p43 induces IP-10 expression through the JAK-STAT signaling pathway in HMEC-1 cells

WEI WANG[1,2*], JUNJIE TAN[1*], YUHUA XING[3], NAIPENG KAN[4], JINGYI LING[1], GUIFU DONG[4], GANG LIU[1] and HUIPENG CHEN[1,2]

[1]Beijing Institute of Biotechnology, Beijing 100071; [2]College of Life Science, Jilin University, Changchun, Jilin 130012; [3]National Institutes for Food and Drug Control, Beijing 100071; [4]School of Life Sciences, Anhui University, Hefei, Anhui 230039, P.R. China

Received August 13, 2015; Accepted July 15, 2016

DOI: 10.3892/ijmm.2016.2710

Abstract. p43 is a cofactor of aminoacyl-tRNA synthetase in mammals that effectively inhibits angiogenesis. However, the role of p43 in angiogenesis remains unclear. In the present study, we examined the effects of p43 on angiogenesis using human microvascular endothelial cells-1 (HMEC-1) cells as a model. Our microarray data showed that p43 regulated a number of cytokines, and the majoity of these are involved in the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. IP-10 was previously shown to inhibit angiogenesis and suppress tumor growth via the JAK-STAT signaling pathway *in vitro* and *in vivo*. Our results showed that p43 induces both the mRNA and protein expression of IP-10. Furthermore, we demonstrated that p43 exerted an effect on the JAK-STAT signaling pathway by regulating key factors of the pathway. Using a JAK inhibitor, AG490, we studied the effect of p43 on HMEC-1 cells by blocking the JAK-STAT pathway. We found that AG490 inhibited the induction of IP-10 expression by p43, and suppressed the inhibitory effect of p43 on tubule formation and cell migration in HMEC-1 cells. We concluded that p43 inhibits tubule formation and cell migration by inducing IP-10 through the JAK-STAT signaling pathway, and blocking the JAK-STAT pathway with AG490 diminishes the inhibitory effects of p43 on angiogenesis.

Introduction

Angiogenesis is the process whereby new blood vessels form from pre-existing vessels via microvascular sprouting (1,2). It is a continuous process, regulated by various angiogenic factors. Abnormal angiogenesis causes a number of diseases, such as diabetes, rheumatoid arthritis, atherosclerosis and malignant tumors (3-5). Tumor angiogenesis has been shown to play an important role in the occurrence, development and metastasis of tumors (6,7). Folkman first attempted to treat tumors by inhibiting angiogenesis and achieved significant clinical outcomes (8). Since then, the development of angiogenesis inhibitors has become a key area of study for cancer treatment.

p43, also known as proEMAPII protein (9,10), is a cofactor of mammalian aminoacyl-tRNA synthetase (ARS). It is a 34-kDa single-chain protein, containing 312 amino acids (11-14). It harbours an N-terminal domain, of which the function remains unclear, and a C-terminal domain, which encodes EMAPII protein (15-19) and is comprised of a super-secondary structure of an OB-fold and a β-barrel structure (10). Previous reports have revealed two primary functions of p43. Firstly, p43 effectively inhibits angiogenesis. p43 was found to suppress development of new blood vessels in chick embryo chorioallantoic membrane at a low dose and inhibit *in vitro* tubule formation and cell migration in human microvascular endothelial cells-1 (HMEC-1) cells (20). Secondly, p43 is secreted into the extracellular space as a cytokine and plays a role in a number of cellular processes. For example, p43 was found to prevent tumor growth by activating tumor necrosis factor (TNF), interleukin (IL)-8 and other TNFs (20,21). Therefore, p43 may potentially serve as a clinical drug target for the treatment of solid tumors such as lung, gastric, prostate and breast cancers as well as nasopharyngeal carcinoma. However, the underlying mechanisms responsible for the inhibition of angiogenesis by p43 remain unclear. Previous findings have suggested that p43 inhibits angiogenesis by blocking the proliferation of endothelial cells and inducing the apoptosis of endothelial cells (22). The C-terminus of p43 is reported to contain a heparin-binding region. By binding to heparin, p43 interacts with the α-subunit of ATP synthase in endothelial cells; thus, repressing blood vessel growth (22).

Although the structure and function of p43 has been identified, the roles of p43 in diverse biological processes, particularly in angiogenesis, remain poorly understood. In the present study, we examined the effects of p43 in HMEC-1 cells using Affymetrix Human Genome chips. Our microarray data showed that p43 upregulated multiple cytokines, the majority of which are associated with the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. Interferon-

Correspondence to: Dr Gang Liu, Beijing Institute of Biotechnology, Liangxiang East Road, Fangshan, Beijing 100071, P.R. China
E-mail: liugang2028@126.com

*Contributed equally

Key words: p43, JAK-STAT, interferon-inducible protein 10, angiogenesis

inducible protein 10 (IP-10), also known as C-X-C motif chemokine 10 (CXCL10), is a member of the chemokine CXC family (23), and it was previously shown to inhibit angiogenesis and repress tumor growth through the JAK-STAT signaling pathway *in vitro* and *in vivo* (24-27). In the present study, we aimed to determine the mechanism through which p43 regulates both angiogenesis in HMEC-1 cells and IP-10 expression as well as the role of the JAK-STAT signaling pathway.

Materials and methods

Cell lines and reagents. The HMEC-1 cell line was purchased from Shanghai Sine Pharmaceutical Laboratories (Shanghai, China) and cultured in MCDB 131 medium (HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS) at 37˚C and at 5% CO_2 in a humidified incubator. AG490 was purchased from Sigma (St. Louis, MO, USA).

Screening for differentially expressed genes using microarray analysis. p43 protein was added to HMEC-1 cells at 80% confluence, and the final p43 concentration was 50 μg/ml (28). PBS-treated cells served as controls. Following 8 h of treatment, the cells were washed once with PBS, followed by the addition of 3 ml TRIzol reagent in order to the digest cells over a 15-min period. Cell lysates (1 ml) were transferred to a 1.5 ml RNase-free centrifuge tube. The collected samples were analyzed by CapitalBio Corporation (Beijing, China) using the Affymetrix Human Genome U133 Plus 2.0 Array. The differentially expressed genes in response to p43 treatment of HMEC-1 cells were analyzed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). When HMEC-1 cells were at approximately 80% confluence, p43 protein was added. PBS was used as a control. Following 8 h of treatment, cells were collected. Total RNAs were isolated using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). cDNA was obtained by reverse transcription using a Transcriptor First Strand cDNA Synthesis kit (Qiagen). The reaction was performed using the LightCycler 2.0 Real-Time PCR system (Roche, Mannheim, Germany). Roche FastStart Universal SYBR-Green Master (Rox) kits were used. The following PCR program was used: 95˚C for 2 min; 45 cycles of 95˚C for 2 sec, 57-60˚C for 3 sec, 72˚C for 11 sec, 80˚C for 3 sec and 65˚C for 30 sec. The reaction for each sample was performed in triplicate. GAPDH was used as a control gene. Gene expression was calculated using the following formula: $2^{-(\Delta Ctsample - \Delta Ctcontrol)}$. The sequences for the PCR primers are presented in Table I.

Western blot analysis. When HMEC-1 cells were at approximately 80% confluence, p43 (Shanghai Sine Pharmaceutical Laboratories) and/or the JAK inhibitor (AG490) at different concentrations were used to treat the cells for 8 h. The cells were then lysed in radioimmunoprecipation buffer (RIPA) supplemented with protease and phosphatase inhibitors. The cell lysates was centrifuged, and the resulting supernatant was loaded onto SDS-PAGE gels. Proteins were resolved on PAGE gels and transferred to PVDF membranes. Membranes were blocked at room temperature for 1 h. Primary anti-JAK1 (ab125051), anti-phospho-JAK1 (ab138005), anti-STAT1 (ab31369), anti-phospho-JAK2 (ab32101) and anti-IP-10 (ab9807) antibodies (AG490) were all 1:1,000 diluted and used to probe membranes for 2 h. The membranes were then washed three times with TBS/Tween-20 (TBST). Goat anti-mouse (ab6789) and goat anti-rabbit (ab6721) IgG H&L [horseradish peroxidase (HRP)]-conjugated secondary antibodies (1:4,000) were then used to probe the membranes (at room temperature for 1 h). β-actin antibody (ab8227) was used as the loading control. All antibodies were from Abcam, Cambridge, UK. Subsequently, the membranes were visualized using chemiluminescent ECL detection reagents (GE Healthcare, Pittsburgh, PA, USA).

Table I. Primer sequences.

Gene	Primer sequences
STAT1	5'-CCCTTCTGGCTTTGGATTGA-3' 5'-GAAGTCAGGTTCGCCTCCGT-3'
GAPDH	5'-TGAAGGTCGGAGTCAACGGAT-3' 5'-CTGGAAGATGGTGATGGGATT-3'
IP-10	5'-AGGAACCTCCAGTCTCAGCA-3' 5'-CAAAATTGGCTTGCAGGAAT-3'
SOCS1	5'-TTTTCGCCCTTAGCGTGAAGA-3' 5'-GAGGCAGTCGAAGCTCTCG-3'
SOCS3	5'-CAGCATCTCTGTCGGAAGACC-3' 5'-GCTGGGTGACTTTCTCATAGGAG-3'
TYK2	5'-TCGCTGTCATCCTCATTGCT-3' 5'-ACACTTGGCGGTTCTTTCG-3'
IRF9	5'-CATCTTCGACTTCAGAGTCTTCTTC-3' 5'-TCGGCACAGCCAGGGTT-3'
SMAD2	5'-AAGGGGTTCGCCGTTCTC-3' 5'-TGGCAGCGCAGTTCAGTG-3'
SMAD3	5'-CTGCGCTGCCAGTGCTT-3' 5'-GGTGCTCCCCTTGTTCAGTATCT-3'
IL-6	5'-ATGAGGAGACTTGCCTGGTGA-3' 5'-GGCATTTGTGGTTGGGTCA-3'
ISG15	5'-ATGGGCTGGGACCTGACG-3' 5'-ACCGCTCGGGTGGACAG-3'
STAT2	5'-CCCCAAACTCCCCATCG-3' 5'-CAAAGTCCCAAATCAAACCCT-3'

Cell migration assays. HMEC-1 cell migration assays were conducted as previously described (29). Briefly, Transwell cell culture chambers were pre-coated with 20 μl fibronectin (500 μg/ml). HMEC-1 cells in the logarithmic growth phase were digested using trypsin, which was followed by re-suspension with serum-free MCDB 131 medium to a final density of $5.5x10^5$ cells/ml. The cell suspension (180 μl) was added to the inner chambers and MCDB 131 medium supplemented with 10% FBS was added to the outer chambers (540 μl/well). Then, 20 μl p43 protein plus JAK inhibitors at different concentrations was added to the inner chambers, and 60 μl of the mixture was added to the outer chambers. PBS was used as a negative control. The system was incubated at 37˚C in 5% CO_2 in an incubator for

16-18 h. The xCELLigence Real Time Cell Analysis (RTCA) DP instrument (ACEA Biosciences, San Diego, CA, USA) was used to record real-time cell migration. When the reaction was complete, the media were removed. The cells were fixed in 90% ethanol and stained with 0.1% crystal violet (Sigma-Aldrich, Shanghai, China). Migration was observed and images were captured using a DP instrument (ACEA Biosciences). Surplus dye was washed off with PBS. Then, 10% acetic acid was applied for cell extraction for 10 min. The absorbance (OD value) was measured at 595 nm using a microplate reader (Thermo Scientific, Pittsburgh, PA, USA). The migration inhibition rate was calculated using the following formula: migration inhibition rate (%) = (OD value$_{\text{control group}}$ - ODvalue $_{\text{drug group}}$)/OD value$_{\text{control group}}$ x100.

Endothelial cell tubule formation assays. ECM Matrigel (Sigma) was placed on ice to melt and then 1:2 diluted with serum-free media. The Matrigel was then added to pre-cooled 96-well plates (60 μl/well) and allowed to solidify at 37˚C for 30 min. Trypsin digestion was applied to the cells, which were then resuspended in MCDB 131 medium supplemented with 10% FBS to attain a final concentration of 3x10^5 cells/ml. Then, 90 μl of cell suspension was added to each well. Moreover, the mixtures of p43 plus AG490 at different concentrations were added to the wells, and PBS was used as a negative control. The plates were cultured at 37˚C in an incubator with 5% CO_2 for 8 h. The fluorescent dye calcein AM (Thermo Scientific) was employed to stain the cells. Tubule formation was observed under a fluorescence microscope (Olympus, Snanghai, China) at x40 magnification. Five fields were randomly selected and images were captured.

Enzyme linked immunosorbent assay (ELISA). Cells in the logarithmic growth phase were treated with p43 protein at different concentrations (0, 10, 30, 50, 70 and 100 μg/ml) at 37˚C in an incubator with 5% CO_2 for 8 h. PBS was used as a negative control. ELISA assays were performed using ELISA kits (Mercodia AB, Uppsala, Sweden) to measure IL-6 and IP-10 protein expression.

Statistical analysis. Data were collected from three or more independent experiments. Statistical significance was assessed using the Student's unpaired t-test. For all analyses, SAS software version 8.0 was used. P<0.05 was considered to indicate a statistically significant difference.

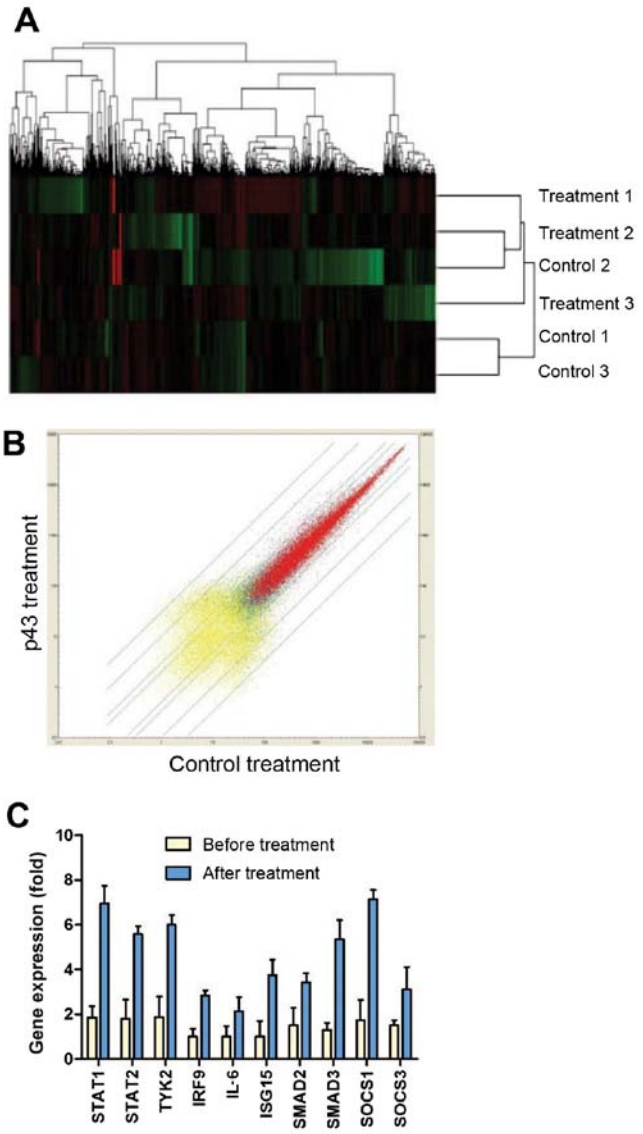

Figure 1. p43 regulates gene expression in human microvascular endothelial cells-1 (HMEC-1) cells. (A) Heatmap shows the differential expression of genes regulated by p43 treatment. Green or red colors on the heat map indicates a decrease or increase in RNA levels, respectively, and color intensities correspond to relative signal levels on a logarithmic scale. (B) Comparative analysis of treatments illustrated in a scatter diagram. Red, increased; yellow, decreased. (C) Validation of microarray results by RT-qPCR. Cells at 80% confluence were treated with 50 μg/ml p43 for 8 h. The mRNA expression of STAT1, STAT2 and other genes was determined using quantitative PCR and error bars represent the means ± SD.

Results

p43 regulates gene expression in HMEC-1 cells. HMEC-1 cells were treated with p43 protein (50 μg/ml) for 8 h. Gene expression was analyzed using the Affymetrix Human Genome U133 Plus 2.0 microarray. A total of 132 differentially expressed genes were identified (Fig. 1A and B). Among them, 123 genes were upregulated and 9 genes were downregulated. These genes were classified according to their functions listed in the Gene Ontology (GO) classification (Table II). Among the GO terms, the JAK-STAT signaling pathway was identified and provided a functional link between p43 and angiogenesis. Furthermore, we validated the identified genes in the JAK-STAT pathway by performing RT-qPCR. GAPDH was used as a reference control. The cells were treated with p43 (50 μg/ml) for 8 h, and total RNA was extracted for qPCR. The PCR results were consistent with the microarray data, showing that the identified genes in the JAK-STAT pathway (STAT1, STAT2, TYK2, IRF9, IL-6, ISG15, SMAD2, SMAD3, SOCS1 and SOCS3) were induced following p43 treatment (Fig. 1C).

p43 regulation of IP-10 expression. To validate the cellular supernatant from the treatment, we measured the protein level of IL-6, a secreted cytokine, by ELISA. The results showed that the IL-6 protein level was 1-fold higher in the drug-treatment group than in the control group (PBS) (Fig. 2A). IP-10 was previously shown to inhibit angiogenesis and repress tumor

Table II. Upregulated genes participating in the JAK-STAT pathway.

Gene	Gene ID	Change ratio
ISG15	NM_005101	3.75
STAT2	NM_005419	3.109
TYK2	NM_003331	3.194
SMAD2	NM_005901	2.28
SOCS1	NM_003745	4.086
IL-6	NM_000600	2.15
STAT1	NM_007315	3.73
IRF9	NM_006084	2.84
SMAD3	NM_005902	4.126
SOCS3	NM_003955	2.061

growth via the JAK-STAT signaling pathway *in vitro* and *in vivo* (20). We examined whether p43 affected IP-10 expression. The HMEC-1 cells were treated with p43 protein at different concentrations (0, 30, 50, 70 and 100 μg/ml) for 8 h. RT-qPCR was performed to evaluate the mRNA expression of IP-10, and ELISA and western blot analysis were applied to evaluate the protein expression of IP-10. Total RNA was extracted from the treated cells and then cDNA was generated by reverse transcription and used for RT-qPCR. The results suggest that IP-10 gene expression was apparently enhanced in a dose-dependent manner (Fig. 2B). RIPA lysis buffer containing protease and phosphatase inhibitors was added to the cells. Following centrifugation, the cell supernatant was collected and used for western blot analysis to determine IP-10 protein expression. Anti-IP-10 antibodies were used to detect endogenous IP-10. The results showed that IP-10 bands appeared at the appropriate molecular weight. IP-10 expression was augmented by p43 (Fig. 2C). IP-10 ELISA kits were used to further detect IP-10 protein expression. IP-10 was upregulated, and the highest expression was detected at a p43 concentration of 50 μg/ml (Fig. 2D).

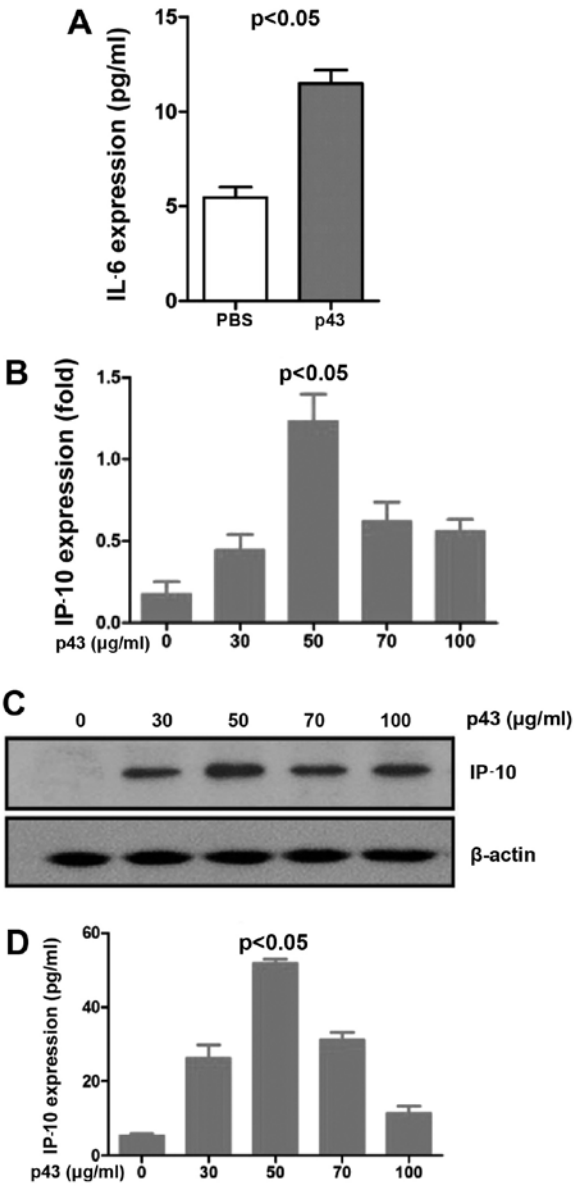

Figure 2. p43 regulates IP-10 expression. (A) Validation of cellular supernatant by detecting IL-6 protein level. IL6 protein expression was measured by ELISA. (B) p43 regulates the mRNA expression of IP-10. Human microvascular endothelial cells-1 (HMEC-1) cells were treated with p43 protein at different concentrations (0, 30, 50, 70 and 100 μg/ml) for 8 h. IP-10 mRNA expression was measured by quantitative PCR. (C) p43 regulates IP-10 protein expression. IP-10 protein expression was measured by western blot analysis. (D) p43 regulates IP-10 protein expression. IP-10 protein expression was measured by ELISA. P<0.05 vs. control.

Expression of key JAK-STAT pathway factors in p43-treated HMEC-1 cells. It was determined that p43 increased IP-10 expression. To determine whether p43 upregulates IP-10 expression through the JAK-STAT pathway, we measured the expression of key JAK-STAT pathway factors in p43-treated HMEC-1 cells.

STAT1 plays an important role in the JAK-STAT pathway. The HMEC-1 cells were treated with p43 at different concentrations (0, 30, 50, 70 and 100 μg/ml) for 8 h and then total RNA was extracted. cDNA was obtained by reverse transcription and used for qPCR in order to detect the mRNA expression of STAT1. The RT-qPCR results indicated that p43 augmented the mRNA expression of STAT1 in a dose-dependent manner (Fig. 3A).

The HMEC-1 cells were treated with p43 at different concentrations (0, 30, 50, 70 and 100 μg/ml) for 8 h. RIPA lysis buffer supplemented with protease and phosphatase inhibitors was added to the cells. Following centrifugation, the cell supernatant was collected and used for western blot analysis to determine the protein levels of JAK1, STAT1, phosphorylated (p-)JAK1 and p-JAK2. All proteins were detected at the appropriate molecular weights (Fig. 3B). Therefore, p43 may function through the JAK-STAT pathway, which supports our hypothesis.

AG490 inhibits the effects of p43 protein on the key factors in the JAK-STAT pathway. AG490 is a JAK inhibitor, which blocks the JAK-STAT pathway. We showed that p43 may function through the JAK-STAT pathway; therefore we hypothesized that AG490 inhibits the effects of p43. We added p43 protein (50 μg/ml) to 80% confluent HMEC-1 cells. The

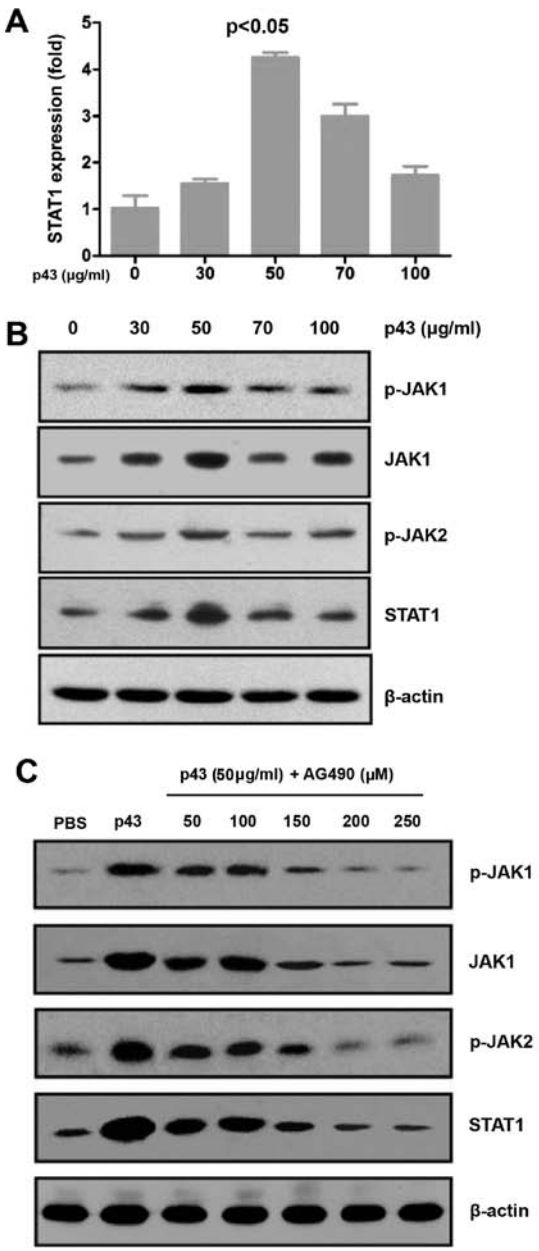

Figure 3. p43 mediates the JAK-STAT signaling pathway in human microvascular endothelial cells-1 (HMEC-1) cells. (A) p43 regulates the mRNA expression of STAT1. HMEC-1 cells were treated with p43 protein at different concentrations (0, 30, 50, 70 and 100 μg/ml) for 8 h. The mRNA expression of STAT1 was determined by quantitative PCR. (B) p43 regulates key regulators in the JAK-STAT signaling pathway. The protein expression of JAK1, STAT1, phosphorylated (p-)JAK1 and p-JAK2 were evaluated by western blot analysis. (C) AG490 inhibits the effects of p43 in HMEC-1 cells. HMEC-1 cells were treated with PBS (negative control), 50 μg/ml p43 (positive control), or p43 (50 μg/ml) + AG490 at 50, 100, 150, 200, 250 and 300 μM, for 8 h. The protein levels of JAK1, STAT1, p-JAK1 and p-JAK2 were measured by western blot analysis.

cells were also treated with AG490 at different concentrations (0, 50, 100, 150, 200 and 250 μM). PBS was used as a negative control. The cells were cultured at 37˚C in 5% CO_2 for 8 h. RIPA lysis buffer supplemented with protease and phosphatase inhibitors was added to the cells. Following centrifugation, the cell supernatant was collected and used for western blot analysis to determine the protein levels of JAK1, STAT1, p-JAK1 and p-JAK2. The results showed that the expression of the above-mentioned proteins was reduced in the presence of AG490; thus, suggesting AG490 inhibits the effect of p43 on these proteins (Fig. 3C).

AG490 suppresses the inhibitory effect of p43 on tubule formation and the migration of HMEC-1 cells. AG490 is an inhibitor of the JAK-STAT pathway, specifically suppressing JAK. To further confirm that p43 increases IP-10 through the JAK-STAT pathway, we performed a tubule formation assay and cell migration experiments.

HMEC-1 cells at an appropriate stage are capable of forming a complete lumen on Matrigel. The HMEC-1 cells were treated with PBS (negative control), p43 or p43+ AG490 for 8 h. The fluorescent dye calcein AM was used to stain the cells. Tubule formation was observed under a fluorescent microscope and images were captured (Fig. 4A). The results showed that p43 significantly inhibited tubule formation in the HMEC-1 cells. AG490 minimally affected tubule formation at concentrations of 25, 50, 75 and 100 μM; however, AG490 markedly induced cell death and decreased tubule formation at a concentration of 150 μM (data not shown). The cells treated with both p43 and AG490 formed more tubules than the cells treated with p43 alone. In addition, the number of tubules formed was increased at higher concentrations of AG490. A concentration of AG490 of 100 μM produced optimal effects, forming a similar amount of tubules compared to the negative control. The findings indicate that p43 functions through the JAK-STAT pathway at least in the range of the tested AG490 concentrations. The higher the AG490 concentration, the weaker the inhibitory effect of p43 on HMEC-1 cell tubule formation. This finding suggests that AG490 suppresses the inhibitory effect of p43 on tubule formation in HMEC-1 cells, and for this effect to occur, the optimal concentration of AG490 is 100 μM.

Cell migration was measured by performing Transwell migration assays (30). As the results of the tubule formation experiment suggested that the optimal concentration of AG490 was 100 μM, we used the same concentration for cell migration experiments. The HMEC-1 cells in the logarithmic growth phase were treated with PBS, AG490 (100 μM), p43 (50 μg/ml), or p43 (50 μg/ml) + AG490 (100 μM) at 37˚C in 5% CO_2 for 16-18 h. The xCELLigence RTCA DP instrument was used to monitor cell migration (Fig. 4B). Crystal violet was used for staining and acetic acid was used for extraction. The absorbance was measured at 595 nm using a microplate reader and the migration inhibition rate was calculated as described in the Materials & methods (Fig. 4C). We determined that AG490 (100 μM) alone had no impact on cell migration; however, p43 (50 μg/ml) significantly inhibited the migration of HMEC-1 cells. The inhibitory effect of p43 on the migration of HMEC-1 cells was reduced in the presence of AG490, suggesting that AG490 decreased the inhibitory effect of p43 on migration. Statistical analysis showed that the differences were significant (P<0.05).

Discussion

Angiogenesis is a complex and continuous process. It is the result of the proliferation, migration and remodeling of vascular endothelial cells. The inhibition of tumor angiogenesis has been a novel approach for the treatment of cancer.

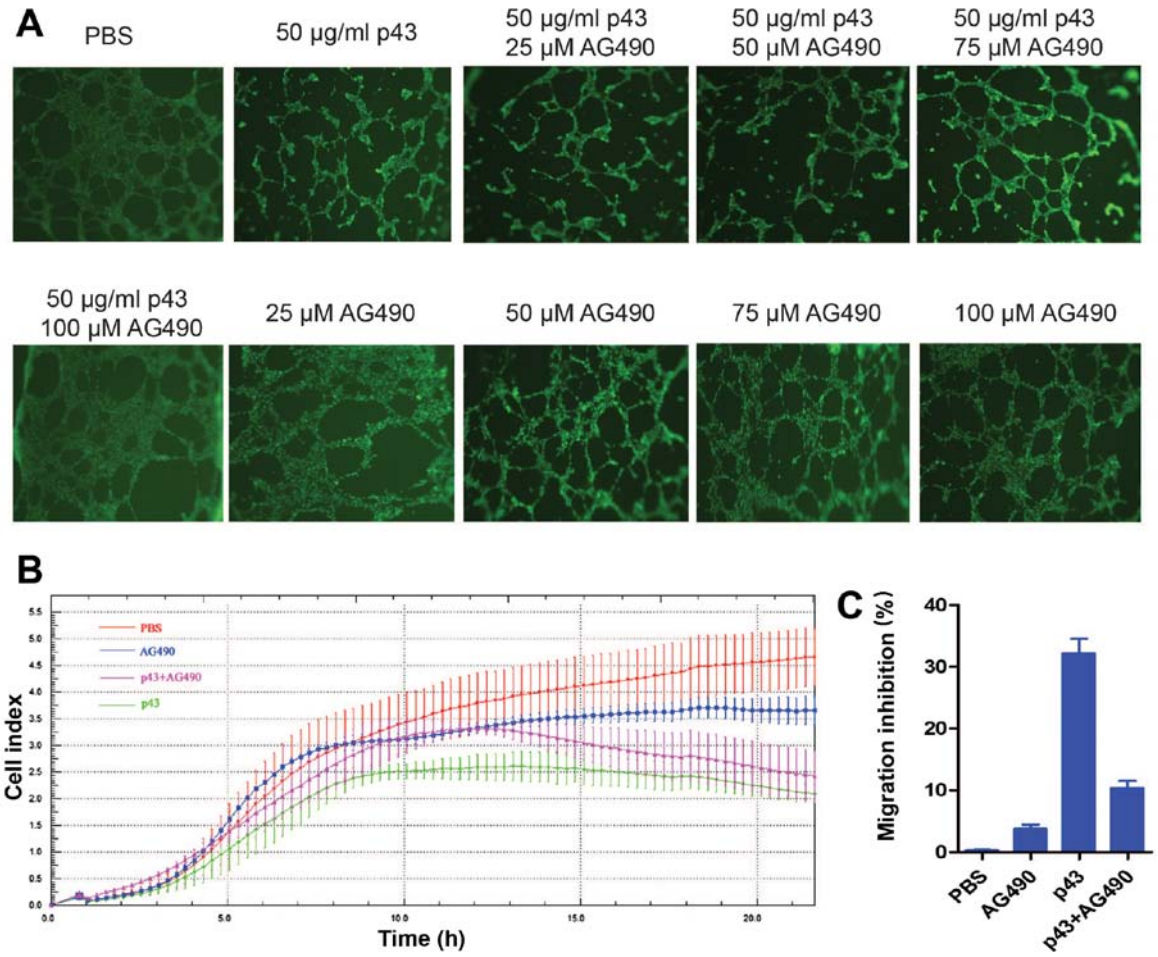

Figure 4. AG490 suppresses the inhibitory effect of p43 on tubule formation and the migration of HMEC-1 cells. (A) p43 inhibition affects tubule formation in human microvascular endothelial cells-1 (HMEC-1) cells. HMEC-1 cells were treated with PBS (negative control), 50 μg/ml p43, 50 μg/ml p43+25 μM AG490, 50 μg/ml p43+50 μM AG490, 50 μg/ml p43+75 μM AG490, 50 μg/ml p43+100 μM AG490, 25 μM AG490, 50 μM AG490, 75 μM AG490 and 100 μM AG490. Tubule formation was measured after 8 h of treatment. Five fields were randomly selected. Each treatment was performed in triplicate. Calcein AM was used to stain cells. The morphology was observed under a fluorescent microscope (x40 magnification). (B) p43 inhibits the migration of HMEC-1 cells. HMEC-1 cells were treated with PBS (negative control), 100 μM AG490, 50 μg/ml p43 (positive control), or p43 (50 μg/ml) + AG490 (100 μM) for 16-18 h. The migration of HMEC-1 cells was measured by real-time monitoring. The data are representative of the results from three independent experiments. Error bars indicate standard deviations; *P<0.05. (C) Inhibitory effects of p43 on the migration of HMEC-1 cells.

It is known that proEMAPII/p43 is a precursor of EMAPII, which is the active polypeptide in endothelial monocytes. p43 is a cofactor of mammalian ARS and it was first identified by Quevillon *et al* in 1997 (9). The p43 protein has multiple biological functions. Previous studies have shown that p43 is also an effective angiogenesis inhibitor (30) exhibiting strong antitumor activity *in vitro* and *in vivo* (31). Therefore, the p43 protein is a potential drug target for the treatment of solid tumors, such as lung, gastric, prostate and breast cancers as well as nasopharyngeal carcinoma.

Some studies have revealed the structure and function of the p43 protein; however, the detailed roles of p43 and the related mechanisms remain undefined. Furthermore, the p43 receptors remain unknown. The mechanism whereby p43 protein inhibits angiogenesis also remains unknown. Thus, we treated HMEC-1 cells with 50 μg/ml p43 protein for 8 h. The Affymetrix Human Genome microarray was applied to analyze differential gene expression. The data showed that p43 regulated many cytokines. The majority of the factors upregulated by p43 are associated with the JAK-STAT pathway. IP-10, also known as CXCL10, is a member of the chemokine CXC family (24). It is activated by the JAK-STAT pathway.

IP-10 significantly inhibits angiogenesis *in vitro*, which is consistent with the role of p43. Therefore, IP-10 was selected as a candidate gene. We performed experiments to reveal the associations among p43, IP-10 and the JAK-STAT pathway. Firstly, quantitative PCR was used to detect the mRNA expression of IP-10 in p43-treated HMEC-1 cells, and ELISA as well as western blot analysis were applied to determine the protein expression of IP-10. The results indicated that p43 increased IP-10 expression in a dose-dependent manner. At a p43 protein concentration of 50 μg/ml, the expression of IP-10 was the highest.

IP-10 functions through the JAK-STAT pathway; therefore, we examined this pathway to find possible mechanisms responsible for the augmentation of IP-10 levels. The JAK-STAT pathway (32) consists of two main families: the JAK family of tyrosine kinases and the STAT family. The STAT family is comprised of transcription factors, which play critical roles in the JAK-STAT pathway. The binding

of cytokines to their specific receptors on the cell surface activates JAK proteins, which are phosphorylated and form dimers. Activated JAKs subsequently activate STATs through particular domains. Functional STATs are transferred into the nucleus to regulate target gene expression. Recent studies have shown that STAT proteins are important regulators of the signaling pathway controlling tumor development (33-36). Various products of cancer genes may continuously activate specific STAT proteins, which play crucial roles in the occurrence of tumors. Therefore, we examined STAT1 expression using quantitative PCR. Western blot analysis was adopted to determine the protein levels of JAK1, STAT1, p-JAK1 and p-JAK2. The results showed that p43 protein upregulated the expression of the above-mentioned genes. They suggest that p43 may increase IP-10 expression through the JAK-STAT pathway; this finding requires confirmation by further studies.

To confirm our hypothesis, we treated cells with p43 as well as an inhibitor of the JAK-STAT pathway and the effect of the inhibitor on the JAK-STAT pathway was detected. AG490 and WP1022 are two common inhibitors of the JAK-STAT pathway, specifically repressing JAK. HMEC-1 cells were treated with p43 (50 μg/ml) + AG490 for 8 h. The inhibitory effect of AG490 on the p43-treated cells was examined using several approaches. Western blot analysis showed that AG490 prevented the phosphorylation of JAK1 and JAK2 by p43. We then performed *in vitro* tubule formation and cell migration experiments in HMEC-1 cells. The tubule formation mediated by p43 was inhibited by AG490. The cells treated with both AG490 and p43 formed more tubules than cells treated with p43 alone. Moreover, the migration inhibition rate was reduced in the presence of the inhibitor. These findings indicate that AG490 inhibits the phosphorylation of JAK1 and JAK2 by p43 and blocks the JAK-STAT pathway. The results suggest that p43 inhibits angiogenesis by increasing IP-10 expression through the JAK-STAT pathway.

Our findings revealed that the p43 protein inhibits angiogenesis mainly through the JAK-STAT pathway. However, whether the JAK-STAT pathway is the only pathway participating in the p43-mediated inhibition of angiogenesis remains unknown. In addition, the p43 receptors remain unclear. These issues warrant further investigation.

References

1. Bdolah Y, Sukhatme VP and Karumanchi SA: Angiogenic imbalance in the pathophysiology of preeclampsia: newer insights. Semin Nephrol 24: 548-556, 2004.
2. Bussolino F, Mantovani A and Persico G: Molecular mechanisms of blood vessel formation. Trends Biochem Sci 22: 251-256, 1997.
3. Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100: 57-70, 2000.
4. Bouck N, Stellmach V and Hsu SC: How tumors become angiogenic. Adv Cancer Res 69: 135-174, 1996.
5. Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. Nat Med 6: 389-395, 2000.
6. Goldmann E: The growth of malignant disease in man and the lower animals, with special reference to the vascular system. Proc R Soc Med 1 (Surg Sect): 1-13, 1908.
7. Algire GH and Legallais FY: Vascular reactions of normal and malignant tissues in vivo. IV. The effect of peripheral hypotension on transplanted tumors. J Natl Cancer Inst 12: 399-421, 1951.
8. Folkman J: Tumor angiogenesis: therapeutic implications. N Engl J Med 285: 1182-1186, 1971.
9. Quevillon S, Agou F, Robinson JC and Mirande M: The p43 component of the mammalian multi-synthetase complex is likely to be the precursor of the endothelial monocyte-activating polypeptide II cytokine. J Biol Chem 272: 32573-32579, 1997.
10. Renault L, Kerjan P, Pasqualato S, Ménétrey J, Robinson JC, Kawaguchi S, Vassylyev DG, Yokoyama S, Mirande M and Cherfils J: Structure of the EMAPII domain of human aminoacyl-tRNA synthetase complex reveals evolutionary dimer mimicry. EMBO J 20: 570-578, 2001.
11. Otani A, Slike BM, Dorrell MI, Hood J, Kinder K, Ewalt KL, Cheresh D, Schimmel P and Friedlander M: A fragment of human TrpRS as a potent antagonist of ocular angiogenesis. Proc Natl Acad Sci USA 99: 178-183, 2002.
12. Kao J, Fan YG, Haehnel I, Brett J, Greenberg S, Clauss M, Kayton M, Houck K, Kisiel W, Seljelid R, *et al*: A peptide derived from the amino terminus of endothelial-monocyte-activating polypeptide II modulates mononuclear and polymorphonuclear leukocyte functions, defines an apparently novel cellular interaction site, and induces an acute inflammatory response. J Biol Chem 269: 9774-9782, 1994.
13. Park H, Park SG, Lee JW, Kim T, Kim G, Ko YG and Kim S: Monocyte cell adhesion induced by a human aminoacyl-tRNA synthetase-associated factor, p43: identification of the related adhesion molecules and signal pathways. J Leukoc Biol 71: 223-230, 2002.
14. Norcum MT and Warrington JA: The cytokine portion of p43 occupies a central position within the eukaryotic multisynthetase complex. J Biol Chem 275: 17921-17924, 2000.
15. Behrensdorf HA, van de Craen M, Knies UE, Vandenabeele P and Clauss M: The endothelial monocyte-activating polypeptide II (EMAP II) is a substrate for caspase-7. FEBS Lett 466: 143-147, 2000.
16. Shalak V, Kaminska M, Mitnacht-Kraus R, Vandenabeele P, Clauss M and Mirande M: The EMAPII cytokine is released from the mammalian multisynthetase complex after cleavage of its p43/proEMAPII component. J Biol Chem 276: 23769-23776, 2001.
17. Wakasugi K and Schimmel P: Highly differentiated motifs responsible for two cytokine activities of a split human tRNA synthetase. J Biol Chem 274: 23155-23159, 1999.
18. Rosen HR, Moroz C, Reiner A, Stierer M, Svec J, Reinerova M, Schemper M and Jakesz R: Expression of p43 in breast cancer tissue, correlation with prognostic parameters. Cancer Lett 67: 35-45, 1992.
19. Park H, Park SG, Kim J, Ko YG and Kim S: Signaling pathways for TNF production induced by human aminoacyl-tRNA synthetase-associating factor, p43. Cytokine 20: 148-153, 2002.
20. Park SG, Kang YS, Ahn YH, Lee SH, Kim KR, Kim KW, Koh GY, Ko YG and Kim S: Dose-dependent biphasic activity of tRNA synthetase-associating factor, p43, in angiogenesis. J Biol Chem 277: 45243-45248, 2002.
21. Ko YG, Park H, Kim T, Lee JW, Park SG, Seol W, Kim JE, Lee WH, Kim SH, Park JE and Kim S: A cofactor of tRNA synthetase, p43, is secreted to up-regulate proinflammatory genes. J Biol Chem 276: 23028-23033, 2001.
22. Chang SY, Park SG, Kim S and Kang CY: Interaction of the C-terminal domain of p43 and the alpha subunit of ATP synthase. Its functional implication in endothelial cell proliferation. J Biol Chem 277: 8388-8394, 2002.
23. Luster AD, Unkeless JC and Ravetch JV: Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature 315: 672-676, 1985.
24. Neville LF, Mathiak G and Bagasra O: The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. Cytokine Growth Factor Rev 8: 207-219, 1997.
25. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD and Strieter RM: CXC chemokines in angiogenesis. J Leukoc Biol 68: 1-8, 2000.
26. Strieter RM, Burdick MD, Gomperts BN, Belperio JA and Keane MP: CXC chemokines in angiogenesis. Cytokine Growth Factor Rev 16: 593-609, 2005.
27. Rosenkilde MM and Schwartz TW: The chemokine system - a major regulator of angiogenesis in health and disease. APMIS 112: 481-495, 2004.
28. Xing YH, Liu DT, Tan JJ, Hu LD, Liu G, Fu XQ and Chen HP: Construction and screening of truncated mutants of recombinant human anti-angiogenic protein proEMAP/p43. Prog Biochem Biophys 41: 567-574, 2014.

29. Staton CA, Stribbling SM, Tazzyman S, Hughes R, Brown NJ and Lewis CE: Current methods for assaying angiogenesis in vitro and in vivo. Int J Exp Pathol 85: 233-248, 2004.
30. Park SG, Jung KH, Lee JS, Jo YJ, Motegi H, Kim S and Shiba K: Precursor of pro-apoptotic cytokine modulates aminoacylation activity of tRNA synthetase. J Biol Chem 274: 16673-16676, 1999.
31. Lee YS, Han JM, Kang T, Park YI, Kim HM and Kim S: Antitumor activity of the novel human cytokine AIMP1 in an in vivo tumor model. Mol Cells 21: 213-217, 2006.
32. Wilks AF and Oates AC: The JAK/STAT pathway. Cancer Surv 27: 139-163, 1996.
33. Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, Harris CC and Herman JG: SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet 28: 29-35, 2001.
34. Lin TS, Mahajan S and Frank DA: STAT signaling in the pathogenesis and treatment of leukemias. Oncogene 19: 2496-2504, 2000.
35. Yu CL and Burakoff SJ: Involvement of proteasomes in regulating Jak-STAT pathways upon interleukin-2 stimulation. J Biol Chem 272: 14017-14020, 1997.
36. Guschin D, Rogers N, Briscoe J, Witthuhn B, Watling D, Horn F, Pellegrini S, Yasukawa K, Heinrich P, Stark GR, *et al*: A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. EMBO J 14: 1421-1429, 1995.