

## N-Myc Downstream-Regulated Gene 2 Induces Angiogenesis

Mina Song and Sayeon Cho\*

College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea. \*E-mail: sycho@cau.ac.kr

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Angiogenesis is the process of generating new capillary blood vessels from pre-existing vessels and a fundamental process in the normal development, reproduction, and wound healing. Since neovascularization in physiological processes is tightly regulated by a balance of angiogenic and anti-angiogenic 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> Tumor growth relies on angiogenesis to receive an adequate supply of nutrients and oxygen. In addition, the newly formed blood vessels provide a way for tumor cells to enter the circulation and to metastasize to distant organs. Therefore, treatment of cancer with anti-angiogenic agents will control cancer cell growth and metastasis. Among a variety of angiogenic factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have a prominent activity in tumor metastasis and mortality.<sup>4</sup> Specially, VEGF expression is induced in various cancers by several stimuli and its transcriptional activation is mediated by hypoxia-inducible factor-1 (HIF-1).<sup>5,6</sup>

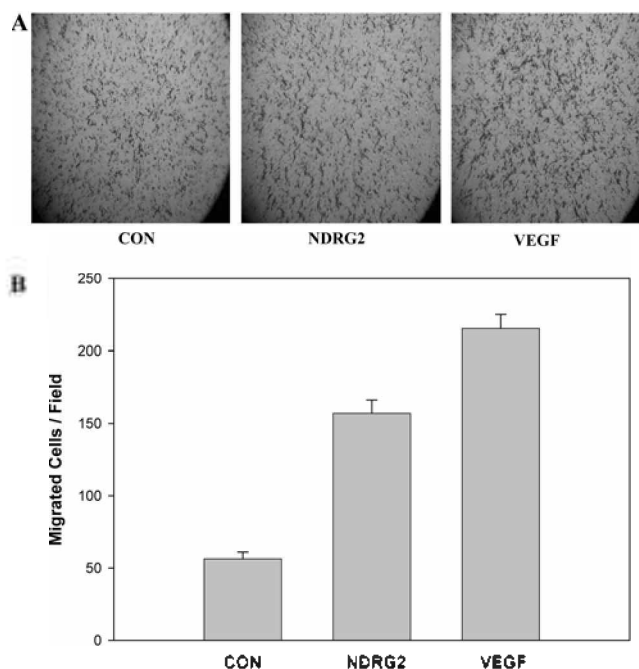
Since angiogenesis requires a multi-step mechanism, the process is mediated by a wide range of angiogenic regulators. In an effort to search for endogenous angiogenic factors, we found that N-myc downstream-regulated gene 2 (NDRG2) is involved in angiogenesis. Human NDRG2 belongs to a family of NDRG. The human NDRG family includes four identified members, NDRG1, NDRG2, NDRG3, and NDRG4, which are highly homologous except for in the C- and N-terminal regions.<sup>7</sup> NDRG2 initially identified as a candidate tumor suppressor gene since its expression is reduced in several cancer cells.<sup>8,9</sup> It has also been suggested that NDRG2 may have roles in cell differentiation,<sup>10</sup> Alzheimer's disease,<sup>11</sup> and neurite overgrowth.<sup>12</sup> However, NDRG2 activity related to angiogenesis was not yet reported.

Since endothelial cell migration through extracellular matrix is one of essential steps for neovascularization, we tested the effect of conditioned medium derived from NDRG2-transfected cells (NDRG2-CM) on endothelial cell migration. To establish cell line expressing NDRG2, human embryonic kidney (HEK) 293 cells were transfected with NDRG2 expression plasmid and transfected cells stably expressing NDRG2 were selected as described in experimental section. After expression of NDRG2 was confirmed by Western blotting analysis, conditioned medium (CM) from the selected cells was obtained. In chemotaxis chamber,

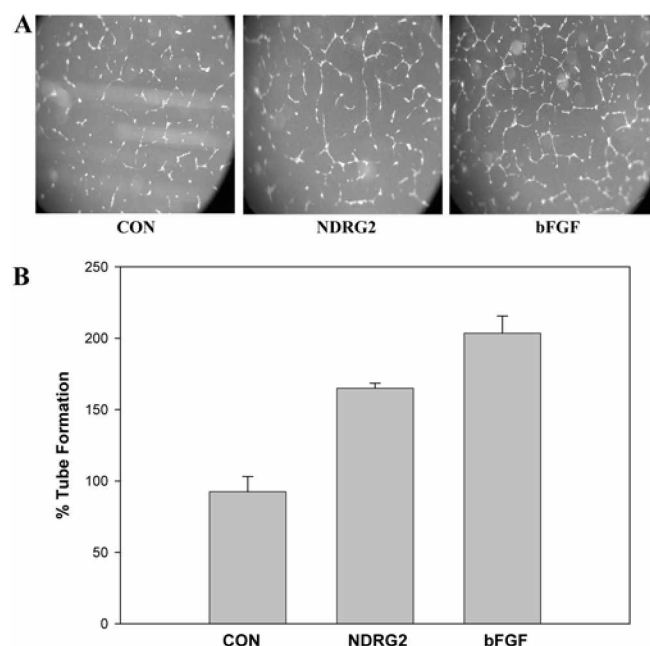
human umbilical vein endothelial cells (HUVECs) were treated with CM from control transfected cells and stably transfected cells for 2 h and HUVECs that had been migrated through membrane pores were counted under microscope. NDRG2-CM showed significantly induced migratory effect, compared to control-CM (Fig. 1A and B). Therefore, these data indicate that NDRG2-CM has the biological activity that can induce endothelial cell migration.

We next examined the ability of NDRG2-CM to promote the mesh-like structures of HUVECs on matrigel. Matrigel is generally used for studying HUVEC attachment, migration and differentiation. The extent of mesh-like structure was more enhanced in HUVECs influenced in NDRG2-CM than that of control CM (Fig. 2A and B). This result indicates that NDRG2-CM has the ability of inducing tube formation of endothelial cells.

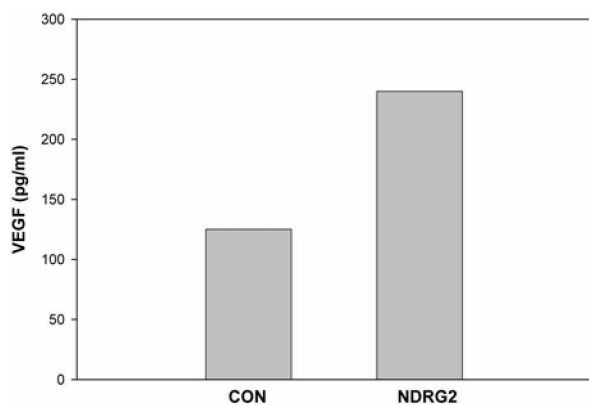
We then measured the protein level of VEGF in NDRG2-



**Figure 1.** NDRG2-CM stimulates endothelial cell migration. (A) HUVECs migration assay was carried out in 48-well microchemotaxis chambers. HUVECs were incubated with control-CM or NDRG2-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.

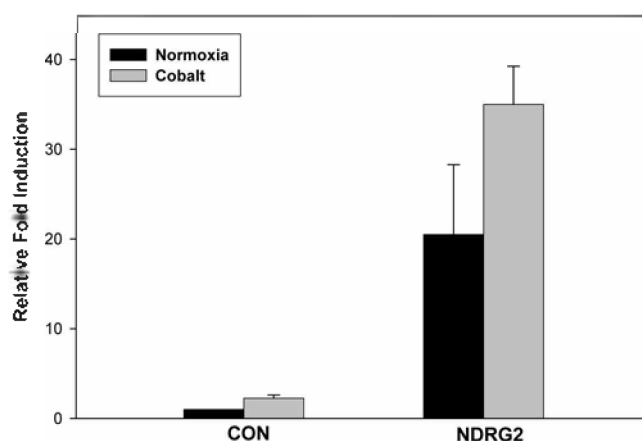


**Figure 2.** NDRG2-CM promotes tube formation of endothelial cells. (A) HUVECs were collected and seeded on Matrigel-coated plates at a density of  $2.4 \times 10^4$  cells/well and then incubated with control-CM or NDRG2-CM. As a positive control, bFGF (10 ng/mL) was used. After 18 h, fields from each sample were photographed. Representative images of tube formation activity are shown. (B) Total tube areas were analyzed 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.



**Figure 3.** NDRG2 increases the level of VEGF in CM. Control or NDRG2-expressing 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 under experimental section.

CM to investigate whether VEGF production is involved in NDRG2-stimulated HUVEC tube formation since it is a major component of angiogenesis. Using ELISA assay kit, we found that NDRG2-overexpressing cells released significantly more VEGF than control cells (Fig. 3). This result suggests that the increased secretion of VEGF may be associated with NDRG2-induced angiogenic activity involved in migration and tube formation.



**Figure 4.** NDRG2 activates the transactivation function of HIF-1. HEK 293 cells that constitutively express NDRG2 and control cells that contain empty plasmid were transfected by lipofectamine with 1  $\mu$ g each of the reporter plasmid HRE-Luc and 0.5  $\mu$ g of pCMV/ $\beta$ -gal. Cells were treated with 125  $\mu$ M  $\text{CoCl}_2$  for 24 h. Luciferase activities were normalized to  $\beta$ -galactosidase activities.

Since NDRG2-CM contains the increased level of VEGF, we examined whether NDRG2 regulates the transcriptional activity of HIF-1 that is stabilized at hypoxia and plays a key role in VEGF transcription. NDRG2-overexpressing cells were transfected with pHRE-Luc reporter plasmid and either grown under normoxic condition or treated with a hypoxia-mimicking reagent (125  $\mu$ M  $\text{CoCl}_2$ ). As shown in Figure 4, NDRG2-overexpressing cells resulted in an increase of the luciferase activity under both normoxic and hypoxia-mimicking conditions, compared to control cells grown at normoxia or hypoxia. Interestingly, the relative ratio of luciferase activities from NDRG2-overexpressing cells incubated under hypoxia-mimicking and normoxic conditions is significantly lower than that of control cells, suggesting that NDRG2 activates HIF-1 activity primarily at normoxia. Taken together, these results suggest that NDRG2 activates the HIF-1 $\alpha$  transcriptional activity and that its activatory function is oxygen-independent.

In the present study, we found that NDRG2 induced angiogenesis although it is a candidate tumor suppressor gene due to its low expression in various cancer tissues. Interestingly, NDRG1 is highly expressed in many types of human tumors including colon, breast, prostate, kidney, liver, and brain cancers compared with normal tissue.<sup>13</sup> Moreover, NDRG1 overexpression in some cancers shows a marked reduction in tumor-induced angiogenesis,<sup>14</sup> suggesting that overexpression of proteins in cancer cells is not always favorable for cancer growth. The distinct expression patterns of NDRG1 and NDRG2 suggest that they have different functions in angiogenesis of cancer tissues. These findings support the possibility that NDRG2 has angiogenic activity. We confirmed that this ability of NDRG2 is involved in the increased VEGF levels and HIF-1 transactivation.

In conclusion, we demonstrated that NDRG2 mediates angiogenesis and VEGF may be the target of NDRG2-medi-

ated process. NDRG2 could be a novel target for developing anti-cancer agent in tumor growth and angiogenesis.

### Experimental Section

**Cell culture.** Human embryonic kidney (HEK) 293 and human umbilical vein endothelial cells (HUVECs) were routinely maintained as previously described.<sup>15,16</sup>

**Plasmid construction.** The N-terminal hemagglutinin (HA)-tagged NDRG2 gene for expression in mammalian cells was constructed by polymerase chain reaction, followed by cloning into the pcDNA3.1/HA Zeo plasmid. The luciferase reporter plasmid (HRE-Luc) containing the promoter of HIF-1 $\alpha$  was purchased from Stratagene.

**Stable transfection and Western blotting.** HEK293 cells were transfected by lipofectamine (Invitrogen) with 1  $\mu$ g each of pcDNA3.1 Zeo-NDRG2 or pcDNA3.1 Zeo-empty vector. After 48 h, transfected cells were selected for two weeks in DMEM containing 10% FBS and zeocin (200  $\mu$ g/mL). After 15 days of selection, resistant cells arising in dishes were propagated in zeocin of low level (150  $\mu$ g/mL). The expression of stably transfected genes was confirmed by Western blotting as previously described.<sup>16</sup>

**NDRG2-CM preparation.** Confluent stable cells were washed and grown in serum free M199 medium. After 20 h of incubation, CM was collected and centrifuged at 500  $\times$  g for 10 min and then at 800  $\times$  g for 20 min to remove debris. The resultant CM was immediately used for migration and tube formation assays or stored  $-70$   $^{\circ}$ C until use.

**HUVEC migration and tube formation assays.** Endothelial cell migration and tube formation assays were carried out as previously described.<sup>17</sup>

**Determination of VEGF level.** To determine VEGF protein expression level, we obtained CM from control or stable cells. The amount of VEGF protein found in the CM was determined as previously described.<sup>15</sup>

**Luciferase assay.** Stable cells were grown to 50-70% confluence in 60 mm dish, and were transfected by lipo-

fectamine with 1  $\mu$ g pHRE-Luc and pCMV/ $\beta$ -gal. and assayed as previously described.<sup>18</sup>

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