

Research Paper

Upregulation of miRNA3195 and miRNA374b Mediates the Anti-Angiogenic Properties of Melatonin in Hypoxic PC-3 Prostate Cancer Cells

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Received: 2014.05.07; Accepted: 2014.08.13; Published: 2015.01.01

Abstract

Recently microRNAs (miRNAs) have been attractive targets with their key roles in biological regulation through post-transcription to control mRNA stability and protein translation. Though melatonin was known as an anti-angiogenic agent, the underlying mechanism of melatonin in PC-3 prostate cancer cells under hypoxia still remains unclear. Thus, in the current study, we elucidated the important roles of miRNAs in melatonin-induced anti-angiogenic activity in hypoxic PC-3 cells. miRNA array revealed that 33 miRNAs (>2 folds) including miRNA3195 and miRNA 374b were significantly upregulated and 16 miRNAs were downregulated in melatonin-treated PC-3 cells under hypoxia compared to untreated control. Melatonin significantly attenuated the expression of hypoxia-inducible factor (HIF)-1 alpha, HIF-2 alpha and vascular endothelial growth factor (VEGF) at mRNA level in hypoxic PC-3 cells. Consistently, melatonin enhanced the expression of miRNA3195 and miRNA 374b in hypoxic PC-3 cells by qRT-PCR analysis. Of note, overexpression of miRNA3195 and miRNA374b mimics attenuated the mRNA levels of angiogenesis related genes such as HIF-1alpha, HIF-2 alpha and VEGF in PC-3 cells under hypoxia. Furthermore, overexpression of miRNA3195 and miRNA374b suppressed typical angiogenic protein VEGF at the protein level and VEGF production induced by melatonin, while antisense oligonucleotides against miRNA 3195 or miRNA 374b did not affect VEGF production induced by melatonin. Also, overexpression of miR3195 or miR374b reduced HIF-1 alpha immunofluorescent expression in hypoxic PC-3 compared to untreated control. Overall, our findings suggest that upregulation of miRNA3195 and miRNA374b mediates anti-angiogenic property induced by melatonin in hypoxic PC-3 cells.

Key words: melatonin; miRNA3195; miRNA374b; VEGF; HIF-1 alpha; PC-3 cells.

Introduction

Melatonin regulates circadian rhythms and modulates various biological functions with anti-cancer (1), anti-aging (2), anti-inflammatory (3), anti-obesity (4) and anti-oxidant (5, 6) activities. Regarding the anti-angiogenic activity, melatonin suppresses tumor angiogenesis by inhibiting hypoxia-inducible factor 1 (HIF-1) and vascular endothelial

growth factor (VEGF) via sphingosine kinase 1 in HCT116 colon cancer cells (7) and in a mouse tumor model using RENCA renal adenocarcinoma cells (8). In contrast, melatonin promotes osteogenesis during the repair of bone defects (9) and wound healing in an indomethacin-induced gastric ulcer (10) to maintain homeostasis.

Recently, microRNAs (miRNAs) were shown to be good targets for several intractable diseases, including cancer (11), diabetes (12) and cardiovascular diseases (13). miRNAs, consisting of noncoding small RNAs (~20-22 nt) and single-stranded RNA molecules, generally regulate gene expression at the post-transcriptional level (14) and play key roles in cell death, hematopoietic lineage differentiation, cell growth, ageing, autophagy (15, 16), angiogenesis (17-19) and cancer biology (20, 21). miRNAs are transcribed by RNA polymerase II as long primary miRNAs, and can be processed by Drosha, RNAase III endonucleases and DGCR8 (DiGeorge critical region 8) in the nucleus (22).

Despite previous reports on 22 miRNAs that are differentially expressed by melatonin treatment in breast cancer cells (23) and EPACs/miRNA-124/Egr1 pathways in neurodegenerative diseases (24), the roles of miRNAs and the anti-angiogenic properties of melatonin remain unclear. In the present study, we examined the profile of differentially expressed miRNAs using a miRNA array in melatonin-treated PC-3 cells and explored the roles of miRNA3195, the most highly upregulated miRNA, and miRNA374b, a predictive biomarker for prostate cancer (25), in the anti-angiogenic activity of melatonin in PC-3 prostate cancer cells using two miRNA overexpression mimics. We demonstrate that melatonin exerts anti-angiogenic activity by upregulating miRNA3195 and miRNA 374b in PC-3 prostate cancer cells under hypoxia.

Material and Methods

Cell culture

Prostate cancer cell line PC-3 cells were obtained from the American Type Culture Collection (ATCC), and cultured in RPMI1640 medium (Welgene, Daegu, South Korea) supplemented with 10% fetal bovine serum (FBS), 2 μ M L-glutamine and penicillin/streptomycin under 5% CO₂ condition.

Hypoxia induction

For the hypoxic stress, the PC-3 cells in the absence or presence of 1mM melatonin were cultured for 4 h under an anaerobic chamber (Forma Scientific, OH, USA) with a humidified atmosphere of 2% O₂ and 5% CO₂ balanced with N₂.

miRNA transfection

The miRNA mimics (200 nM) of miRNA2190, miRNA3195 and miRNA 374b and antisense oligonucleotides (50 nM) against miRNA3195 and miRNA were transfected into PC-3 cells using lipofetamine 2000 (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacture's protocol. Two days or

three days after transfection, PC-3 cells were collected for RNA isolation or Protein expression.

Isolation and expression of miRNA

Total RNA from PC-3 cells in the absence or presence of melatonin (1mM) (Sigma, St. Louis, MO, USA) under hypoxia was isolated by QIAzol (Invitrogen). One microgram of total RNA was used to make cDNA using GenoExplorer™ miRNA cDNA kit (GenoSensor Corporation, Arizona, USA) according to the manufacture's protocol. To determine the expression of miRNAs, qRT-PCR was performed with the LightCycler™ instrument (Roche Applied Sciences, Indianapolis, USA). miRNA primers were purchased from GenoExplorer™ (GenoSensor Corporation, Arizona, USA). U6 or RNU44 was used to normalize the expression of interesting miRNAs.

miRNA microarray analysis

Total RNA was extracted from the PC-3 cells in the presence or absence of 1mM melatonin under hypoxia by QIAzol (Invitrogen). For analysis of miRNA expression profile, total RNA sample (100 ng) was labeled with Cyanine 3-pGp (Cy3) using the Agilent miRNA Complete Labeling and Hyb kit (Agilent Technologies, Foster City, CA, USA). The sample was placed on an Agilent Human miRNA v14 (AMDID 026867) and covered by a Gasket slide (Agilent Technologies). Slides were hybridized for 16 h at 42°C in the Agilent hybridization system. Hybridized slides were washed in the first wash buffer (0.0005% Triton X-102) for 5 min and the second wash buffer for 5 min. After the washing steps, the slides were dried by centrifugation at 900 g at room temperature. Data were obtained as a median of replicated fluorescent signal measurements of the same miRNA from each slide. Slides were analyzed using Exiqon's protocol. Normalization included background subtractions with a global LOWESS regression algorithm. Spots were flagged and analyzed for signal quality (empty spot, spot signal less than background, less than optimal spot morphology, or saturated spot).

Wound healing assay

Two days after transfection with miRNA3195 and miRNA374b in PC-3 cells, a plastic pipette tip was used to scrape a line between PC-3 cells~ 2 to 3 mm wide. The migratory activity of PC-3 cells was evaluated in the miRNA negative control or miRNA mimics. After incubation for 24 h, the PC-3 cells were stained with Diff-Quick solution II (Sysmex CO., Japan). Randomly chosen fields were photographed under an Axiovert S 100 light microscope (Carl Zeiss, Weimar, Germany) at \times 100 magnification, and the migrated cells were counted.

Quantitative real-time PCR assay (qRT-PCR)

A quantitative real-time PCR was used to detect the expression and the quantification of the targeted molecules such as HIF-1 alpha, HIF-2 alpha and VEGF in 1 mM melatonin-treated PC-3 cells transfected by miRNA3195 and miRNA374b under hypoxia or normoxia. 1 µg of total RNA was used to construct the template cDNA with reverse transcription kit (Promega, WI, USA). To amplify the reaction of DNA, RT-qPCR was used with the LightCycler™ instrument (Roche Applied Sciences, Indianapolis, USA) according to the manufacturer's protocol. The mRNA level of GAPDH was used to normalize the expression of genes of interest. All reactions were carried out twice, and the final analysis was based on the mean of the reactions. The primers used to be as follows: HIF-1 alpha forward primer 5'-CTGCCACC ACTGATGAATTA-3', reverse primer 5'-GTATGTGG GTAGGAGATGGA-3', HIF-2 alpha forward primer 5'GCGCTAGACTCCGAGAACAT-3', reverse primer 5'-TGGCCACTTACTACCTGACCCTT-3', VEGF forward primer 5'-CCAGCAGAAAGAGGAAAGAG GTAG-3', reverse primer 5'-CCCCAAAAGCAGGTC ACTCAC-3'; GAPDH forward primer: 5'-CCA CTC CTC CAC CTT TGA C-3', reverse primer: 5'-ACC CTG TTG CTG TAG CCA-3'.

Western blotting

PC-3 cells were exposed to 1 mM melatonin for 24 h and cell lysates were prepared on ice in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and 1X protease inhibitor cocktail (Roche, Mannheim, Germany). The lysates were spun at 14,000 × g for 20 min at 4°C and the supernatants were collected. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA), and proteins (50–100 µg) were separated by electrophoresis on 4–12% NuPAGE Bis-Tris gels (Novex, Carlsbad, CA, USA). The proteins were then transferred to a Hybond ECL transfer membrane and immunoblotted with antibodies of HIF-1 alpha, VEGF (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and β-actin (Sigma, St. Louis, MO, USA).

Measurement of VEGF production by enzyme-linked immunosorbent assay (ELISA)

Vascular endothelial growth factor (VEGF) level from the supernatant of PC-3 cells transfected by control, miRNA374b or miRNA3196 mimics or miRNA inhibitors against miRNA374b or miRNA3196 in the presence or absence of melatonin under hypoxia was measured by using VEGF ELISA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunofluorescence assay for HIF-1 alpha

PC-3 cells were exposed to 1 mM melatonin under hypoxia for 4 h after transfection with miRNA3195 and miRNA374b mimics. PC-3 cells were washed and fixed with 3% paraformaldehyde and permeabilized in 0.1% Triton X-100. Incubation with monoclonal anti-HIF1α antibody (Novus Biologicals, clone number H1alpha67) for 30 min was followed by incubation with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA). The fixed cells were stained with 5 mg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma). Images were photographed under a Delta Vision imaging system (Applied Precision, Issaquah, WA, USA).

Statistical analyses

Statistical analysis of the data was carried out using Prism (GraphPad software, Inc. CA, USA). All data were presented as means ± standard error of the mean (SEM). The statistically significant differences between control and melatonin-treated groups were evaluated by the Student's t test and one way Anova Tukey's multiple comparison test.

Results

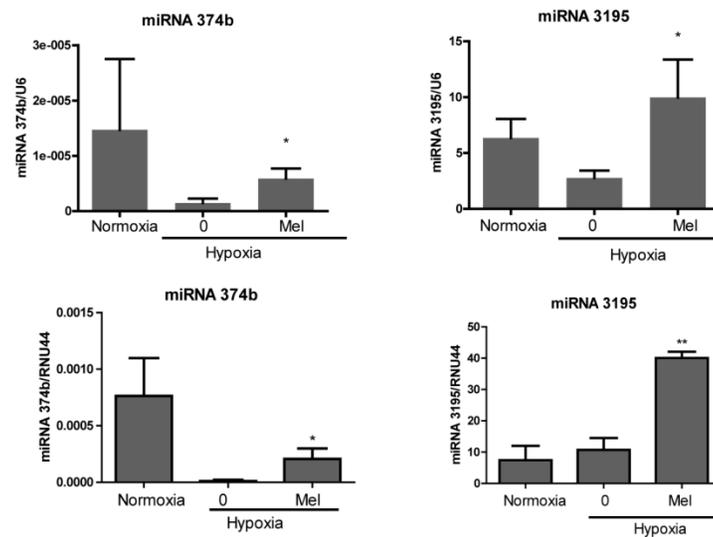
Previous studies have shown that melatonin has anti-angiogenic activity by regulating HIF-1alpha and sphingosine kinase 1 pathways (26, 27), despite weak cytotoxicity in HCT116 and PC-3 cancer cells. Recently, it was shown that miRNAs play critical roles as modulators of angiogenesis (19). To explore whether melatonin affects angiogenesis by regulating miRNAs, we used a miRNA array to determine miRNA profiles in melatonin-treated PC-3 cells under hypoxia. As shown in Table 1, the levels of 33 miRNAs were significantly upregulated, while those of 16 miRNAs were downregulated in melatonin-treated PC-3 cells based on a miRNA array. The differentially expressed miRNAs were further subjected to gene ontology (GO) and Kyoto Encyclopedia of gene and genomes (KEGG) analysis. Bioinformatic software packages, including TargetScan, miRNAwalk, miRNADB and RNA2-HAS, predicted that the biological processes involved in melatonin-treated PC-3 cells were metabolic process, cellular process, cell communication, developmental process, and transport, whereas the signaling pathways included Wnt, gonadotropin releasing hormone, inflammation mediated by chemokine and cytokine, integrin, cadherin, and angiogenesis-related pathways (Additional file 1: Supplementary Figure 1). Among them, HIF-1 alpha, HIF-2 alpha and VEGF target the angiogenic-signaling pathway. In this study, we found that miR-374b, miR-663 and miR130b are associated with VEGFA; miR-3613-3p

and miR-3074-3p are associated with HIF-1alpha; hsa-miR-3195_st, miR-21-5p and Let7f are associated with HIF-2alpha; and miR-3613-3p and miR-4710 are associated with HIF-3alpha (Table 2). Among them, we selected two potent microRNAs (miRNA3195 and miRNA374b) for further study based on bioinformatic information and our miRNA data.

We validated upregulation of two miRNAs using qRT-PCR. Consistent with the miRNA array, the levels of miRNA3195 and miRNA374b were upregulated in melatonin-treated PC-3 cells under hypoxia compared to hypoxia control (Figure 1A). We also confirmed that miRNA3195 was upregulated by melatonin in hypoxic DU145 cells compared to normoxic control (Additional file 1: Supplementary Figure 2), while miRNA374b expression was not detected in DU145 cells using RT-qPCR. In addition, miR222 was downregulated in melatonin-treated PC-3 cells under hypoxia, while miR27a was not significantly repressed (Figure 1B).

Furthermore, we evaluated mRNA expression of angiogenesis-related proteins, such as HIF-1alpha, HIF-2alpha and VEGF in melatonin-treated PC-3 cells under hypoxia by qRT-PCR. As expected, melatonin at 1 mM attenuated mRNA levels of the angiogenesis-related genes HIF-1alpha, HIF-2alpha and VEGF in melatonin-treated PC-3 cells under hypoxia compared to hypoxia control (Figure 2). To further investigate the angiogenic properties of two potent microRNAs, we used miRNA mimics for overexpression in melatonin-treated PC-3 cells by transfection with control vector or miRNA3195 and miRNA374b mimics. We confirmed that two miRNAs were overexpressed in PC-3 cells by qRT-PCR (Figure 3A). To determine whether two miRNAs affect migratory activity, wound healing assay was performed in PC-3 cells 48 h after miRNA transfection. As shown in Figure 3B,C and 3D, overexpression of miRNA3195 and miRNA374b significantly inhibited the motility of PC-3 cells compared to untreated control.

A



B

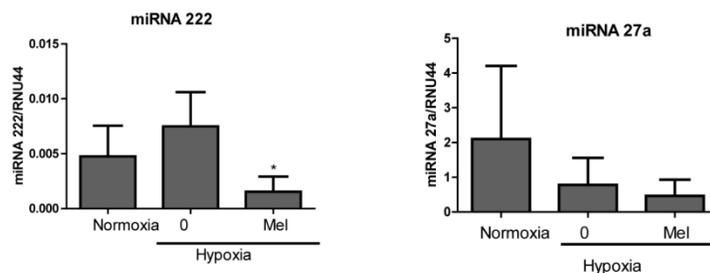
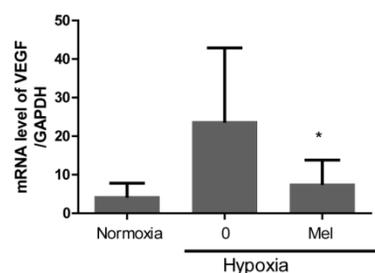
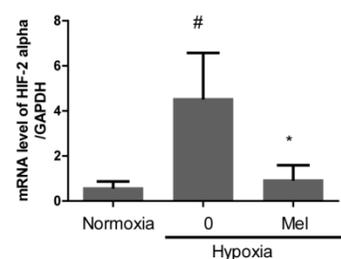
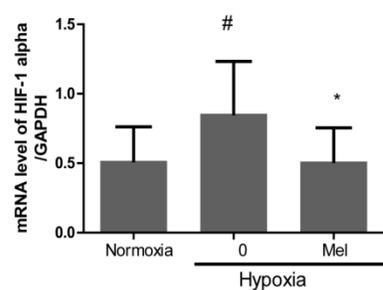


Figure 1. Melatonin upregulated the expression of miRNA3195 and miRNA374b in hypoxic PC-3 cells. PC-3 cells were treated in the absence or presence of melatonin (Mel) (1mM) for 4 h under hypoxia or normoxia (normal oxygen level condition). Total RNA was isolated and analyzed by qRT-PCR for determining the level of miRNA3195, miRNA374 (A), miRNA222 and miRNA27a (B). U6 or RNU44 was used to normalize the expression of miRNA. Data are presented as means \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs hypoxia control.

Table 1. MicroRNA profile differentially expressed in melatonin treated PC-3 cells under hypoxia.

Transcript ID	p >2 fold	Transcript ID	p <0.5 fold
hsa-miR-3195_st	4.252	hsa-miR-222-star_st	0.181
hsa-miR-4665-5p_st	3.711	hsa-miR-27a-star_st	0.206
hsa-miR-1246_st	3.504	hsa-miR-92a-1-star_st	0.245
hsa-miR-130b-star_st	3.317	hsa-let-7a-2-star_st	0.309
hsa-miR-4649-5p_st	3.048	hsa-miR-3177-3p_st	0.325
hsa-miR-4492_st	2.941	hsa-miR-1226_st	0.352
hsa-miR-374b_st	2.674	hsa-miR-4304_st	0.356
hsa-miR-4667-5p_st	2.639	hsa-miR-23a-star_st	0.359
hsa-miR-3937_st	2.618	hsa-miR-30c-2-star_st	0.385
hsa-miR-663_st	2.491	hsa-miR-1972_st	0.387
hsa-miR-3074-3p_st	2.460	hsa-miR-4436b-5p_st	0.424
hsa-miR-3175_st	2.456	hsa-miR-193a-3p_st	0.435
hsa-miR-1184_st	2.394	hsa-miR-3162-3p_st	0.445
hsa-miR-3613-3p_st	2.351	hsa-miR-1237_st	0.458
hsa-miR-4488_st	2.350	hsa-miR-424-star_st	0.484
hsa-miR-3621_st	2.342	hsa-miR-4671-3p_st	0.486
hsa-miR-4486_st	2.340		
hsa-miR-4710_st	2.327		
hsa-miR-192-star_st	2.320		
hsa-miR-2116_st	2.320		
hsa-miR-1587_st	2.294		
hsa-miR-3944-5p_st	2.284		
hsa-miR-3196_st	2.280		
hsa-miR-4467_st	2.234		
hsa-miR-21_st	2.230		
hsa-miR-26b_st	2.218		
hsa-miR-1973_st	2.187		
hsa-miR-4498_st	2.182		
hsa-miR-943_st	2.104		
hsa-miR-4324_st	2.104		
hsa-miR-4750_st	2.069		
hsa-miR-3682-3p_st	2.068		
hsa-let-7f_st	2.055		

**Figure 2.** Melatonin attenuated mRNA expression of HIF-1 alpha, HIF-2 alpha and VEGF in hypoxic PC-3. PC-3 cells were treated in the absence or presence of melatonin (1mM) for 4 h under hypoxia. Total RNA was analyzed by qRT-PCR to determine the mRNA expression of HIF-1 alpha, HIF-2 alpha and VEGF. GAPDH was used to normalize the target gene. Data are presented as means \pm SEM of three independent experiments. # p < 0.05 vs normoxia control, * p < 0.05 vs hypoxia control.**Table 2.** Predicted target genes of selected upregulated miRNAs related to angiogenesis.

	Fold(up)	Predicted molecular target	Source
hsa-miR-3195	4.251	HIF2alpha	RNA22-HAS
has-miR-374b	2.674	VEGFA	miRwalk, Targetscan
hsa-miR-663	2.674	VEGFA	miRDB
hsa-miR-3613-3p	2.351	HIF1alpha	miRDB
hsa-miR-4710	2.327	HIF3alpha	miRDB
hsa-miR-21-5p	2.230	VEGF,HIF1alpha,HIF2alpha	miRwalk
hsa-miR-3074-3p	3.317	VEGFA	RNA22-HAS
hsa-miR-4710	3.327	HIF3alpha	miRDB
hsa-miR-1973	2.187	IL-8	miRDB
has-miR-943	2.104	HIF3alpha	RNA22-HAS
Let7f	2.055	HIF2alpha,HIF3alpha	RNA22-HSA

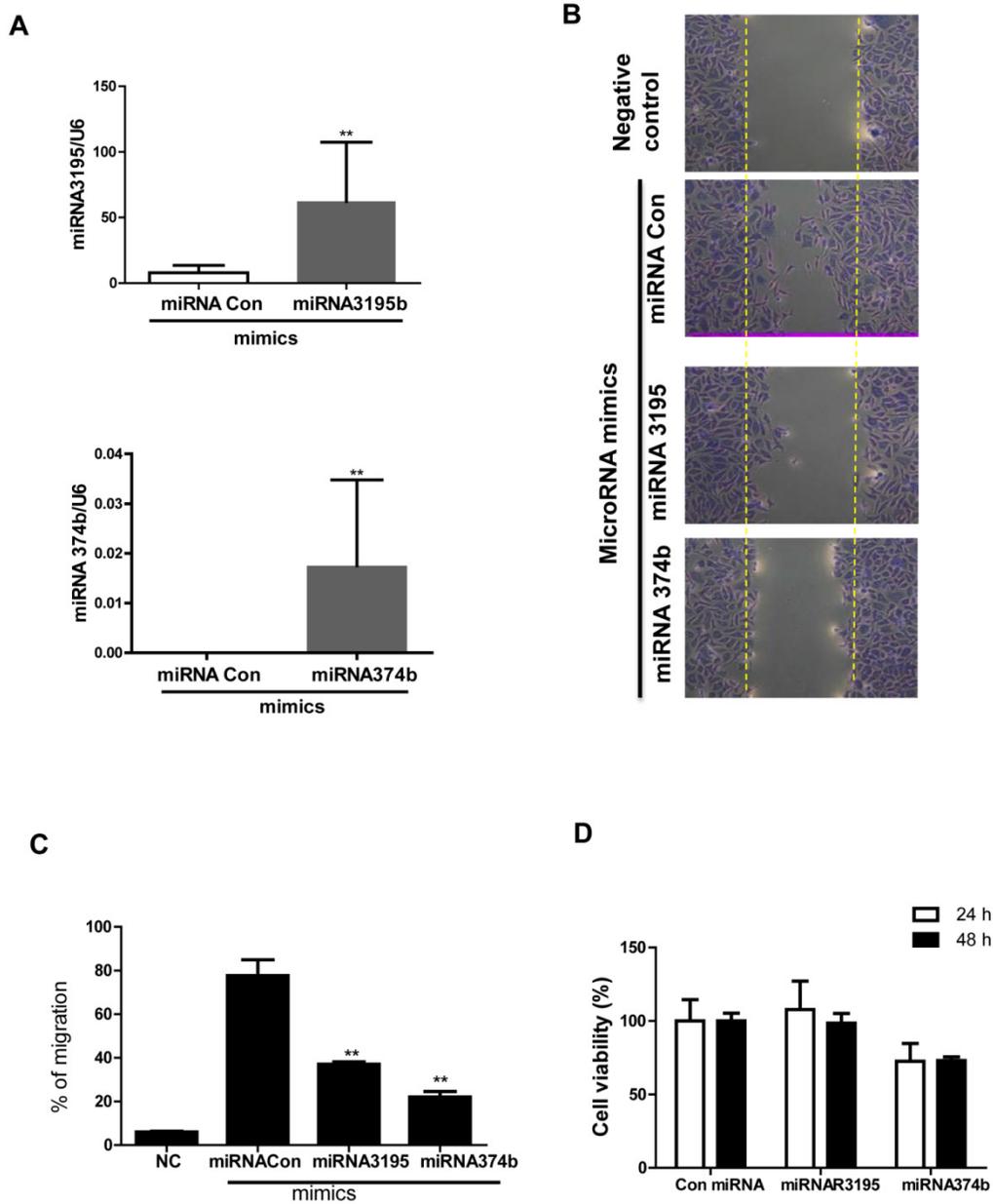


Figure 3. Overexpression of miRNA3195 and miRNA374b suppressed the migratory activity of hypoxic PC-3 cells by wound healing assay. Mimics of miRNA3195 and miRNA374b were transfected into PC-3 cells. Two days after transfection, the cells were harvested. (A) Overexpression level of miRNA3195 and miRNA374b after transfection with miRNA3195 and miRNA374b mimics. Total RNAs for miRNA3195 and miRNA374b were analyzed by qRT-PCR. (B) Effect of overexpressed miRNA3195 and miRNA374b on migratory activity of PC-3 cells under normoxia condition. Two days after transfection with miRNA mimics, the confluent PC-3 cells were scratched with a tip and the migratory activity of PC-3 cells was evaluated under an inverted microscopy. Negative control (NC) represents non-migratory status right after wound scratch in the PC-3 cells. (C) Bar graphs showed quantification of migrated PC-3 cells. (D) Cell viability in miRNA3195 and miRNA374b transfected PC-3 cells. After transfection with control vector, miRNA3195, or miRNA374b mimics, MTT assay was performed at 24 or 48 h after transfection. Data are presented as means \pm SEM of triplicate samples. **, $p < 0.01$ vs. miRNA control

Likewise, overexpression of miRNA3195 and miRNA374b effectively inhibited mRNA expression of HIF-1 α , HIF-2 α and VEGF in hypoxic PC-3 cells compared to untreated hypoxia control (Figure 4A, 4B, 4C). In addition, Western blotting revealed that expression levels of HIF-1 α and VEGF were attenuated in miRNA3195 and miRNA374b mimic-transfected PC-3 cells under hypoxia, but not in miRNA1290-transfected PC-3 cells (Figure 4D). Of note, melatonin significantly reduced VEGF produc-

tion (as determined by ELISA) and VEGF protein (as determined by Western blotting) in hypoxic PC-3 cells transfected with miRNA3195 and miRNA374b mimics compared to miRNA vector control or only melatonin treated control (Figure 5A and C), while antisense oligonucleotides against miRNA374b or miRNA3195 did not attenuate production of VEGF in the presence of melatonin (Figure 5B). Antisense oligonucleotides against miRNA374b without melatonin rather increased the production of VEGF (Figure 5B).

In contrast, the miRNA1290 mimic (used as the negative control) did not affect VEGF protein expression (Figure 5C). Similarly, immunofluorescence assay revealed that melatonin attenuated HIF-1 α expression in miRNA3195 and miRNA 374b mimic-transfected PC-3 cells under hypoxia, whereas the miRNA1290 mimic did not affect VEGF immunofluorescent expression (Figure 5D).

Discussion

Many studies have demonstrated a dual effect of melatonin on angiogenesis in cancer and endothelial cells. Ganguly *et al.* (10) reported that melatonin promotes angiogenesis during protection and healing of *indomethacin*-induced gastric ulcers, and Soybir *et al.* (28) suggested that melatonin enhances neovascularization and wound healing in rats with 5-cm incision damage. In contrast, melatonin suppressed tumor angiogenesis in HepG2 hepatocellular cancer (29) and PANC-1 pancreatic cancer (30) by inhibiting VEGF or signal transducer and activator of transcription 3 (STAT3) and HIF-1 α , and in PC-3 prostate cancer by inhibiting the sphingosine kinase pathway

(26). Nevertheless, the mechanism underlying the association between melatonin and miRNAs remains unknown. In the current study, we found that 33 miRNAs (changes of at least twofold), including miRNA3195 and miRNA 374b, were significantly upregulated, and 16 miRNAs were downregulated, in melatonin-treated PC-3 cells under hypoxia based on miRNA expression profiling using microarrays, implying the important roles of miRNAs in melatonin-treated PC-3 cells under hypoxia. Considering our previous report that melatonin suppresses HIF-1 α accumulation by inhibiting the sphingosine kinase 1 (SPHK1) pathway and reactive oxygen species (ROS) generation in hypoxic PC-3 cells, the anti-angiogenic activity may be the result of blockade of HIF-1 α /VEGF/SPHK1 axis in association with some key miRNAs in hypoxic PC-3 cells. We selected two potent miRNAs (miRNA3195 and miRNA374b) for further mechanistic studies since miRNA3195 was the most highly upregulated based on our microRNA array data analysis and miRNA374b is downregulated in human prostate cancer tissues (25).

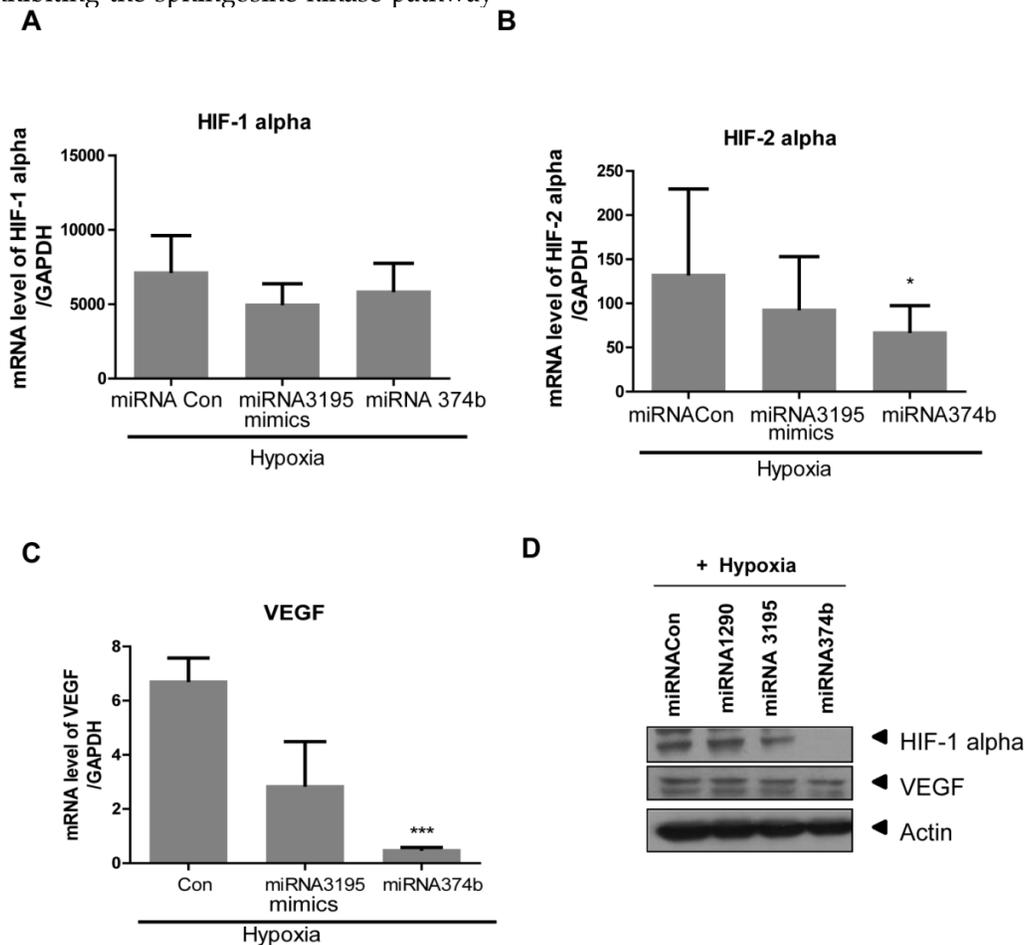


Figure 4. Overexpression of miRNA3195 and miRNA374b inhibited the expression of HIF-1 α , HIF-2 α and VEGF in hypoxic PC-3 cells. Effect of miRNA3195 and miRNA374b mimics on HIF-1 α (A), HIF-2 α (B) and VEGF (C) in hypoxic PC-3 cells by qRT-PCR. Mimics of miRNA3195 and miRNA374b were transfected into PC-3 cells. Two days after transfection, PC-3 cells were cultured for 4 h under hypoxia. Total RNAs were isolated and analyzed to determine the mRNA expression of HIF-1 α , HIF-2 α and VEGF by qRT-PCR. GAPDH was used to normalize the target gene. Data are presented as means \pm SEM of three independent experiments. (D) Effect of miRNA1290 (negative control), miRNA3195 and miRNA374b mimics on HIF1 α and VEGF in hypoxic PC-3 cells by Western blotting.

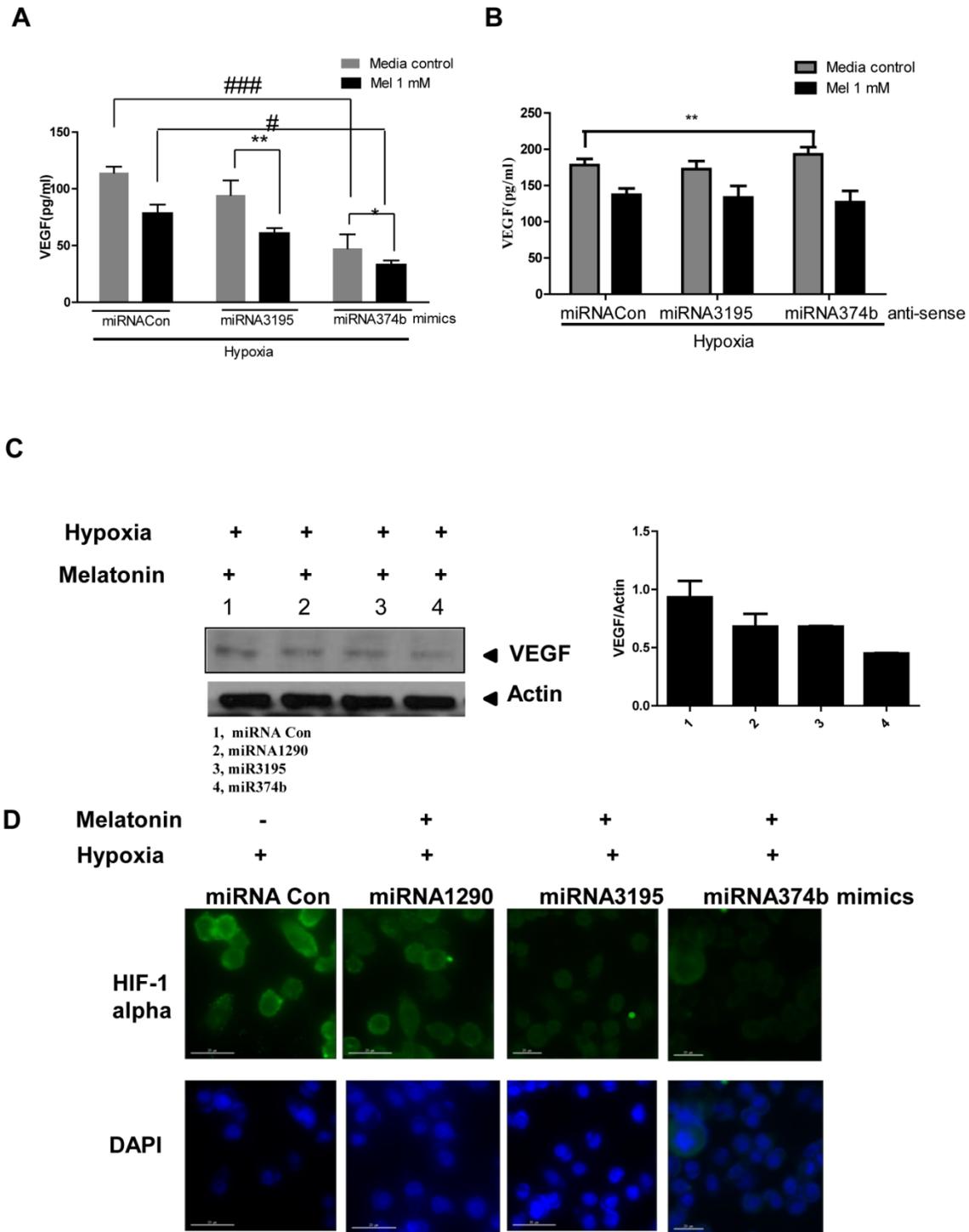


Figure 5. Melatonin significantly reduced VEGF secretion and HIF-1 alpha green color expression in miRNA3195 and miRNA374b mimics treated in hypoxic PC-3 cells. Effect of melatonin on VEGF production in hypoxic PC-3 cells transfected with miRNA3195 or miRNA374b mimics(A) or miRNA374b mimics against miRNA3195 or miRNA374b(B). Mimics or anti-sense oligonucleotides against miRNA3195 or miRNA374b were transfected into PC-3 cells. Two days after transfection, the cells were cultured in the presence of melatonin at 1 mM for 4 h under hypoxia. The level of VEGF was measured by using VEGF ELISA kits. Statistical significance was evaluated using Tukey's multiple comparison test: # p<0.05, ### p<0.001 vs. hypoxic control, and * p<0.05, ** p<0.01 vs. microRNA control. Data are presented as mean ± SEM for triplicate experiment. (C) Effect of melatonin on VEGF production assessed by Western blotting in hypoxic PC-3 cells when miRNA3195 and miRNA374b were overexpressed using mimics. PC-3 cells were exposed to 1 mM melatonin for 24 h and Western blotting was performed as shown in Materials and methods. 1st lane; hypoxia, 2nd lane; hypoxia + miRNA1290 mimic as negative control. 3rd lane; hypoxia + miRNA3195 mimic, 4th lane; hypoxia + miRNA374b. (D) Effect of melatonin on HIF-1 alpha by immunofluorescent green color expression in miRNA3195 and miRNA374b mimics treated in hypoxic PC-3 cells by immunofluorescence assay.

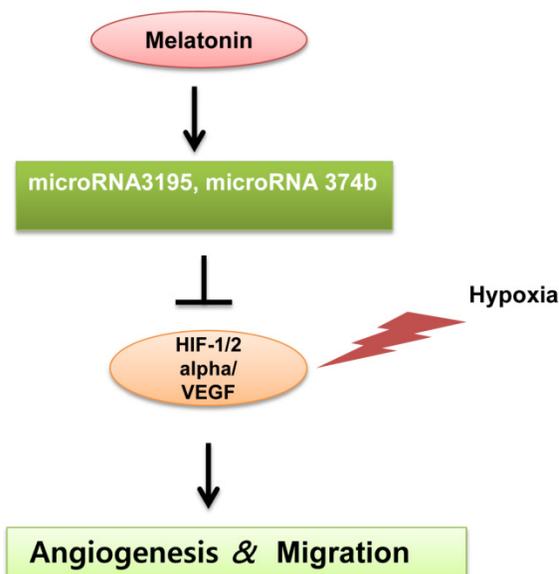


Figure 6. A schematic diagram for anti-angiogenic property of melatonin via regulation of miRNA3195 and miRNA374b in hypoxic PC-3 cells.

Consistent with the miRNA array data, qRT-PCR analysis demonstrated that melatonin enhanced the expression of miRNA3195 and miRNA374b. In addition, wound-healing assays revealed that melatonin significantly suppressed the repair motility of hypoxic PC-3 cells, indicating that melatonin has antimigratory activity, since angiogenic capillary sprouts invade the fibrin/fibronectin-rich clot during wound healing and within a few days to organize into a microvascular network throughout the granulation tissue (31-33). HIF-1 α promotes the expression of pro-angiogenic genes, such as *VEGFA*, *PDGFB* and *IL-8* (34, 35), and both HIF-1 α and HIF-2 α regulate the expression of genes involved in angiogenesis (36). Thus, qRT-PCR analysis revealed that melatonin significantly attenuated the expression of HIF-1 α , HIF-2 α and VEGF at the mRNA level in hypoxic PC-3 cells, which was supported by previous reports that melatonin exerts an anti-angiogenic effect by decreasing VEGF or HIF-1 α expression under hypoxic conditions *in vitro* (7) and *in vivo* (37).

Previous studies demonstrate that miRNAs play important roles in angiogenesis, which involves the formation of new blood vessels from pre-existing blood vessels (38, 39). Furthermore, several studies have shown that miRNAs are upregulated or downregulated in hypoxic breast and colon cancer cells (40). In addition, the hypoxia-regulated miRNAs miRNA-15b, miRNA-16b, and miRNA20a/b are known to control VEGF production, because VEGF (a pro-angiogenic molecule) has been identified as a potential miRNA target (41). Nevertheless, no report of the relationship between melatonin and miRNAs has been published to date except one report on the

regulation of melatonin synthesis by miR-483 (42). In the present study, overexpression mimics of miRNA3195 and miRNA374b in PC-3 cells abrogated the mRNA levels of the angiogenesis-related proteins HIF-1 α , HIF-2 α and VEGF under hypoxia, demonstrating that miRNA3195 and miRNA374b target the HIF-1/2 α /VEGF axis. Consistently, melatonin attenuated VEGF expression at the protein level, reduced VEGF secretion, and also reduced HIF-1 α immunofluorescent expression in hypoxic PC-3 cells transfected with miRNA3195 and miRNA374b mimics compared to untreated control, indicating the antiangiogenic property of miRNA3195 and miRNA374b. Also, we confirmed that miRNA3195 was upregulated by melatonin in hypoxic DU145 cells. Furthermore, melatonin upregulated miRNA3195 and miRNA374b as determined by miRNA microarray and qRT-PCR analysis and overexpression of miRNA3195 and miRNA374b synergistically reduced VEGF production by hypoxic PC-3 prostate cancer cells, implying important role of miRNA3195 and miRNA374b in melatonin induced antiangiogenic activity.

In summary, melatonin significantly attenuated the expression of HIF-1 α , HIF-2 α and VEGF at mRNA level and also enhanced the expression of miRNA3195 and miRNA 374b in hypoxic PC-3 cells by qRT-PCR analysis. Notably, overexpression of miRNA3195 and miRNA374b mimics attenuated the mRNA levels of HIF-1 α , HIF-2 α and VEGF in hypoxic PC-3 cells. Furthermore, overexpression of miRNA3195 and miRNA374b suppressed VEGF protein expression and production and also reduced HIF-1 α immunofluorescent expression in melatonin treated PC-3 cells under hypoxia. Taken together, our findings demonstrate that upregulation of miRNA3195 and miRNA374b mediates anti-angiogenic property induced by melatonin in hypoxic PC-3 cells. However, further mechanistic studies are required to confirm the anti-angiogenic effects of miRNA3195 and miRNA374b in animals.

Supplementary Material

Additional File 1:

Supplementary Figures 1-2.

<http://www.jcancer.org/v06p0019s1.pdf>

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. No. 2012-0005755).

Conflict of Interest

The authors have declared that there is no conflict of interest.

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