

Effects of the knockdown of hypoxia inducible factor-1 α expression by adenovirus-mediated shRNA on angiogenesis and tumor growth in hepatocellular carcinoma cell lines

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Background/Aims: Hypoxia-inducible factor-1 α (HIF-1 α) is a central transcriptional factor involved in the cellular responses related to various aspects of cancer biology, including proliferation, survival, and angiogenesis, and the metabolism of the extracellular matrix in hypoxia. This study evaluated whether adenovirus-mediated small hairpin RNA (shRNA) against HIF-1 α (shHIF-1 α) inhibits cell proliferation and angiogenesis in hepatocellular carcinoma (HCC) cell lines. **Methods:** Knockdown of HIF-1 α expression was constructed by adenovirus-mediated RNA interference tools, and HCC cell lines infected with shHIF-1 α coding virus were cultured under a hypoxia condition (1% O₂) for 24 hours. Following infection, the expression levels of HIF-1 α , angiogenesis factors, and matrix metalloproteinase (MMP) were examined using Western blotting. Cell proliferation and angiogenesis were measured by a cell proliferation assay (MTT assay) and an angiogenesis-related assay (invasion and tube-formation assay), respectively. **Results:** Adenovirus mediated inhibition of HIF-1 α induced suppression of tumor growth in HCC cell lines. It also down-regulated the expression of angiogenesis factor and MMP proteins. Angiogenesis as well as mobility of vascular cells to tumor was suppressed by adenovirus-mediated shHIF-1 α -infected groups in human umbilical vein endothelial cells (HUVECs). **Conclusions:** These data suggest that adenovirus-mediated inhibition of HIF-1 α inhibits the invasion, tube formation, and cell growth in HUVECs and HCC cells. ([Korean J Hepatol 2010;16:280-287](#))

Keywords: Angiogenesis; Hypoxia-inducible factor-1 α (HIF-1 α); Small hairpin RNA (shRNA); Human umbilical vein endothelial cells (HUVECs); Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the 5th most common malignant tumor and approximately 500,000 new cases are reported every year. Furthermore, the mortality rate of primary hepatic malignancies ranks 3rd place on the tumor related mortality rate scale.¹ It is one of the most well-known hypervascular tumors in which lots of blood vessels are formed as the tumor grows.^{2,3}

Angiogenesis is a major *in vivo* phenomenon which generates capillaries from existing blood vessels.^{4,5} Also, this phenomenon can appear when a wound occurs in a particular organ or tissue. As well, angiogenesis can also play an important role in growth and metastasis of malignant tumors.^{4,6} It is known that the generation of blood vessels is regulated by various growth factors.⁵⁻⁷

A cell overcomes the hypoxic state by activating the transcription of genes related to glycogen degradation, blood cell generation, blood vessels formation, infiltration, etc.⁸⁻¹⁰ The

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Abbreviations: Ad-, Adenovirus mediated; DMEM, Dulbecco Modified Eagle's medium; DMSO, Dimethylsulfoxide; ECL, Enhanced chemoluminescence; HCC, Hepatocellular carcinoma; HIF-1 α , Hypoxia inducible factor-1 α ; HRE, Hypoxia responsive element; HUVEC, Human umbilical vein endothelial cell; miRNA, Micro RNA; MMP, Matrix metalloproteinase; MOI, Multiplicity of infection; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PAGE, Polyacrylamide Gel Electrophoresis; RNAi, RNA interference; SDS, Sodium Dodecyl Sulfate; shRNA, Small hairpin RNA; VEGF, Vascular endothelial growth factor; VHL, von Hippel-Lindau

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hypoxia inducible factor-1 α (HIF-1 α) is known as an important transcription factor in this state. HIF-1 α is activated when it is bound to HIF-1 β (ARNT), and the activated factor further activates target genes that are related to angiogenic effect, tumor growth, apoptosis, etc, by activating the hypoxia responsive element (HRE) sequence.¹¹⁻¹³ The HIF-1 α maintains a low concentration under normal oxygen levels due to the von hippel-lindau (vhl)-ubiquitin dependent protein degradation process. In the hypoxic level, the vhl-ubiquitin dependent protein degradation process is hindered so target genes are activated due to the increased concentration of HIF-1 α and increased binding of HIF-1 α to HRE.^{14,15} During the growth process of cancer cells, localized hypoxia is caused due to the excessive division of cancer cells and HIF-1 α stimulates angiogenesis and plays a role in the metastasis of cancer cells.^{4,14}

In this study, an adenovirus mediated small hairpin HIF-1 α (shHIF-1 α) which efficiently inhibits HIF-1 α expression was constructed, and also the HIF-1 α inhibition effect on the proliferation of HCC cell lines and angiogenesis was studied by inducing infection in HCC cell lines (HepG2, Huh7).

MATERIALS AND METHODS

1. Construction of an adenovirus mediated shHIF-1 α for the HIF-1 α gene

The 19 nucleic acid sequence of small hairpin RNA (shRNA) which targets the HIF-1 α gene sequence was constructed by the method reported in previous studies.^{14,15,20} Also, the shRNA inserted in the vector of adenovirus was constructed by inserting the loop sequence which forms a small hairpin structure in the

middle of the vector.^{16,17} The E1 site, which is responsible for the proliferation of adenovirus, was removed by BamHI and Hind III (Biolabs, Ipswich, MA, USA) and the shRNA bound to pSilencer-2.1-hygro-U6 vector was inserted into the pSP72-E3 Ad vector. Essentially, a replication-incompetent adenovirus mediated shHIF-1 α with a U6 promoter and specific shHIF-1 α sequence was created. Before infecting the cells with the constructed virus, quantification with 1×10^{12} vp/ml through DNA titering. Through the above procedures, replication-incompetent adenovirus mediated shHIF-1 α was produced by inserting the shRNA (Fig. 1).

2. Infection of adenovirus mediated shHIF-1 α

After cultivating certain numbers of HCC cell lines (HepG2, Huh7) in the culture medium, the medium was changed to a 5% serum medium (Dulbecco Modified Eagle's Medium, GIBCO, Invitrogen, CA, USA) before infecting it with the virus. The cell lines were then infected with the virus at desired MOI (multiplicity of infection, the number of infected virus per one cell) by simply adding in the media. It was cultivated for a 24 hour period in a normoxia condition (normoxia condition-21% O₂), and after changing the 5% serum DMEM (non-antibiotics) medium it was cultured for a 24 hour period in a hypoxia condition (hypoxia condition-1% O₂, 5% CO₂, 94% N₂). The change of status was observed by optical microscope.

3. Immunoblot assay

The Western blot was performed in order to determine the effect of HIF-1 α expression inhibition on the protein expression related to proliferation of HCC cell lines and angiogenesis. The

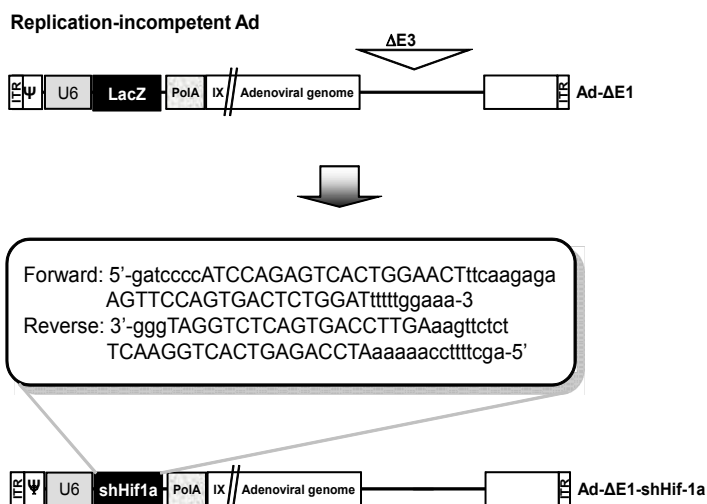


Figure 1. Construction of adenovirus mediated small hairpin hypoxia inducible factor-1 α (HIF-1 α). Schematic representation of the genomic structure of the adenoviral vectors (Ad) used. Ad-shHIF-1 α , adenoviral-mediated small hairpin HIF-1 α ; Ad- Δ E1, adenoviral-mediated deletion of the E1 site.

adenoviral-mediated shHIF-1 α (Ad-shHIF-1 α) infected HCC cell lines were incubated in a hypoxia condition during a 24-hour period. Protein was extracted after cell lysis [NP-40 Lysis buffer: 5M NaCl-30 mL, 10% NP-40-100 mL, 50 mL 1M Tris (pH8.0) per liter]. All cell lysis took place on ice for an hour using the lysis buffer. Also, the proteins were separated according to their molecular weight by SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis). The separated proteins on the gel were transferred to the PVDF membrane (GE healthcare, Amersham, Buckinghamshire, UK), and then bound with mouse monoclonal antibodies to HIF-1 α (1:500 dilution, BD bioscience, CA, USA), vascular endothelial growth factor (VEGF, 1:500 dilution, Millipore, CA, USA), matrix metalloproteinase-2 (MMP-2, 1:500 dilution, Invitrogen, CA, USA), and β -actin (1:3,000 dilution, Santacruz, CA, USA). A secondary goat anti mouse antibody tagged Horseradish peroxidase (1:5,000 dilution, Santacruz, CA, USA) was bound to the primary antibody and developed by enhanced chemo-illumination (Perkinelimer, Boston, MA, USA). The quantification of each protein expression was comparatively analyzed.

4. Cell growth assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT: Amresco, OH, USA) was treated to cells and the growth rate of the cell was measured by observing the increased rate of cell number. After seeding in a 96 well plate with 5×10^3 cell/well it was incubated at 37°C during a 24-hour period. It was infected with adenovirus (Ad-shHIF-1 α , Ad- Δ E1) after transferring it to a serum free media. After cultivation in a normoxia condition (normoxia condition-21% O₂) for a 24-hour period, it was transferred to a Serum 10% medium and a hypoxia induction (hypoxia induction-1% O₂) was given. The stimulated well was cell dyed with MTT and cultivated in 37°C for a 3~4 hour period for efficient cell dyeing. After removing the MTT mixed media, it was treated with DMSO (Dimethylsulfoxide, Sigma-Aldrich, MO, USA) 150 μ L for 5 minutes at room temperature and the proliferation was measured by spectrophotometry (Molecular Devices) 595 nm.

5. Production of condition media

After cultivating the adenovirus infected HCC cell lines group and adenovirus uninfected HCC cell lines group in a hypoxia condition during a 24-hour period, 500 μ L culture media was injected in the amicon (Millipore, Billerica, MA, USA), and it

was repeatedly concentrated at 4°C, 10,000 rpm during 30 minutes. It was washed by centrifugation with sterile distilled water until the amicon membrane was transparent. After injecting 3rd sterile distilled water 100 μ L, the plate of the amicon membrane was conversed and the 100 μ L concentrated culture media was collected after centrifugation. The collected culture media was used in various experiments such as invasion, tube formation, migration assay, etc.

6. Angiogenesis assay

The migration assay which detects the mobility of the cell, invasion assay which confirms the cell invasion capacity, and the tube formation assay which measures the tube formation of vascular cells were done by using the HUVEC (human umbilical vein endothelial cell).^{2,3,17} Each experimental result was observed by optical microscope ($\times 40$).

7. Statistical analysis

Each experiment was expressed in average \pm standard deviation, and frequency (%). Statistical analysis was done by independent-*t*-test SPSS 12.0 (SPSS Inc, Chicago, USA). Statistically significant level between the control group and experiment group was set at *P* value<0.05.

RESULTS

1. Construction of adenovirus mediated shHIF-1 α and inhibition of tumor growth

An *in vivo* replication-incompetent Ad-shHIF-1 α which efficiently inhibits the HIF-1 α expression was constructed. The quantitative value for the *in vivo* infection was calculated by the MOI and the growth inhibition effect was confirmed through MTT assay by the treatment with increasing MOI of the virus. After 72 hours of treatment, the increased growth inhibitions of HCC cell lines were observed as the MOI increases (Fig. 2A). In the HepG2 cell line, a 40.3 \pm 3.4% (0.79 \pm 0.05 *p*-value:0.05) proliferation inhibition effect was seen in 100 MOI and 45.0 \pm 7.6% was seen in 200 MOI. In the Huh7 cell line, 44.7 \pm 14.4% (0.87 \pm 0.35, *p*-value:0.05) proliferation inhibition effect was seen in 100 MOI and 41.6 \pm 0.7% (0.62 \pm 0.30, *p*-value: 0.05) was seen in 200 MOI. But at infections higher than 200 MOI, cytotoxicity was confirmed through the viability assay in both cell lines (data not shown). Also the time-dependency of the growth inhibition rate of HCC cell lines was confirmed (Fig. 2B).

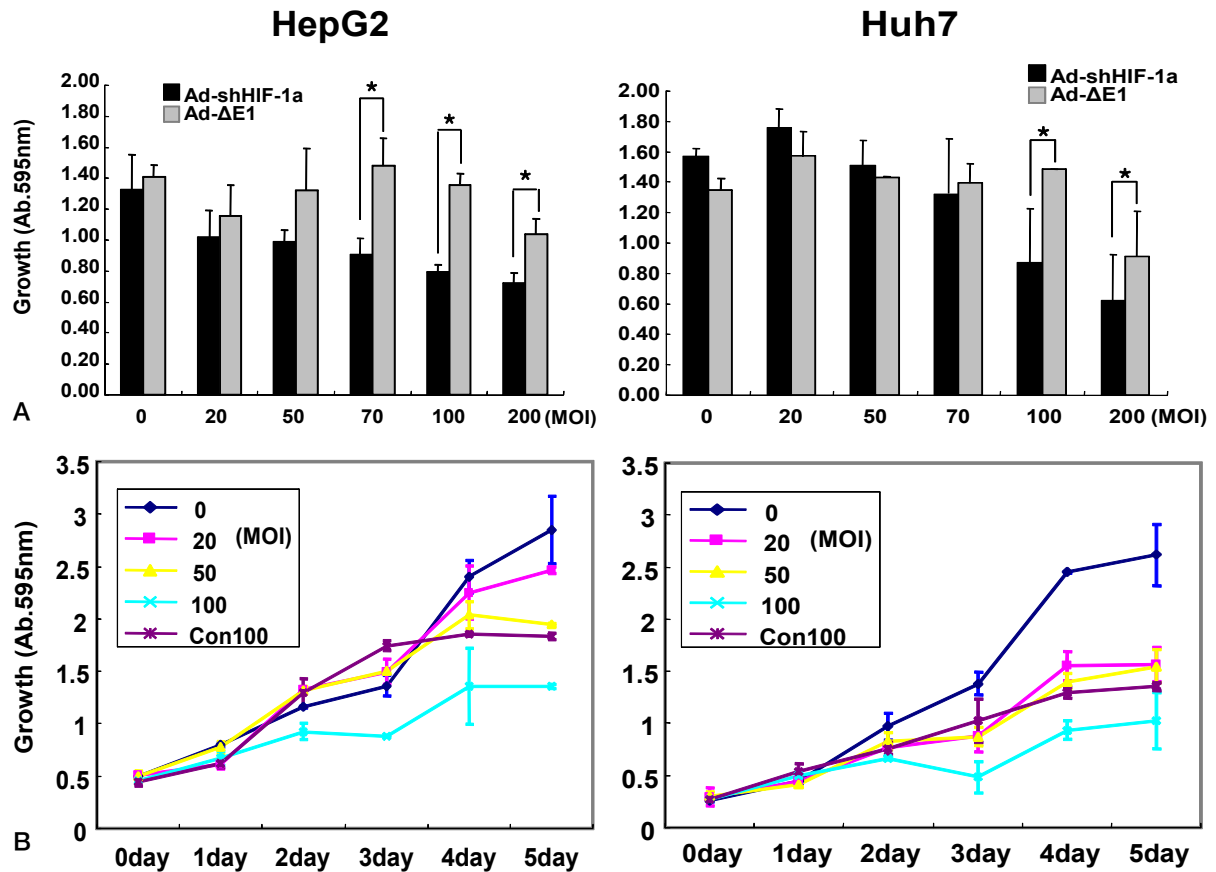


Figure 2. Inhibition of tumor cell growth by shHIF-1 α . HIF-1 α -knockdown HCC cell lines were incubated under a hypoxia condition (0.1% O₂). (A) After 72 hours, tumor cell growth (HepG2 and Huh7 cells) was inhibited by MOI-dependent Ad-shHIF-1 α . (B) The daily growth was greater in the non-infected group than in the adenovirus-mediated HIF-1 α -knockdown group (* P <0.05 versus Ad- Δ E1 treatment).

shHIF-1 α , small hairpin hypoxia inducible factor-1 α ; HCC, hepatocellular carcinoma; MOI, multiplicity of infection; Ad-shHIF-1 α , adenoviral-mediated small hairpin HIF-1 α ; Ad- Δ E1, adenoviral-mediated deletion of the E1 site.

After Ad-shHIF-1 α infection, the growth rates of the two infected HCC cell lines were inhibited compared to the control groups. From the experimental data, it was ascertained that adenoviral vector expressing shHIF-1 α transduced in hypoxic conditions induced an inhibition effect of the cell proliferation.

2. Knockdown of HIF-1 α affects expression of variable proteins

The amounts of expressed proteins in uninfected Huh7 and Huh7 infected with Ad-shHIF-1 α and Ad- Δ E1 independently were measured. The MOI dependent knockdown of HIF-1 α by shHIF-1 α was confirmed by Western blot (Fig. 3A). According to the comparative analysis of the density of HIF-1 α expression band, the expression decreased up to 50% at 100 MOI. The expression rate of VEGF and MMP-2 were measured in order to observe the effect of the inhibition of HIF-1 α on factors involved

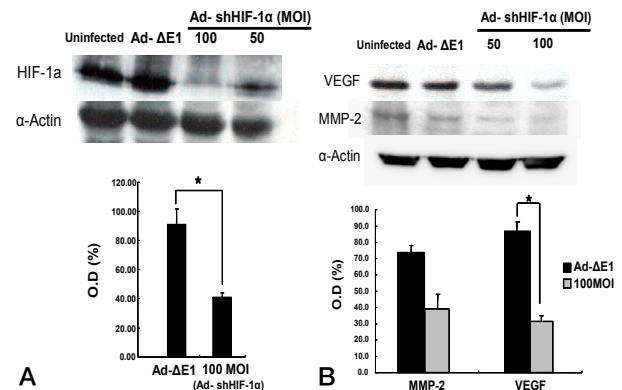


Figure 3. Expression level of angiogenic factor by suppressing HIF-1 α in Huh7 cells. (A) The expression of HIF-1 α decreased in an MOI-dependent manner under the hypoxia condition. (B) The hypoxic induction of angiogenesis factors and MMP was partially blocked in the HIF-1 α -blocked HCC cell line (* P <0.05 versus Ad- Δ E1 treatment).

HIF-1 α , hypoxia inducible factor-1 α ; MOI, multiplicity of infection; MMP, matrix metalloproteinase.

in angiogenesis. According to the observation, the VEGF protein expression rate decreased approximately 68% compared to the Ad-ΔE1 control group, and the pre-transition related MMP-2 protein expression rate decreased approximately 57% (Fig. 3B). According to the experiment results, it was confirmed that the expressions of VEGF and MMP-2 were down-regulated by the knockdown of HIF-1α in a hypoxia condition.

3. Inhibition of migration by adenovirus-mediated shHIF-1α

After a 24-hour period, migration of HUVEC in the overall well occurred in the non-treated control group and Ad-ΔE1 virus treated group after the treatment with condition media. However, a condition media from the sample treated with Ad-shHIF-1α at 50 MOI induced a decrease of migration by 50% compared to the initial migration as seen by a decrease of approximately 50% of the bar representing 0h compared the 24h bar, and at the 100 MOI almost no migration of HUVEC was observed (Fig. 4). The

data strongly suggest that the inhibition of HIF-1α expression further inhibits the mobility of tumor vascular cells from the blood vessel to the tumor.

4. Inhibition of angiogenesis by adenovirus mediated shHIF-1α

Based on the Western blot result where the expression of VEGF and MMP-2 decreased, the cell infiltration induced by the growth factors expressed in the HCC cells and the tube formation was measured using the special media (condition media) obtained from the HCC cell lines. Using the hematoxylin-eosin dye, it was confirmed that in the untreated group and the Ad-ΔE1 virus infected group, most of the vascular cells distributed in the gelatin region through the membrane. In the group treated with condition media of 50 MOI, cell infiltration was partially inhibited, and at 100 MOI approximately 60% of cell infiltration inhibition was observed (Fig. 5A). In the untreated group and the Ad-ΔE1 virus infected group, uniform tube formation of the vascular cell occurred and the tube was thick and clear. But in the

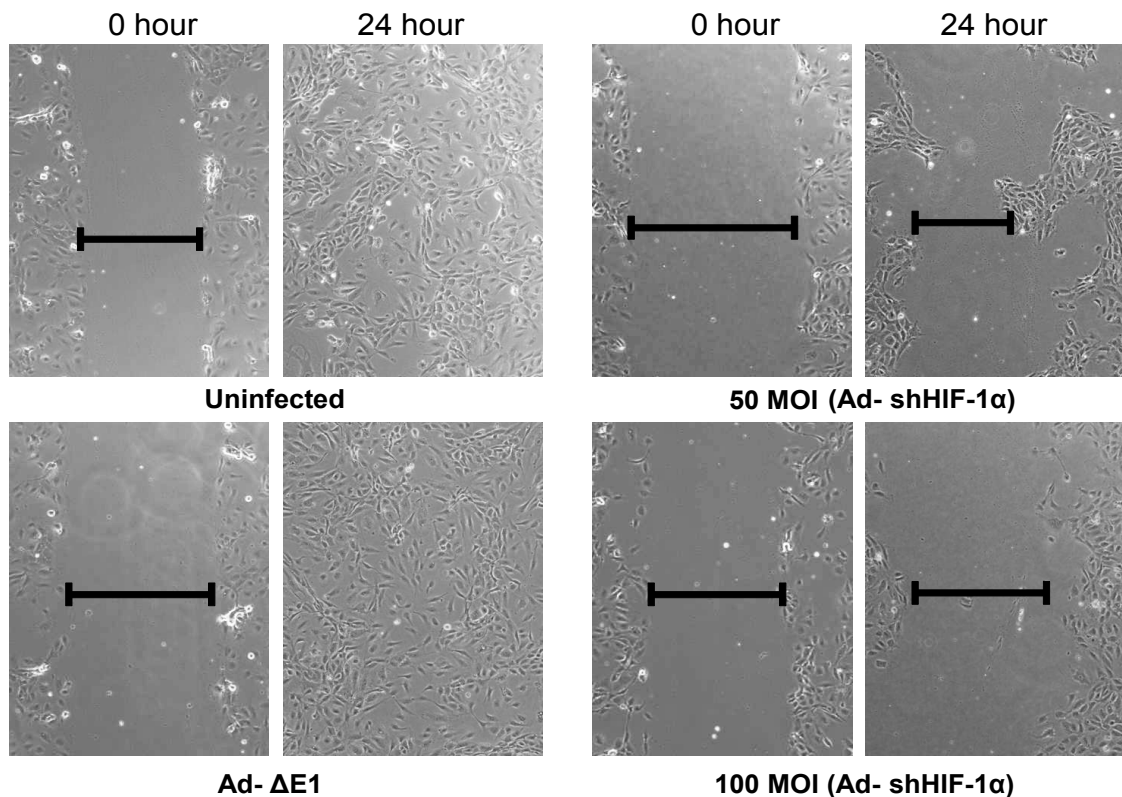


Figure 4. Effects of adenovirus-mediated shHIF-1α expression on migration. The conditioned medium collected from Ad-ΔE1-transduced Huh7 cells induced an increase in motility. However, the conditioned medium from Ad-shHIF-1α-transduced cells inhibited migration on HUVECs (bar length indicates the extension of HUVEC motility). shHIF-1α, small hairpin hypoxia inducible factor-1α; Ad-ΔE1, adenoviral-mediated deletion of the E1 site; Ad-shHIF-1α, adenoviral-mediated small hairpin HIF-1α; HUVEC, human umbilical vein endothelial cells.

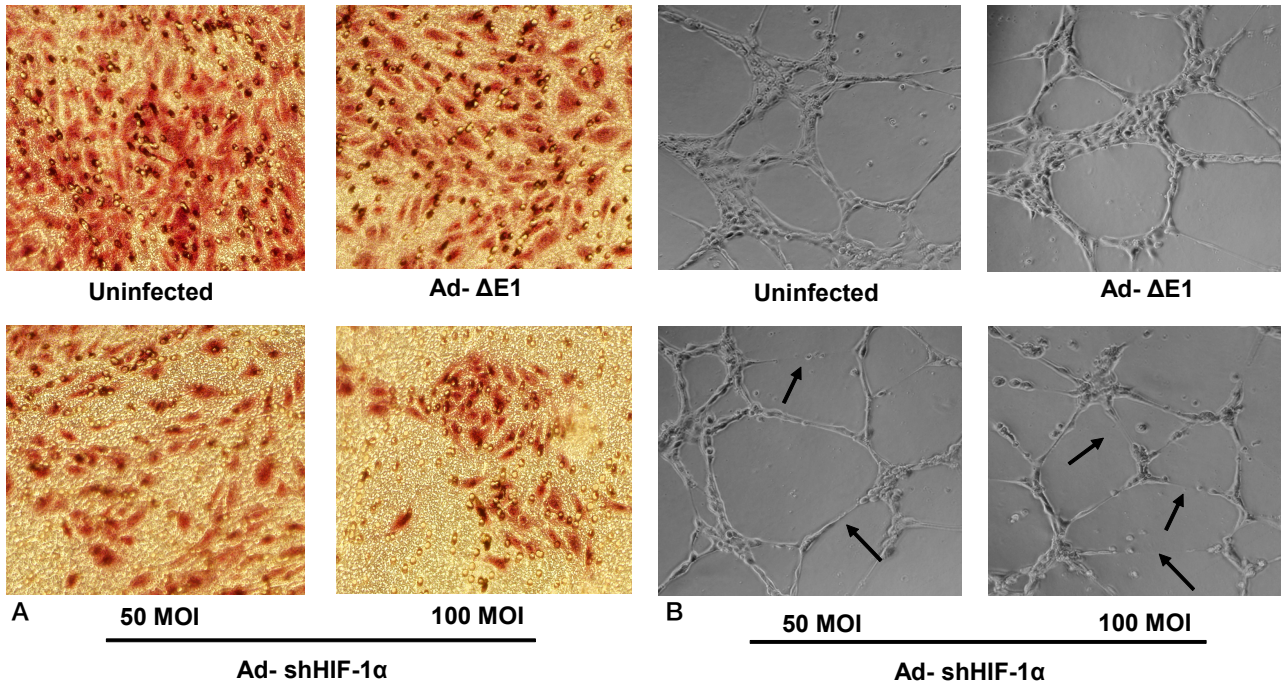


Figure 5. Effects of adenovirus-mediated shHIF-1 α expression on invasion and tube formation. (A) The conditioned medium collected from Ad- Δ E1-transduced Huh7 cells induced invasion, whereas that from Ad-shHIF-1 α -transduced Huh7 cells inhibited MOI-dependent inroads. (B) The conditioned medium collected from Ad- Δ E1-transduced Huh7 cells induced the formation of an extensive network of organized, elongated tube-like structures that resembled capillaries. However, the conditioned medium from Ad-shHIF-1 α -transduced cells inhibited tube-forming structures, with the plate instead becoming filled with an incomplete network of a capillary-like structure on HUVECs (arrows). shHIF-1 α , small hairpin hypoxia inducible factor-1 α ; Ad- Δ E1, adenoviral-mediated deletion of the E1 site; MOI, multiplicity of infection; HUVEC, human umbilical vein endothelial cells.

50 MOI treated group, the diameter of some tubes decreased and in the 100 MOI treated group most of the tubes became thin and also broken and therefore did not form good tube structure (Fig. 5B). This experiment confirmed that the inhibition of HIF-1 α expression induces the inhibition of angiogenic effect.

DISCUSSION

Excessive localized hypoxia during the division of cancer cells is one of the singularities in the growth phase of the cancer cell.⁸ Severe cell damage can occur if the hypoxic condition is maintained and cell growth is inhibited due to lack of oxygen and nutrition.^{8,10} Also, the HIF-1 α which receives the hypoxic signal becomes accumulated in the cell and if it is transferred to the nucleus, it induces the expression of angiogenesis-related genes and the expression of genes related to the metastasis of cancer cells.¹¹ The series of tumor growth programs suppresses cell damage by supplying oxygen and nutrition to the tumor and induces tumor growth. The inhibition of HIF-1 α expression blocks the HIF-1 α function as a transcription factor, so as a result, the angiogenic effect, tumor growth, metastasis effect

which are manipulated by the HIF-1 α are all inhibited step by step.^{19,20}

The RNAi is an experimental method which hinders the *in vivo* translation of mRNA in order to control the expression rate of a certain gene. In the previous papers, it was reported that the *in vivo* and *in vitro* RNAi tool experiment has an effect on survival and proliferation of tumor,^{25,30} and the possibility of application as cancer treatment is suggested.²⁵ But there has to be various modifications such as sustainability, safety, and efficiency in order for the RNAi to be applied in clinical treatment. The transfer into the cell is known to be the greatest problem and by using the virus vector, an efficient transfer of RNAi into the cell can be possible. Actually, efficient transfer of RNAi into the cell by the virus vectors such as, retrovirus and adenovirus have already been confirmed.^{26,27} Also, one of the weak points of siRNA is that the *in vivo* duration time is short,²⁸ but recently it is reported that the short duration time can be solved by using the virus vector.^{28,29} The adenovirus mediated small hairpin HIF-1 α was manufactured based methods delineated in previous studies. shHIF-1 α is transcribed into ssRNA similar to the hairpin loop of pre-miRNA which is regulated by RNA polymerase III and

transferred to the cytoplasm by Exportin-5 to knock down the function of the target gene following a series of processes, playing the same role as siRNA.^{17,25} When the HCC cell lines are treated MOI dependently with adenovirus mediated shHIF-1 α , the numbers of *in vivo* activating shHIF-1 α increases as the MOI increases, so it was concluded that it inhibits the expression of HIF-1 α and cell proliferation. Also by measuring the inhibition rate as time passed by, the inhibition rate increased compared to the control group. It was reported that the inhibition effect decreases after 48 hours in a previous study which conducted an experiment using siRNA,^{28,30} and the short duration time had been one of its defects, but in this study the inhibition effect on the virus was maintained even after 5 days. In this study, the inhibition effect induced by the virus remained even after 5 days. This study was carried out for a total of 7 days and there was no significant change in experimental data on the 7th day compared to the 5th. After the 5th day, the minimum surface area needed for cell proliferation was insufficient and further study in a larger culture area is needed (data not shown). By confirming the expression change in VEGF and MMP-2 on a molecular level, the HIF-1 α of the adenovirus mediated shHIF-1 α treated group was inhibited dependently of MOI and as a result, the expression of VEGF and MMP-2 were also partially inhibited. It was confirmed that the long-term inhibition of the proliferation of cancer cells and angiogenesis activity regulated by the HIF-1 α is possible in a hypoxic condition by inhibiting the expression of HIF-1 α using the adenovirus. Also during the *in vitro* experiment, the efficient transfer of shRNA which plays a similar role in siRNA is possible by using the virus instead of other transfection agents. According to this experiment, it was confirmed that the HIF-1 α expression affects the proliferation, angiogenesis, and metastasis of cancer cells and the inhibition of the expression of virus mediated HIF-1 α inhibits the continuous proliferation of cancer cells. It also inhibits angiogenesis for a long-term duration.

Based on the data on a molecular level, the shHIF-1 α inhibited the angiogenesis and proliferation of the blood vessel during the angiogenesis and cell mobility experiment, as expected. The vascular endothelial cell migration plays an important role in angiogenesis. The proteins expressed by the tumor such as VEGF and MMP-2 plays an important role in transferring the endothelial cells to the tumor. The transfer of endothelial cells to the tumor can be inhibited by inhibiting the proteins expressed by the tumor. However, it is difficult to describe the effect of angiogenesis in detail by just the *in vitro* experiment due to

HIF-1 α expression inhibition on the tumor. Animal experiments that show the regulation of tissue growth factor expression in hepatocellular carcinoma by HIF-1 α expression and angiogenesis has also been conducted. In addition, angiogenesis is regulated by more than one factor and is a combination reaction inside cells or tissues, so additional research on factors other than HIF-1 α is required.^{23,24}

The regulation of HIF-1 α expression is an important mechanism in tumor growth and angiogenesis. The hypoxia induced by the tumor growth is an important key to the expression of HIF-1 α and the induced HIF-1 α in a hypoxia state becomes activated as a transcription factor and regulates the growth factors related to tumor growth and angiogenesis. It also regulates the control factors which regulates the growth factors. For example, if the HIF-1 α expression is inhibited, the expression of sub-genes such as, VEGF and MMP-2 are also repressed.

Taken together, it can be confirmed that the inhibition of HIF-1 α expression ultimately represses the growth of tumor or transition, and this concept is key in developing an efficient treatment model which does not damage normal cells.

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