

## Angiopoietin-like 3 (ANGPTL3) Stimulates Human Umbilical Vein Endothelial Cell Tube Formation and Vascular Endothelial Growth Factor Production

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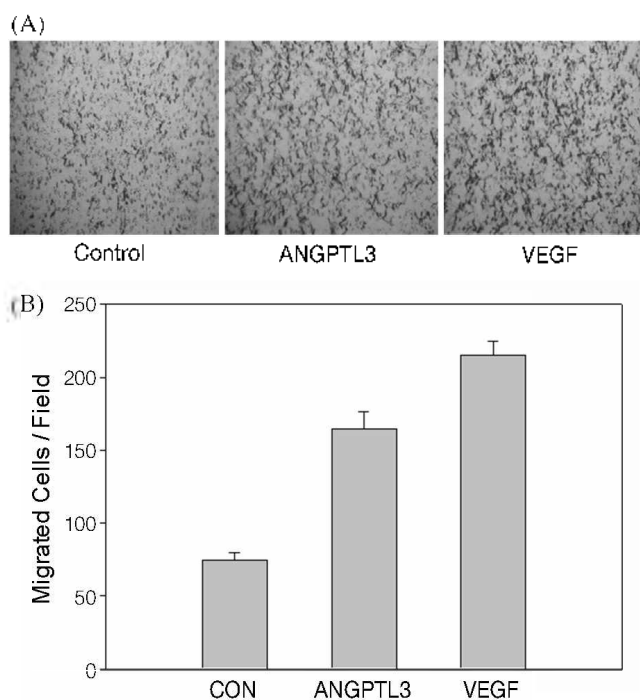
Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels and a fundamental process in the normal development, reproduction, and wound healing. Since angiogenesis in physiological processes is tightly regulated by a balance of stimulatory factors and inhibitory factors, disrupted balance effect plays a leading role in the progress of diseases such as tumor growth, rheumatoid arthritis and various blood vessel related pathology.<sup>1-3</sup> Progressive tumor growth is dependent on angiogenesis to receive a supply of oxygen and other essential nutrients. In addition, growth of new vascular capillaries from preexisting vessels provides a way for tumor cells to enter the circulation and to metastasize to distant organs. Among a variety of angiogenic factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have a synergistic effect in the induction of angiogenesis.<sup>4</sup> VEGF expression is induced in various cancer by stimuli including hypoxia, activation of IGF-1R, or p53 loss of function and transcriptional activation of VEGF gene is mediated by hypoxia-inducible factor-1 (HIF-1)<sup>5,6</sup> or nuclear factor kappa B (NF- $\kappa$ B).<sup>7</sup>

Since angiogenesis process is composed of multi-steps that are regulated by a wide range of angiogenic regulators, there might be more angiogenic regulators. In an effort to search for endogenous angiogenic factors, we found that angiopoietin-like 3 (ANGPTL3) is involved in angiogenesis. ANGPTL3 is a member of the angiopoietin-like family of secreted factors. Angiopoietin family consists of ANGPT1, ANGPT2, ANGPT4, ANGPTL1, ANGPTL2, ANGPTL3, ANGPTL4, ANGPTL5, ANGPTL6, and ANGPTL7. ANGPTL3 is expressed predominantly in the liver during development<sup>8</sup> and it was found to be involved in the regulation of serum lipid levels in mice.<sup>9</sup> ANGPTL3 has the characteristic structure of angiopoietins, the fibrinogen (FBN)-like domain in this protein was shown to bind alpha-5/beta-3 integrins, and this binding induced endothelial cell adhesion and migration. However, its role in HUVEC tube formation and VEGF induction has not been studied.

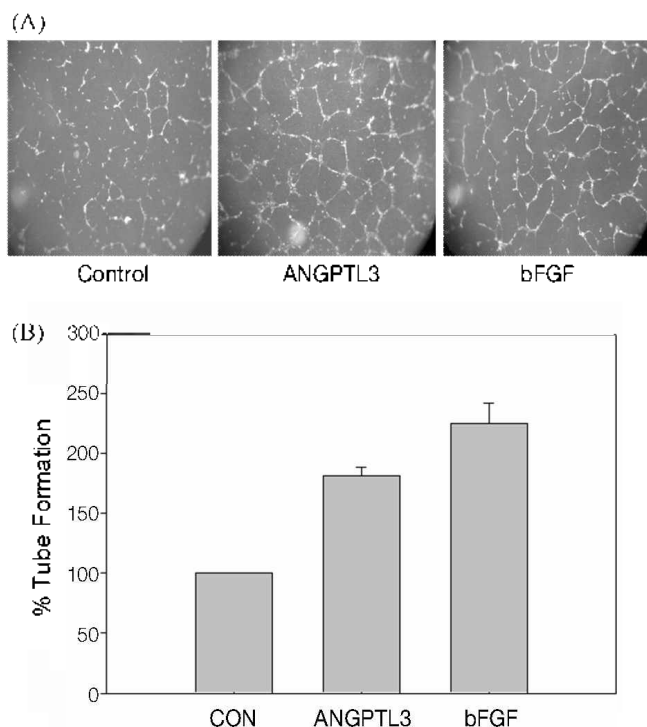
Since endothelial cell migration through extracellular matrix is essential to angiogenesis, we investigated the effect of ANGPTL3 on endothelial cell migration. Human embryonic kidney (HEK) 293 cells were transfected with ANGPTL3 expression plasmid and transfected cells were selected as described in experimental section. After completion of selection procedure, conditioned medium (CM) from the selected cells was obtained. In chemotaxis chamber, human umbilical vein

endothelial cells (HUVECs) were treated with CM from control transfected cell and stably transfected cell for 2 h, and HUVECs that had been migrated through membrane pores were counted under microscope. We used VEGF (10 ng/mL) as positive control. CM derived from stably ANGPTL3-transfected cells (ANGPTL3-CM) showed significantly induced migratory effect, compared to that of control-CM (Fig. 1A and B). Taken together, these results suggest that ANGPTL3-CM has the biological activity that can induce endothelial cell migration.

We next tested the ability of ANGPTL3-CM to promote the mesh-like structures of HUVECs on matrigel. The matrigel angiogenesis assay is generally used for studying HUVEC attachment, migration, and differentiation. The extent of mesh-like structure was more enhanced in HUVECs influenced in ANGPTL3-CM than that of control CM (Fig. 2A and B). Collectively, these data present that ANGPTL3-CM has angio-



**Figure 1.** ANGPTL3-CM stimulates endothelial cell migration. (A) HUVECs migration assay was carried out in 48-well microchemotaxis chambers. HUVECs were incubated with control-CM or ANGPTL3-CM for 2 h. Representative images of cell migration are shown. (B) After fixation and staining, the number of migrated cells was determined by counting two regions of each well under a microscope. VEGF (20 ng/mL) was used as a positive control.

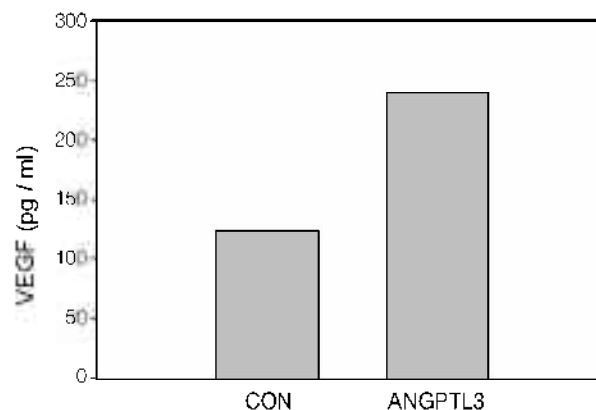


**Figure 2.** ANGPTL3-CM enhanced tube formation of endothelial cells. (A) HUVECs were collected and seeded on Matrigel-coated plates at a density of  $2.4 \times 10^4$  cells/well and then incubated with control-CM or ANGPTL3-CM. As a positive control, bFGF (10 ng/mL) was used. After 18 h, fields from each sample were photographed. Representative images of tube formation activity are shown. (B) Total tube areas were analyzed and quantitated by the Scion Image program. The control tube areas were defined as 100% tube formation, and the percent increase in tube formation as compared with control was calculated for each sample.

genic activity involved in migration and tube formation of endothelial cells.

We then measured the protein level of VEGF in ANGPTL3-CM to investigate whether VEGF production is involved in ANGPTL3-stimulated HUVEC tube formation because it is a major component of angiogenesis. Using ELISA assay kit, we found that ANGPTL3-overexpressing cells released significantly more VEGF than control cells (Fig. 3). This result suggests that the increased secretion of VEGF may be associated with ANGPTL3-induced angiogenic activity involved in migration and tube formation.

Angiogenesis is regulated by the balance between angiogenic activators and angiogenic inhibitors. ANGPTL4 showed a proangiogenic response in chicken chorioallantoic membrane assays,<sup>10</sup> and inhibits angiogenesis and VEGF-induced vascular leakiness.<sup>11</sup> Another study demonstrated that ANGPTL4 prevents metastasis by inhibiting vascular activity as well as tumor cell motility and invasiveness.<sup>12</sup> ANGPTL6 promotes angiogenesis.<sup>13,14</sup> ANGPTL1 inhibited VEGF-induced angiogenesis.<sup>15</sup> Recently, ANGPTL1 and ANGPTL2 have been shown to exhibit antiapoptotic activity.<sup>16</sup> Recent reports showed that ANGPTL3 stimulates adhesion and migration of endothelial cells as well induces blood vessel formation.<sup>17</sup> Despite its importance, however, the role of ANGPTL3 in angiogenesis remains largely unclear. In the present study, we



**Figure 3.** ANGPTL3 increases the level of VEGF in CM. Control or ANGPTL3 stable cells were incubated in serum-free M199 for 20 h, and aliquots of the CM were collected. The level of VEGF in CM was measured by ELISA as described in experimental section.

examined whether ANGPTL3 has angiogenic activity using several assays. First, we tested endothelial cell migration and tube formation of HUVECs. Next, we confirmed that ANGPTL3 induced VEGF secretion. In conclusion, we demonstrated that ANGPTL3 mediates angiogenesis and VEGF may be the target of ANGPTL3-mediated process. ANGPTL3 could be a candidate for developing anti-cancer agent in tumor growth and angiogenesis.

### Experimental Section

**Cell culture.** HEK 293 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 0.375% sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS in humidified 5% CO<sub>2</sub> incubator at 37 °C. Primary HUVECs were obtained from POSTEC1 and maintained on gelatin-coated dishes in M199 medium supplemented with 20% FBS, 5 U/mL of heparin, 3 ng/mL bFGF, and penicillin/streptomycin. The HUVECs used in this study were from passages 5 to 8.

**Plasmid constructs.** The N-terminal hemagglutinin (HA)-tagged gene for expression in mammalian cells was constructed by polymerase chain reaction, followed by cloning into the pcDNA3.1/HA Zeo plasmid.

**Transfection and selection of transfected cells.** HEK 293 cells were transfected and selected as previously described.<sup>18</sup>

**ANGPTL3-CM preparation.** Confluent cells were washed and grown in serum free M199 medium. After 20 h of incubation, CM was collected and centrifuged at 500 X g for 10 min and then at 800 X g for 20 min to remove debris. The resultant CM was immediately used for migration and tube formation assays or stored at -70 °C until use.

**HUVEC migration assays.** Endothelial cell migration assays were carried out in a 48-well microchemotaxis chamber (Neuro Probe Inc., Cabin John, MD) as described.<sup>19</sup> Polycarbonate membrane with 12-µm pore was coated with 0.1% gelatin. HUVECs were resuspended in 0.1% BSA/M199. The bottom chamber was loaded with 30,000 cells and the membrane was laid over the cells. Inveration and incubation of the chamber were carried out in sequence. After 2 h incubation, upper

wells were loaded with serum free M199 and CM samples. The chamber was reincubated for 2 h and membrane filter was fixed and stained using Diff-Quick (Baxter Healthcare). The number of cells that migrated through the filter was counted under a microscope.

**Capillary-like tube formation assays.** Tube structure assays were performed with commercial GFR matrigel (BD bioscience). Forty-eight-well plates were coated with 150  $\mu$ L GFR matrigel (10 mg/mL) and incubated at 37 °C for 30 min to promote polymerization. Briefly, total 0.3 mL mixtures of HUVECs (24,000 cells/well) and CM samples were added to each well coated with GFR matrigel. After 18 h incubation, fields from each sample were photographed, and total tube areas were analyzed by the Scion Image program. The control tube areas were defined as 100% tube formation, and the percent increase in tube formation as compared with control was calculated for each sample.

**Determination of the VEGF level.** To determine the VEGF protein expression level, we obtained CM from control or stable cells. The amount of VEGF in the CM from the cells was determined as previously described.<sup>19</sup>

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