Identification of activated signal transduction pathways and molecules in the HMGB1 induced angiogenesis

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Identification of activated signal transduction pathways and molecules in the HMGB1 induced angiogenesis

Directed by Professor Hoguen Kim

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설렘과 꿈을 안고 대학원 생활을 시작한 것이 엊그제 같은데, 어느 새 석사과정을 마치고 학위 논문을 제출하게 되었습니다. 학위논문을 마무리하는 이 글을 쓰며 돌아보니, 지나온 세월이 가슴 벅차게 다가옵니다. 부족한 제게 너무나 많은 분들의 관심 어린 사랑과 이해 그리고 도움과 함께하는 진정한 마음이 있었습니다. 미흡하지만 학위 논문을 마치면서 그 분들께 감사의 말씀을 전합니다.

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마지막으로 그 누구보다 이루 다 말씀드릴 수 없을 만큼 깊고 무한한 사랑으로 한없이 부족한 저를 위해 모든 것을 믿고 아낌없이 지원해 주신 부모님께 존경과 감사를 드립니다. 힘들고 지칠 때 마다 부모님이 제게 주신 깊고 넓은 사랑과 믿음이 있어 이겨 낼 수 있었고, 지금의 제가 있을 수 있었습니다. 항상 새벽마다 저를 위해 기도해 주시는 사랑하는 외할머니, 아빠 같은 국장님, 친 딸 이상으로 아껴주시는 막내이모, 이모부, 또 저를 자랑스럽게 생각해주고 항상 최고라 말해주는 가장이 된 믿음직한 오빠들과 친언니처럼 챙겨주는 새언니들께도 감사함과 사랑을 전합니다. 마지막으로 저에게 지혜와 명철을 주시고, 감사함으로 살아갈 수 있게 해주신 하나님 아버지께 감사드립니다.

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ABSTRACT

Identification of activated signal transduction pathways and molecules in the HMGB1 induced angiogenesis

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(Directed by Professor Hoguen Kim)

High-mobility group box-1 (HMGB1), a non-histone chromosomal protein, is expressed in almost all cells with nuclei, and its dysregulated expression correlates with pathological conditions such as inflammatory diseases, ischemia, and cancer. Some of these conditions accompany abnormal angiogenesis induced by HMGB1-mediated activation of downstream signaling pathways. Thus far, the underlying mechanism by which HMGB1 induces angiogenesis remains largely unknown.

In this study, I performed time-dependent gene expression microarray analysis of endothelial cells (ECs) after HMGB1 or VEGF treatment. According to the pathway analysis of each gene set upregulated by HMGB1 or VEGF, I found that most HMGB1-induced angiogenic pathways were also activated by VEGF, although the activation time and gene sets belonging to the pathways differed. In addition, HMGB1 upregulated some VEGFR signaling-related conventional angiogenic factors including EGR1 and,

importantly, novel angiogenic factors, such as ABL2, CEACAM1, KIT, and VIPR1, which are reported to independently promote angiogenesis under physiological and pathological conditions.

My gene expression profiling suggests that HMGB1 independently induces angiogenesis by activating HMGB1-specific angiogenic factors and also functions as an accelerator for VEGF-mediated conventional angiogenesis under physiological and pathological conditions.

Key words : HMGB1, VEGF, angiogenesis, endothelial cell, gene expression profile

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I. INTRODUCTION

The highly conserved protein high-mobility group box-1 (HMGB1) plays various roles according to cellular localization and is expressed in almost all mammalian cells.[1] Nuclear HMGB1 contributes to the structural formation of chromatin and is involved in transcriptional regulation, while cytosolic HMGB1 functions as a regulator for autophagy and intracellular immune reactions.[2] Secreted HMGB1 functions as an immune cell-associated cytokine that induces systemic inflammation, cell proliferation, signal transduction, and activation of endothelial cells (ECs).[3] Secreted HMGB1 activates downstream signaling pathways (CDC42/Rac, MAPK, NF-κB, and PI3K-Akt) by binding to several known receptors, such as the receptor for advanced glycation end products (RAGE) and the Toll-like receptors TLR-2, TLR-4, and TLR-9.[4-7]

The pathogenic functions of HMGB1 have been documented in a wide range of diseases such as neurodegeneration, aging, ischemia, and cancers.[8,9] In particular, HMGB1 overexpression is now considered a hallmark of various types of cancers including colon, breast, and lung cancer and hepatocellular carcinoma, and its

expression correlates with invasion, metastasis, and poor prognosis.[10] In vitro and in vivo studies have shown that secreted HMGB1 directly or indirectly promotes pathological or physiological angiogenesis by activating ECs, cytokine release, and immune cell stimulation.[11-16] Therefore, HMGB1-targeted cancer therapeutic strategies using siRNA and antibodies have been developed and applied to *in vitro* and *in vivo* disease models.[17,18] HMGB1 inhibition by these agents potently suppresses the progression of cancers through the perturbations of migration, proliferation, and angiogenesis.[7,19] The inhibition of angiogenesis is particularly important in advanced cancers because angiogenesis is a crucial step for the acquisition of malignant properties such as metastasis and invasion.[20] While HMGB1-mediated angiogenesis is disturbed by siRNA-mediated downregulation of RAGE or by HMGB1 neutralizing antibody,[13,21] the mechanisms of HMGB1-mediated angiogenesis are not fully understood in terms of the transcriptomic changes caused by exogenous HMGB1 stimulation. Moreover, the differences between HMGB1-mediated angiogenesis and conventional VEGF-mediated angiogenesis are essentially unknown.

In this study, I aimed to characterize the angiogenic pathways and genes activated in ECs treated with HMGB1 or VEGF at various time points and how they contribute to angiogenesis. I found that HMGB1 as well as VEGF significantly induced proangiogenic features in ECs, accompanying time-dependent activation of angiogenic pathways. HMGB1 treatment also resulted in HMGB1-specific upregulation and VEGF-like upregulation of oncogenic and angiogenic genes. These findings indicate that HMGB1 can function as a potent angiogenic signal both independently and synergistically in combination with other angiogenic signals.

II. MATERIALS AND METHODS

1. Cell culture and treatment

Primary human umbilical vein endothelial cells (HUVECs) were isolated from human placental microvessels according to previous methods[22,23] and maintained in M199 (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS), 1% penicillin/streptomycin, 10 μg/mL bFGF (Sigma, St. Louis, MO, USA), and 2500 units/mL heparin (Sigma). This study was approved by the Institutional Review Board of Yonsei University College of Medicine (No.12-genebank-11). Written informed consent was obtained from all patients. HUVECs between the second and fifth passages were used. HUVECs were treated with recombinant HMGB1 (ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel) at 1 μg/mL and recombinant VEGF (ProSpec) at 50 ng/mL for 20 minutes, 45 minutes, 2 hours, 6 hours, or 12 hours.

2. Cell proliferation and wound healing assays

For the cell proliferation assay, HUVECs were seeded into 12-well cell culture plates (~30% confluence). Cells were starved with M199 containing 2% FBS for 6 hours, stimulated with HMGB1 (2 μg/mL) or VEGF (50 ng/mL) for 48 hours, and then harvested and manually counted. For the wound healing assay, HUVECs were seeded into 6-well cell culture plates with serum-containing medium and cultured to ~100% confluence. After 6 hours of starvation, an artificial, homogeneous wound was created by scratching the monolayer with a sterile 200-μL pipette tip. Images of cells migrating into the wound were captured after 12 hours using a microscope.

3. Invasion assay

Invasion assays were performed using the Matrigel invasion chamber (Corning Inc., Corning, NY, USA) according to the manufacturer's protocol. HUVECs were seeded in the upper compartment (~80% confluence) and allowed to migrate for 18 hours after HMGB1 or VEGF treatment. M199 complete medium was used as a chemoattractant.

4. Tube formation assay

Matrigel (BD Biosciences, Bedford, MA, USA) was added to 12-well plates and incubated for 1 hour at 37℃. HUVECs in low serum medium (M199 containing 5% FBS) were seeded on the top of the polymerized Matrigel and treated with HMGB1 or VEGF. After 2, 6, and 12 hours of incubation, tube formation was imaged under a light microscope. The effect of HMGB1 and VEGF on tube formation was calculated by measuring the length of the capillary-like network and number of tubules.

5. Gene expression microarray

For DNA microarray hybridization, RNA was extracted from HUVECs using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was pooled by mixing equal amounts of total RNA, and biotin-labeled cRNA targets were synthesized from 1.5 μg of total RNA. Double-stranded cDNA synthesis was performed using the Illumina® TotalPrep RNA Amplification Kit (Illumina, Inc., San Diego, CA, USA), while biotin-UTP-labeled antisense RNA was transcribed in vitro using the Ambion MEGAscript kit (Ambion Life Technologies, Carlsbad, CA, USA). All steps of the labeling procedure were performed according to the manufacturers' protocols. Microarray experiments were conducted on the

HumanHT-12 v4 Sentrix Expression BeadChip (Illumina) containing 47,231 probes representing 31,332 annotated genes.

6. Gene enrichment, functional annotation, and statistical analyses

Data are expressed as mean ± standard deviation (SD). $P < .05$ was considered significant. Student's t-tests were performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA). DAVID software (Database for Annotation, Visualization and Integrated Discovery, v6.8; http://david.abcc.ncifcrf.gov) was used for pathway and gene ontology analysis.

7. siRNA and Transfection

siRNAs against ABL2, CEACAM1, VIPR1, and KIT and nontargeting siRNA (siCtrl) were purchased from Bioneer (Bioneer Corporation, Daejeon, South Korea). All transfection experiments were performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

8. Quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA synthesis was performed using M-MLV reverse transcriptase (Invitrogen Life Technologies). qRT-PCR primer sequences were obtained from the Primer Bank database (http://pga.mgh.harvard.edu/primerbank/). Expression of each transcript (CCL2, CCND2, EPHB1, PCDH12, VASH1, TMEM140, CD34, EGR1, ABL2, CEACAM1, VIPR1, KIT, β-actin, and GAPDH) was measured using SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Japan) and the ABI PRISM 7500 Sequence Detector (Applied Biosystems, Carlsbad, CA, USA). The level of each

transcript as normalized to that of β-actin mRNA. The sequences of the primers used are listed in Table 1.

Table 1. Primers used for qRT-PCR

Gene	Direction	Sequence
CCL2	Forward	5'- CAGCCAGATGCAATCAATGCC
	Reverse	5'- TGGAATCCTGAACCCACTTCT
CCND2	Forward	5'- ACCTTCCGCAGTGCTCCTA
	Reverse	5'- CCCAGCCAAGAAACGGTCC
EPHB1	Forward	5'- GGCTGCGATGGAAGAAACG
	Reverse	5'- CTGGTTGGGCTCGAAGACATT
PCDH12	Forward	5'- GTCAAGGTCAACGTCTTGGAC
	Reverse	5'- GTTTTATGAGAAGCGTACCAGGT
VASH1	Forward	5'- GGTGGGCTACCTGTGGATG
	Reverse	5'- CACTCGGTATGGGGATCTTGG
TMEM140	Forward	5'- TCGGCTTCTATAACTTCTGCCT
	Reverse	5'- CTGTTGCACTGGGCTAGGAG
CD34	Forward	5'- CTACAACACCTAGTACCCTTGGA
	Reverse	5'- GGTGAACACTGTGCTGATTACA
EGR1	Forward	5'- GGTCAGTGGCCTAGTGAGC
	Reverse	5'- GTGCCGCTGAGTAAATGGGA
ABL2	Forward	5'- GTTGAACCCCAGGCACTAAAT
	Reverse	5'- CAACGAAGAGATTAGGGTCACTC
CEACAM1	Forward	5'- TGCTCTGATAGCAGTAGCCCT
	Reverse	5'- TGCCGGTCTTCCCGAAATG
VIPR1	Forward	5'- TCATCCGAATCCTGCTTCAGA
	Reverse	5'- AGGCGAACATGATGTAGTGTACT
KIT	Forward	5'- CGTTCTGCTCCTACTGCTTCG
	Reverse	5'- CCCACGCGGACTATTAAGTCT
β-actin	Forward	5'-TGGGTCAGAAGGATTCCTATGTG
	Reverse	5'-TCGTCCCAGTTGGTGACGAT
GAPDH	Forward	5'- AAGGTGAAGGTCGGAGTCAAC
	Reverse	5'- GGGGTCATTGATGGCAACAATA

9. Western blotting

Protein extracts were prepared using a passive lysis buffer (Promega, Madison, WI, USA) with a protease inhibitor cocktail (Roche, Penzberg, Germany). The membranes were incubated with primary antibodies against CCL2 (Cell Signaling Technology, Beverly, MA, USA), PCDH12 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD34 (Santa Cruz Biotechnology), EGR1 (Santa Cruz Biotechnology), and β-actin (Cell Signaling Technology) overnight at 4℃. Western blot images were recorded using an LAS 4000 imager (Fujifilm Co. Ltd., Tokyo, Japan).

III. RESULTS

1. HMGB1 treatment confers proangiogenic features on ECs

Given the expected roles of HMGB1 in angiogenesis, I first tested whether exogenous HMGB1 treatment stimulated proangiogenic features in ECs by evaluating proliferation, migration, wound healing, and tube formation after HMGB1 treatment in a time-dependent manner. ECs were counted after 48 hours of HMGB1 or VEGF treatment, and relative proliferation was measured. HMGB1 treatment induced an approximately twofold increase in EC proliferation compared to that of VEGF treatment (Fig. 1A). The migration assay also showed that HMGB1 treatment increased migrated EC numbers 2.5-fold (Fig. 1B). The wound healing assay to further evaluate both migration and cell–cell interaction showed that wound healing was significantly promoted by HMGB1 treatment (Fig. 1C and D) and that HMGB1-treated ECs were more likely to form capillary-like structures (Fig. 1E and F). Although VEGF-mediated proangiogenic activation of ECs is well known, I further confirmed that exogenous VEGF treatment strongly induced the proangiogenic features of ECs in my system (Fig. 1A–F).

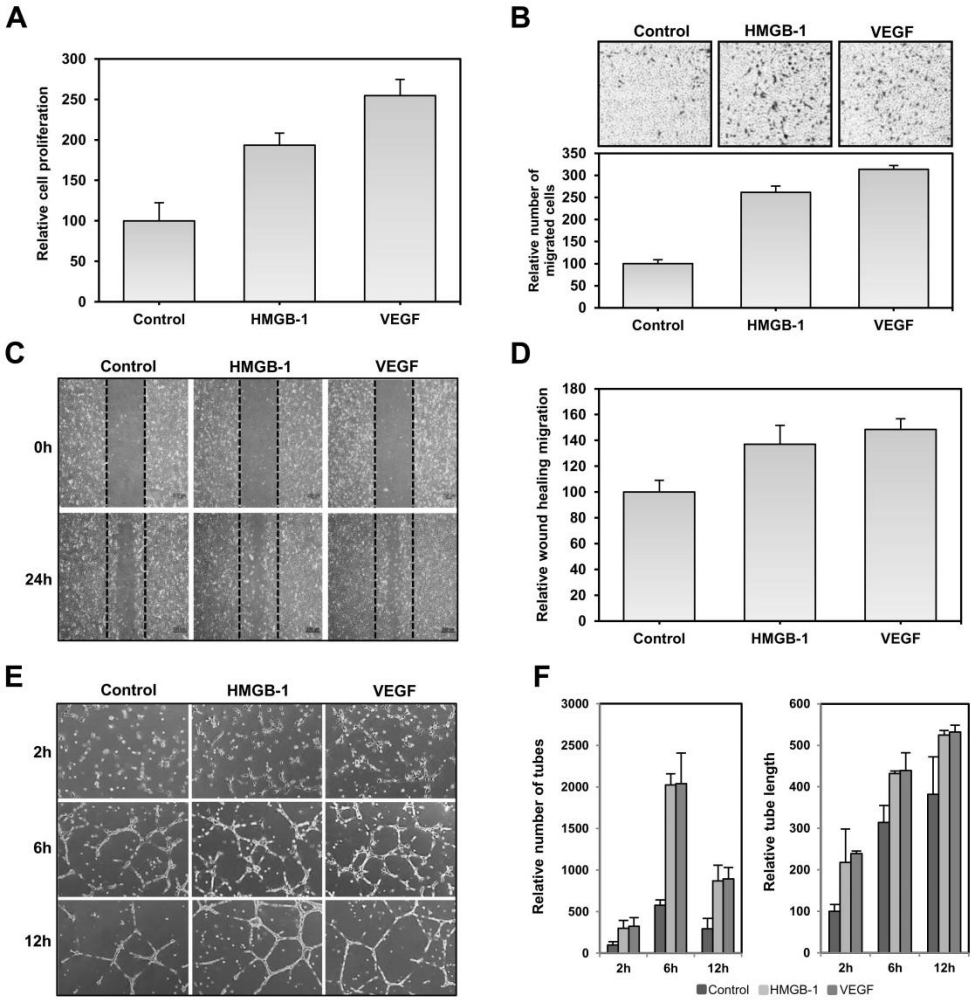


Figure 1. HMGB1 and VEGF stimulate proangiogenic features in ECs. (A) Proliferation assay. ECs were counted after 48 hours of HMGB1 or VEGF treatment. (B) Invasion assay. Migrated ECs were stained and counted after 18 hours of treatment with HMGB1 or VEGF. (C and D) Wound healing assay. Relative wound healing rates were measured in ECs after wound creation and 24 hours of treatment with HMGB1 or VEGF. (E and F) Tube formation assay. The relative number of tubes and tube length were measured manually after ECs were

incubated on Martrigel with HMGB1 or VEGF for 2, 6, and 12 hours.

2. Microarray profiling of gene expression in ECs activated by HMGB1 or VEGF

I performed gene expression microarray analysis to clarify the molecular mechanism of HMGB1-induced EC activation at the gene expression level. ECs were treated with HMGB1 or VEGF at various time points to investigate time-dependent gene expression changes. RNAs were extracted from ECs harvested at early time points (20 and 45 minutes after HMGB1 or VEGF treatment) and late time points (2, 6, and 12 hours after HMGB1 or VEGF treatment), and gene expression microarray profiling was performed. By pre-processing the raw data, I obtained 19,389 and 19,335 probes in HMGB1-treated and VEGF-treated samples, respectively. Further selection of probes that showed more than 1.5-fold up- or downregulation resulted in 507 probes in HMGB1-treated samples and 1,212 probes in VEGF-treated samples (Fig. 2).

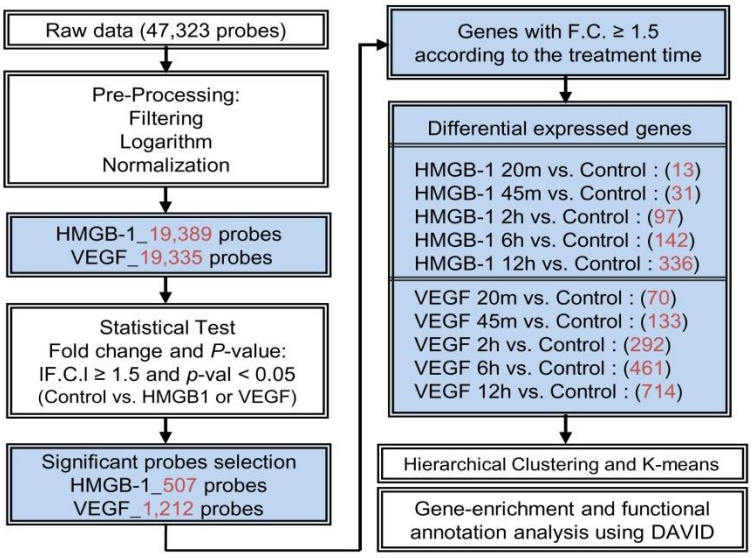


Figure 2. Analysis pipeline for time-dependent gene expression microarray in ECs treated with HMGB1 or VEGF.

Selected probes were mapped to official genes and K-means, and hierarchical clustering was performed using the list of obtained genes. K-means cluster analysis showed that gene expression was distinctively altered in a time-dependent manner in ECs treated with HMGB1 compared to that in ECs treated with VEGF. Gene expression patterns changed dramatically between 45 minutes and 2 hours after HMGB1 treatment, while VEGF treatment induced more gradual gene expression changes (Fig. 3).

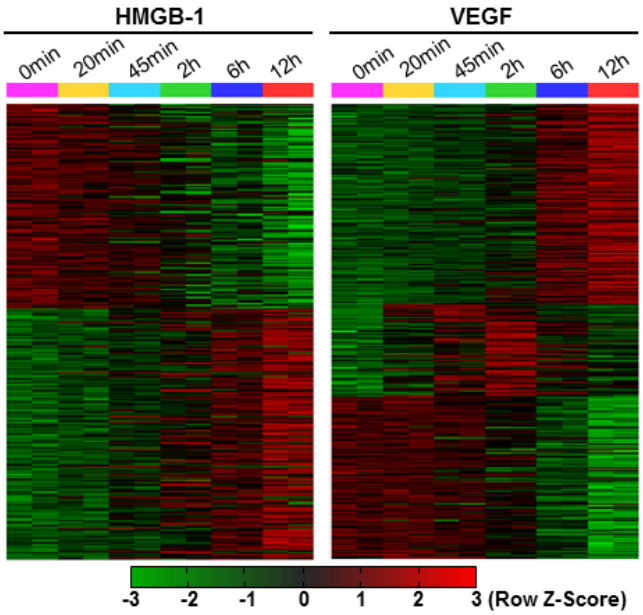


Figure 3. K-means cluster analysis in ECs treated with HMGB1 or VEGF for various periods of time.

To validate my microarray data, eight genes that showed distinct expression levels at each time point after HMGB1 or VEGF treatment were selected (*CCL2*, *CCND2*, *EPHB1*, *PCDH12*, *VASH1*, *TMEM140*, *CD34*, and *EGR1*), and qRT-PCR was performed in another set of EC samples treated with HMGB1 or VEGF in the same manner as in the microarray analysis. To accurately compare my microarray data with qRT-PCR data, expression intensities of the eight genes obtained from my microarray data were normalized to β-actin intensities. Expression patterns as

determined by qRT-PCR were similar to those observed in the microarray data (Fig. 4).

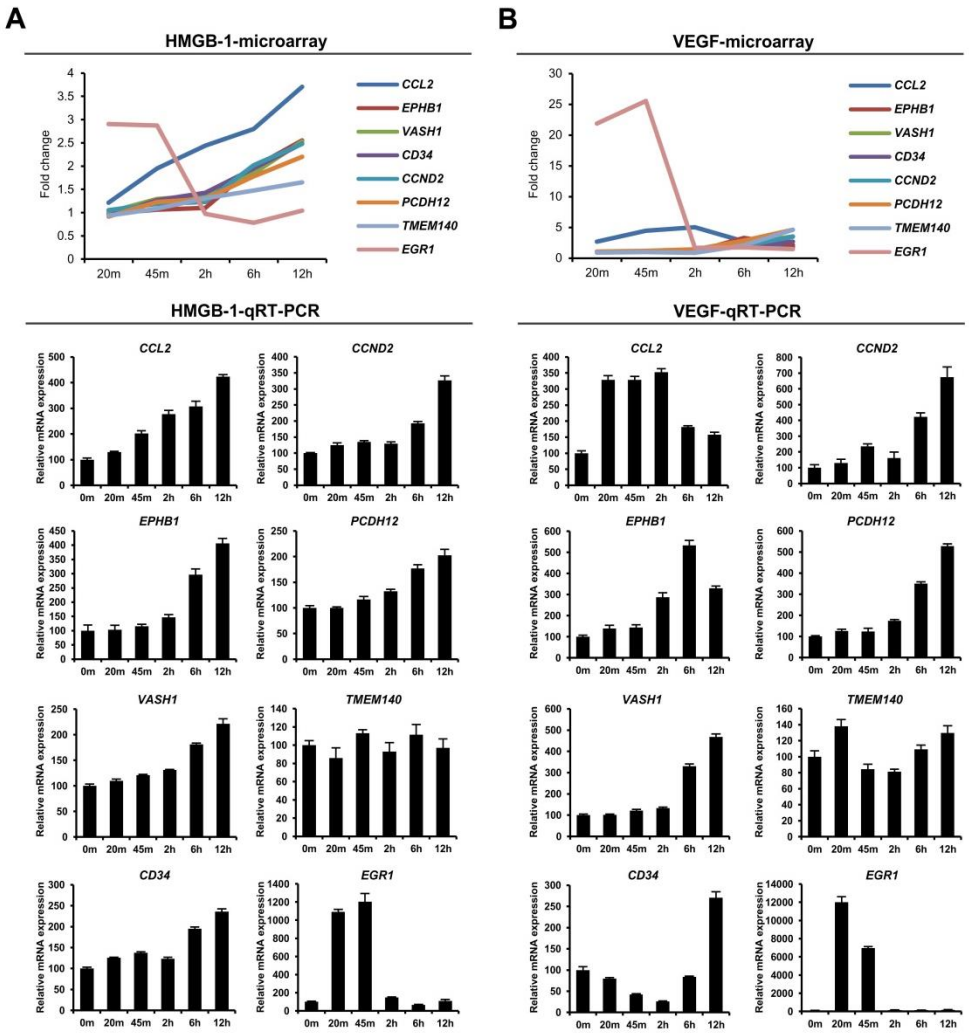


Figure 4. Microarray results were validated by qRT-PCR analysis of eight genes that showed characteristic expression changes following HMGB1 and VEGF treatment. (A and B) The expression patterns of eight genes that showed more than 1.5-fold upregulation during at least one time point according to the microarray (normalized to *β-actin* intensity) were compared with those of the same

genes obtained by qRT-PCR analysis (normalized to *β-actin* expression) in another set of ECs treated with HMGB1 and VEGF.

However, there were some discrepancies in *TMEM140, CD34*, and *EGR1* between the results from microarray and qRT-PCR in both HMGB1-treated and VEGF-treated samples. I, therefore, further performed qRT-PCR analysis of the three genes using *GAPDH*, another highly stable housekeeping gene, and compared the results with my microarray data normalized to *GAPDH* intensities, which allowed us to observe minimal discrepancies between the results from the microarray and qRT-PCR in all three genes (Fig. 5A and 5B). My microarray data were further validated at the protein level. I obtained commercially available antibodies against CCL2, PCDH12, CD34, and EGR1 and performed western blotting analysis with the same EC samples used for the qRT-PCR analysis. In my microarray data, the expression of *CCL2, PCDH12*, and *CD34* gradually increased after HMGB1 treatment with the highest expression at 12 hours, whereas *EGR1* expression was specifically upregulated at 20 and 45 minutes of HMGB1 treatment. Western blotting analysis showed that protein expression of CCL2, PCDH12, and CD34 started to increase after 6 hours of HMGB1 treatment and that EGR1 expression was upregulated after 45 minutes of HMGB1 treatment. On the other hand, our microarray data showed that the expression of PCDH12 and CD34 gradually increased after VEGF treatment, while the expression of CCL2 and EGR1 was specifically upregulated at 20 minutes, 45 minutes, and 2 hours of VEGF treatment and 20 and 45 minutes of VEGF treatment, respectively. Western blotting analysis showed that the expression of PCDH12 and CD34 started to increase after 6 hours of VEGF treatment and that the expression of CCL2 and EGR1 increased at 45 minutes and 2 hours of VEGF treatment and 45 minutes of VEGF treatment, respectively. Western blotting analysis showed that the mRNA expressions of the four genes were highly proportional to their protein expressions, which validates my microarray data (Fig. 5C and 5D).

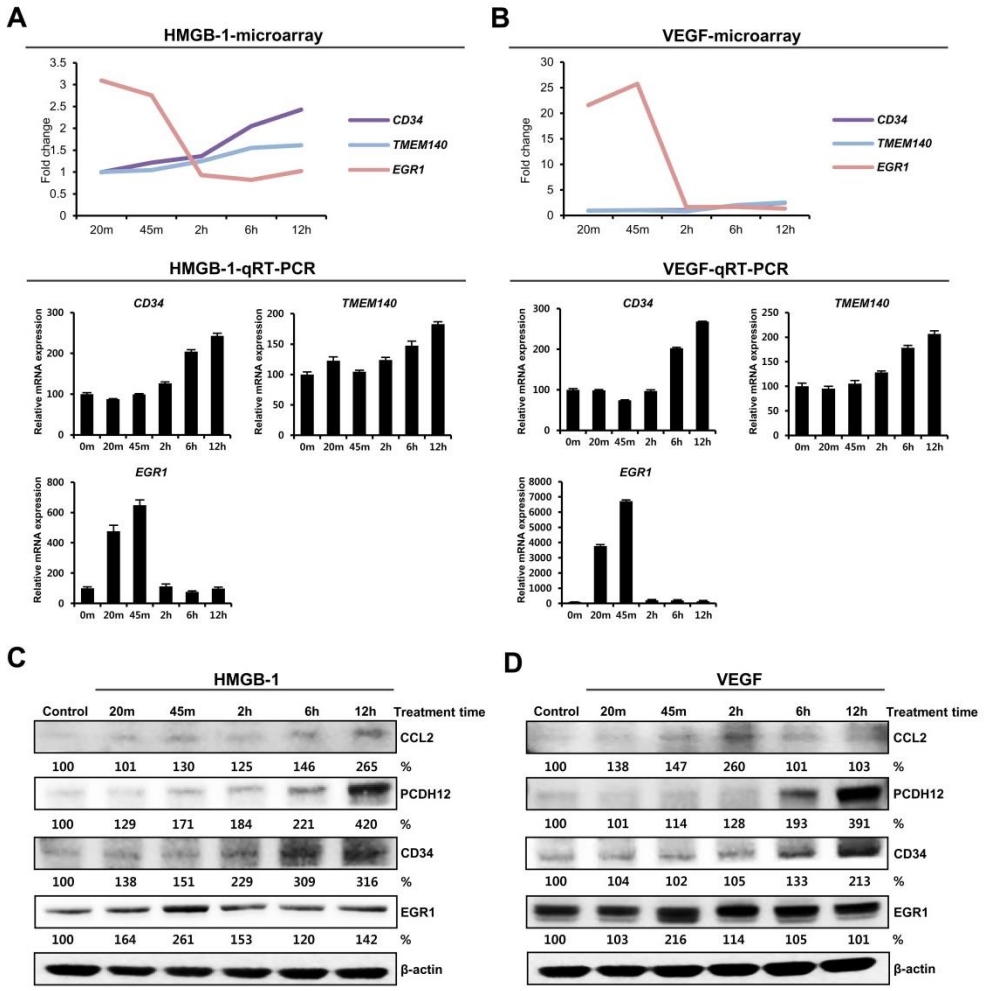


Figure 5. Validation of microarray results by qRT-PCR analysis of *TMEM140, CD34,* and *EGR1* using *GAPDH* as a control gene and additional validation of microarray results by western blotting analysis of CCL2, PCDH12, CD34, and EGR1. (A and B) The expression patterns of *TMEM140*, *CD34*, and *EGR1* that showed more than a 1.5-fold upregulation during at least one time point according to the microarray data (normalized to *GAPDH* intensity) were compared with those of the same genes obtained by qRT-PCR analysis (normalized to *GAPDH* expression) in another set of ECs treated with HMGB1 and VEGF. (C and D) To evaluate the relationship between mRNA and protein expressions of eight selected

genes in Figure 4, CCL2, PCDH12, CD34, and EGR1 were selected for western blotting analysis. Relative band intensities were indicated under each band.

3. HMGB1 and VEGF induce characteristic activation of angiogenic pathways

The number of genes upregulated by HMGB1 or VEGF by more than 1.5-fold gradually increased in a time-dependent manner, with more genes upregulated by VEGF than HMGB1 at each time point. Using this list of upregulated genes at each time point, I performed gene enrichment and functional annotation analyses with DAVID.

Most upregulated genes after 20 minutes of VEGF treatment belonged to well-documented VEGF-dependent angiogenic pathways, such as the MAP kinase, hypoxia, chemotaxis, cell migration, cell proliferation, apoptosis, angiogenesis, vasculogenesis, tumor necrosis factor (TNF) signaling, and transforming growth factor beta receptor (TGFBR) signaling pathways.[3,24] Most of these pathways remained activated after 45 minutes of VEGF treatment, at which point the unfolded protein response (UPR), cell adhesion, NF-κB signaling, and cell differentiation pathways were newly activated. No significant changes in the activated pathways were found after 2 hours of VEGF treatment except for the upregulation of some genes related to the canonical Wnt and Notch signaling pathways, which are also known to be involved in vessel branching. Similarly, no major changes in the activated pathways were found after 6 hours and 12 hours of VEGF treatment, but increased activation of UPR and new activation of the Rap1 signaling and PI3K-Akt signaling pathways were noted. UPR signaling has been recently highlighted due to its involvement in HIF1α-mediated VEGF expression and angiogenesis in cancers. These findings indicate that angiogenic pathways are concomitantly and strongly induced promptly after VEGF treatment and remain activated for an extended period. Overall, the TNF, TGFBR2, NF-κB, and MAP kinase signaling pathways

showed early activation after VEGF treatment, whereas the canonical Wnt, Notch, Rap1, and PI3K-Akt signaling pathways showed relatively late activation (Table 2).

Table 2. Time-dependent activation of direct and indirect angiogenic pathways induced by HMGB1 or VEGF

	20m (10 genes)	45m (18 genes)	2h (49 genes)	6h (85 genes)	12h (182 genes)
HMGB1	Inflammatory response	Cell adhesion	Cell proliferation	Response to wounding	Response to wounding
	Cell adhesion	Transcription	Hypoxia	Blood vessel development	Hypoxia
	Response to molecule of bacterial origin	Signal transduction	Response to progesterone	Cell proliferation	Cell proliferation
	Regulation of neutrophil Chemotaxis	Response to progesterone	Transcription	Apoptosis	Apoptosis
	Chemokine-mediated Signaling	Response to mechanical Stimulus	Response to mechanical Stimulus	Vasculogenesis	Angiogenesis
	Movement of cell of subcellular component	Response to glucose	Marginal zone B cell Differentiation	Angiogenesis	Inflammatory response
		Cellular response to interleukin-1	Regulation ubiquitin-protein transferase activity	Inflammatory response	Lamellipodium assembly
			Cytokine-cytokine receptor interaction	Signal transduction	Glomerular filtration
				Endothelium development	Esophagus smooth muscle contraction
				Chemotaxis	Defense response
				EGFR signaling	Embryonic skeletal system Development
				Morphogenesis	Endothelium development
				Cytokine-cytokine receptor interaction	Glial cell-derived neurotrophic factor receptor signaling
	20m (66 genes)	**45m (115 genes)**	**2h (234 genes)**	**6h (330 genes)**	**12h (402 genes)**
VEGF	MAP kinase	MAP kinase	MAP kinase	MAP kinase	MAP kinase
	Hypoxia	Hypoxia	Hypoxia	Hypoxia	Hypoxia
	Chemotaxis	Chemotaxis	Chemotaxis	Chemotaxis	Chemotaxis
	Cell migration	Cell migration	Cell migration	Cell migration	Cell migration
	Cell proliferation	Cell proliferation	Cell proliferation	Cell proliferation	Cell proliferation
	Apoptosis	Apoptosis	Apoptosis	Apoptosis	Apoptosis
	Angiogenesis	Angiogenesis	Angiogenesis	Angiogenesis	Angiogenesis
	Vasculogenesis	Vasculogenesis	Vasculogenesis	Vasculogenesis	Vasculogenesis
	TNF signaling	TNF signaling	TNF signaling	TNF signaling	TNF signaling
	TGFBR signaling	TGFBR signaling	TGFBR signaling	UPR signaling	TGFBR signaling
	Transcription	UPR signaling	UPR signaling	Cell adhesion	UPR signaling

	Skeletal muscle cell diffrentiation	Cell adhesion	Cell adhesion	Cell differentiation	Cell adhesion
	Cellular response to interleukin-1	NF-kappa B signaling	NF-kappa B signaling	PI3K-Akt signaling	Wnt signaling pathway
	Response to progesterone	Cell differentiation	Cell differentiation	Palate development	PI3K-Akt signaling
	Chemokine-mediated Signaling	Transcription	Wnt signaling pathway	Protein glycosylation	Rap1 signaling
	Signal transduction	Response to mechanical Stimulus	Notch signaling pathway	Immune response	Signal transduction
		Chemokine-mediated Signaling	Signal transduction	Chemokine-mediated signaling	Renal tubule morphogenesis
		Cellular response to interleukin-1	Transcription	Fibrinolysis	Blood coagulation
		Signal transduction	Immune response	Glomerulus morphogenesis	Immune response
		Response to progesterone	VEGFR signaling	Potassium ion transport	Erythropoietin-mediated signaling
		Inflammatory response	Neurogenesis	Post-embryonic development	Bone development
			Morphogenesis	Protein secretion	Regulation of fibroblast growth factor receptor signaling
			Cellular response to interleukin-1	Response to vascular endothelial growth factor stimulus	Embryonic skeletal system Development
				Response to glucose starvation	Post-embryonic development
				Blood coagulation	Regulation of calcium ion import

TNF, tumor necrosis factor signaling; TGFBR, transforming growth factor beta receptor; UPR, unfolded protein response

At early time points (20 and 45 minutes after HMGB1 treatment), 28 genes were upregulated by HMGB1, and these were related to inflammatory response, cell adhesion, transcription, and signal transduction, which are pathways activated in the early process of HMGB1 binding to its receptor. After 2 hours of HMGB1 treatment, 49 genes were upregulated, and some of which belonged to angiogenic pathways such as cell proliferation and hypoxia. My functional annotation analysis further showed that 85 and 182 genes were upregulated 6 and 12 hours after HMGB1 treatment, respectively, and these were closely related to both direct and indirect angiogenic pathways, such as response to wounding, blood vessel development, cell proliferation, apoptosis, vasculogenesis, angiogenesis, and inflammatory response. These findings suggest that prolonged exposure to HMGB1 initiates strong activation of direct angiogenic pathways including migration, proliferation, and

sprouting in ECs, accompanying early activation of the inflammatory response, an indirect angiogenic pathway. Although HMGB1 and VEGF commonly induced EC activation and subsequent angiogenesis, HMGB1-mediated activation of ECs was shown to be quite different from VEGF-mediated activation in terms of the order of pathway activation and the upregulated genes involved (Table 2).

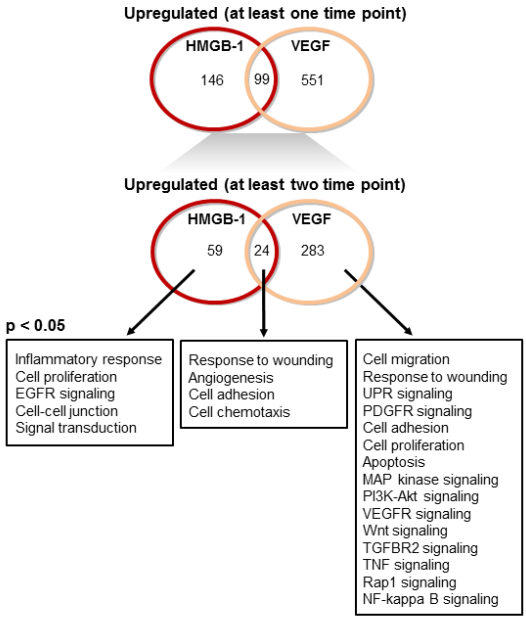
4. HMGB1 induces novel angiogenic and oncogenic genes

Integrative gene ontology analysis was performed using the 245 genes upregulated more than 1.5-fold during at least one time point after HMGB1 treatment (Fig. 6A). Of these, 99 genes were upregulated by both HMGB1 and VEGF treatment. Ontology analysis of the 146 genes specifically upregulated by HMGB1 revealed no significant angiogenesis-relevant pathways, while the 99 genes upregulated by both HMGB1 and VEGF were closely related to direct and indirect angiogenic pathways, such as response to wounding, inflammatory response, angiogenesis, cell chemotaxis, and cell proliferation (Table 3). To analyze the time-dependent expression changes of each gene, I further selected the 83 genes upregulated more than 1.5-fold during at least two time points (Fig. 6A). Importantly, expression of most genes (n = 62) started to increase after 6 hours of HMGB1 treatment and remained upregulated until 12 hours of HMGB1 treatment (Fig. 6B).

Table 3. Aangiogenic pathways associated with 99 commonly up-regulated genes by HMGB1 and VEGF

Category	Term	p-value	Benjamini
GOTERM_BP_DIRECT	Response to wounding	1.40E-04	9.30E-02
GOTERM_BP_DIRECT	Inflammatory response	1.10E-03	1.40E-01
GOTERM_BP_DIRECT	Angiogenesis	2.50E-03	2.50E-01
GOTERM_BP_DIRECT	Cell chemotaxis	2.60E-03	2.20E-01
GOTERM_BP_DIRECT	Cell proliferation	7.80E-03	4.40E-01

A



B

HMGB-1 specifically up-regulated 59 genes

Gene Symbol	20m	45m	2h	6h	12h
TXNIP					
FAM89A					
DDIT4					
KIT					
LFNG					
RNF150					
CEACAM1					
MAN1C1					
ABCA1					
CHRNA1					
OAS2					
LHX6					
TNFSF10					
PLA2G4C					
KIAA1671					
GALNTL4					
SNORD13					
MMP28					
GPR116					
TMEM107					
ABLIM1					
LAP3					
AFAP1L2					
SESN1					
MYO5C					
RNF144					
ANKRD47					
CHST1					
GJA4					
PRND					
MX1					
RNF144A					
TNFRSF21					
ADAM19					
GDPD5					
TIMP3					
ANGEL1					
SDSL					
SPG3A					
CORO2B					
C9orf61					
RHBDF2					
SLC46A3					
APOL3					
VLDLR					
ADCY4					
NUDT7					
LMCD1					
AQP1					
CLDN5					
FHL3					
PARP12					
VIPR1					
FAM74A3					
LOC201175					
ARRDC3					
LOC653123					
KLF6					
ABL2					

HMGB-1 and VEGF commonly up-regulated 24 genes

Gene Symbol	20m	45m	2h	6h	12h
ANKRD37					
C8orf4					
CCL2					
CCND2					
CD34					
CD97					
CSMD1					
DENND2A					
EPHB1					
KANK3					
KIAA1274					
LOC100132810					
LOC441179					
MTSS1					
MYO1B					
PCDH12					
TMEM140					
TMEM178					
TNFRSF4					
VASH1					
WSCD1					
BHLHB2					
EGR1					
F2RL3					

Up (Fold change > 1.5)
Up (Fold change > 2)
Up (Fold change > 3)

Figure 6. HMGB1 induces angiogenic pathways via genes specifically activated by HMGB1 and those activated by both HMGB1 and VEGF. (A) Ontology analysis of genes upregulated by HGMB-1 or VEGF during at least two time points. (B) Time-dependent expression patterns of genes specifically upregulated by HMGB1 (n = 59) and those upregulated by both HMGB1 and VEGF (n = 24).

Out of the 83 genes, 59 were specifically upregulated by HMGB1, and some of these genes were related to angiogenic pathways including inflammatory response, cell proliferation, epidermal growth factor receptor (EGFR) signaling, and cell–cell junction. Specifically, *ABL2, AFAP1L2, APOL3, CEACAM1, CHST1, CLDN5, KIT, PLA2G4C, RHBDF2, TNFRSF21, GJA4,* and *VIPR1* are reported to play a role in physiological or pathological angiogenesis (Table 4). I further selected *ABL2, CEACAM1, VIPR1*, and *KIT* to demonstrate the angiogenic roles of the HMGB1 specifically upregulated genes. Wound healing and tube formation assays were performed after HMGB1 treatment when HMGB1 specifically upregulated genes were inhibited by RNAi. Efficient downregulation of each gene was confirmed by qRT-PCR in ECs transfected with respective RNAi (Fig. 7A). The wound healing assay showed that the wound healing process promoted by HMGB1 treatment was significantly dysregulated by inhibition of ABL2, CEACAM1, and VIPR1 and most potently by inhibition of KIT (Fig. 7B and 7C). The tube formation assay also showed a similar result in which HMGB1 treated ECs barely formed capillary-like structures when HMGB1 specifically up-regulated genes were inhibited. Consistent with the wound healing assay results, inhibition of KIT most dramatically led to a reduction in capillary formation of ECs (Fig. 7D and 7E).

Table 4. General roles of genes specifically upregulated by HMGB1 and those upregulated by both HMGB1 and VEGF and their involvement in angiogenesis

	Gene symbol	Description	General role	Previously reported angiogenic relevance
HMGB1 specifically up-regulated genes	**ABL2**	ABL proto-oncogene 2 (A non-receptor tyrosine kinase)	Regulator of the cell growth, survival, adhesion, and migration	Plays a role in the survival and activation of ECs
	AFAP1L2	Actin filament associated protein 1-like 2	Mediator in the PI3K/Akt pathway	Activates Src kinases that play an important role in lymphokine-mediated cell survival and VEGF-induced angiogenesis
	APOL3	Apolipoprotein L3	Regulator of the cholesterol transport	Up-regulated by tumor necrosis factor-alpha in endothelial cells and activates NF-κ B signaling
	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	Mediator in the immune system and cell adhesion	Induces blood vessel remodeling through ECs differentiation
	CHST1	Carbohydrate sulfotransferase 1	Catalizer in the biosynthesis of selectin ligands	Predominantly expressed in vascular endothelial cells and involved in lymphocyte homing at sites of inflammation
	CLDN5	Claudin 5	Component of the tight junction strands	Enriched in endothelial cell and maintains the integrity of endothelial cell sheets
	KIT	KIT (Proto-Oncogene Receptor Tyrosine Kinase)	Receptor tyrosine kinase associated with multiple oncogenic downstream signaling pathways	Leads to increment of VEGF expression and inhibition of KIT disturbs tumor angiogenesis
	PLA2G4C	Phospholipase A2 group IVC	Enzyme that hydrolyzes phospholipids into fatty acids and other lipophilic molecules	Related to the stress response of endothelial cells and plays an important role in chemotaxis of cancer cells
	RHBDF2	Rhomboid 5 homolog 2	Regulator of several ligands of the epidermal growth factor receptor	May promote angiogenesis in association with ADAM17 by increasing blood vessel sprouting and the pericyte number during microvessel development
	TNFRSF21	TNF receptor superfamily member 21	Member of the tumor necrosis factor receptor that induces apoptosis	Predominantly expressed in tumour vascular cells and regulates vascular development
	GJA4	Gap junction protein alpha 4	Component of the gap junction	Involved in the growth regulation of microvascular ECs
	VIPR1	Vasoactive intestinal peptide receptor 1	G-protein coupled receptor	Simulate angiogenesis through the transactivation of epidermal growth factor receptor (EGFR) and the expression of vascular endothelial growth factor (VEGF)

	Gene symbol	Description	General role	Previously reported angiogenic relevance
HMGB1 specifically up-regulated genes	**ABL2**	ABL proto-oncogene 2 (A non-receptor tyrosine kinase)	Regulator of the cell growth, survival, adhesion, and migration	Plays a role in the survival and activation of ECs
	AFAP1L2	Actin filament associated protein 1-like 2	Mediator in the PI3K/Akt pathway	Activates Src kinases that play an important role in lymphokine-mediated cell survival and VEGF-induced angiogenesis
	APOL3	Apolipoprotein L3	Regulator of the cholesterol transport	Up-regulated by tumor necrosis factor-alpha in endothelial cells and activates NF-κ B signaling
	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	Mediator in the immune system and cell adhesion	Induces blood vessel remodeling through ECs differentiation
	CHST1	Carbohydrate sulfotransferase 1	Catalizer in the biosynthesis of selectin ligands	Predominantly expressed in vascular endothelial cells and involved in lymphocyte homing at sites of inflammation

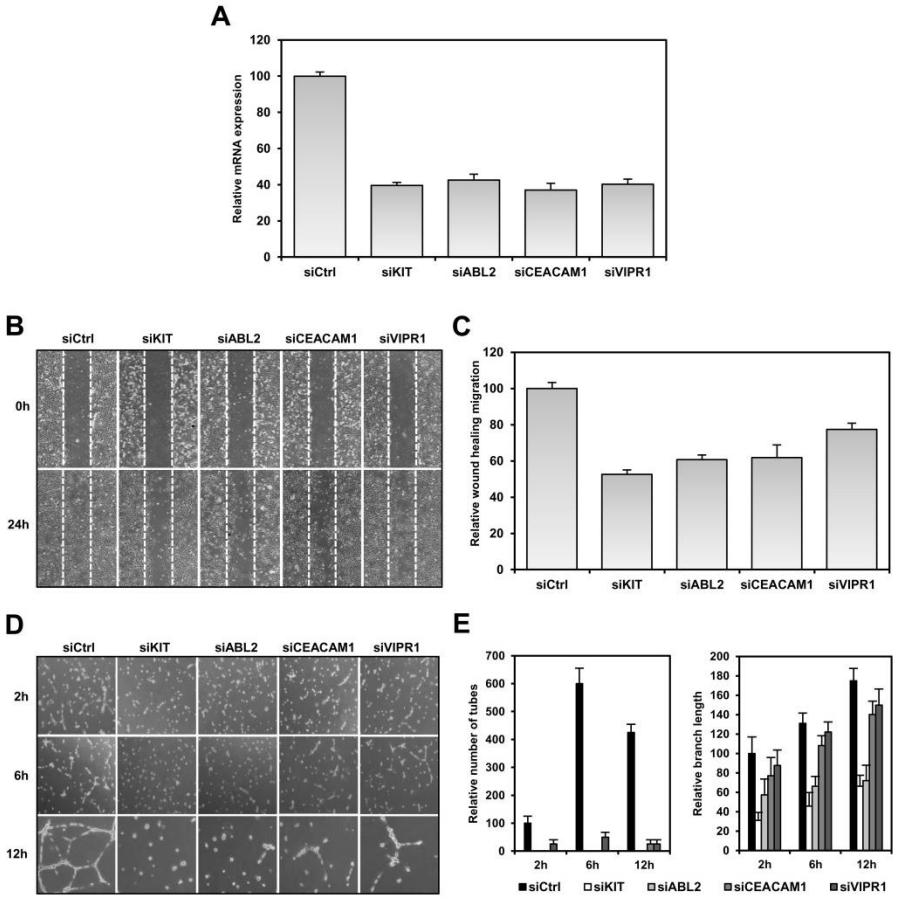


Figure 7. Involvement of HMGB1 specifically upregulated genes in the activation of angiogenic features in ECs. (A) Efficiency of RNAi against *ABL2, CEACAM1, VIPR1,* and *KIT* was confirmed by qRT-PCR. (B-E) Wound healing and tube formation assays were performed using ECs treated with HMGB1 when *ABL2, CEACAM1, VIPR1,* and *KIT* were inhibited by RNAi, respectively.

Out of the 83 genes, the remaining 24 genes were upregulated in both HMGB1-treated and VEGF-treated ECs, and some of these were related to response to wounding, angiogenesis, cell adhesion, and cell chemotaxis, which were similarly identified in the analysis of the 99 genes upregulated by both HMGB1 and VEGF during at least one time point. Specifically, *CCL2, EPHB1, PCDH12, EGR1,* and *CD34* are involved in physiological or pathological angiogenesis (Table 4). In contrast, 551 and 283 genes were found to be specifically upregulated (more than 1.5-fold) by VEGF during at least one time point and at least two time points, respectively. As expected, most of these genes were related to well-documented VEGF-induced direct and indirect angiogenic pathways, including cell migration, response to wounding, UPR signaling, PDGFR signaling, cell adhesion, cell proliferation, apoptosis, MAP kinase signaling, PI3K-Akt signaling, VEGFR signaling, Wnt signaling, TGFBR2 signaling, TNF signaling, Rap1 signaling, and NF-κB signaling (Fig. 6A).

IV. DISCUSSION

Angiogenesis is the physiological or pathological proliferation of capillaries from pre-existing blood vessels. Importantly, pathological conditions such as tumor growth and metastasis accompany the dysregulation of angiogenesis, which can be induced by several major molecular mechanisms including hypoxia and abnormally activated VEGFR signaling.[25] Although a significant fraction of patients with

cancer respond to VEGF-targeted therapies, clinical use of VEGF blockers results in different outcomes according to cancer type.[26,27] Moreover, angiogenesis in patients with advanced cancer is regulated not only by VEGF but also by other proangiogenic molecules, making it difficult to block angiogenesis using VEGF inhibitors.[24] HMGB1, a cytokine broadly overexpressed in most cancers, is known to be a potent inducer of angiogenesis and has been studied as a target molecule for anti-angiogenic therapy.[7,10] Under both physiological and pathological conditions, HMGB1 binds to RAGE, and the HMGB1/RAGE axis activates the MAP kinase/NF-κB and CDC42/Rac pathways, contributing to EC proliferation, migration, and sprouting.[7] However, most of these findings were derived from unrelated in vitro experiments, and the molecular mechanism by which HMGB1 activates ECs and subsequently induces angiogenesis remains unclear.

In this study, I performed time-dependent gene expression analysis of ECs to characterize HMGB1- or VEGF-mediated changes in the transcriptome. Ontology analysis showed that genes upregulated by VEGF mostly belonged to conventional VEGF/VEGFR axis-mediated pathways such as cell proliferation, migration, death, angiogenesis, and hypoxia. Most of these angiogenic pathways were activated promptly after VEGF treatment and showed increased activation over time. HMGB1-induced angiogenic pathways mostly overlapped with those activated by VEGF, even though the upregulated genes in these common pathways differed. Moreover, angiogenic pathways began to be highly activated after 6 hours of HMGB1 treatment. These findings suggest that HMGB1-induced angiogenesis might require a longer period of exposure than VEGF-induced angiogenesis and involve some HMGB1-specific angiogenic factors.

I found that 83 genes were upregulated by HMGB1 during at least two time points, and most of these were continuously upregulated from 6 to 12 hours after HMGB1 treatment. Out of these 83 genes, 24 were upregulated by both HMGB1 and VEGF, and most of these were closely related to angiogenic pathways including response to wounding, angiogenesis, cell adhesion, and cell chemotaxis, suggesting that

HMGB1 at least partially induces angiogenesis in a VEGF-like manner. Several reports have shown that HMGB1 plays angiogenic roles by regulating expression of VEGF (especially, VEGF-A). In detail, Biscetti *et al*[28] has reported that HMGB1 administration restores blood flow recovery and capillary density in the ischemic muscle of diabetic mice, accompanying increased expression of VEGF. The increase of VEGF expression was demonstrated by western blotting using the ischemic legs of HMGB1-treated mice. In addition, there has been another report that exogenous HMGB1 treatment induces angiogenesis via increased expression of HIF-1α and VEGF in EC-associated fibroblasts.[29] These findings suggest that HMGB1 is capable of activating ECs in a fibroblast dependent manner and might directly activate ECs by up-regulating VEGF expression in ECs which in turn leads to the increased secretion of VEGF and activation of the VEGF/VEGFR pathway in an autocrine manner. In this study, I aimed to evaluate the direct effect of exogenous HMGB1 on EC activation without being affected by uncertain factors, including cytokines released from fibroblasts. In this relatively closed system, my microarray data showed no significant changes in the expression of *VEGF* and *HIF-1α* even after prolonged HMGB1treatment, which has been further confirmed by qRT-PCR analysis (Fig. 8). As described in the above results, some angiogenic pathways, such as cell proliferation, hypoxia, angiogenesis, and cell adhesion, were found to be commonly activated in ECs treated with HMGB1 or VEGF, which suggests that HMGB1 and VEGF signaling pathways might share some downstream effector molecules. For example, EGR1 (a well-known effector molecule in both HMGB1 and VEGF mediated angiogenesis) showed highly similar expression patterns in ECs treated with HMGB1 or VEGF. Based on these findings, I believe that synergistic and complementary relationships might exist between the HMGB1 and VEGF pathway, rather than the direct involvement of VEGF-dependent signaling in HMGB1 mediated angiogenesis.

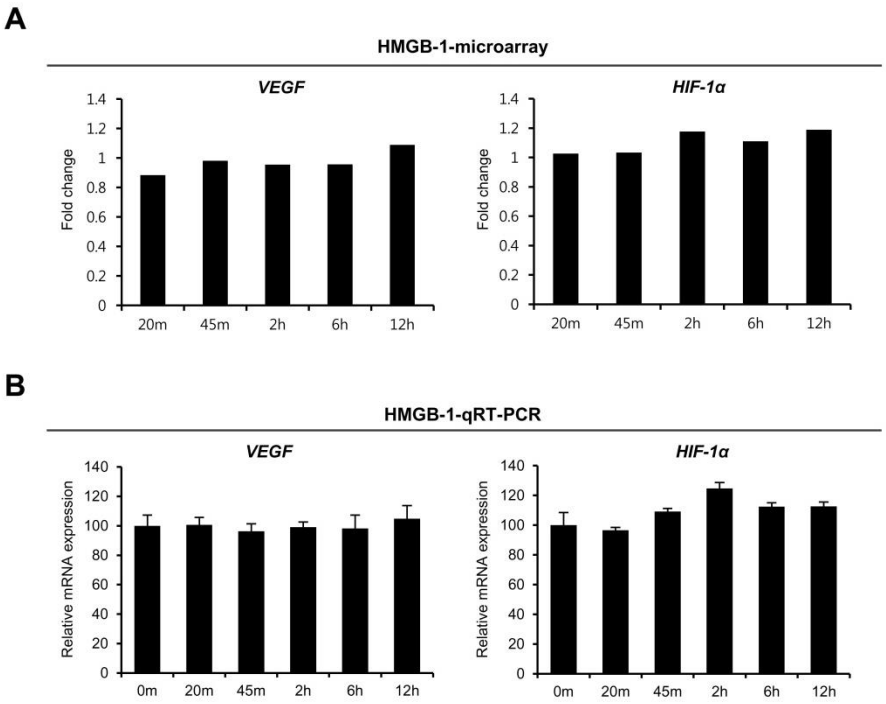


Figure 8. Validation of microarray results by qRT-PCR analysis of *VEGF* and *HIF-1α*. (A and B) The expression patterns of *VEGF* and *HIF-1α* that showed more than 1.5-fold upregulation during at least one time point according to the microarray (normalized to *β-actin* intensity) were compared with those of the same genes obtained by qRT-PCR analysis (normalized to *β-actin* expression) in another set of ECs treated with HMGB1 and VEGF.

Based on the known functions of these 24 genes, 13 genes were related to physiological or tumor angiogenesis. Notably, four genes (*CCL2*, *EGR1*, *EPHB1*, and *PCDH12*) are reported to independently promote angiogenesis in vitro or in vivo. *CCL2* accelerates cancer metastasis by promoting angiogenesis, and its expression is also regulated by the HMGB1-mediated activation of TLR4 signaling.[30,31] *EGR1* is a well-known angiogenic factor whose expression is reportedly regulated by HMGB1/RAGE signaling and VEGF/VEGFR signaling.[32,33] In addition, *EGR1* is reportedly involved in endothelial cell growth,

tumor angiogenesis, and tumor growth and has even been considered as an anti-angiogenic therapeutic target.[33] *EPHB1*, another well-known angiogenic factor, is expressed in the developing vasculature and promotes angiogenesis.[34] *PCDH12* is required for normal angiogenesis and is highly expressed in angiogenic ECs.[35] I here report the relationships between HMGB1 and the genes *EPHB1* and *PCDH12* for the first time.

The characterization of the 59 genes that were specifically upregulated by HMGB1 during at least two time points is crucial, as these genes explain HMGB1-specific angiogenesis. I found that 12 genes were functionally related to angiogenesis, and four (*ABL2, CEACAM1, KIT,* and *VIPR1*) are reported to directly induce in vitro and in vivo angiogenesis. *ABL2*, an oncogenic non-receptor tyrosine kinase, is reported to be critical for the survival and activation of ECs.[36] *CEACAM1*, a cell adhesion protein, functions as an activator in both physiological and tumor angiogenesis by inducing blood vessel remodeling through EC differentiation.[37] *KIT* is a well-known oncogenic receptor tyrosine kinase with reported involvement in physiological and tumor angiogenesis.[38] *VIPR1*, a vasoactive intestinal peptide receptor, plays a major role in progression and angiogenesis in a number of cancers.[39,40] In my wound healing and tube formation assays, I demonstrated that inhibition of these four genes dramatically disturbed HMGB1 mediated angiogenic features in ECs, which suggests that HMGB1 specifically upregulated genes play critical roles in evoking angiogenic features in ECs. In this context, HMGB1-mediated upregulation of these four genes could cause VEGF independent angiogenesis, which might explain drug resistance to anti-angiogenesis therapy based on VEGF inhibitors in cancer patients.

HMGB1 induces the upregulation of HMGB1-specific angiogenic factors, accompanying VEGF-like upregulation of some conventional angiogenic factors. This suggests that HMGB1 can independently induce angiogenesis and also synergistically enhance VEGF-mediated angiogenesis. In future studies, the angiogenic genes induced by HMGB1 should be further characterized to understand

how they contribute to angiogenesis in physiological and tumor microenvironments and how to inhibit abnormal angiogenesis in various cancers.

V. CONCLUSION

To identify the molecular mechanism of HMGB1-mediated angiogenesis, I performed gene expression microarray analysis of ECs after HMGB1 or VEGF treatment at various time points, and subsequent *in vitro* functional assays and gene ontology analysis using bioinformatics methods.

In this study, I found that;

1. HMGB1 and VEGF induce proangiogenic features in ECs, accompanying gradual activation of angiogenic pathways in a time-dependent manner.
2. Most HMGB1-induced angiogenic pathways overlapped with those activated by VEGF, although the activation time and upregulated genes belonging to the pathways substantially differed.
3. HMGB1 induced HMGB1-specific upregulation and VEGF-like upregulation of angiogenic and oncogenic genes, 121 genes and 5 genes out of the 83 genes upregulated more than 1.5-fold during at least two time points, respectively.
4. Inhibition of the four genes (ABL2, CEACAM1, KIT, and VIPR1) among the HMGB1 specifically upregulated genes dramatically disturbed HMGB1 mediated angiogenic features in ECs.

In summary, HMGB1 can independently induce angiogenesis by activating HMGB1-specific angiogenic factors and also synergistically enhance VEGF-mediated angiogenesis.

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ABSTRACT (IN KOREAN)

HMGB1 의 혈관형성 유도에 관여하는 신호전달체계 및 물질 규명

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권 유 진

비 히스톤 염색체 단백질인 고 이동성 그룹 박스 -1 (high-mobility group box-1, HMGB1)은 핵을 가진 거의 모든 세포에서 발현되며, HMGB1 의 이상 발현은 염증성 질환, 허혈 및 암과 같은 병리학적 상태와 관련이 있다. 이러한 조건 중 일부는 HMGB1 이 매개하는 하위 신호전달체계의 활성화에 의해 유발되는 비정상적인 혈관 신생을 동반한다. 그러나 HMGB1 이 혈관 신생을 유도하는 근본적인 기전에 대해서는 아직 많이 알려져 있지 않다.

본 연구에서는 HMGB1 또는 혈관내피성장인자 (vascular endothelial growth factor, VEGF) 처리 후 내피세포의 시간 의존적인 유전자 발현 마이크로 어레이 분석을 수행하였다. HMGB1 또는 VEGF 에 의해 상향 조절된 각 유전자의 신호전달계 분석을 통해 신호전달계에 속하는 활성화 시간 및 유전자 세트가 상이 함에도 불구하고, HMGB1 에 의해 유도되는 대부분의 혈관신생 신호전달계가 VEGF 에 의해 활성화되는 것을 확인하였다. 또한,

HMGB1 이 EGR1 을 비롯한 일부 VEGFR 신호전달관련 혈관신생인자들의 발현을 증가 시키는 것을 확인하였고, 더 중요하게는 생리학적 및 병리학적 조건하에 독립적으로 혈관 신생을 촉진하는 것으로 보고된 ABL2, CEACAM1, KIT 및 VIPR1 과 같은 새로운 혈관신생인자들을 활성화 시키는 것을 규명하였다.

결론적으로, HMGB1 은 독립적으로 HMGB1 특이적 혈관신생 인자를 활성화시킴으로써 혈관 신생을 유도하고, 또한 생리학적 및 병리학적 조건 하에서 VEGF 매개 혈관 신생에 대한 촉진제로서 중요한 역할을 한다.

핵심되는 말: 고 이동성 그룹 박스-1 (HMGB1), 혈관 내피 성장 인자 (VEGF), 혈관 신생, 내피 세포, 유전자 발현 프로파일

PUBLICATION LIST

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