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Original Article

Up-regulation of miR-221 mediates the pioglitazone-induced inhibition of vascular endothelial cell proliferation

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Abstract: Given that angiogenesis plays a vital role in development of cancer, a becoming anti-tumor strategy is anti-angiogenic. Although the anti-angiogenic effect of pioglitazone has been known already, the detailed molecular mechanism, which accounts for the endothelial cells (ECs) to proliferation inhibited by pioglitazone, is limited, particularly concerning their posttranscriptional regulation involving miRNA. In present study, we provide the mechanistic link between pioglitazone and its anti-angiogenic activity. Primarily, using immumofluorescence and CCK-8 assay, we find pioglitazone inhibits the ECs proliferation. During this inhibiting process, 12 microRNAs (miRNAs) show significant differences at relative gene abundance in which miR-221 presents a highest up-regulated expression. In following experiment, we demonstrate that expression of miR-221 mediated by pioglitazone positively correlate with anti-angiogenic action of pioglitazone. At last, we discover that pioglitazone augments the reduction of p27 mRNA level in ECs by up-regulating miR-221. Collectively, we have exploited a valuable mechanism by which up-regulation of miR-221 contributes to the pioglitazone-induced inhibition vascular EC proliferation, which raise a possibility that inhibition of miR-221 may be a potential strategy for therapeutic intervention in excessive vascular EC proliferation.

Keywords: Pioglitazone, miR-221, vascular endothelial cell, angiogenesis

Introduction

The process of angiogenesis is that pre-existing vasculature produces the new vessels, in which endothelial cells (ECs)-dependent proliferation, migration, and differentiation are mainly responsible for this process [1, 2]. Multiple normal physiological processes including embryonic development, wound healing and menstrual cycle rely on angiogenesis [3]. Besides, angiogenesis plays a vital role in numerous pathological conditions, especially in development of cancer [4]. In the vast majority of cancers, blood vessel growth is motivated and these vessels are unnatural in almost all aspects of their structure and function, thereby nourishing cancers [5]. Given that, a becoming concept is that anti-angiogenic strategies through inhibiting vessel growth and normalizing vessels are potential therapeutic intervention in cancers, which were consistently evidenced in recent studies [6-8]. Thus, current main task is still to further screen the anti-angiogenic agents although some functional agents have been determined [9].

Increasing reports have revealed that mammalian genomes contain an emerging class of highly conserved small non-coding RNAs [10], microRNA (miRNA), which involves in various biological processes such as cardiogenesis, skeletal muscle proliferation, oncogenesis and angiogenesis, and negatively regulates gene expression on the post-transcriptional level through degradation or translational inhibition of their target mRNAs [11]. So far, some miRNAs presenting positive or negative role in angiogenesis have been partially revealed. Two miRNA, let-7f and miR-27b, which highly express in ECs, and possess pro-angiogenic effects based on suppression of angiogenesis using according miRNA inhibitors [12]. In contrast, highly expressed miR-221 and miR-222 exert anti-angiogenic effects in vitro as evidenced by targeting the stem cell factor receptor c-kit and indirectly regulating endothelial nitric oxide synthase expression [13, 14]. Moreover, a few miRNAs including the miR-17-92 cluster, miR-378, miR-155 and miR-107 involved in tumour angiogenesis are identified [15-18]. Although above-mentioned results have presented the

dual anti-tumour and anti-angiogenic effect of miRNA, their value to cancer therapy remains to be established.

Besides miRNA, pioglitazone, a prescription drug of class thiazolidinedione, has also been reported to associate with anti-angiogenesis [19]. Traditionally, pioglitazone was utilized for the therapy of type-II diabetes mellitus in clinical because of their ability to reverse insulin resistance [20]. In spite of this application, pioglitazone have also been presented a comprehensive package of biological activities such as anti-inflammatory [21], anti-proliferative [22] and anti-tumor effects [23-25]. More importantly, the anti-cancer effect of pioglitazone is positively related to its anti-angiogenesis [19] and may be regulated through peroxisome proliferator-activated receptor-γ (PPAR-γ)-dependent or -independent way [19, 23-26]. Although the potential anti-tumour and anti-angiogenic activity of pioglitazone has been speculated, molecular mechanism by which pioglitazone acts is poorly understood, particularly concerning their posttranscriptional regulation involving miRNA. Here we attempted to investigate whether the miRNAs are involved in the anti-angiogenic effect of pioglitazone in human microvascular endothelial cell line HMEC-1.

Materials and methods

Cell culture

The human microvascular endothelial cell line HMEC-1 was cultured in Dulbecco's modified eagle medium (DMEM, Hyclone) supplemented with 10% (V/V) FBS (Hyclone), 10 ng/ml epidermal growth factor and 1% penicillin-streptomycin at 37°C, 5% CO_2 incubator. Cells grown in 60 mm cell culture dish were allowed approximately to reach 85% confluence. The culture medium was changed every two day and then were rinsed and removed from the dishes by incubating them with a trypsin-EDTA solution (Hyclone), and harvested in a 15 mL centrifuge tube for subsequent study.

MiRNA mimic

Chemically modified double-stranded RNAs engineered to mimic the endogenous mature miR-221 and negative control miRNA were purchased from Ambion. miRNA mimics were transfected using RNAi Max (Invitrogen) according to the manufacturer's indication at 0.3 or 3 nM as directed. MiR-221 inhibitor (2'OMe-miR-221) were chemically synthesized and purified by high-performance liquid chromatography (Gene Pharma, Shanghai, China). HMEC-1 transfected with scrambled 2'-OMe oligonucleotides (scramble) were used as a negative inhibitor control. These oligonucleotides were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's direction at 300 pmol as indicated.

Cell proliferation assay

HMEC-1 cells were directly cultured at a density of 1×10^5 cells/well in 6 well plates. For experiment with the effect of pioglitazone on proliferation, the cells cultured overnight were incubated with DMSO (negative control) and pioglitazone (prepared in DMSO) respectively for indicated time at 37°C. For experiment with administrations of miR-221 mimic, negative mimic, miR-221 inhibitor, negative inhibitor or synergy of each of above-mentioned molecules with pioglitazone, after overnight cultivation, the cells were stimulated with these reagents for indicated concentration and time. Cell proliferation was primarily investigated by immunofluorescence. For immunofluorescence observation, we incubated HMEC-1 cells with antifade mounting medium containing carboxyfluorescein diacetate (CFDA, Invitrogen) for cytoplasmic staining and DAPI (Invitrogen) for nuclear staining, followed by analysis with a laser scanning confocal microscope (Leica 224).

Cell proliferation was analyzed additionally by using CCK-8 assay kit (Dojindo) according to manufacturer introductions. In particular, HMEC-1 cells were incubated in the medium containing DMSO or pioglitazone in 96-well plates. After that, 5 μl CCK-8 reagent was added to each well and incubated at 37°C for 1 h. The cell numbers were evaluated by measurement of absorbance at 450 nm. Proliferation assays for cells pretreated with miR-221 mimic, negative mimic, miR-221 inhibitor, negative inhibitor or each of above-mentioned molecules plus pioglitazone were also assessed by utilizing CCK-8 assay. All the experiments were carried out in triplicate.

MiRNAs array screening

MiRNAs were extracted from HMEC-1 by using the mirVana miRNA isolation kit (Ambion). The

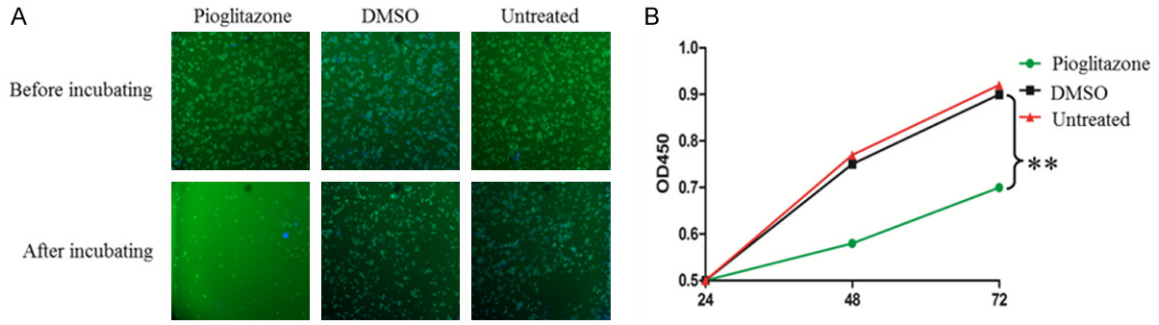

Figure 1. Pioglitazone inhibits the proliferation of HMEC-1 cell. A. Immunofluorescence showing cell proliferation of HMEC-1 cell in untreated, DMSO-treated and pioglitazone-treated group. HMEC-1 cells were incubated with CFDA for cytoplasmic staining and DAPI for nuclear staining. B. CCK-8 assay of HMEC-1 cell in untreated, DMSO-treated and pioglitazone-treated group. Error bars ± SD, $^{**}P<0.01$.

expression profiling of miRNA was investigated by miRNA microarray analysis using the human miRNA array probes including 15 mature human miRNAs. These miRNAs are let-7a, let-7b, let-7d, miR-1, miR-221, miR-222, miR-122, miR-375, miR-24, miR-133, miR-126, miR-16, miR-21, miR-155 and miR-320.

Quantitative RT-PCR assay

For quantitative RT-PCR assay, RNA was isolated from HMEC-1 cells using the TRIzol reagent according to the manufacturer's instructions. 4 μg of total RNA were provided to generate the first-strand cDNAs by using commercially available kits (Applied Biosystems). All subsequent PCR reactions were carried out using the 7 Universal PCR Master Mix (Applied Biosystems). Thermal cycling and fluorescence detection of mRNA were analyzed by 7500 real-time PCR System (Applied Biosystems). To normalize mRNA concentrations, mRNA levels of β-actin gene were identified in parallel for each sample, and relative mRNA level of Notch1 was adjusted by standardization based on the β-actin transcriptional levels. Samples for each experimental condition were run in triplicate.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) and subjected to analysis of variance (ANOVA) to assess the treatment effects by using SPSS 13.0 software. The Student *t* test was used to determine the statistically significant differences in numbers with two significant levels (0.05 and 0.01).

Results

Pioglitazone inhibits the efficient proliferation of HMEC-1 cell

To understand the anti-angiogenic effect of pioglitazone in ECs, HMEC-1 cell was used to treat. Cell proliferation was measured by utilizing the immunofluorescence. Prior to incubating the pioglitazone, the identical cell population was plated on coverslips (**Figure 1A**). After that, pioglitazone was used to stimulate the cells and then immune stained with CFDA and DAPI. Interestingly, the results showed that cell numbers between untreated and DMSO-treated group had no difference, however, HMEC-1 cell treated with pioglitazone presented a significant reduction in the cell population in contrast to untreated and DMSO treated group (**Figure 1A**). Then, we explored the cell proliferation using CCK-8 assay, as shown in **Figure 1B**. Similarly, the cell proliferation rate was differentially decreased in the HMEC-1 cell treated with pioglitazone compared with untreated and DMSO-treated cells ($P<0.01$), indicating that pioglitazone efficiently suppressed the proliferation of HMEC-1 cell.

Pioglitazone induces the miR-221 expression

To further explore the anti-angiogenic molecular mechanism, we use the miRNA array to identify the downstream miRNA molecules of pioglitazone. Fifteen vital angiogenesis-related miRNAs were selected to analysis and, undoubtedly, 12 miRNAs showed significant differences at relative gene abundance in pioglitazone-treated cells compared with DMSO-

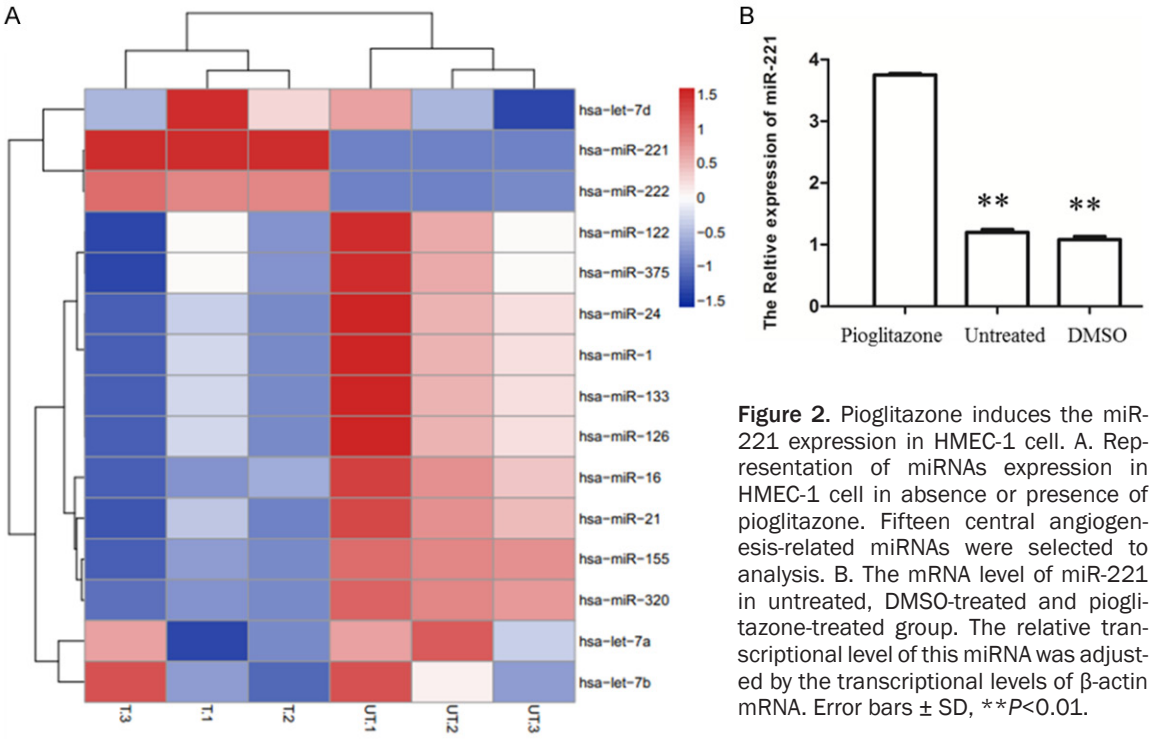

Figure 2. Pioglitazone induces the miR-221 expression in HMEC-1 cell. A. Representation of miRNAs expression in HMEC-1 cell in absence or presence of pioglitazone. Fifteen central angiogenesis-related miRNAs were selected to analysis. B. The mRNA level of miR-221 in untreated, DMSO-treated and pioglitazone-treated group. The relative transcriptional level of this miRNA was adjusted by the transcriptional levels of β-actin mRNA. Error bars ± SD, $^{**}P<0.01$.

treated cells (**Figure 2A**). Out of them, two containing miR-221 and miR-222 were up-regulated in pioglitazone-treated cells while ten including miR-1, miR-122, miR-375, miR-24, miR-133, miR-126, miR-16, miR-21, miR-155 and miR-320 were down-regulated. Among these 12 miRNAs, we found that miR-221 presented the most significant difference in pioglitazone-treated cells in contrast to DMSO-treated cells. Then, we further confirmed the miR-221 expression in pioglitazone-treated cells from transcriptional level. As **Figure 2B** showed, the expression of miR-221 was significantly improved in HMEC-1 cells treated with pioglitazone compared to the untreated and DMSO-treated cells, respectively ($P<0.01$). This finding suggested that pioglitazone have an ability to promote the miR-221 expression in HMEC-1 cell and miR-221 may be a central downstream target of pioglitazone.

miRNA-221 contributes to pioglitazone-induced inhibition of EC proliferation

Next, to examine the mechanism that links miR-221 to anti-angiogenic activity of pioglitazone, miR-221 mimic oligonucleotides, nontargeting negative control mimic, miR-221 inhibitor and scrambled negative inhibitor were transfected respectively into cells and then cell proliferation were measured by using immunofluorescence and CCK-8 assay in absence or presence of pioglitazone. Because administration of pioglitazone promotes the expression of miR-221, the effect of miR-221 mimic and inhibition on anti-angiogenic action of pioglitazone was analyzed. First, we investigate the cell proliferation of HMEC-1 cell with miR-221 mimic and miR-221 mimic plus pioglitazone treatment (**Figure 3**). As expected, the miR-221 mimic-transfected HMEC-1 cells have a lower proliferation in contrast to negative mimic-transfected cell (**Figure 3A** and **3B**) and further enhance the inhibition of cell proliferation in the presence of pioglitazone (**Figure 3A** and **3C**), indicating the anti-angiogenic activity of miR-221. Moreover, HMEC-1 cells stimulated by miR-221 inhibitor plus pioglitazone show the similar proliferation when compare with miR-221 inhibitor-treated cells but have an obviously increased proliferation as compared to the pioglitazone-treated cells (**Figure 3A** and **3C**), and HMEC-1 cells treated by negative inhibitor plus pioglitazone could not prevent the declining cell proliferation resulted from pioglitazone treatment through immunofluorescence and CCK-8 assay (**Figure 3A** and **3C**), thereby revealing the specific ability of restored proliferation for miR-221 inhibi-

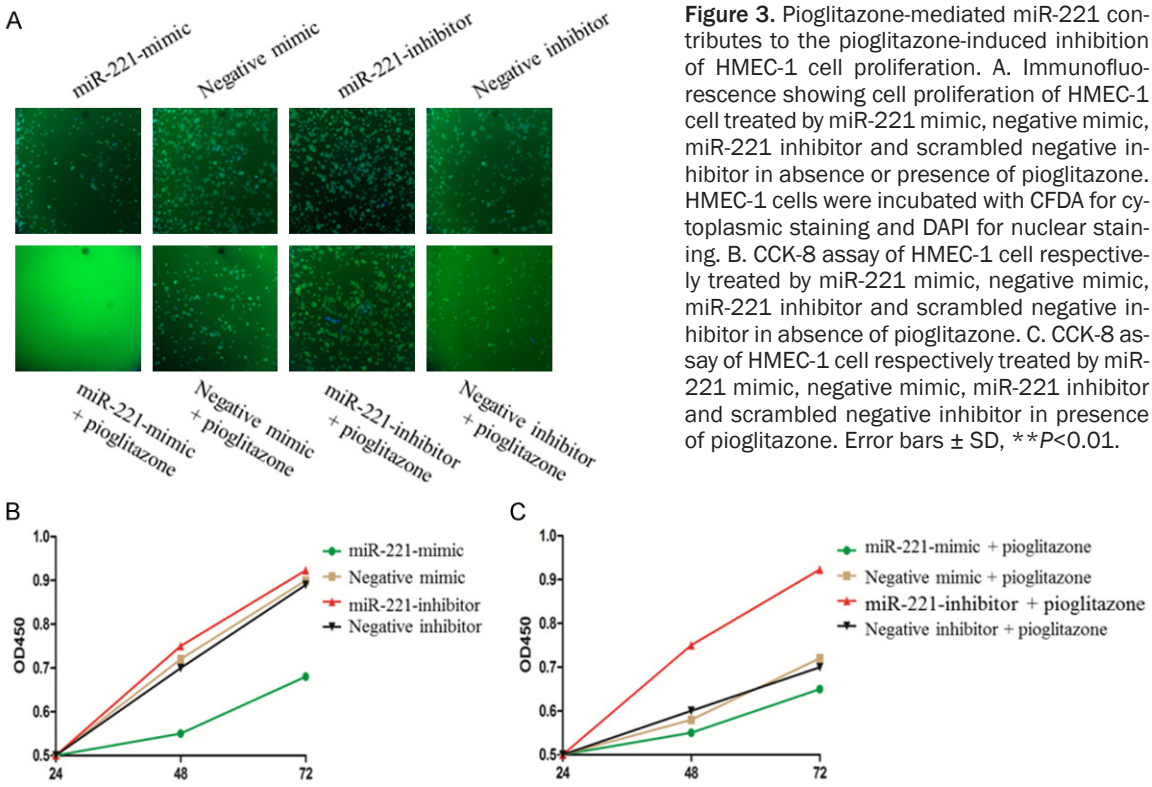

Figure 3. Pioglitazone-mediated miR-221 contributes to the pioglitazone-induced inhibition of HMEC-1 cell proliferation. A. Immunofluorescence showing cell proliferation of HMEC-1 cell treated by miR-221 mimic, negative mimic, miR-221 inhibitor and scrambled negative inhibitor in absence or presence of pioglitazone. HMEC-1 cells were incubated with CFDA for cytoplasmic staining and DAPI for nuclear staining. B. CCK-8 assay of HMEC-1 cell respectively treated by miR-221 mimic, negative mimic, miR-221 inhibitor and scrambled negative inhibitor in absence of pioglitazone. C. CCK-8 assay of HMEC-1 cell respectively treated by miR-221 mimic, negative mimic, miR-221 inhibitor and scrambled negative inhibitor in presence of pioglitazone. Error bars ± SD, $^{**}P<0.01$.

tor. These observations indicate that a pioglitazone-mediated miRNA, miR-221, play a significantly positive role in pioglitazone-induced inhibition of HMEC-1 cell proliferation.

Pioglitazone-mediated miR-221 reduces the p27 expression in HMEC-1 cell

In a follow-up experiment, we evaluated whether the pioglitazone have a capacity to regulate the expression of p27 in HMEC-1 cells through up-regulation of miR-221. To this end, we primarily detected the transcriptional expression of p27 in HMEC-1 cell in response to pioglitazone treatment, as shown in **Figure 4A**. The untreated and DMSO-treated HMEC-1 cell relatively highly expressed p27, while the expression of p27 markedly reduced in HMEC-1 cell treated by pioglitazone ($P<0.01$). This finding suggested that pioglitazone have an ability to inhibit the p27 expression in ECs. Given that miR-221 mimic and miR-221 inhibitor could also respectively suppress and enhance the p27 mRNA level ($P<0.01$, **Figure 4B**), presenting the targeting activity of miR-221 on p27 in ECs, it is therefore intriguing to suggest that the effect of pioglitazone on expression of p27 is likely to be accomplished by up-regulation of miR-221. As expected, pioglitazone-stimulated cells treated by miR-221 inhibitor had a specific restoration in expression of p27 as compared to pioglitazone-stimulated cells treated by scrambled inhibitor ($P<0.01$, **Figure 4C**). Meanwhile, the transcriptional level of p27 was further reduced in cells response to miR-221 mimic plus pioglitazone treatment in contrast to negative mimic plus pioglitazone treatment ($P<0.01$, **Figure 4C**). Altogether, these data indicate that pioglitazone boosts the reduction of p27 expression in vitro by up-regulating miR-221.

Discussion

A growing body of evidence indicates that pioglitazone has an anti-angiogenic effect in vitro, which is positively related to its anti-cancer effect [19]. Although these effects of pioglitazone may be regulated through PPAR-γ-dependent or -independent way [19, 23-26], the detailed molecular mechanism, which accounts for the ECs to proliferation inhibited by pioglitazone, was elucidated incompletely, particularly about their posttranscriptional regulation involving miRNA. In present study, we not only enrich the pioglitazone as an anti-angiogenic reagent that inhibits HMEC-1 cell proliferation but also, in follow-up experiments, pro-

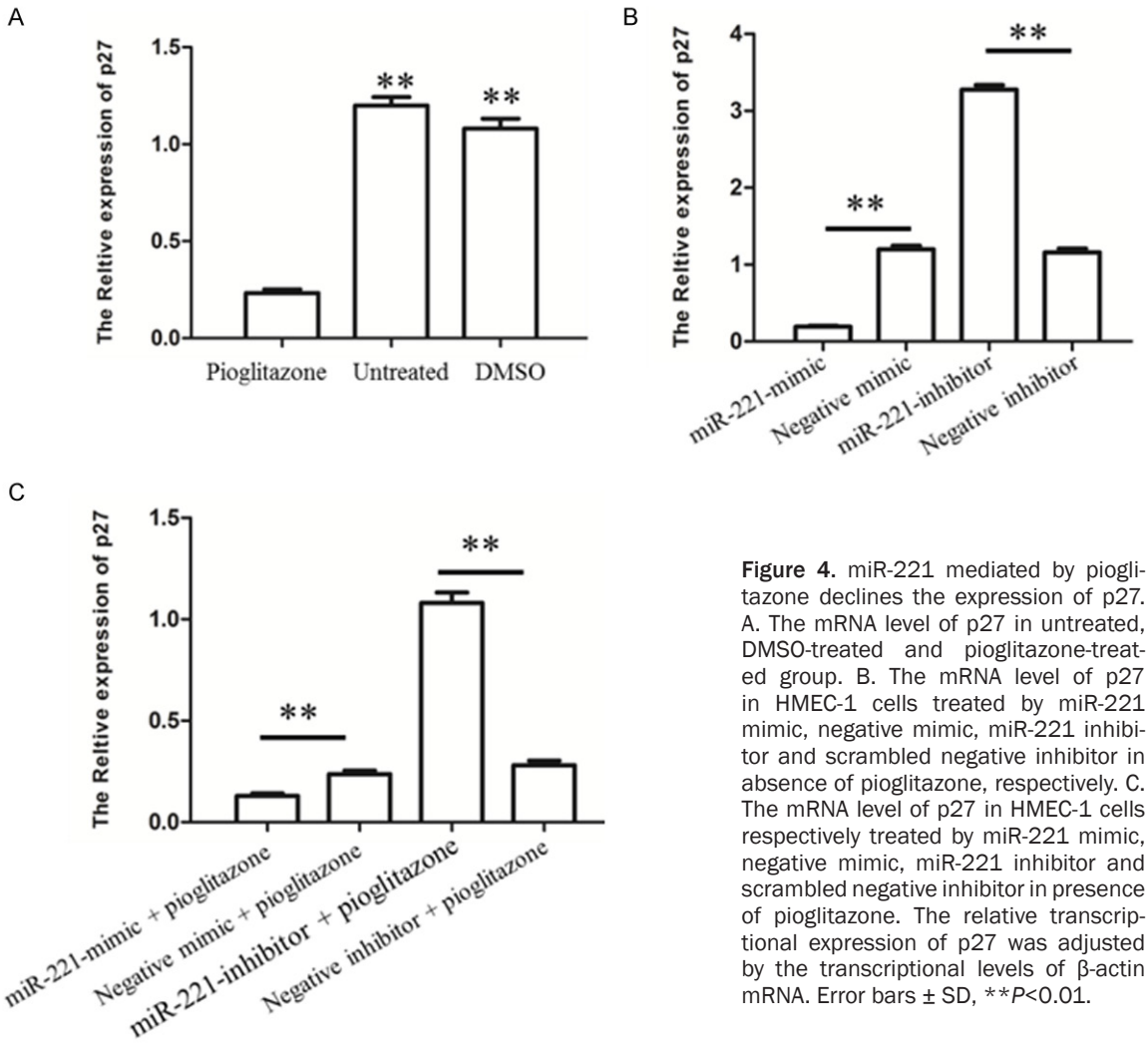

Figure 4. miR-221 mediated by pioglitazone declines the expression of p27. A. The mRNA level of p27 in untreated, DMSO-treated and pioglitazone-treated group. B. The mRNA level of p27 in HMEC-1 cells treated by miR-221 mimic, negative mimic, miR-221 inhibitor and scrambled negative inhibitor in absence of pioglitazone, respectively. C. The mRNA level of p27 in HMEC-1 cells respectively treated by miR-221 mimic, negative mimic, miR-221 inhibitor and scrambled negative inhibitor in presence of pioglitazone. The relative transcriptional expression of p27 was adjusted by the transcriptional levels of β-actin mRNA. Error bars ± SD, $^{**}P<0.01$.

vide the mechanistic link between pioglitazone and its inhibiting function of cell proliferation.

The core discovery in our study is the potent ability of exogenous pioglitazone to regulate the expression of miRNA, such as miR-221, in HMEC-1 cell. This is a novel finding, since previous studies did not disclose how any miRNA might be reliable for pioglitazone-induced inhibition of cell proliferation. Currently, we present that miR-221 contributes to pioglitazone-induced inhibition of cell proliferation, demonstrating a plausible miR-221-regulated mechanism. The cell proliferation regulated by miRNA and pioglitazone have been extensively evidenced in the literature [12-19]. However, the amazed capacity of pioglitazone to facilitate the up-regulation of miR-221 and in turn reduces cell proliferation in ECs was previously unrecognized. Additionally, previous studies indicated that silencing of tumor suppressor gene p27 is an important role of miR-221 [27, 28]. Given that, a becoming hypothesis is that pioglitazone treatment may lead to the down-regulation of p27. This is consistent with current observation that pioglitazone-treated cells represent the declined transcriptional level of p27 through up-regulation of miR-221 (**Figure 4**), which historically leaded to cancer cell proliferation, exhibiting a prognostic significance in human cancer [27, 28]. This apparent contradiction has raised a conundrum regarding the role of p27 in pioglitazone-mediated inhibition of ECs proliferation and therefore need to be further investigated in future.

Besides miR-221, we also found that other 11 miRNAs containing miR-222, miR-1, miR-122, miR-375, miR-24, miR-133, miR-126, miR-16, miR-21, miR-155 and miR-320 were differentially altered in pioglitazone-treated ECs. Similar to miR221, miR-222 also has anti-angiogenic

effect for ECs in vitro [13, 14]. miR-1/126 involve in the cross-talk between the muscle and vasculature to regulation of developmental angiogenesis [29]. miR-122 functions as a tumor suppressor in liver cancer partly because of its anti-angiogenic potential in ECs [30]. By directly targeting vascular endothelial growth factor (VEGF), miR-16 is a key negative regulator of angiogenic signaling [31] while activation of miR-21 induces tumor angiogenesis through enhancing VEGF expression [32]. Recent reports reveal that overexpression of miR-155 contributes to placental angiogenesis [33], and miR-320 augments the degradation of insulin-like growth factor (IGF)-1 in mediating angiogenesis in diabetic rats [34]. However, the potential role of remaining miRNA, miR-24, miR-375 and miR-133, in angiogenesis awaits future studies although it has been shown to regulate apoptosis and inhibit cell proliferation [35-37]. Therefore, a suitable possibility is that EC angiogenesis inhibited by pioglitazone is likely realized in part by altering the expression of these miRNAs, and certainly this inference requires to be further checked in subsequent study.

Taken all together, we represents substantial improve toward elucidating the miRNA-regulated mechanism corresponding to pioglitazone-mediated anti-angiogenesis. These findings raise a possibility that inhibition of miR-221 may be a potential strategy for therapeutic intervention in excessive vascular EC proliferation.

Disclosure of conflict of interest

None.

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