3 amplify across splice junctions of the mature RNA. qPCR value was normalized to GAPDH. Rest as in Fig. 1A

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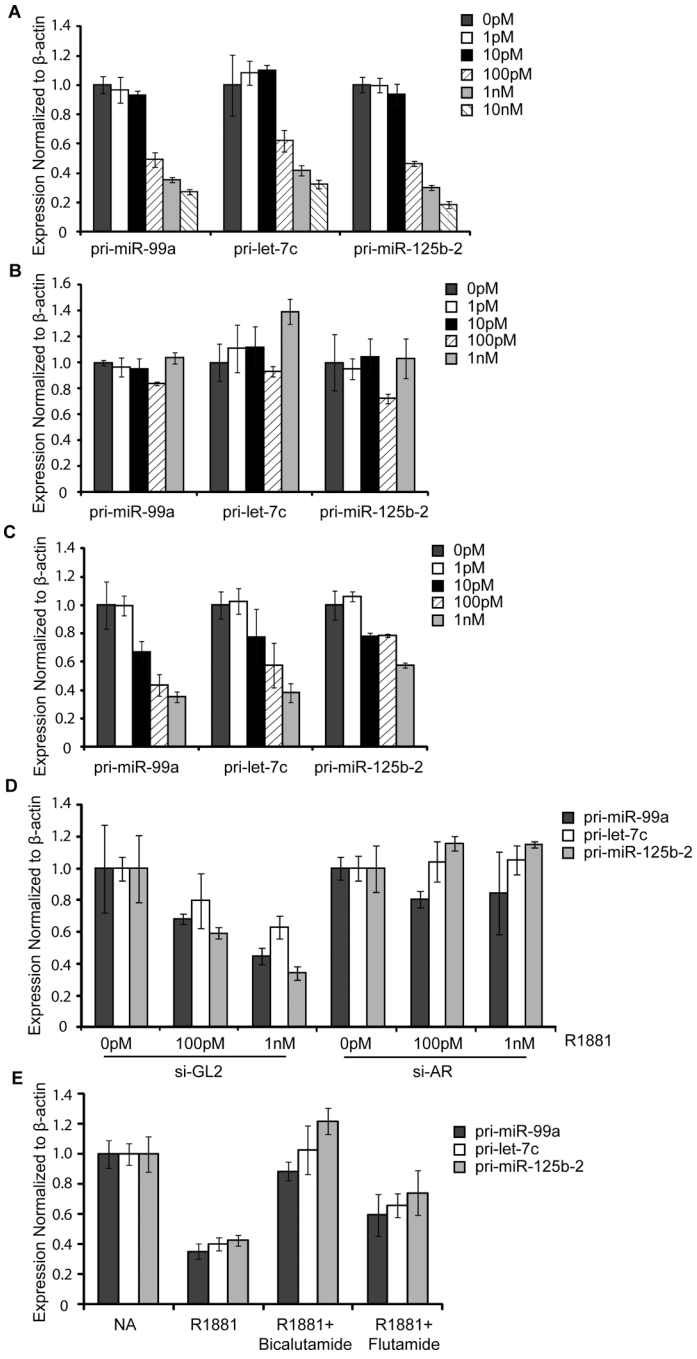

Figure 2. AR is required for androgen-repression of miR-99a/let7c/125b-2 cluster

A. The expression of primary miRNAs in C4-2 cells was measured by qPCR and normalized to β-actin. Rest as in Fig. 1A. **B.** The expression of primary miRNAs in PC-3 cells. Rest as in Fig. 2A. **C.** The expression of primary miRNAs in PC-3 cells expressing wild type AR. Rest as in Fig. 2A. **D.** The expression of primary miRNAs in LNCaP cells after knocking down AR by siRNA. LNCaP cells were treated with no androgen, 100pM or 1nM R1881. Rest as in Fig. 1A. **E.** The expression of primary miRNAs in LNCaP cells was measured by

qPCR and normalized to β-actin. LNCaP cells were treated with no androgen, 1nM R1881, 1nM R1881 plus 30μM Bicalutamide or 1nM R1881 plus 30μM Flutamide.

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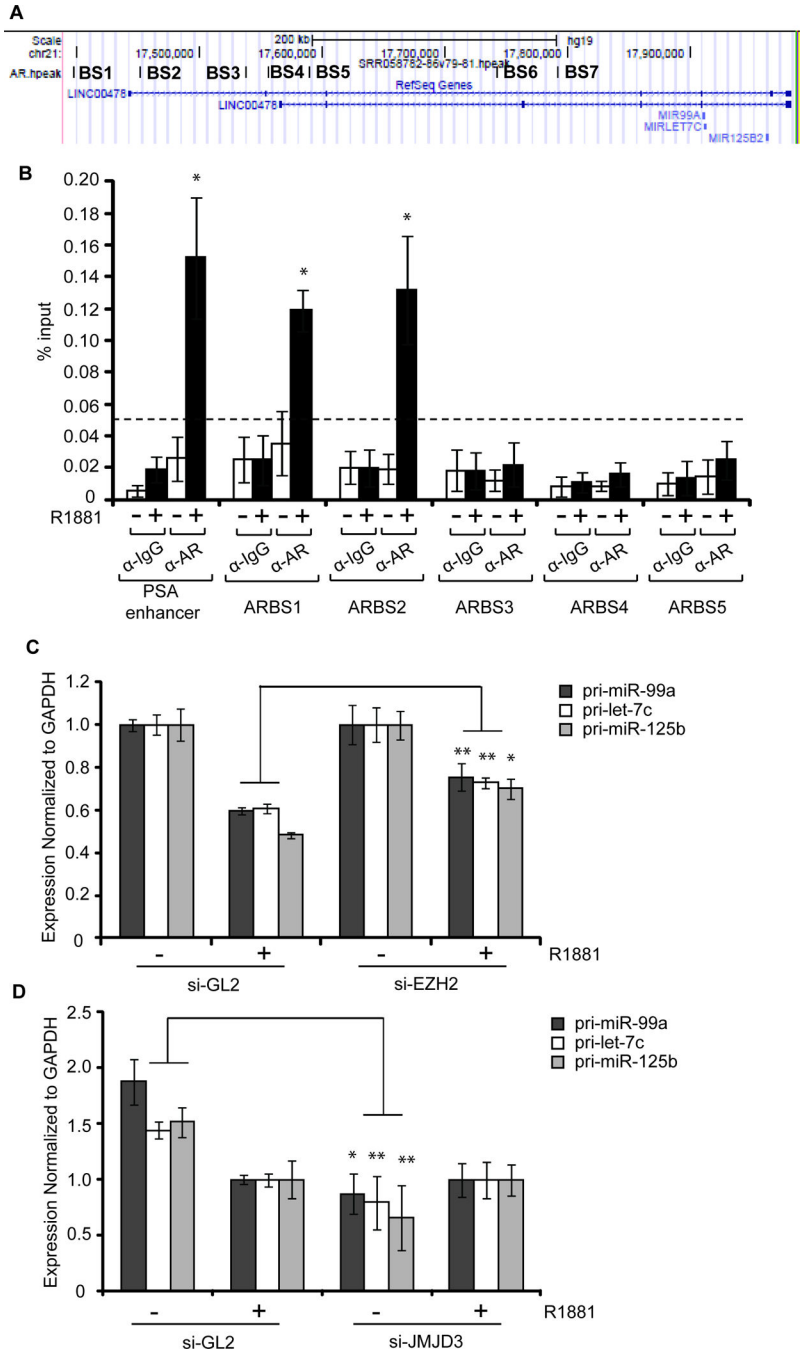

Figure 3. AR directly binds to host gene of miR-99a/let7c/125b-2 cluster and the repression is attenuated when EZH2 is knocked down

A. The AR binding sites at the LINC00478 locus from published AR ChIP-seq data are shown. Seven sites shown as vertical lines are labeled as BS1–7. The two primary transcripts and the microRNAs are shown. **B.** AR ChIP was performed and followed by qPCR. Primers were designed to detect AR ChIP-seq peaks ARBS1–5. A PSA enhancer containing an ARE was used as a positive control. ChIP grade IgG antibody was used as negative control. The mean and standard deviation from three independent experiments are indicated. The value was expressed as percentage of input DNA. The dashed line (---) marks

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the background threshold at 0.05% of input DNA. The statistical difference between "−"and "+" R1881 for AR ChIP was assessed by t-test. * indicated p-value less than 10^{-6}. **C.** The expression of primary miRNAs in LNCaP cells was measured by qPCR and normalized to GAPDH. LNCaP cells were transfected with siRNA against EZH2 or control si-GL2, and treated without or with 1nM R1881. Mean±s.d. n=3. The expression in no androgen is set as 1. * indicates p-value of difference from si-GL2 <0.05 and ** indicates p-value <0.01. **D.** The expression of primary miRNAs in LNCaP cells was measured by RT-qPCR and normalized to GAPDH. LNCaP cells were transfected with siRNA against JMJD3 or control si-GL2, and treated without or with 1nM R1881. Mean±s.d. n=3. The expression in 1nM R1881 is set as 1. * indicates p-value of difference from si-GL2 <0.05 and ** indicates p-value <0.01.

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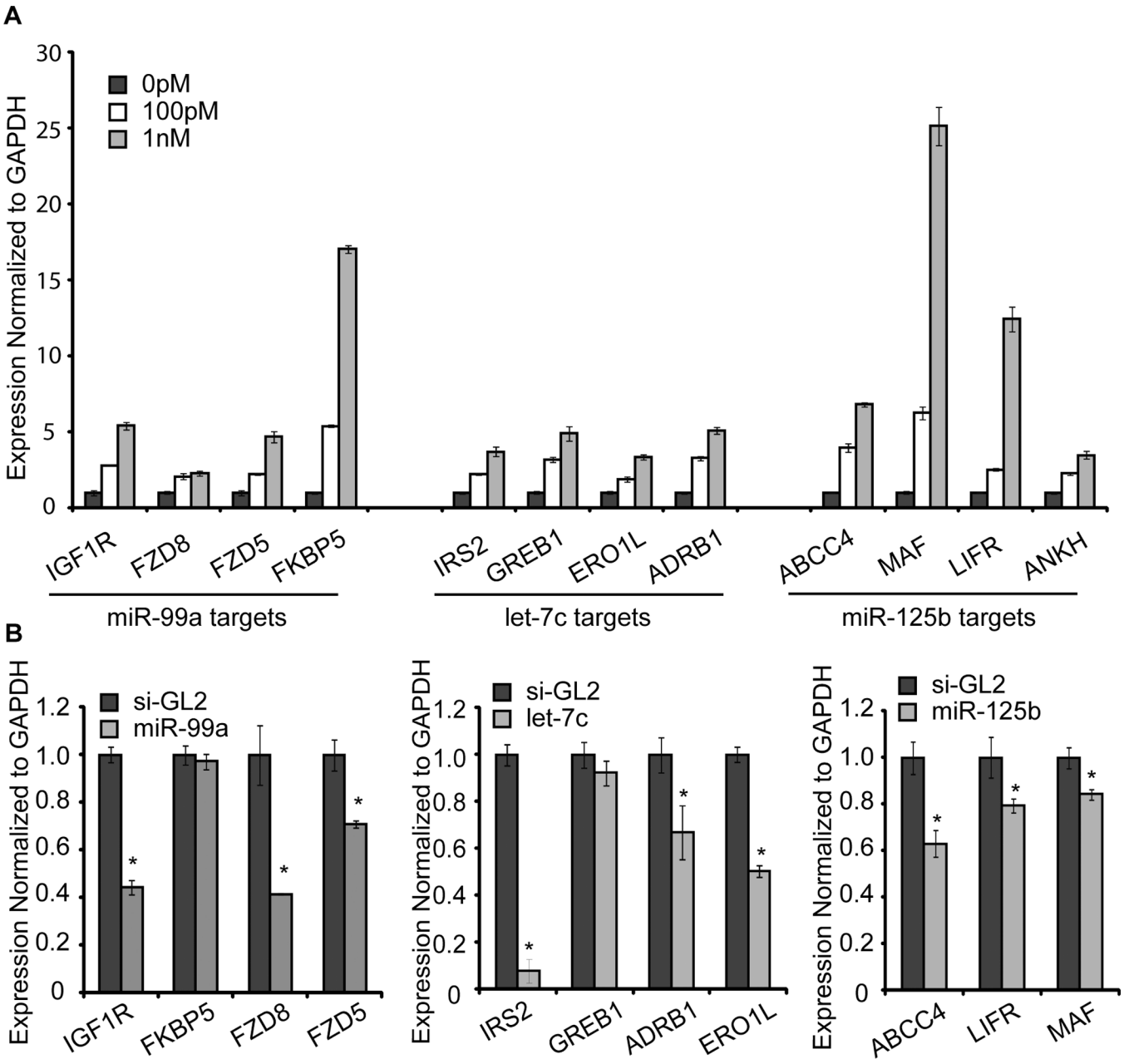

Figure 4. Validation of androgen-induced targets of miR-99a/let7c/125b-2 cluster

A. The expression of four predicted targets each of miR-99a, let-7c or miR-125b respectively was measured by RT-qPCR and normalized to GAPDH. LNCaP cells grown in charcoal stripped serum were treated with 0pM, 100pM or 1nM R1881. Mean±s.d. n=3. The expression in no androgen is set as 1. **B.** The expression of targets of miR-99a/let7c/125b-2 cluster after transfecting LNCaP cells with miR-99a, let-7c, miR-125b miRNA duplex or control siRNA duplex si-GL2. Expression was measured by RT-qPCR and normalized to GAPDH. Rest as in Fig. 4A. * indicates p-value of difference from si-GL2 <0.01.

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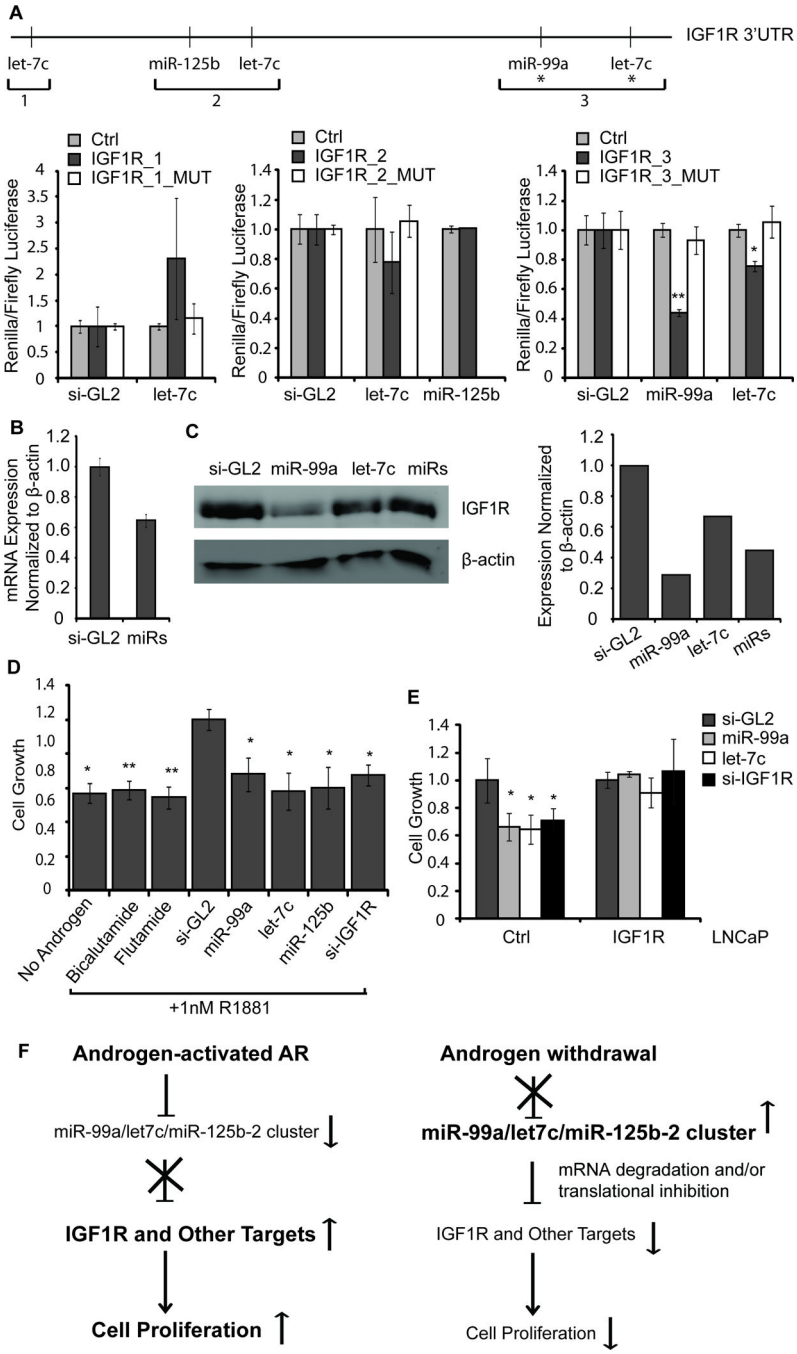

Figure 5. IGF1R is a key target molecule regulated by miR-99a/let7c/125b-2 cluster

A. Top: The 3'UTR of IGF1R, the relative positions of each miRNA binding site and the fragments inserted in the luciferase vectors. * indicates miRNA binding sites validated by luciferase assays. Bottom: Luciferase assay was performed with control luciferase vector, vector with indicated 3'UTR fragment of IGF1R, or 3'UTR fragments with mutation in the predicted target sites (indicated by MUT). The ratio of the renilla luciferase to firefly luciferase (transfection control) was normalized to that in the si-GL2 transfection. Mean ±s.d. n=3 * indicates p-value of difference from si-GL2 <0.005 and ** indicates p-value

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<0.001. **B.** The level of IGF1R mRNA after transfecting miRNA duplex mixture of miR-99a and let-7c (marked as miRs) or control si-GL2 was measured by RT-qPCR and normalized to β-actin. Mean±s.d. n=3. The value in si-GL2 treated cells was set as 1. **C.** Western blot was used to detect IGF1R protein after transfecting miR-99a, let-7c, miRNA duplex mixture of miR-99a and let-7c (miRs) or control si-GL2. β-actin was used as a loading control. **D.** LNCaP cells were treated with no androgen or 1nM R1881 during the experiment. 30μM Bicalutamide and Flutamide were added to LNCaP cells for 2 hours before addition of 1nM R1881. miR-99a, let-7c, miR-125b, siRNA against 3'UTR of IGF1R or si-GL2 was transfected twice at 0 hr and 48 hrs. At 96 hrs, cell number was counted using an automated cell counter. The mean and standard deviation from triplicate samples are shown. Cell numbers were normalized to that of cells in 1nM R1881 and transfected with si-GL2. * indicates p-value<0.01 and ** indicates p-value <0.001. **E.** miR-99a, let-7c, siRNA against 3'UTR of IGF1R or si-GL2 was transfected twice at 0 hr and 48 hrs. LNCaP cells were treated with 1nM R1881 during the experiment. At 96 hrs, cell number was counted in an automated cell counter. The mean and standard deviation from triplicate samples are shown. Cell numbers were normalized to that of cells transfected with si-GL2. * indicates p-value<0.05. **F.** Schematic of how the repression of miR-99a/let-7c/miR-125b-2 by androgen stimulates prostate epithelial cell proliferation.

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Table 1

Two miRNA clusters containing miR-99a, miR-100 and miR-125b.

Cluster Name	miRNAs	Genomic Locus	Host Gene
mir-99a/let-7c/miR-125b-2	miR-99a	chr21	LINC00478
	let-7c		
	miR-125b-2		
mir-100/let-7a-2/miR-125b-1	miR-100	chr11	MIR100HG
	let-7a-2		
	miR-125b-1		

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Table 2

Enrichment of target genes of miR-99alet7c125b-2 cluster in androgen-induced gene sets. (by Monte Carlo Simulation)

Dataset 1	Number of miRNA targets	Observed Number of Androgen-induced miRNA targets	Expected Number of Androgen-induced genes	Fold Enrichment (Observed/Expected)	P-value
let-7c	845	58	37.0±6.12	1.58	0.001
miR-125b	626	43	27.5±5.24	1.64	0.003
miR-99a	38	8	1.7±1.22	4.92	0.001
Dataset 2					
let-7c	845	28	14.0±3.79	1.89	0.003
miR-125b	626	14	10.3±3.07	1.3	0.149
miR-99a	38	4	0.6±0.75	6.63	0.004

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Table 3

Androgen-induced targets genes of miR-99alet7c125b-2 cluster

Gene Name	Gene Description	Fold change (GSE22606/GSE17044)
miR-99a targets		
FZD8	frizzled homolog 8 (Drosophila)	1.40/1.27
FZD5	frizzled homolog 5 (Drosophila)	2.77/1.25
FKBP5	FK506 binding protein 5	1.92/1.47
IGF1R	insulin-like growth factor 1 receptor	1.60/1.46
let-7c targets		
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	1.25/1.30
GREB1	GREB1 protein	1.37/1.68
BCAP29	B-cell receptor-associated protein 29	1.83/1.46
ADRB1	adrenergic, beta-1-, receptor	1.67/1.42
SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.89/1.36
IRS2	insulin receptor substrate 2	1.39/1.25
IGF1R	insulin-like growth factor 1 receptor	1.60/1.46
C14orf28	chromosome 14 open reading frame 28	1.28/1.23
TMPRSS2	transmembrane protease, serine 2	1.60/1.52
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	1.23/1.24
EDEM3	ER degradation enhancer, mannosidase alpha-like 3	1.47/1.34
PNKD	paroxysmal nonkinesigenic dyskinesia	1.26/1.23
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	1.46/1.32
GALE	UDP-galactose-4-epimerase	1.24/1.25
CPEB3	cytoplasmic polyadenylation element binding protein 3	1.46/1.27
ERO1L	ERO1-like (S. cerevisiae)	1.60/1.24
ANKH	ankylosis, progressive homolog (mouse)	1.91/1.37
STYK1	serine/threonine/tyrosine kinase 1	1.21/1.21
miR-125b targets		
LIFR	leukemia inhibitory factor receptor alpha	2.07/1.41
ANKH	ankylosis, progressive homolog (mouse)	1.91/1.37
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	2.28/1.52
ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	2.13/1.29
GTPBP2	GTP binding protein 2	1.22/1.32
ALDH1A3	aldehyde dehydrogenase 1 family, member A3	1.35/1.28
DNAJC10	DnaJ (Hsp40) homolog, subfamily C, member 10	1.61/1.30
FLVCR2	feline leukemia virus subgroup C cellular receptor family, member 2	1.41/1.24
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	1.23/1.24
SLC1A5	solute carrier family 1 (neutral amino acid transporter), member 5	1.21/1.22