Interrelation of androgen receptor and miR-30a and miR-30a function in ER$^-$, PR$^-$, AR$^+$ MDA-MB-453 breast cancer cells

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Abstract. The association between androgen-induced androgen receptor (AR) activating signal and microRNA (miR)-30a was investigated, as well as the function of miR-30a in estrogen receptor-negative (ER$^-$), progesterone receptor-negative (PR$^-$), and AR-positive (AR$^+$) MDA-MB-453 breast cancer cells. Androgen-induced AR activating signal upregulated the expression of AR, and downregulated the expression of miR-30a, b and c. Bioinformatics analysis indicated a putative miR-30a, b and c binding site in the 3'-untranslated region of AR mRNA. It was confirmed that the AR gene is a direct target of miR-30a, whereas AR does not target the miR-30a promoter, and AR activating signal may indirectly down-regulate miR-30a through other cell signaling pathways. In this positive feedback mechanism AR is then upregulated through miR-30a. Overexpression of miR-30a inhibited cell proliferation, whereas inhibition of miR-30a expression by specific antisense oligonucleotides, increased cell growth. Previously, androgen-induced AR activating signal was demonstrated to inhibit cell proliferation in ER$^-$, PR$^-$ and AR$^+$ MDA-MB-453 breast cancer cells, but AR activating signal downregulated the expression of miR-30a, relieving the inhibition of MDA-MB-453 cell growth. Therefore, in MDA-MB-453 breast cancer cells, miR-30a has two different functions regarding cell growth: Inhibition of cell proliferation through a positive feedback signaling pathway; and the relative promotion of cell proliferation through downregulation of miR-30a. Thus, the association between AR activating signal and microRNAs is complex, and microRNAs may possess different functions due to different signaling pathways. Although the results of the present study were obtained in one cell line, they contribute to subsequent studies on ER$^-$, PR$^-$ and AR$^+$ breast cancer.

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Abbreviations: ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor

Key words: estrogen receptor-negative, progesterone receptor-negative, androgen receptor-positive, breast cancer, miR-30a, MBA-MD-453 cells

Introduction

Sex steroid hormones and sex steroid hormone receptors play a critical role in breast cancer development and progression. In particular, androgen and androgen receptor (AR) have special importance, especially for estrogen receptor-negative, progesterone receptor-negative, AR-positive (ER$^-$, PR$^-$, AR$^+$) breast cancers. As patients with ER$^-$, PR$^-$, AR$^+$ breast cancer obtain little benefit from anti-estrogen therapy (1), more and more studies are focusing on AR as a useful therapeutic target, with some encouraging results (2).

AR is an androgen-dependent transcription factor activated by binding androgens. Activated AR recognizes androgen response elements (AREs) located in or near the promoter and enhancer regions of androgen-dependent genes, thereby activating the transcriptional machinery, including microRNA transcription (3-5).

MicroRNAs are evolutionarily conserved ~22-nucleotide-long short non-coding RNA molecules. These molecules repress target gene expression by binding to complementary sequences in the 3'-untranslated regions (UTRs) of target mRNAs. MicroRNAs participate in diverse biological functions, including development, cell proliferation, differentiation, and apoptosis (6-9). As microRNAs play a central role in the regulation of gene expression, aberrant expression is found in almost all types of human cancer (10).

As both AR and microRNA have the ability to regulate genes, their interaction within a cancer cell can take on many forms. Some studies have suggested a role for AR in the transcriptional regulation of microRNA expression (11-14), whereas other studies have shown a role for the regulation of AR expression by microRNA (15-17). However, most of these regulatory interactions remain unknown and almost all of the research has been focused in the field of prostate cancer.

Here, we explored the interaction between miR-30a and AR, as well as the function of miR-30a, in ER$^-$, PR$^-$, AR$^+$ MDA-MB-453 breast cancer cells on the basis of our previous studies (14,18).

Materials and methods

Cell culture and treatment. We chose to use MDA-MB-453 cells [American Type Culture Collection (ATCC)] because they express high levels of AR but not ER or PR (19-21). Cells were maintained in 75-cm^2 flasks containing minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were passaged every 3-4 days (80% confluence) and harvested using 0.25% trypsin/EDTA. Before each experiment, the cells were grown for 3 days in phenol red-free (PRF) DMEM containing 5% charcoal-treated fetal calf serum (PRF-CT). To achieve synchronization, the cells were serum-starved in PRF DMEM for 24 h. All experiments were performed in 2.5% PRF-CT.

Cells were treated with 5α-dihydrotestosterone (DHT; Sigma-Aldrich, MO, USA), a non-aromatizable androgen with the highest affinity for AR among natural androgens, or vehicle alone at 10^{-8} M (14). DHT was dissolved in 100% ethanol and added to the media immediately before use.

Western blot analysis. After 48 h, RIPA lysis buffer was used to lyse charcoal-stripped MDA-MB-453 cells treated with DHT or vehicle. Proteins were harvested, resolved on 10% SDS denaturing polyacrylamide gel, and transferred to a nitrocellulose membrane. The membranes were incubated at 4˚C overnight in Blotto with anti-AR or anti-GAPDH antibody (Saier Biotech, Tianjin, China) and then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Saier Biotech). Protein expression was assessed utilizing enhanced chemiluminescence. Band intensity was determined by Lab Works™ Image Acquisition and Analysis Software (UVP).

MicroRNA microarray analysis. KangChen Bio-tech, Inc., (Shanghai, China) performed the microRNA microarray experiments using total RNA extracted from MDA-MB-453 cells treated with DHT or vehicle alone. TRIzol reagent (Invitrogen, CA, USA) was used for the extraction according to the manufacturer's instructions. The quantity and quality of RNA were assessed using a NanoDrop ND-1000. Denaturing agarose gel electrophoresis was used to assess RNA integrity and DNA contamination.

The miRCURY™ Array Power Labeling kit (cat no. 208032-A; Exiqon, Vedbaek, Denmark) was used to label total RNA with Cy3 or Cy5 fluorescent dye according to the manufacturer's instructions. Each miRCURY LNA microRNA array (v.11.0; Exiqon), which consists of 847 capture probes for mature human microRNAs, was hybridized with a single Cy3- or Cy5-labeled sample. Each group was hybridized with three miRCURY LNA arrays in triplicate using independent samples of DHT- or vehicle-treated cells. Images were scanned by a Gene Pix 4000B scanner using GenePix Pro 6.0 software (Axon Instruments, Union City, CA, USA). The background was subtracted and normalization performed. We selected microRNAs with expression intensities (ForeGround-BackGround) >50 and expression levels that differed by at least 1.5-fold between DHT- and vehicle-treated cells.

Predicted AR-targeting microRNA. Computer-aided algorithms to predict microRNAs targeting AR were obtained from miRanda (August 2010 release, http://www.microrna.org) and TargetScan (release 5.2, http://www.targetscan.org).

Real-time reverse transcription PCR. Stem-loop RT-PCR was performed to determine the level of mature miR-30a as described previously (22). Briefly, 2 μg of small RNA was reverse-transcribed to cDNA using M-MLV reverse transcriptase (Promega Corp., Madison, WI, USA) and the following primers synthesized by BGI, Inc.: miR-30a-RT,5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCTTCCAG-3'; miR-30b-RT, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAGCTGAG-3'; miR-30c-RT, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACGCTGAGAG-3'; and U6-RT, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAAC-3', which can fold into a stem-loop structure. The specific miR-30a,b,c cDNA fragment and an endogenous control (U6 snRNA) were amplified using the following primers synthesized by BGI, Inc.: miR-30a-Fwd, 5'-TGCGGTGTAAACATCCTCGACTG-3'; miR-30b-Fwd, 5'-TGCGGTGTAAACATCCTACACTCA-3'; miR-30c-Fwd, 5'-TGCGGTGTAAACATCCTACACTCTC-3'; U6-Fwd, 5'-TGCGGGTGCTCGCTTCGGCAGC-3'; and Reverse, 5'-CCAGTGCAGGGTCCGAGGT-3', a universal downstream primer. The PCR reactions were heated to 94˚C for 4 min, followed by 40 cycles of 94˚C for 30 sec, 50˚C for 30 sec, 72˚C for 40 sec. The SYBR Premix Ex Taq™ kit (Takara Bio, Inc., Otsu, Japan) was used as instructed by the manufacturer and real-time PCR carried out on a 7300 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA).

For real-time RT-PCR to detect the relative level of AR transcription, cDNA was generated using M-MLV reverse transcriptase (Promega) with 5 μg of the large RNA. PCR to amplify the AR gene, and β-actin gene as an endogenous control, used the following primers: AR sense, 5'-AAGACGCTTCTACCAGCTCACCAA-3'; AR antisense, 5'-TCCCAGAAAGGATCTTGGGCACTT-3'; β-actin sense, 5'-CGTGACATTAAGGAGAAGCTG-3'; and β-actin antisense, 5'-CTAGAAGCATTTGCGGTGGAC-3'. The PCR reaction was heated to 94˚C for 5 min, followed by 40 cycles of 94˚C for 1 min, 56˚C for 1 min, and 72˚C for 1 min. After real-time PCR, the expression of the AR gene was normalized to the expression of the β-actin gene in the same sample.

Vector construction. The pcDNA3.1/pri-miR-30a expression vector was constructed by amplifying a 487 bp DNA fragment carrying pri-miR-30a from genomic DNA and inserting it into the pcDNA3.1 (+) vector at the BamHI and EcoRI sites. The PCR primers were miR-30a-sense, 5'-CGCGGATCCGGAAACACTTGCTGGGATTAC-3', and miR-30a-antisense, 5'-CCGGAATTCAACTGCAGAAAGGGCAGGACA-3'.

The enhanced green fluorescent protein (EGFP) reporter vector (pcDNA3/EGFP/AR) was constructed by amplifying the EGFP coding region from pEGFP-N2 (Clontech) and inserting it into pcDNA3.1 (+) at the HindIII and BamHI sites.

The AR 3'UTR fragment containing the predicted miR-30a binding site was then inserted into pcDNA3.1 (+)/EGFP at the BamHI and XhoI sites. The PCR primers were EGFP sense, 5'-GCAGCCAAGCTTGCCACCATGTGTAGCAAG GGC-3'; EGFP antisense, 5'-CGCGGATCCTTTACTTGT ACAGCTCGTCC-3'; AR sense, 5'-CGCGGATCCTTTAAA TCTGTGATGATCCTC-3'; and AR antisense, 5'-GAGGCC TCGAGTTTGTGTGGCTGGCACAGAG-3'. The mutant enhanced green fluorescent protein (EGFP) reporter vector (pcDNA3/EGFP/AR-mut) was constructed by inserting the mutant AR 3'UTR fragment into pcDNA3.1 (+)/EGFP at the same sites. The fragment of mutant AR 3'UTR was annealed by the following primers. Top-primer: 5-GATCCTTTAAAT CTGTGATGATCCTCATATGGCCCAGTGTCAAGTTGTG CTTCTATTCTGCACTACTCTGTGCCAGCCACACAAA C-3' Bot-primer: 5'-TCGAGTTTGTGTGGCTGGCACAGA GTAGTGCAGAATAGAAGCACAACTTGACACTGGGCC ATATGAGGATCATCACAGATTTAAAG-3'.

Transfection. Lipofectamine 2000 (Invitrogen) was used to transfect MDA-MB-453 cells with vectors (0.5 mg/l) or antisense oligonucleotide (ASO; final concentration 10^{-9} M) 24 h after plating and transfection complexes prepared according to the manufacturer's instructions. The transfection medium was replaced 4 h after transfection. Oligonucleotides complementary to miR-30a were synthesized by IDT (Coralville, IA, USA): miR-30a ASO, 5'-CTTCCAGTCGAGGATGTTTACA-3', and control ASO, 5'-TGACTGTACTGAGACTCGACTG-3'.

EGFP reporter assay. To confirm the direct action of miR-30a on AR, wild-type and mutant AR experimental groups were further divided into three subgroups: group 1, cells transiently transfected with pcDNA3/EGFP/AR; group 2, cells transiently co-transfected with pcDNA3/EGFP/AR and pcDNA3.1 (+) (control vector); and group 3, cells transiently co-transfected with pcDNA3/EGFP/AR and pri-miR-30a. The loading control was RFP expression vector pDsRed2-N1. Forty-eight h after transfection, the cells were lysed using radio-immunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH Q2 7.2, 1% Triton X-100, and 0.1% SDS). The intensities of EGFP and RFP were measured using a fluorescence spectrophotometer (F4500; Hitachi, Tokyo, Japan).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) to determine whether activated AR directly up-regulates miR-30a expression in MDA-MB-453 cells was performed as described previously (23). Briefly, chromatin DNA was extracted from harvested cells by sonication and incubated with normal rabbit serum (IgG) and protein A/G-Sepharose beads. Rabbit anti-AR antibody (PG-21, Upstate) was used for immunoprecipitation. The -2843/-2623 and -1513/-1286 fragments were amplified using the following primers: 5'-CCTAGATTTCTTGCCTTTAG (forward) and 5'-TCCACATAACCTTCTCGCTC (reverse) for -2843/-2623, and 5'-GTAGGGGACCTGTCACTTTG (forward) and 5'-CTT GAGTCAATCCACCAAAC (reverse) for -1513/-1286.

Cell proliferation assay. MDA-MB-453 cells were plated in 96-well microtiter plates (7000 cells/well) in replicates of six. Twenty-four h after seeding, the cells were transfected with vectors or ASO. The 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was performed to measure cell viability and proliferation. Over seven consecutive days, MTT was added to the wells and dimethyl sulfoxide (DMSO) added 4 h later to dissolve the resulting formazan crystals. The plates were shaken for 20 min and the absorbance detected at 570 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, USA).

Flow cytometry. Forty-eight h after transfection with vectors or ASO, the MDA-MB-453 cells were detached from the plates using trypsin, rinsed with PBS, and fixed in 70% (v/v) ethanol. The cells were rehydrated in PBS and incubated with RNase (100 μg/ml) and propidium iodide (60 μg/ml; Sigma-Aldrich, MO, USA) prior to analysis by the FACSCalibur System (BD Biosciences, San Jose, CA, USA). The cell cycle phase was determined by Cell Quest analysis. The proliferation index (PI) was calculated as (S+G2/M) /G1, where S, G2/M, and G1 refer to the percentage of cells in S phase, G2/M phase, and G1 phase, respectively (24).

Statistical analysis. The data are reported as the mean ± standard deviation of three independent determinations. The Student's t test was used for comparisons with corresponding controls. $P<0.05$ was considered to indicate a statistically significant difference. SPSS17.0 was used for all statistical analyses.

Results

Effect of DHT on AR and miR-30a,b,c expression. AR mRNA and protein were overexpressed when MDA-MB-453 cells were exposed to 10^{-8} M DHT for 48 h compared to vehicle-treated cells (Fig. 1A and B). To identify critical microRNAs potentially related to androgen-induced AR activating signal in breast cancer, we examined global microRNA expression in MDA-MB-453 cells. The microRNA array identified 43 up-regulated microRNAs and 51 down-regulated microRNAs, including miR-30a,b,c (Table I), in DHT-treated cells compared to vehicle-treated cells.

AR may be a target of miR-30a,b,c. Among the microRNAs predicted to target AR, miR-30a,b,c were identified because the 3'UTR of AR mRNA contained a putative binding site for miR-30a,b,c (Fig. 1C). In ER^-, PR^-, AR^+ MDA-MB-453 cells, the validity of ectopic miR-30a,b,c expression was confirmed by real-time RT-PCR, which revealed a 5-fold, 2.5-fold, and 2-fold decrease in DHT-treated MDA-MB-453 cells compared to the vehicle-treated groups (Fig. 1D). We focused on miR-30a and investigated the relationship between miR-30a and AR.

MiR-30a regulates AR at both the mRNA and protein level. AR expression was elevated at both the mRNA and protein level in MDA-MB-453 cells transfected with miR-30a ASO compared to control ASO (Fig. 2A). AR mRNA and protein levels were also assessed by real-time RT-PCR and Western blot 48 h after transfecting MDA-MB-453 cells with pcDNA3.1 (+)/30a vector expressing miR-30a or control vector (pcDNA3.1 (+)). Overexpression of miR-30a reduced the expression of AR at both the mRNA and protein level (Fig. 2B).

Table I. Differentially expressed miR-30a,b,c in MDA-MB-453 cells.

		Expression intensity (ForeGround-BackGround)		Normalized	
MiRNA	Fold change	DHT	Vehicle	DHT	Vehicle
Hsa-miR-30a	0.591493493	1646.5	923.5	7.925391095	4.687817259
Hsa-miR-30b	0.457648129	1919.5	833	9.239470517	4.228426396
Hsa-miR-30c	0.488154896	822	380.5	3.9566787	1.931472081

DHT, 5α-dihydrotestosterone.

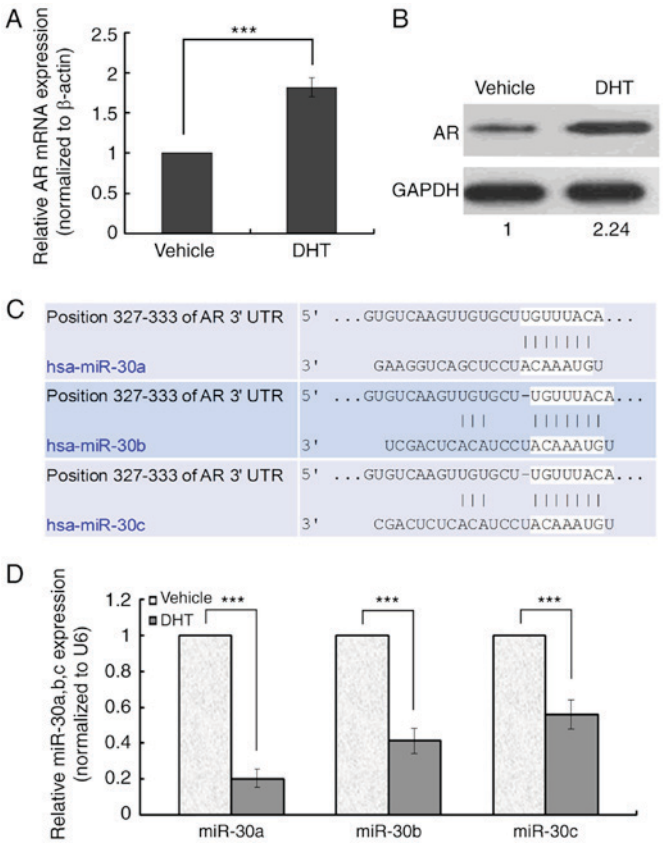

Figure 1. Effect of activated AR signaling on AR and miR-30a,b,c expression and TargetScan prediction outcomes. (A and B) Real-time RT-PCR and Western blot of AR expression. (C) TargetScan programs predicted that miR-30a,b,c are upstream microRNAs of AR. (D) The relative miR-30a,b,c mRNA expression level in DHT- and vehicle-treated MDA-MB-453 cells. In real-time RT-PCR, the expression level of miR-30a,b,c in the control group was set to 1 and U6 was regarded as an endogenous normalizer. In Western blots, the expression level of AR protein in the control group was set to 1 and GAPDH protein regarded as an endogenous normalizer. Error bars indicate standard deviation. ***P<0.001 compared to control.

Direct effect of miR-30a on the AR 3'UTR. The alignment of miR-30a with the wild-type or mutant AR 3'UTR is illustrated in Fig. 1C. Using a vector containing the EGFP gene upstream of the wild-type AR 3'UTR, EGFP fluorescence was strongly reduced in the presence of miR-30a overexpression. However, the overexpression of miR-30a did not affect the fluorescence intensity of the mutant AR 3'UTR (Fig. 2C). Thus, miR-30a regulates wild-type AR expression directly through the UTR.

Activated AR cannot load the promoter of miR-30a. We hypothesized that androgen-induced AR activating signal loaded onto the 5'DNA region of the miR-30a locus may serve as a transcription factor. A typical TATA box was found in the 5' region of the miR-30a gene when the length up to 4.0 kb was analyzed. Using the PROMO 3.0 program, six potential AREs were identified in the 5' region of miR-30a, suggesting the presence of a promoter in the 5' region. Among these AREs, ARE2 and ARE6 are the most likely to be loaded by activated AR. To determine AR loading, ChIP was performed with two primer pairs to amplify the -2843/-2623 fragment corresponding to ARE2 and the -1513/-1286 fragment corresponding to ARE6. Treatment of MDA-MB-453 cells with 10^{-8} M DHT did not induce an increase in AR loading at ARE2 or ARE6. Taken together, the results suggest that androgen-AR signaling does not directly mediate the regulation of miR-30a in MDA-MB-453 cells.

MiR-30a inhibits the growth of MDA-MB-453 cells. The MTT assay was used to measure viable proliferating cells 1-7 days after MDA-MB-453 cells were transfected. Blocking miR-30a with miR-30a ASO increased cell growth (Fig. 3A). Overexpressing miR-30a inhibited cell growth, an opposite effect as that observed with low miR-30a expression (Fig. 3B). Forty-eight h after transfection, the number of cells in S phase significantly increased and the PI increased when miR-30a was blocked (Fig. 3C). When miR-30a was overexpressed, the number of cells in G1 phase significantly increased, G1-S arrest was obvious, and the PI decreased. An opposite effect was observed in cells with low levels of miR-30a overexpression (Fig. 3D).

Discussion

In this study, we found that miR-30a,b,c are down-regulated by androgen-induced AR activating signal and AR is up-regulated in MDA-MB-453 cells affected by DHT. Two bioinformatics programs, miRanda and TargetScan, predicted miR-30a,b,c as upstream microRNAs of AR because the AR mRNA 3'UTR contained a putative binding site for miR-30a,b,c. We then chose miR-30a for further experiments because it is the most down-regulated among

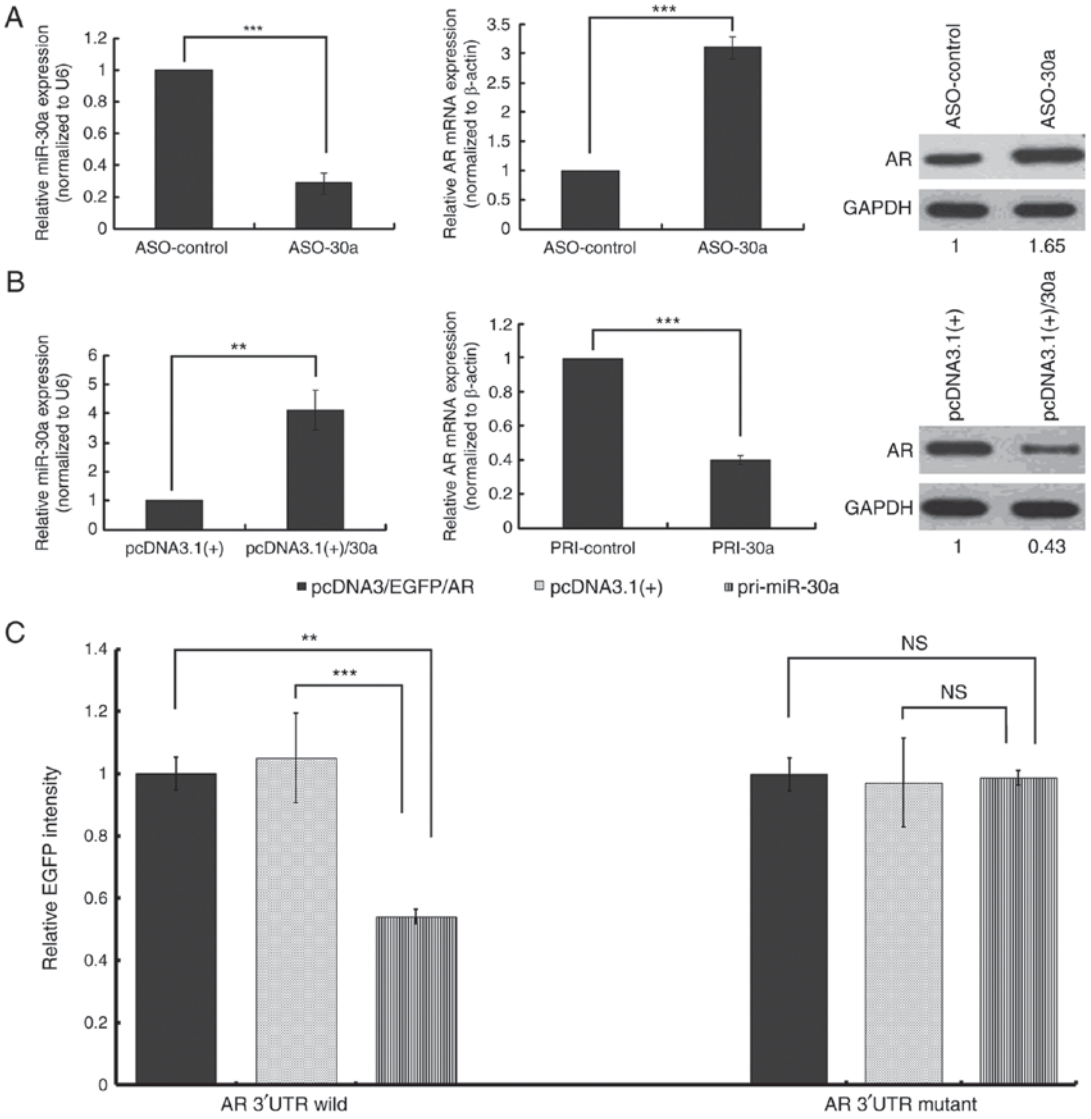

Figure 2. AR is a direct target gene of miR-30a. (A and B) RT-PCR and Western blot of miR-30a and AR expression 48 h after transfection with vector or ASO. (C) EGFP reporter assay to confirm the direct regulation of AR mRNA by miR-30a. The data are the mean ± standard deviation normalized fluorescence intensity from three independent experiments. **P<0.01, ***P<0.001 compared to control.

miR-30a,b,c. EGFP reporter assay confirmed that AR is the downstream target of miR-30a, but ChIP assay showed that androgen-AR signaling does not directly mediate the regulation of miR-30a. Therefore, other mechanisms potentially lead to the down-regulation of miR-30a by AR activating signal. Some studies have shown that AR and miR-30a are involved in the epithelial-mesenchymal transition in breast cancer (25-28), and vimentin is a specific bridge between AR and miR-30a (26,27). Feng *et al* reported that AR activated by DHT potentiates the promoter activity of vimentin and upregulates vimentin expression (27), whereas Cheng *et al* showed that vimentin is the target of miR-30a (26). Activated AR may upregulate vimentin, resulting in downregulated miR-30a because of the binding between miR-30a and vimentin. In addition, Guo *et al* reported that miR-30a is a novel downstream target of p53 R273H mutant, which binds to the promoter region to repress miR-30a expression (29). Therefore, we can assume that p53 is regulated by AR, and AR indirectly downregulates miR-30a via p53. These assumptions are worthy of study in the future.

The positive feedback signaling pathway that finally activates AR activating signal, up-regulating AR, follows a specific process. First, DHT activates the AR activating signal, down-regulating miR-30a. Second, the down-regulation of miR-30a relieves the down-regulating effect of miR-30a on AR. Our previous study revealed that DHT decreases MDA-MB-453 cell proliferation (14). In the present study, MTT assay and flow cytometry revealed that miR-30a inhibits the growth of MDA-MB-453 cells. The results are similar to other studies in which miR-30a functioned as a cancer suppressor gene in breast cancer (26,30,31). However, in this study, because AR activating signal down-regulates miR-30a expression, the growth of MDA-MB-453 cells should be promoted because miR-30a inhibits their growth.

We conclude that miR-30a has two different effects on MDA-MB-453 cell proliferation via AR activating signal.

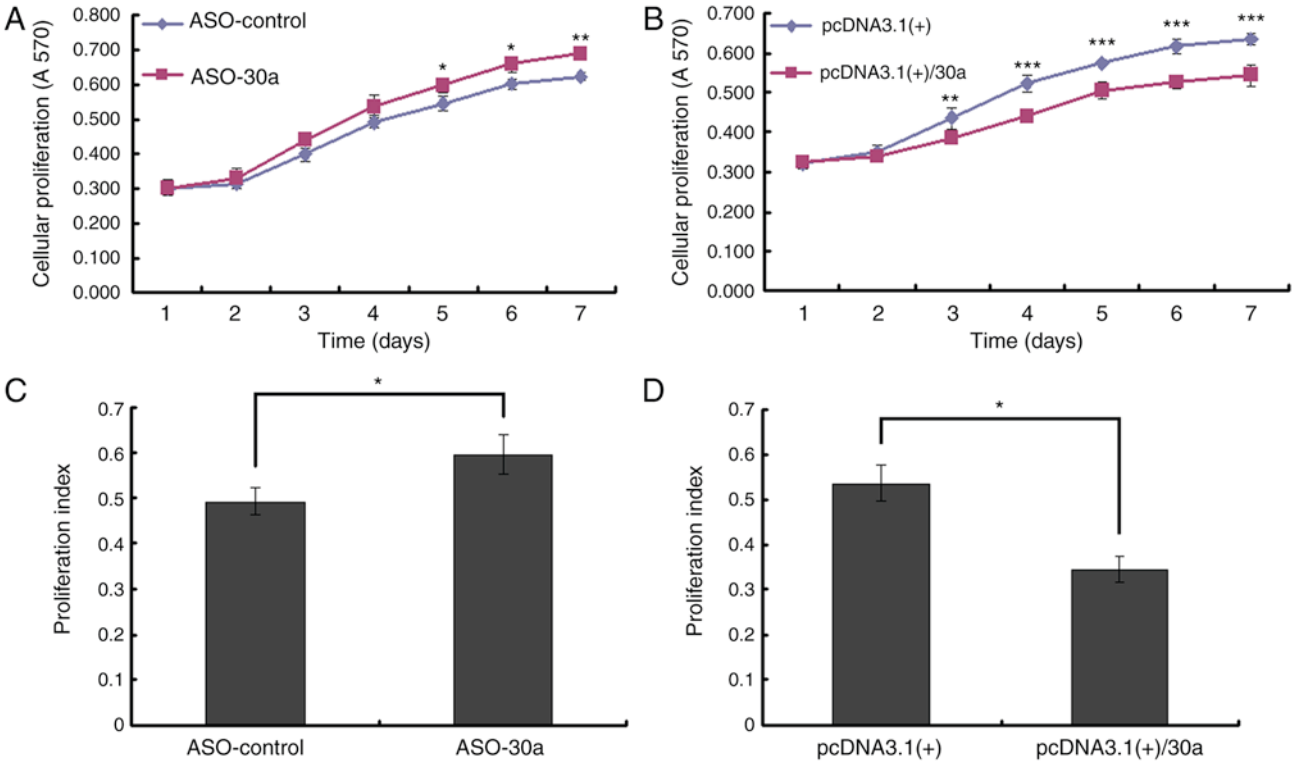

Figure 3. MiR-30a inhibits cell proliferation and causes G1-S cell cycle arrest in MDA-MB-453 cells. (A and B) MTT assay of cell proliferation 1-7 days after transfection with vector or antisense oligonucleotide (ASO). (C and D) The cell cycle phase distribution and proliferation index 48 h after transfection with vector or ASO. Error bars indicate standard deviation. *P<0.05, **P<0.01, ***P<0.001 compared to control.

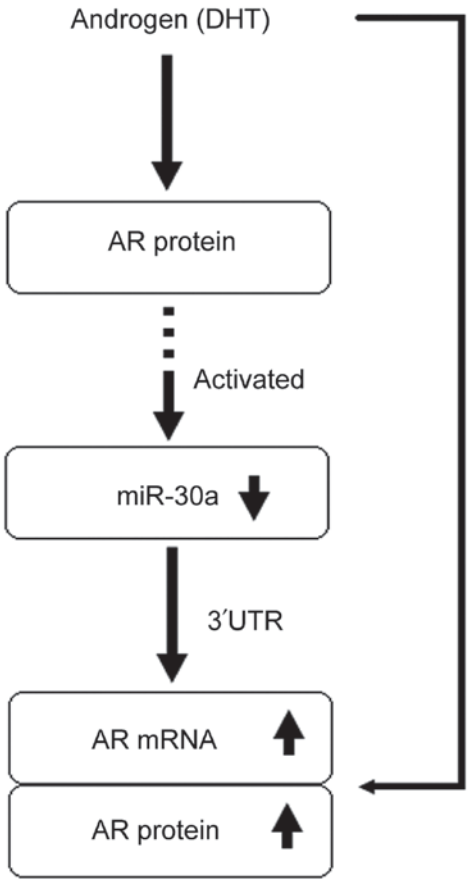

Figure 4. Schematic model of regulation including DHT, AR, and miR-30a. The dotted arrow indicates the indirect effect. The solid arrow indicates the direct effect.

One effect is through increased AR expression, as AR activating signal down-regulates miR-30a expression and then relieves the inhibition of AR expression by miR-30a. The other effect is through activating AR signal down-regulating miR-30a expression and relieving the inhibition of cell growth by miR-30a. The first effect is more likely than the latter when treating cells with DHT. We also found that AR activating signal inhibits the proliferation of MDA-MB-453 cells.

In conclusion, we found an interrelation between AR and miR-30a in human ER$^-$, PR$^-$, AR$^+$ MDA-MB-453 breast cancer cells (Fig. 4). MiR-30a and AR create a positive feedback mechanism in that leads to AR activating signal inhibiting cell proliferation. In addition, as a cancer suppressor gene, miR-30a inhibits the growth of MDA-MB-453 cells and is down-regulated to promote their growth. The opposite effects of miR-30a in the function of AR activating signal in MDA-MB-453 cells are very interesting. The results show that the mechanism underlying AR activating signal regulation of related microRNAs and AR is complex and waiting to be explored. Even though the results are for only one cell line, they contribute to subsequent studies of ER$^-$, PR$^-$, AR$^+$ breast cancer.

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