



IL-28A/IL-10R β axis promotes angiogenesis via eNOS/AKT signaling and AP-1/NF- κ B/MMP-2 network by regulating HSP70-1 expression

Jun-Hui Song^{a,1}, Byungdoo Hwang^{a,1}, Sung Lyea Park^a, Hoon Kim^a, Soontag Jung^a, Changsun Choi^a, Hwan Myung Lee^b, Seok-Joong Yun^c, Yung Hyun Choi^d, Eun-Jong Cha^e, Cam Patterson^f, Wun-Jae Kim^{c,g}, Sung-Kwon Moon^{a,*}

^a Department of Food and Nutrition, Chung-Ang University, Anseong 456-756, Korea

^b Department of Cosmetic Science, Hoseo University, Asan-si 31499, Republic of Korea

^c Personalized Tumor Engineering Research Center, Department of Urology, Chungbuk National University, Cheongju, Chungbuk 361-763, South Korea

^d Department of Biochemistry, College of Oriental Medicine, Dongguk University, Busan 614-052, South Korea

^e Department of Biomedical Engineering, Chungbuk National University, Cheongju 361-763, Korea

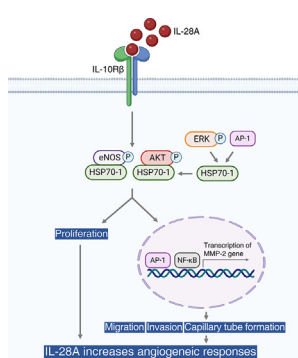
^f University of Arkansas for Medical Sciences, Little Rock, AR, USA

^g Institute of Urotech, Cheongju, Chungcheongbuk-do 361-763, Korea

HIGHLIGHTS

- IL-28A enhances angiogenesis via use of *in vitro* and animal models.
- IL-28A increases angiogenic responses via eNOS/AKT/ERK1/2 signaling.
- IL-28A promotes angiogenesis through induction of AP-1/NF- κ B/MMP-2 network.
- IL-28A/HSP70-1 axis leads angiogenesis via eNOS/AKT and AP-1/NF- κ B/MMP-2 cascade.
- IL-28A/IL-10R β dyad extends angiogenesis via eNOS/AKT and AP-1/NF- κ B/MMP-2 network.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 18 November 2023

Revised 6 August 2024

Accepted 7 August 2024

Available online 9 August 2024

Keywords:

IL-28A
IL-10R β
Angiogenesis
HSP70-1

ABSTRACT

Introduction: Angiogenesis plays a significant role in the development of tumor progression and inflammatory diseases. The role of IL-28A in angiogenesis and its precise regulatory mechanisms remain rarely elucidated.

Objectives: We report the novel regulatory role of IL-28A in physiological angiogenesis. The study aimed to elucidate the regulatory mechanisms involved in IL-28A-mediated angiogenesis and identify key genes associated with IL-28A-induced angiogenic responses.

Methods: To know the effect of IL-28A on angiogenesis, HUVECs were applied to perform proliferation, migration, invasion, tube formation, immunoblot, and EMSA. Gene expression changes in HUVECs following IL-28A treatment were analyzed by NGS. The functional role of HSP70-1 and IL-10R β in IL-28A-induced angiogenic responses was evaluated using PCR and siRNA knockdown. Animal studies were conducted by aortic ring *ex vivo* assays, Matrigel plug *in vivo* assays, and immunochemistry using HSP70-1 knockout and transgenic mice models. The efficacy of IL-28A in angiogenesis was confirmed in a hind-limb ischemia model.

* Corresponding author at: Department of Food and Nutrition, Chung-Ang University, Anseong 456-756, Republic of Korea.

E-mail address: sumoon66@cau.ac.kr (S.-K. Moon).

¹ These authors contributed equally to this work.

Results: Autocrine/paracrine actions in HUVECs regulated IL-28A protein expression. Exogenous IL-28A increased the proliferation of HUVECs via eNOS/AKT and ERK1/2 signaling. IL-28A treatment promoted migration, invasion, and capillary tube formation of HUVECs through induction of the AP-1/NF- κ B/MMP-2 network, which was associated with eNOS/AKT and ERK1/2 signaling. The efficacy of IL-28A-induced angiogenic potential was confirmed by aortic ring and Matrigel plug assay. HSP70-1 was identified as an IL-28A-mediated angiogenic effector gene using bioinformatics. Knockdown of HSP70-1 abolished angiogenic responses and eNOS/AKT signaling in IL-28A-treated HUVECs. IL-28A-induced microvessel sprouting formation was testified in HSP70-1-deficient and HSP70-1 transgenic mice. Flow recovery in hind-limb ischemia mice was accelerated by IL-28A injection. Finally, ablation of the IL-10R β gene impeded the angiogenic responses and eNOS/AKT signaling stimulated by IL-28A in HUVECs.

Conclusion: HSP70-1 drives the progression of angiogenesis by the IL-28A/IL-10R β axis via eNOS/AKT signaling and the AP-1/NF- κ B/MMP-2 network.

© 2024 The Authors. Published by Elsevier B.V. on behalf of Cairo University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Angiogenesis, the neovessel formation process, is a prominent step in the development of tumor progression and inflammatory diseases [1]. The exquisite network communication between proangiogenic factors and complicated signaling pathways in the intricate microenvironment regulates the promotion of neovessel formation [2,3]. Angiogenic factors, such as growth factors and cytokines released from endothelial cells as well as inflammatory cells and cancer cells, are the main contributors to the progression of angiogenesis [1–5]. Angiogenesis also necessitates the migration, tube formation, and proliferation of endothelial cells via multiple signaling pathways in response to angiogenic factors [2–6]. Human interferon (IFN)- λ s, class II cytokine receptor ligands or Type III IFNs, have antiviral activities and antitumor effects [7–11]. IFN- λ 1, -2, and -3 were initially denoted as interleukin (IL)-29, IL-28A, and IL-28B that were highly similar to the several IL-10-related cytokines (IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26) on the basis of sequence and structure of gene and protein [7–9]. Three cytokines, namely IL-29, IL-28A, and IL-28B, interact with an apparent heterodimeric receptor system composed of IL-28 receptor α (IL-28R α) chain in charge of unique ligand specificity and IL-10 receptor β (IL-10R β) chain shared with the other IL-10 family members (IL-10, IL-22, and IL-26) [7–9]. Although IL-10R β expression is ubiquitously distributed in most cells, expression of IL-28R α is restricted to, epithelial cells, B cells, plasmacytoid dendritic cells, macrophages, and hepatocytes [12,13]. Engagement of IL-28A with its receptors induced activation of AKT, MAPK, and JAK/STAT signaling involved in the antitumor and antiviral effects [7,14]. By contrast, we have previously demonstrated that IL-28A-induced migration was associated with the induction of the MAPKs/NF- κ B/MMP-9 signaling cascade in bladder cancer cells [15]. IL-28 produced by canine myeloid-derived suppressor cells (MDSCs) induced tumor angiogenesis, EMT, and invasion and migration of tumor cells via stimulation of STAT3 [16]. However, the exact regulatory mechanism of IL-28A in the promotion of angiogenesis is rarely elucidated.

Heat shock protein 70 (HSP70) functions as a molecular chaperone to rescue cells from harmful effects in response to stressors [17,18]. HSP70 maintains physiological homeostasis regulating proliferation, cell cycle, cell death, migration, and cell signaling [17,18]. The HSP70 family is divided into stress-induced (HSP70-1, HSP70-2, HSP70-3, and HSPA6) and non-stress-induced members (HSPA1L, HSPA2, HSPA5, HSPA13, and HSPA14) [19]. The HSP70-1 and HSP70-3 genes, which are referred to together as HSP70-1 due to high homology in their gene locus, are induced by environmental stress [20]. HSP70-2 is a spermatid-specific HSP70 gene in mice. HSP70-2 knockout mice are male-sterile because of the enlargement of apoptotic testis cells [21]. HSPA6 is expressed in the human genome and lacking in mice and rats

[22]. Evidence from our group demonstrated that IL-5-induced HSP70-1 is involved in the progression of angiogenesis through eNOS signaling [23].

The various members of the cytokine family are reported to regulate both negative and positive angiogenic responses [4,23–30]. However, whether the IL-28 family regulates the angiogenic progression involved in HSP70 expression remains to be investigated. In this study, we examined the novel regulatory mechanism of IL-28A as an angiogenic factor using *in vitro* experiments and an animal model. Furthermore, we also report the potential role of molecular chaperone HSP70-1 in IL-28A/IL-10R β axis-promoted angiogenesis through modulating eNOS/AKT signaling and the AP-1/NF- κ B/MMP-2 network using *in vitro* experiments and a genetic mouse model.

Results

IL-28A expression is promoted in human muscle-invasive bladder cancer (MIBC) tissues

Previously, we addressed that IL-28A mRNA levels were elevated in MIBC tissues in comparison to the adjacent normal bladder tissues [31,32]. Our subsequent data suggested that IL-28A induced invasion and migration of bladder cancer cells [15]. In the present study, we conducted an immunohistochemistry (IHC) experiment to verify the expression level of IL-28A between MIBC and surrounding bladder tissues. IL-28A expression was significantly upregulated in MIBC (Fig. 1A), suggesting that IL-28A might be participated in the progression and development of bladder cancer. To establish the relationship between IL and 28A expression and angiogenesis in MIBC tissue, we performed IHC staining for CD31. Our findings indicated that CD31 expression was markedly higher in MIBC tissues compared to surrounding bladder tissues (Fig. 1B). This observation supports our hypothesis that the upregulation of IL-28A in MIBC tissues is associated with enhanced angiogenesis. These results led us to investigate whether IL-28A functions as an angiogenic activator during tumor occurrence or progression.

IL-28A stimulates the proliferation of HUVECs by inducing eNOS/AKT/ERK1/2 signaling

We examined the level of IL-28A protein expression in HUVECs. While a low level of endogenous IL-28A expression was observed in HUVECs, the level was significantly induced in IL-28A-treated cells (Fig. 1C and D). These data indicated that autocrine/paracrine actions in HUVECs mediated IL-28A expression. To investigate whether IL-28A induces angiogenic responses, we first assessed the proliferation ability of HUVECs using the MTT assay. The prolif-

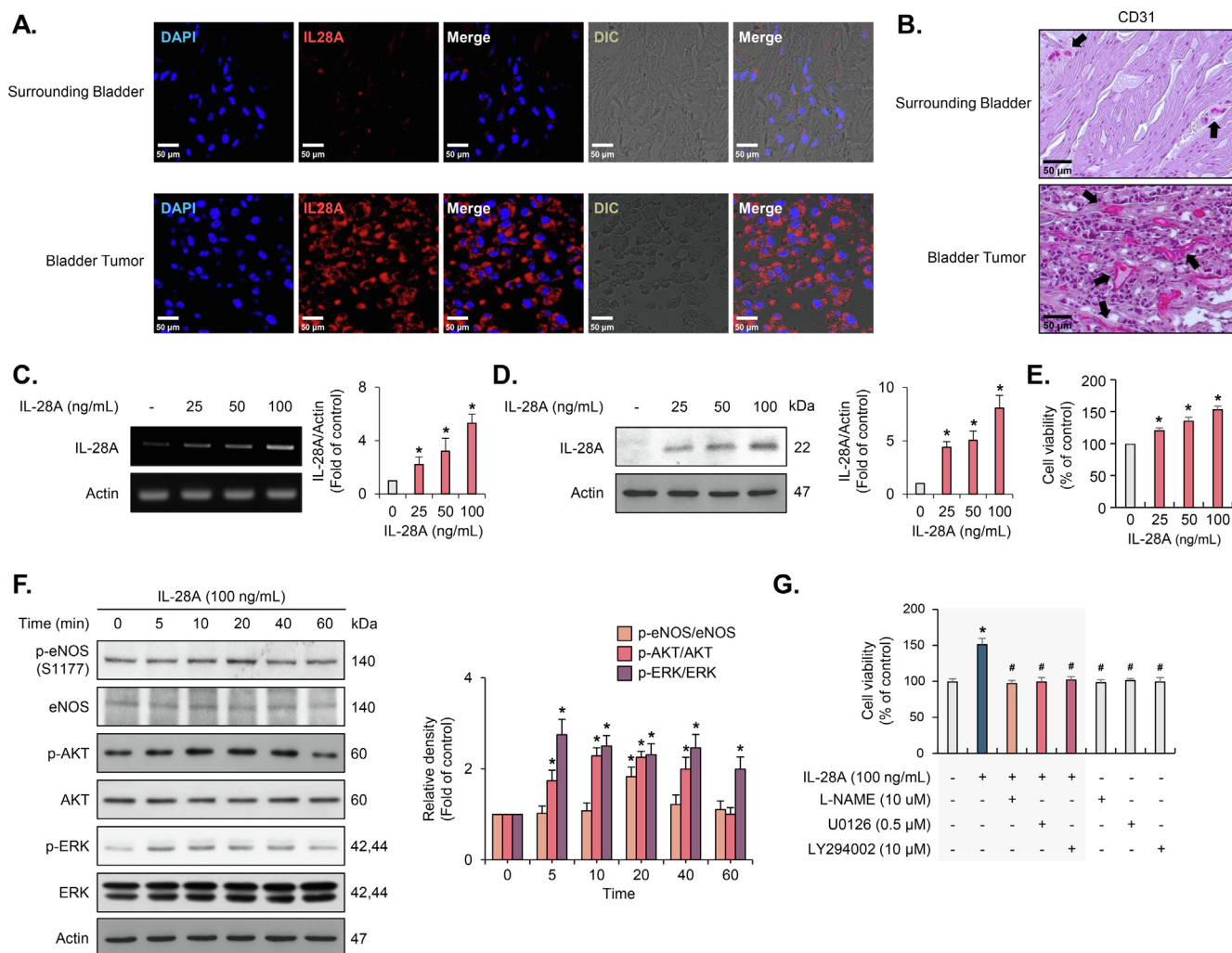


Fig. 1. IL-28A induced proliferation of HUVECs via eNOS/AKT/ERK1/2 signaling. A Immunofluorescence staining of IL-28A in surrounding bladder and muscle-invasive bladder cancer (MIBC) tissues. The images show DAPI staining for nuclei (blue), IL-28A staining (red), and merged images to indicate co-localization. Differential interference contrast (DIC) images provide detailed structural context. The merged images combine DAPI, IL-28A, and DIC to show the overall localization and expression of IL-28A. The scale bar represents 50 μ m. B Immunohistochemical (IHC) staining for CD31 in surrounding bladder and MIBC tissues. The scale bar represents 50 μ m. C RT-PCR analysis was performed to measure IL-28A mRNA expression in HUVECs treated with IL-28A compared to control. D IL-28A treatment induced production of IL-28A in HUVECs. E MTT assay was performed in IL-28A-treated HUVECs. F Phosphorylation level of eNOS, AKT, and ERK1/2 was examined using immunoblot analysis. The non-phosphorylation level of each protein was used as an internal control. G Inhibition of IL-28A-induced proliferation of HUVECs in the presence of specific signaling inhibitors, such as L-NAME (eNOS), LY294002 (AKT), and U0126 (ERK1/2). Data are presented as mean values $\pm$ SE from three independent experiments. * $P < 0.05$ compared with control, # $P < 0.05$ compared with IL-28A treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

eration of HUVECs was increased by IL-28A treatment (Fig. 1E). Next, the signaling molecules involved in the IL-28A-induced proliferation of HUVECs were determined. The eNOS enzyme plays a crucial role in the production of nitric oxide (NO), a key mediator in endothelial cell proliferation, migration, and angiogenesis [33,34]. NO enhances vascular permeability and blood vessel formation, thereby facilitating angiogenesis [29,35]. Similarly, the activation of AKT and ERK1/2 leads to increased endothelial cell proliferation and migration, which are vital processes for angiogenesis [14,29,36]. The immunoblot result showed that IL-28A promoted eNOS, AKT, and ERK1/2 phosphorylation (Fig. 1F). To explore the specificity of the signaling molecules in IL-28A-treated HUVECs, their specific inhibitors L-NAME (eNOS), LY294002 (AKT), and U0126 (ERK1/2) were tested. The phosphorylation level of eNOS, AKT, and ERK1/2 induced by IL-28A was suppressed in the addition of its specific inhibitors (Fig. 4B). To know the direct correlation between cell proliferation and signaling molecules, the HUVECs were treated with specific inhibitors, followed by IL-28A treatment. Treatment of the specific inhibitors

in HUVECs led to a significant decline in the IL-28A-stimulated cell proliferation (Fig. 1G). Our results indicate that IL-28A stimulates HUVEC proliferation through induction of eNOS/AKT/ERK1/2 signaling.

IL-28A induces the migration, invasion, and tube formation of HUVECs by upregulating AP-1/NF- κ B/MMP-2 axis through stimulation of eNOS/AKT/ERK1/2 signaling

We next examined the mobility and tube formation of HUVECs after treatment with IL-28A. Wound closure of scratched HUVEC monolayers was promoted in the presence of IL-28A (Fig. 2A). Furthermore, IL-28A elevated the number of invaded cells across the membrane (Fig. 2B) and enhanced the tube formation by HUVECs (Fig. 2C and Supplemental Fig. S2A). Transcription factors, such as AP-1 and NF- κ B, are known to regulate the expression of MMP-2 and MMP-9, which play a crucial role in extracellular matrix remodeling and are vital for angiogenesis [15,37,38]. We subsequently explored the expression level of MMPs and their

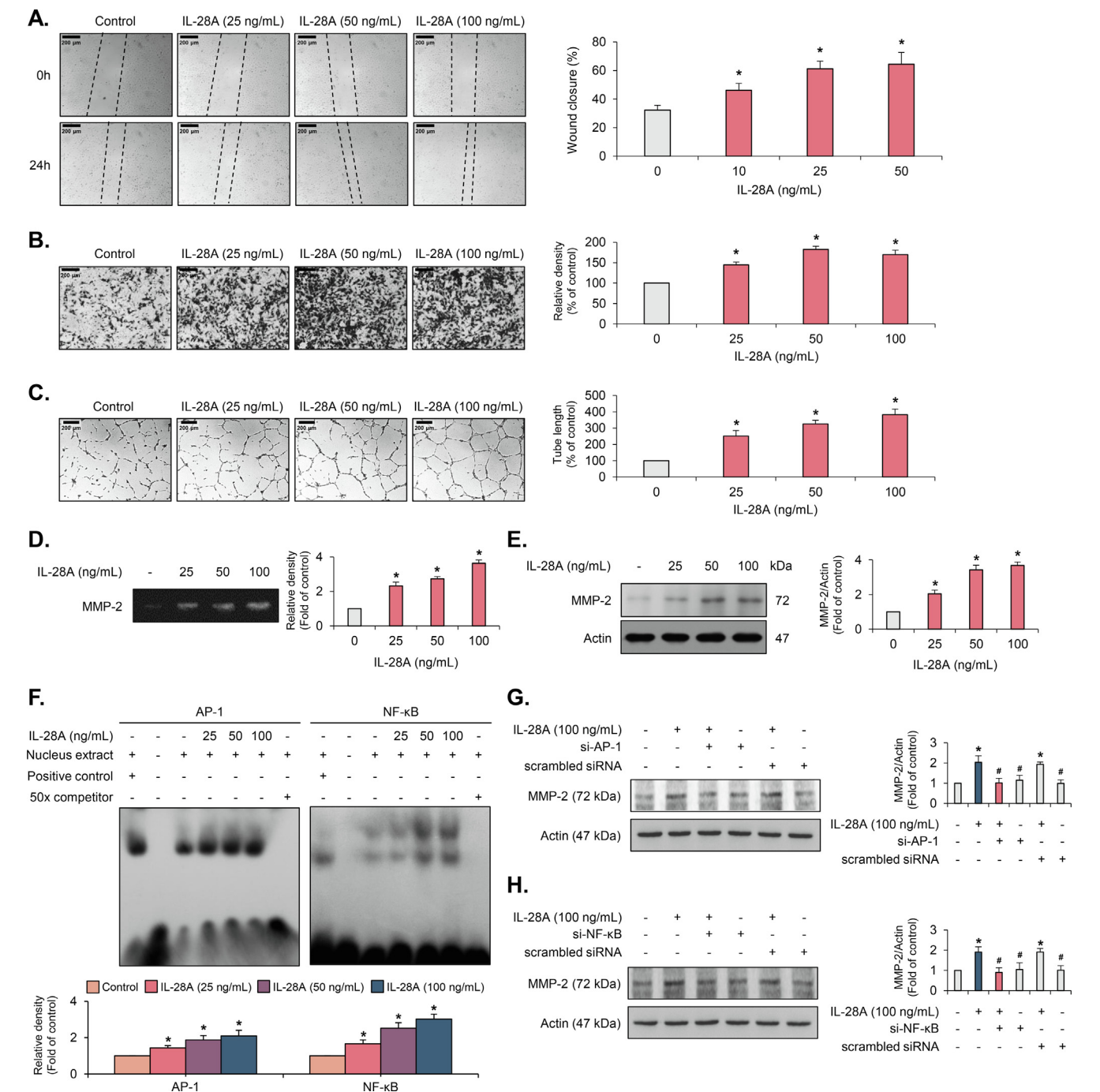


Fig. 2. IL-28A promoted migration, invasion, and tube formation via transcription factor-mediated MMP-2 expression in HUVECs. A Wound-healing assay was performed to determine the wound closure ability of HUVECs after IL-28A treatment for 24 h. The scale bar represents 200 μ m. B Result of invasive potential induced by IL-28-A in HUVECs for 24 h. The scale bar represents 200 μ m. C Colony tube formation assay in HUVECs treated with IL-28A for 24 h. The scale bar represents 200 μ m. D, E MMP-2 level was examined in IL-28A-treated HUVECs using gelatin zymography and immunoblot analysis. F IL-28A-promoted binding ability of AP-1 and NF- κ B was determined from the nuclear extract. G HUVECs were transfected with AP-1 siRNA or scrambled siRNA and then exposed to IL-28A for the analysis of MMP-2 levels through immunoblot. H Analysis of MMP-2 protein levels through immunoblot in HUVECs transfected with NF- κ B siRNA or scrambled siRNA following IL-28A treatment. Data are presented as mean values $\pm$ SE from three independent experiments. * P <0.05 compared with control.

related transcription factors in IL-28A-treated HUVECs. IL-28A treatment notably increased the expression and activity level of MMP-2, as evidenced by immunoblot and zymography assay (Fig. 2D and E). Moreover, the electrophoretic mobility shift assay (EMSA) analysis revealed that IL-28A upregulated the binding ability of AP-1 and NF- κ B motif (Fig. 2F). To provide direct evidence for the involvement of AP-1 and NF- κ B in IL-28A-induced MMP-2 expression, we used siRNA to silence AP-1 and NF- κ B in HUVECs

(Supplemental Fig. S1A, B). Our results showed that silencing AP-1 and NF- κ B significantly reduced the upregulation of MMP-2 protein levels in response to IL-28A treatment (Fig. 2G and H). Additionally, we assessed the regulatory role of eNOS/AKT/ERK1/2 signaling in the IL-28A-mediated mobility and tube formation of HUVECs. The L-NAME treatment significantly inhibited the capacity of IL-28A to induce migration, invasion, and tube formation of HUVECs (Fig. 3A–F and Supplemental Fig. S2B). Similar results

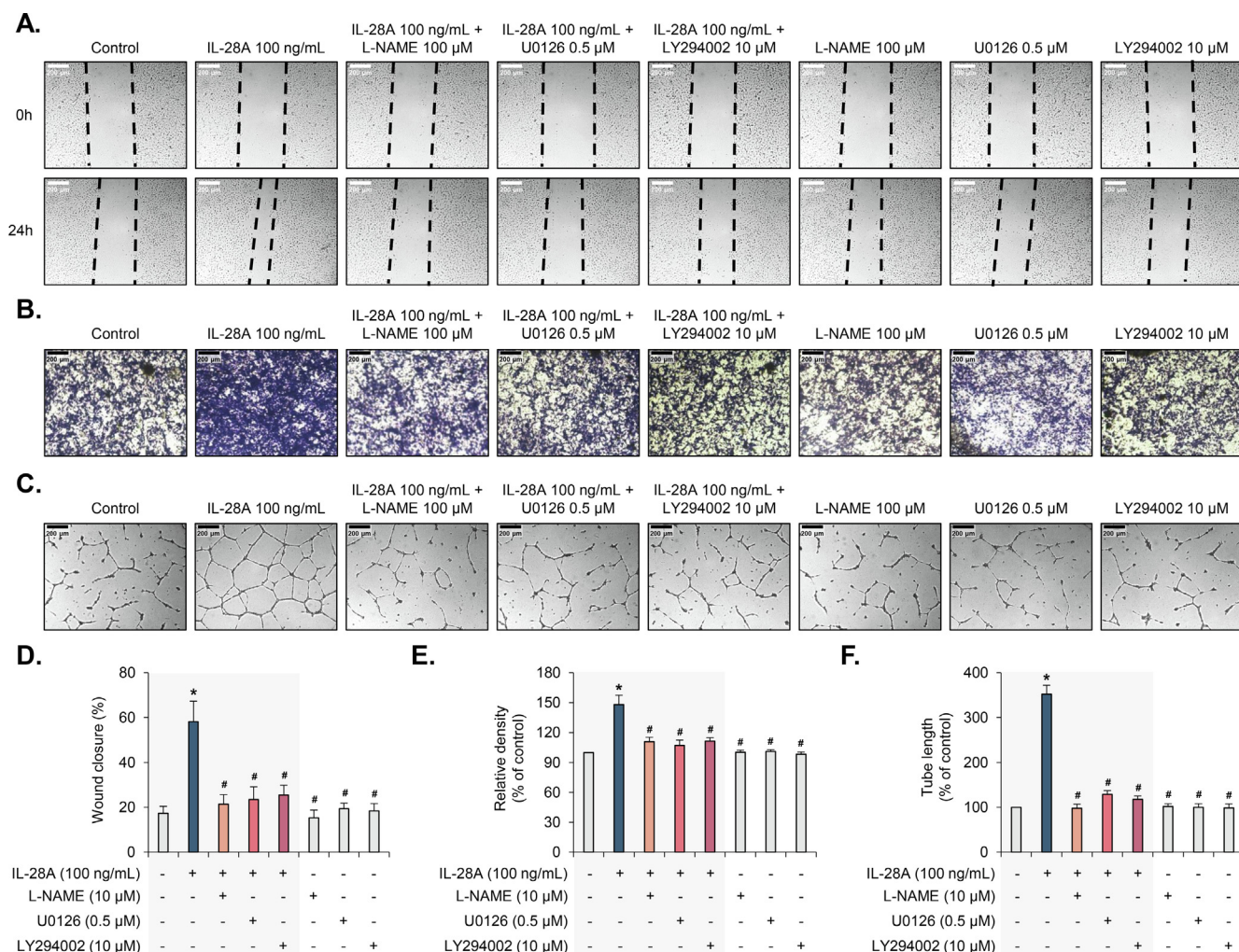


Fig. 3. Enos/akt/erk1/2 signaling is involved in migration, invasion, and tube formation in il-28a-treated huvecs. cells were treated with il-28a for 24 h in the presence or absence of L-NAME, LY294002, and U0126. A, D Wound-healing migration, B, e invasion, and C, F colony tube forming assay was performed in HUVECs. All scale bars represent 200 μ m. Data are presented as mean values $\pm$ SE from three independent experiments. * P <0.05 compared with control, # P <0.05 compared with IL-28A treatment.

were obtained in the presence of LY294002 and U0126 (Fig. 3A–F and Supplemental Fig. S2B). The elevated level of MMP-2 in IL-28A-treated HUVECs was also suppressed by treatment with each of the three inhibitors (Fig. 4A and B). The IL-28A-induced binding activity of AP-1 and NF- κ B was abolished by blockade of eNOS, AKT, and ERK1/2 signaling (Fig. 4C). Our findings demonstrate that IL-28A-mediated tube formation and mobility of HUVECs are linked to up-regulation of the AP-1/NF- κ B/MMP-2 axis through increased phosphorylation levels of the eNOS/AKT/ERK1/2 signaling.

IL-28A promotes microvessel formation ex vivo and in vivo

To examine the angiogenic responsive effect of IL-28A using an animal model, an aortic ring *ex vivo* assay was performed. IL-28A treatment increased the total length of micro-neovessels emerging from the isolated aortic ring compared with the control (Fig. 5A and B). Further evidence supporting IL-28A-mediated angiogenesis was obtained by performing a Matrigel plug *in vivo* assay. Implanted Matrigel plugs infused with IL-28A retained better vessel perfusion and contained more hemoglobin (an indicator of neovessel formation) compared to control plugs containing PBS buffer (Fig. 5C and D). The formation of micro-neovessels in the capillary tubes was certified through immunostaining experiment

with the endothelial cell marker CD31 (Fig. 5E). Furthermore, IL-28A-stimulated neovessel formation was verified with the addition of IL-28A antibody under both animal experiments (Fig. 5F–J). These data strongly demonstrated the potent angiogenic potential of IL-28A *in vivo* and *ex vivo*.

Selection of HSP70-1 in IL-28A-treated HUVECs under gene expression file

We carried out a NGS analysis to identify the IL-28A-mediated angiogenic effector genes. After treatment of HUVECs with or without IL-28A for 6, 12, and 24 h, a data analysis technique was applied to analyze the differentially expressed gene (DEG) expression profiles. Twenty-eight genes were significantly upregulated, and 22 genes were downregulated among the genes changed by more than ± 1.5 -fold when comparing between HUVECs treated with IL-28A at any time and control cells (Supplemental Table S1). Hierarchical clustering of genes and gene ontology (GO) analysis revealed the molecular functions of the genes represented in the sets of DEGs in the two groups (Fig. 6A and B). Both analyses revealed 7 clusters associated with biological functions (Fig. 6B). These clusters belonged to the functional categories of response to stress, response to stimulus, response to biotic stimulus, response to unfolded protein, response to protein stimulus,

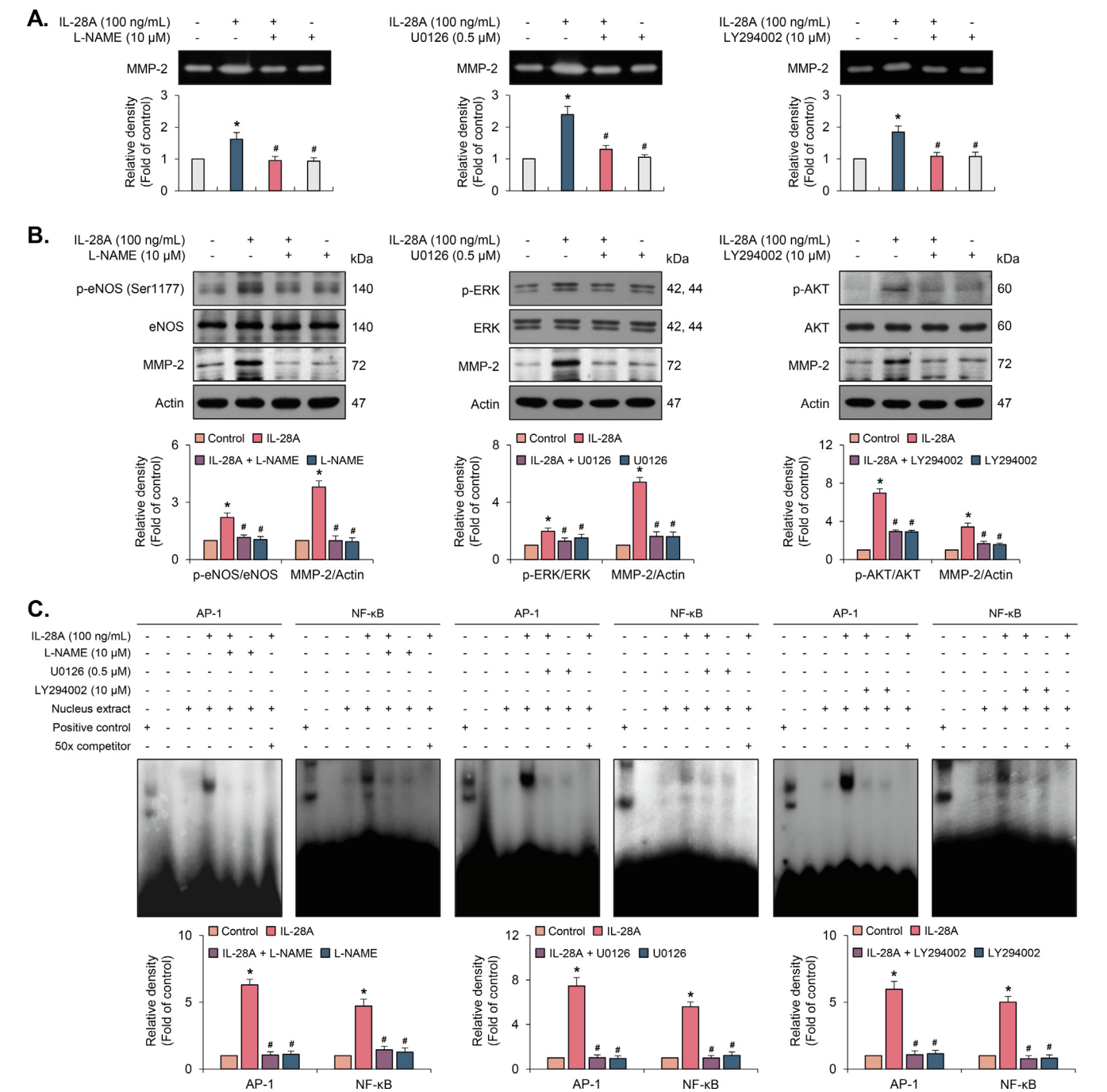


Fig. 4. Transcription factor-mediated MMP-2 expression is abolished following inhibition of eNOS/AKT/ERK1/2 signaling in HUVECs treated with IL-28A. After pretreatment of HUVECs with L-NAME, LY294002, and U0126 for 40 min, cells were further incubated with IL-28A for 24 h. A Enzyme activity of MMP-2 was determined by zymographic assay. B Immunoblot results of protein level of MMP-2 and phosphorylation level of signaling molecules (eNOS, AKT, and ERK1/2). C Binding ability of AP-1 and NF- κ B was performed using EMSA. Data are presented as mean values $\pm$ SE from three independent experiments. * P <0.05 compared with control, # P <0.05 compared with IL-28A treatment.

interferon-mediated immunity, and protein complex assembly (Fig. 6B). Interestingly, the HSP family genes, such as HSP70-1, HSPA6, and HSP70-2, were the most upregulated genes in cells treated with IL-28A; they displayed a maximal fold-change at 24 h (Fig. 6A and Supplemental Table S1), whereas the most down-regulated genes in HUVECs stimulated by IL-28A were GP109 and DYRK2 at 24 h (Fig. 6A and Supplemental Table S1). In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and the gene network diagram of gene interaction analyzed by

Ingenuity Pathway Analysis (IPA) based on the genes upregulated by more than 1.5-fold revealed a set of candidate hub genes that included HSP70-1, HSPA6, and HSP70-2, which are mainly enriched in the legionellosis, longevity regulating pathway, and antigen processing and presentation (Fig. 6C and Supplemental Table S2). Because of the deficient reproductive ability of HSP70-2 and the non-expression pattern of HSPA6 in mice [21,22], our subsequent experiments focused on the HSP70-1 gene as a novel angiogenic factor that responds to IL-28A.

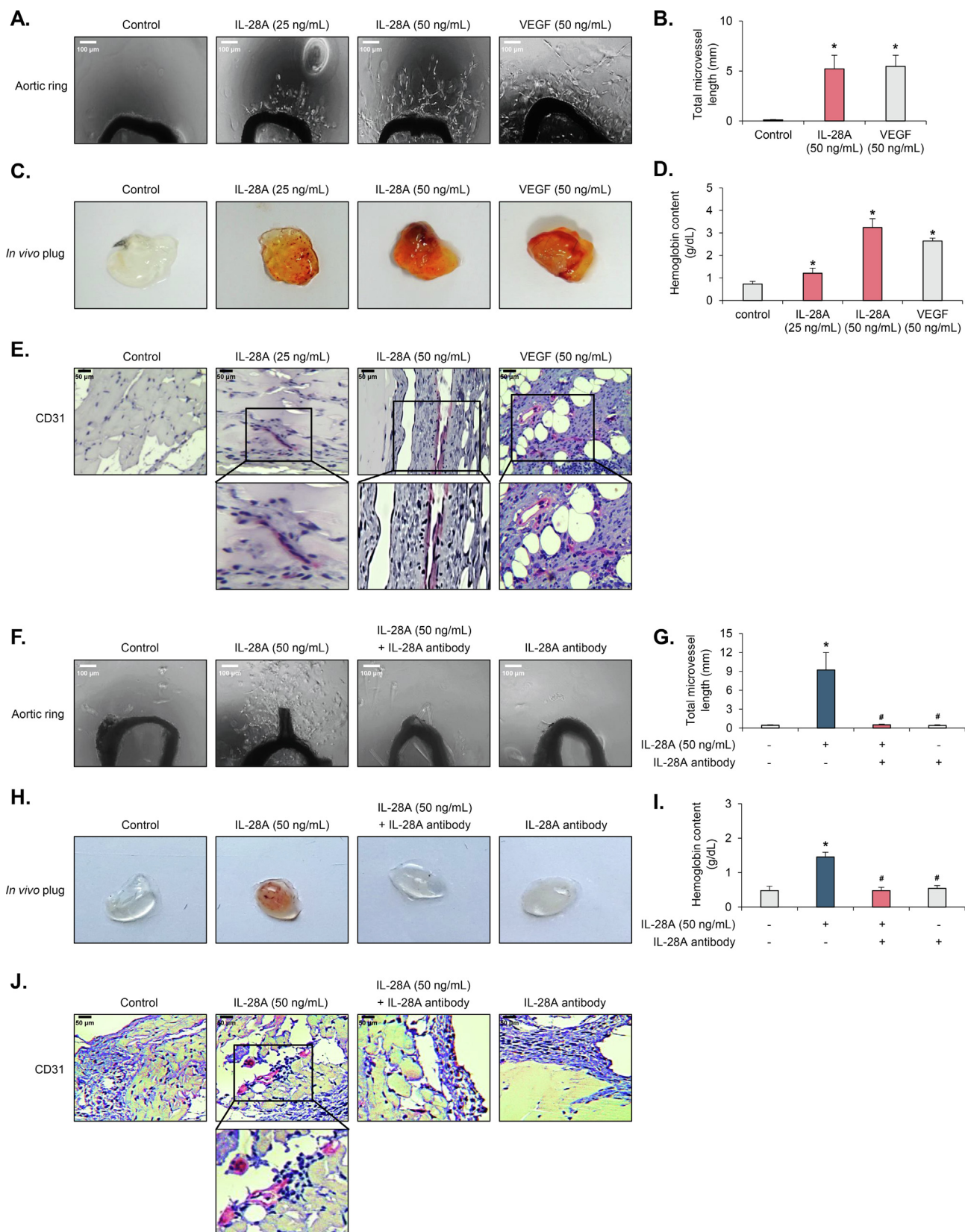


Fig. 5. IL-28A induced microvessel outgrowth of angiogenesis *ex vivo* and *in vivo*. A Image of microvessel formation, followed by IL-28A treatment using aortic ring assay *ex vivo*. B Quantification of the number of vessels sprouting after IL-28A treatment. C Angiogenic effect of IL-28A in Matrigel plug *in vivo* assay. D Quantitative analysis of hemoglobin content. E Image of CD31 immunostaining in Matrigel plug. Effect of IL-28A antibody in IL-28A-mediated angiogenesis was examined via F, G aortic ring assay *ex vivo*, H, I Matrigel plug *in vivo* assay, and J immunostaining of CD31 antibody. The aortic ring assay *ex vivo* has a scale bar of 100 μ m. The immunostaining of the CD31 antibody has a scale bar of 50 μ m. Data are presented as mean values $\pm$ SE from three independent experiments. * P <0.05 compared with control, # P <0.05 compared with IL-28A treatment.

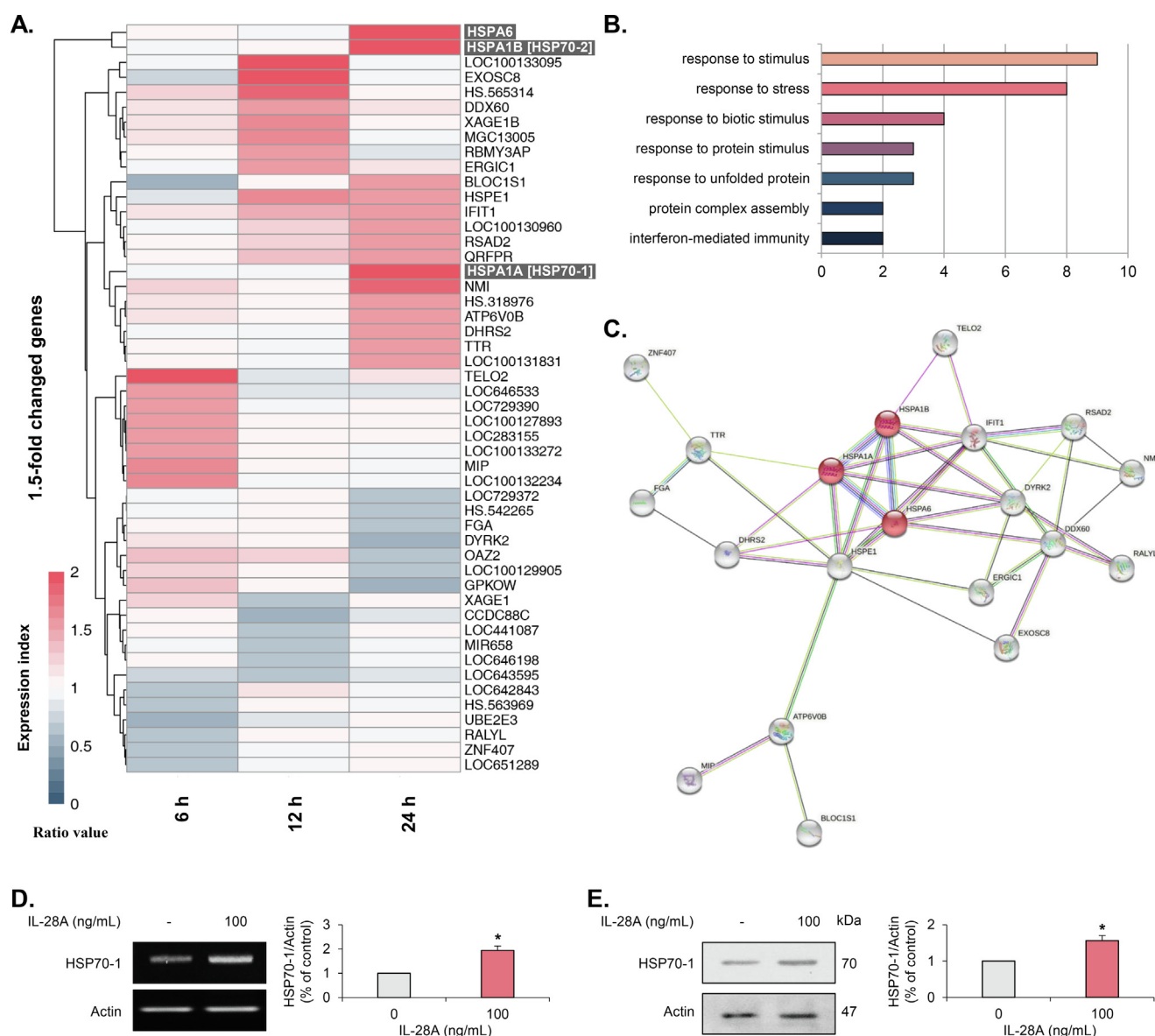


Fig. 6. Identification of HSP70-1 as a potential target gene in IL-28A-mediated angiogenesis. A Hierarchical clustering of RNA-seq expression data for IL-28A-induced genes. Red areas represent 1.5-fold upregulated DEGs, and green areas represent 1.5-fold downregulated DEGs between nontreated- and IL-28A-treated HUVECs. B Gene ontology annotations of upregulated DEGs. C Gene network analysis of 1.5-fold upregulated DEGs in IL-28A-induced HUVECs. The most significant modules of DEGs represent top three genes (red color), which were identified as HSP70-1, HSPA6, and HSP70-2. D, E Expression level of HSP70-1 was confirmed by RT-PCR and immunoblot analysis in IL-28A-treated HUVECs. Data are presented as mean values $\pm$ SE from three independent experiments. * $P < 0.05$ compared with control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HSP70-1 regulates the IL-28A-stimulated proliferation of HUVECs through phosphorylation of eNOS/AKT signaling

The expression level of HSP70-1 in IL-28A-treated HUVECs was validated by reverse transcriptase–polymerase chain reaction (RT-PCR). Addition of IL-28A elevated the level of HSP70-1 mRNA in HUVECs at 24 h (Fig. 6D). Up-regulation of HSP70-1 protein was observed by immunoblot in HUVECs treated with IL-28A using HSP70 antibody, which designed to bind specific epitope for HSP70-1 (Fig. 6E). To further comprehend the functional role of HSP70-1 in the angiogenic responses induced by IL-28A, we used a small-interfering RNA (siRNA)-specific HSP70-1 (si-HSP70-1) or a scrambled siRNA (scramble) (Supplemental Fig. S1C). Knockdown of HSP70-1 reduced the IL-28A-induced proliferation of HUVECs (Fig. 7A). Next, we explored whether HSP70-1 regulates the signaling pathways in IL-28A-treated HUVECs. The increased phosphorylation of eNOS and AKT stimulated by IL-28A was suppressed in the

HUVECs transfected with si-HSP70-1 (Fig. 7B). However, IL-28A-stimulated ERK1/2 phosphorylation was not affected by si-HSP70-1 transfection (Fig. 7B). To investigate the potential affinity of HSP70-1 for signaling molecules eNOS and AKT in IL-28A-treated HUVECs, an immunoprecipitation assay was performed. IL-28A treatment significantly induced the association between HSP70-1 and eNOS as well as AKT in HUVECs (Fig. 7C). Our results suggest that HSP70-1 is a crucial effector in the IL-28A-induced proliferation of HUVECs via upregulation of eNOS/AKT phosphorylation.

HSP70-1 modulates IL-28A-mediated mobility and tube formation of HUVECs by enhancing the AP-1/NF- κ B/MMP-2 axis

To elucidate the role of HSP70-1 in motility and tube formation of HUVECs following IL-28A treatment, we performed a wound-healing assay, invasion assay, and colony tube formation assay.

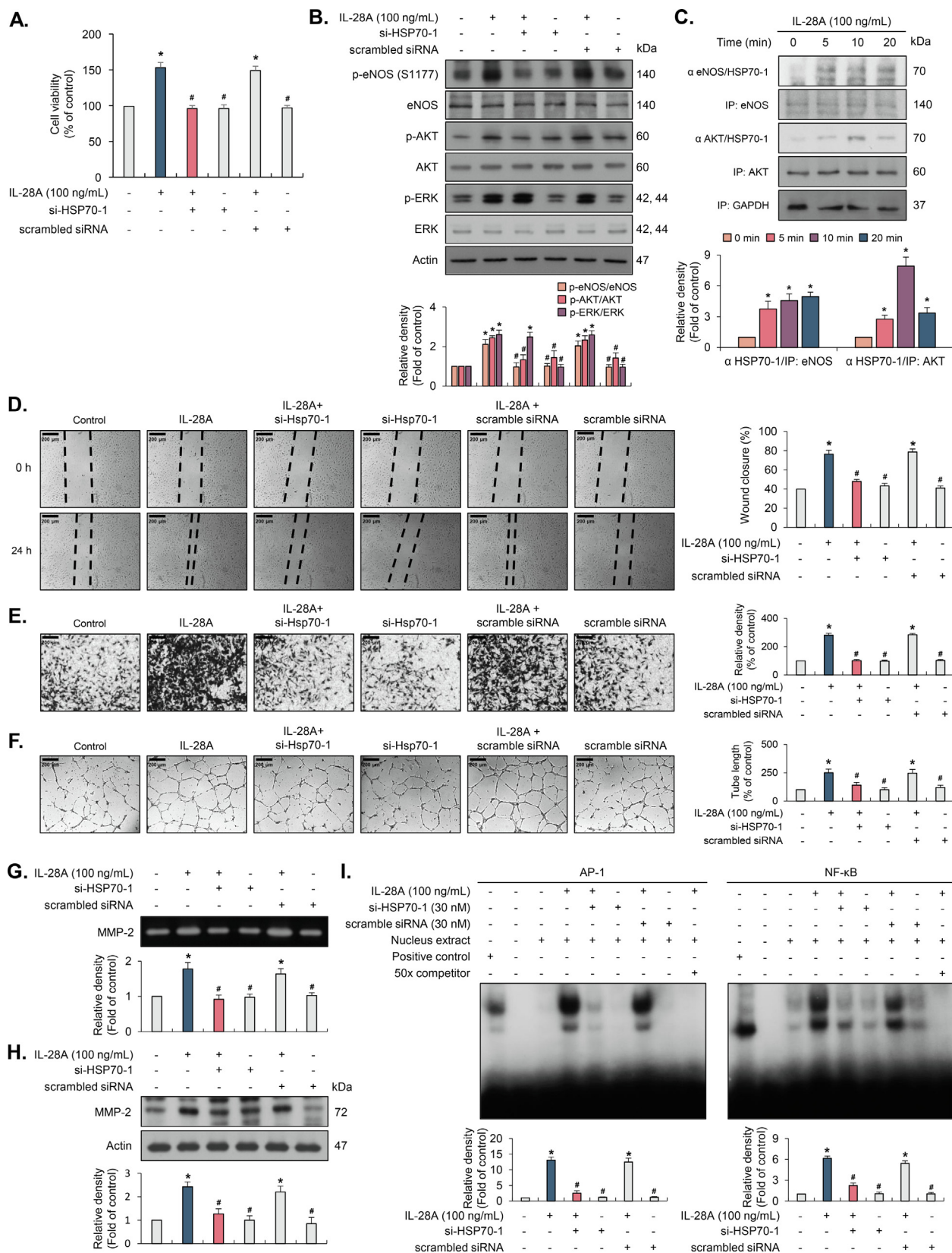


Fig. 7. Knockdown of HSP70-1 attenuated IL-28A-induced angiogenic programs *in vitro*. A, D–F IL-28A-mediated proliferation, migration, invasion, and tube formation was suppressed in HUVECs transfected with HSP70-1 siRNA (si-HSP70-1). All scale bars represent 200 μ m. B Phosphorylation level of eNOS, AKT, and ERK1/2 induced by IL-28A was measured by immunoblot analysis in si-HSP70-1-transfected HUVECs. C HUVECs were treated with IL-28A for 24 h. Cell lysates were immunoprecipitated with the anti-eNOS antibody or anti-AKT antibody, followed by immunoblot analysis using HSP70-specific antibody. GAPDH was used as an internal control. G, H IL-28A-induced MMP-2 level was performed in si-HSP70-1-transfected cells by analysis of gelatin zymography (enzyme activity) and immunoblot (protein expression). I EMSA experiment was performed to determine the IL-28A-promoted AP-1 and NF- κ B binding activity in HUVECs transfected with si-HSP70-1. Data are presented as mean values $\pm$ SE from three independent experiments. * P <0.05 compared with control, # P <0.05 compared with IL-28A treatment.

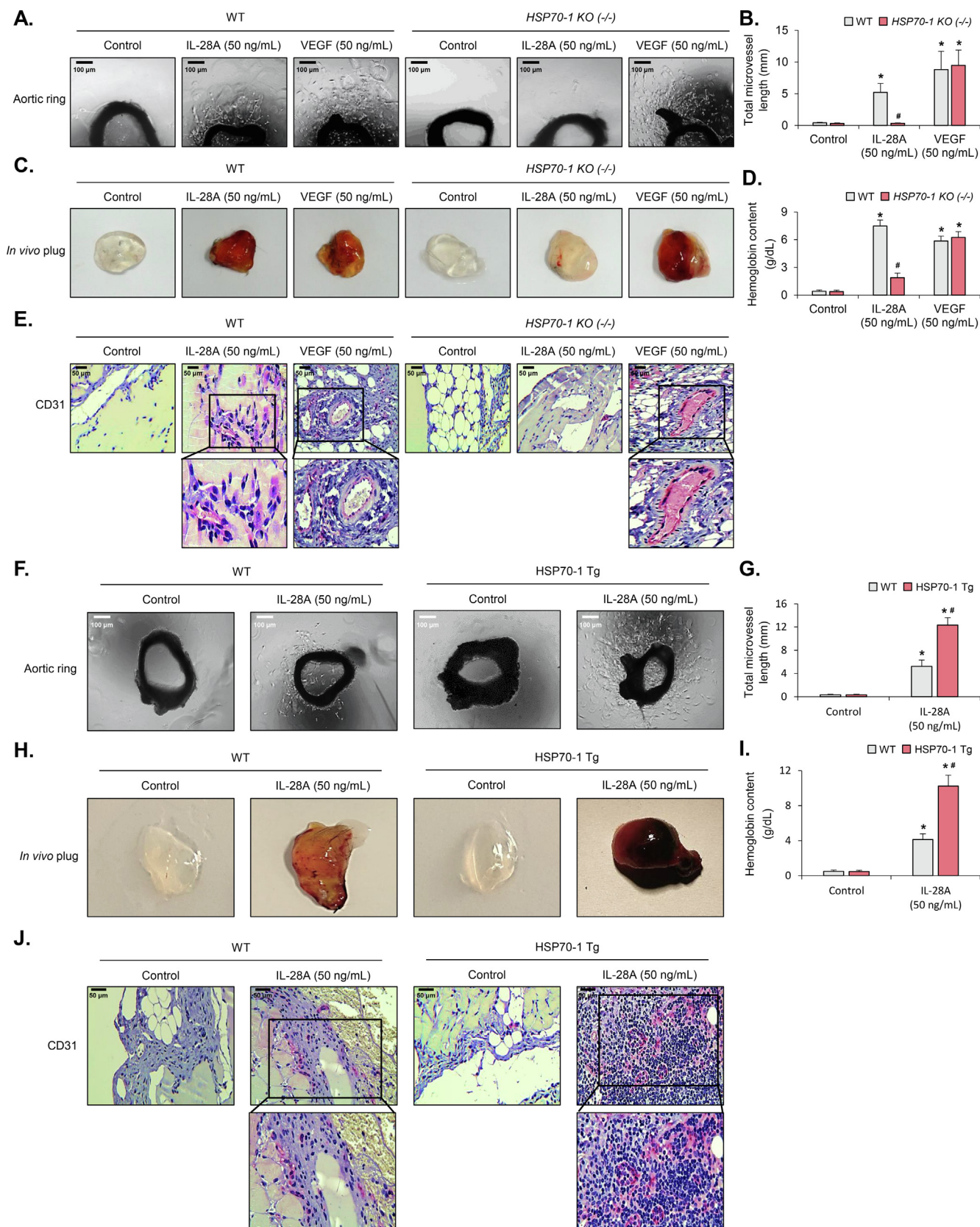


Fig. 8. Effect of angiogenesis induced by IL-28A in HSP70-1 mutant animal models. HSP70-1 knockout mice (KO) and HSP70-1 transgenic mice (Tg) were used. A Microvessel outgrowth at 9 days using aortic ring assay in KO and wild-type mice (WT). B Quantification of microvessel sprouting. C Image of angiogenic effect from Matrigel plug *in vivo* assay at 7 days. D Quantitative analysis of hemoglobin content. E Image of CD31 immunostaining from Matrigel plug. F Aortic assay experiment in Tg and WT. G Quantitative analysis of microvessel sprouting. H Matrigel plug *in vivo* assay in Tg and WT. I Statistic analysis of hemoglobin content. J Immunostaining of Matrigel plug against CD31 antibody. The aortic ring assay *ex vivo* has a scale bar of 100 μ m. The immunostaining of the CD31 antibody has a scale bar of 50 μ m. Data are presented as mean values $\pm$ SE from three independent experiments. * P <0.05 compared with control, # P <0.05 compared with IL-28A treatment in WT.

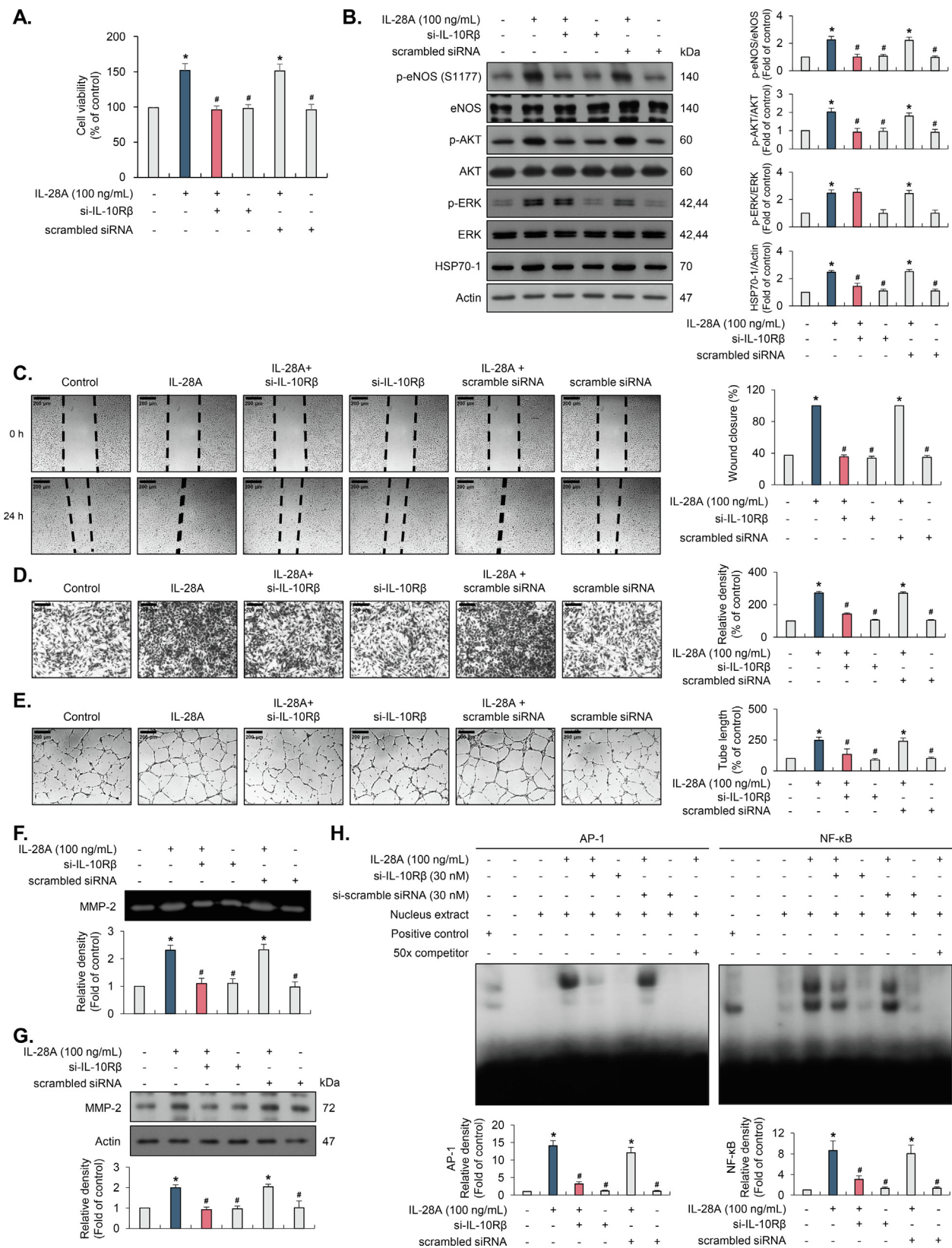


Fig. 9. IL-28A/IL-10R β axis is essential for the potential angiogenic effect of HUVECs. A, C–E IL-10R β siRNA (si-IL-10R β) or scrambled siRNA were transfected into HUVECs, then cells were examined for extent of proliferation, migration, invasion, and tube formation stimulated by IL-28A. All scale bars represent 200 μ m. B Subsequently, IL-28A-induced phosphorylation of eNOS, AKT, and ERK1/2 was investigated in cells transfected with si-IL-10R β or scrambled siRNA using immunoblot. Additionally, the expression of HSP70-1 was examined under the same conditions. F, G IL-28A-stimulated MMP-2 level was studied in the presence of si-IL-10R β or scrambled siRNA transfectants. H After transfection of cells with si-IL-10R β or scrambled siRNA, EMSA was performed to determine the binding ability of AP-1 and NF- κ B motifs. Data are presented as mean values $\pm$ SE from three independent experiments. * P <0.05 compared with control, # P <0.05 compared with IL-28A treatment.

Down-regulation of HSP70-1 suppressed the ability of wound closure rate, invasiveness, and capillary-like tube formation in IL-28A-induced HUVECs (Fig. 7D–F and Supplemental Fig S2C). In addition, the IL-28A-promoted MMP-2 level was abrogated in the presence of si-HSP70-1, as evidenced by immunoblot and zymographic studies (Fig. 7G and H). HSP70-1 knockdown inhibited the binding ability to AP-1 and NF- κ B motifs stimulated by IL-28A in HUVECs (Fig. 7I). Our results indicate that HSP70-1 expression is correlated with IL-28A-induced mobility and tube formation of HUVECs via increased MMP-2 expression by upregulating both AP-1 and NF- κ B transcription factors.

HSP70-1 is an indispensable factor for IL-28A-mediated angiogenesis ex vivo and in vivo

To confirm the direct regulatory role of HSP70-1 in IL-28A-stimulated angiogenesis derived from *ex vivo* and *in vivo*, we used both HSP70-1 knockout and transgenic mice models. In the aortic ring experiments, IL-28A induced the ability of neovessel sprouting in wild-type (WT) mice (Fig. 8A and B). In addition, the IL-28A-stimulated induction of neovessel sprouting was impaired in HSP70-1 knockout mice (Fig. 8A and B). Moreover, comparing the mice models, the increased neovessel formation stimulated by IL-28A in implanted Matrigel plugs was attenuated in HSP70-1 knockout mice using a Matrigel plug assay (Fig. 8C and D). The intensity of neovessel formation was verified by immunostaining using the CD31 antibody (Fig. 8E). As suggested by our previous report [23], VEGF treatment enhanced the capacity of neovessel sprouting in both WT and *HSP70-1*^{−/−} mice via use of aortic ring explant assay and subcutaneous Matrigel plug assay (Fig. 8A–E). Our data also showed that no alterations were observed in the mRNA and protein expression levels of VEGF-A in HUVECs treated with IL-28A (Supplemental Fig. S1D). We next examined whether overexpression of HSP70-1 can affect the IL-28A-induced angiogenesis using HSP70-1 transgenic mice. HSP70-1 overexpression did not affect on the angiogenic potential in both aortic ring and Matrigel plug experiments (Fig. 8F and J). However, it is notable that the IL-28A-induced ability of neovessel sprouting was promoted in the HSP70-1 transgenic mice in comparison with WT mice (Fig. 8F and G). Similarly, the increased neovessel formation of implanted Matrigel plugs in response to IL-28A was also promoted in HSP70-1-overexpressing mice (Fig. 8H and I). Similar results for CD31 immunostaining were detected in Matrigel plugs derived from the HSP70-1 transgenic mouse model (Fig. 8J). Our findings exhibit that HSP70-1 is an indispensable regulator of the IL-28A-mediated angiogenesis, but VEGF-stimulated angiogenesis is independent of HSP70-1 expression.

IL-28A modulates HSP70-1 expression through ERK1/2 and AP-1 signaling

HUVECs were treated with IL-28A, which resulted in significant phosphorylation of AKT and ERK1/2 (Fig. 1F). To determine whether IL-28A-induced HSP70-1 is involved in the AKT and ERK1/2 pathways, we used inhibitors to block the phosphorylation of AKT and ERK1/2 in HUVECs. IL-28A-induced HSP70-1 expression was suppressed by ERK1/2 inhibitor U0126 (Supplemental Fig. S3B). However, inhibition of AKT did not significantly affect HSP70-1 levels in IL-28A-treated cells (Supplemental Fig. S3A). Our previous study identified the transcription factor AP-1 as a key regulator of HSP70-1 expression [23]. To further confirm the regulatory role of IL-28A on HSP70-1 expression, HUVECs were treated with siRNA specific to AP-1, which effectively silenced its expression (Supplemental Fig. S1A). Knockdown of AP-1 significantly reduced IL-28A-induced HSP70-1 expression (Supplemental Fig. S3C). The findings suggest that IL-28A regulates HSP70-1

expression through phosphorylation of ERK1/2 and activation of AP-1.

IL-28A/IL-10R β axis promotes angiogenic responses via eNOS/AKT signaling and AP-1/NF- κ B/MMP-2 network

The receptor complex of IL-28A is consisted of IL-28R α and IL-10R β [7–9]. Because no IL-28R α expression was observed in endothelial cells [12,13], we subsequently examined the role of IL-10R β on IL-28A-mediated angiogenic responses in HUVECs. To this end, IL-10R β -specific siRNA (si-IL-10R β) was used in HUVECs, and the results were compared with scrambled siRNA (Supplemental Fig. S1E). Blockade of IL-10R β inhibited the proliferation of HUVECs in response to IL-28A (Fig. 9A). IL-28A-induced phosphorylation of eNOS and AKT was abrogated in HUVECs transfected with si-IL-10R β (Fig. 9B). However, the phosphorylated level of ERK1/2 stimulated by IL-28A was not affected by the exposure of si-IL-10R β (Fig. 9B). The expression of HSP70-1 was significantly reduced in HUVECs transfected with si-IL-10R β upon IL-28A treatment (Fig. 9B). We found a significant decline in the ability of IL-28A-stimulated wound-healing migration, invasion, and tube formation in IL-10R β -silenced HUVECs compared to scrambled siRNA-transfected cells (Fig. 9C–E and Supplemental Fig S2D). In addition, IL-10R β silencing impaired the IL-28A-promoted MMP-2 level in HUVECs (Fig. 9F and G). Moreover, IL-10R β knockdown attenuated the binding affinity of NF- κ B and AP-1 motifs in IL-28A-treated HUVECs (Fig. 9H). Our findings indicate that IL-10R β is required for the IL-28A-induced angiogenic responses in HUVECs.

IL-28A induces the ischemia-driven angiogenesis in vivo

To provide more convincing evidence of the angiogenic function of IL-28A, a surgically induced hind-limb ischemia model was evaluated using laser Doppler blood flowmetry (LDBF). After right hind-limb ischemia, the ischemic/normal limb LDBF ratio was persistently higher in mice injected with IL-28A over 21 days compared with PBS-treated mice (Supplemental Fig S4A and B). Our results provided the first demonstration that ischemia-induced angiogenesis is significantly enhanced in the presence of IL-28A. These findings from the hind-limb ischemia model strongly support the role of IL-28A in promoting angiogenesis.

Discussion

Angiogenesis is precisely regulated by the exquisite balance between various angiogenic activators and several angiogenic inhibitors [1–5]. Angiogenic activators, including VEGF members, FGF families, and various growth factors contributing to vascular remodeling, have been depicted [1–5]. It has also been previously demonstrated that some pro-inflammatory cytokines, such as IL-1 α , IL-1 β , IL-5, IL-17, IL-18, IL-32, IL-33, and IL-37, are closely associated with the promotion of angiogenesis [4,23–30]. Here, we suggest the functional study of IL-28A and its molecular regulation in physiological angiogenesis. IL-28A has previously been described as an antiviral and antitumor activator and also as a regulator of adaptive immunity and allergic airway diseases, including asthma [7–11,14]. However, our group found a high expression level of IL-28A mRNA in patients with MIBC in comparison with bladder tissues from control patients using microarray analysis [31,32]. In addition, we suggested that IL-28A induced the migratory and invasive ability of bladder cancer cells [15]. Subsequently, some report revealed that IL-28A-induced tumor induction was dependent on cell type, such as MDSCs [16]. The above results led us to investigate the potential of IL-28A as a proangiogenic activator

and its regulatory mechanism using an *in vitro* system and several types of animal models. First, based on the result from microarray assay [31], we confirmed the up-regulation of IL-28A and CD31 protein expression in MIBC tissues by an IHC staining experiment. Next, we examined whether IL-28A induces angiogenesis in HUVECs. The present results uncovered that IL-28A induced the proliferation of HUVECs. Results from the signaling pathway analysis indicated that IL-28A increased HUVEC proliferation through ERK1/2, eNOS, and AKT signaling. Furthermore, IL-28A promoted the ability of capillary tube formation, wound-healing closure, and invasive potential of HUVECs. Previous studies have shown that endothelium-derived NO plays a pivotal role in modulating angiogenic responses by triggering smooth muscle relaxation and altering cytoskeleton organization through the cGMP-AKT and MAPK pathways [39–41]. VEGF contributes to angiogenesis in endothelial cells by stimulating NO production through the upregulation and phosphorylation of eNOS [39–41]. Moreover, eNOS regulates the activity and function of MMPs, including MMP-2 and MMP-9 [42,43]. There is a strong correlation between the level of MMPs and the migration and capillary tube formation [1–4,6,37]. Our study indicated that IL-28A exposure boosted the up-regulation of MMP-2 levels in HUVECs. Moreover, IL-28A showed strong transcriptional binding affinity to AP-1 and NF- κ B motifs, which are the main elements in the MMP-2 promoter. The blockade of eNOS, AKT, and ERK1/2 signaling suppressed the IL-28A-mediated migration, invasion, and capillary tube formation. Inhibition of eNOS, AKT, and ERK1/2 signaling also impeded transcription factor (AP-1 and NF- κ B)-mediated MMP-2 expression. The efficacy of IL-28A-mediated angiogenesis in an animal model was confirmed by an aortic ring *ex vivo* assay, a Matrigel plug *in vivo* assay, and a hind-limb ischemia model. Our results demonstrate that eNOS/AKT/ERK1/2 signaling might be associated with the IL-28A-stimulated angiogenesis, most likely by promoting the proliferative capacity and enhancing the capillary tube formation, migration, and invasion relevant to the AP-1/NF- κ B/MMP-2 axis in HUVECs.

To further investigate the molecular mechanism of IL-28A-induced angiogenesis, we performed a NGS analysis. Based on the comprehensive list of genes changed by more than 1.5-fold in HUVECs, 28 genes were upregulated in HUVECs treated with IL-28A. The genes were divided into seven major gene clusters by GO analysis. Interestingly, we found that, among the NGS analysis, the most upregulated genes (by more than 2-fold) by IL-28A were the HSP70 family, such as HSP70-1, HSPA6, and HSP70-2. These three HSP70 family members were functionally associated with response to stimulus, response to stress, response to biotic stimulus, response to unfolded protein, response to protein stimulus, protein complex assembly, and interferon-mediated immunity as the six major functional categories. Finally, the enrichment results from KEGG analysis and gene network analysis identified three hub genes: HSP70-1, HSPA6, and HSP70-2. These genes were significantly enriched in legionellosis, the longevity-regulating pathway, and antigen processing and presentation. Therefore, we focused on the three HSP70 family members to elucidate the role and function of novel target genes in response to IL-28A-promoted angiogenesis.

The molecular chaperone HSP70 acts as a cellular repair agent to protect cells under stressful conditions induced by damage from external environmental stimuli [17–19]. Conversely, some studies have suggested that HSP70 expression leads to tumor progression and metastasis [44–47]. Several studies have reported that mice lacking HSP70-1 are susceptible to radio-sensitivity, osmotic stress, neuroprotective effects after cardiac dysfunction, and spinal cord and acute focal cerebral ischemia, while abundant HSP70-1 gene expression in mice and cells provided a protective effect against myocardial ischemia and UVB irradiation [48–53]. It was not possible to obtain sufficient *HSP70-2*^{-/-} mice because disrup-

tion of HSP70-2 in mice leads to male infertility during spermatogenesis [21]. Moreover, gene expression of HSPA6 was detected in humans, goats, and swine but not in rats and mice [22]. Therefore, we focused on HSP70-1, and then the role and function of HSP70-1 in IL-28A-induced angiogenesis were investigated using a siRNA technique and animal models, including knockout mice and transgenic mice. Molecular chaperones, such as HSP27, HSP16, HSP70, and mtHSP70, have a contradictory dual function, regulating the signal between cell survival and cell death [54,55]. Cumulated studies have suggested the role of HSP members during angiogenesis [37,56,57]. In EC, Akt-FOXO3a-induced apoptosis was rescued by HSP70 [58]. HSP induced angiogenesis by up-regulation of AKT/eNOS signaling [59]. Heat shock cognate protein 70 (HSC70) is essential for the VEGF-induced EC responses through the phosphorylation of AKT [60]. HSP70-1 has been reported to be involved in angiogenesis through the phosphorylation of ERK1/2 [61]. Extracellular HSP70-1, secreted from tumor cells, binds to C-type lectin domain family 14 member (CLEC14a) on tumor vessels via a C-type lectin-like domain of CLEC14a [62]. This interaction promotes endothelial cell – cell contacts and stimulates angiogenic functions [62]. Despite the link between molecular chaperone HSP members and angiogenic responses [56–63], the pivotal role of HSP members in the IL-28A-mediated progression of angiogenesis remains unreported. Our results from siRNA knockdown of HSP70-1 indicated that HSP70-1 regulated IL-28A-induced proliferation of HUVECs. In addition, HSP70-1 was essential for IL-28A-mediated migration, invasion, and capillary tube formation by inducing the AP-1/NF- κ B/MMP-2 axis. Moreover, eNOS/AKT signaling appears to be connected with the HSP70-1-dependent IL-28A-associated angiogenesis cascade in HUVECs, but not ERK1/2 signaling. The efficacy of HSP70-1 in IL-28A-mediated angiogenesis was verified in both HSP70-1-deficient mice and HSP70-1 transgenic mice. Unexpectedly, our data from HSP70-1 knockout mice demonstrated that HSP70-1 was not involved in VEGF-promoted angiogenesis. These findings reveal that HSP70-1 might be an essential regulator in IL-28A-mediated angiogenesis but is independent of the action of VEGF. Given our data, IL-28A-induced angiogenesis is likely to be mediated by direct HSP70-1 gene expression.

Our study demonstrated that IL-28A-mediated HSP70-1 expression is involved in the ERK1/2 signaling and AP-1 activation in HUVECs. AKT signaling is not affected by IL-28A-stimulated HSP70-1. Both ERK1/2 and AP-1 may be the primary targets of HSP70-1, which is stimulated by IL-28A in HUVECs. It is possible that ERK1/2 signaling might be responsible for IL-28A-induced HSP70-1 expression through activation of AP-1 in HUVECs. Other studies have demonstrated similar molecular mechanisms. IL-5 has been shown to induce HSP70-1 expression in HUVECs via the ERK1/2 and AKT signaling pathways, with AP-1 playing a pivotal role [23]. Previous research has also revealed the involvement of AP-1 in the regulation of HSP70 expression in HepG2 cells exposed to cadmium [64]. Additionally, IL-17A has been found to induce HSP47 expression in intestinal fibrosis associated with Crohn's disease through the JNK signaling pathway [65]. Our findings suggest that IL-28A regulates HSP70-1 expression through the phosphorylation of ERK1/2 and the involvement of AP-1 signaling. However, further studies are required to clarify the detailed regulatory mechanism of HSP70-1 expression mediated by IL-28A.

The binding of inflammatory cytokines to their receptors generates signals that modulate immune responses [7–9]. Previous studies have shown that IL-28A binds to IL-28R α as well as IL-10R β [7–9]. Because IL-28R α expression is rarely detected in EC [12,13], we used siRNA knockdown of IL-10R β (si-IL-10R β) to investigate the angiogenic responsive signaling in HUVECs treated with IL-28A. In this study, IL-28A-induced proliferation of HUVECs was blocked by the inhibition of IL-10R β . In addition, IL-10R β knockdown diminished the migration, invasion, and tube formation by sup-

pressing the AP-1/NF- κ B/MMP-2 axis in IL-28A-treated HUVECs. eNOS/AKT signaling was involved in IL-28A/IL-10R β dyad-mediated angiogenic responses, whereas ERK1/2 signaling did not affect the angiogenic responses stimulated by IL-28A in the presence of IL-10R β gene silencing. IL-28A-stimulated HSP70-1 expression was suppressed in the absence of IL-10R β . Our present data revealed that the binding of IL-28A to IL-10R β might induce angiogenic responses by activating survival signals relevant to the eNOS/AKT pathway via upregulation of HSP70-1.

IL-28A mediates antiviral and antitumor effects at intestinal surfaces and some immune cells, such as neutrophils, dendritic cells, and B cells [7–11,14]. In addition, our group and another group have suggested that IL-28A induces tumor progression in bladder cancer cells and MDSCs [15,16]. Many studies have shown that inflammatory cytokines act as angiogenic regulators, which have a dual function by promoting direct angiogenic responses in HUVECs or by recruiting inflammatory cells that indirectly guide the secretion of EC mitogen [4,23–30]. In the present study, we found a low level of IL-28A expression in HUVECs and, interestingly, the level was significantly induced by external treatment with IL-28A, suggesting that IL-28A might be produced in both an autocrine and a paracrine fashion. The low level of IL-28A released by endogenous HUVECs had no effect on angiogenic function. However, the exogenous addition of IL-28A stimulated strong angiogenic signaling and function. The addition of IL-28A antibody or knockdown of IL-10R β abolished IL-28A-stimulated angiogenic responses. The present studies represent that IL-28A produced by viruses, immune cells, and HUVECs functions as a potent regulator for proangiogenic signaling and function. Our data demonstrated that the binding of IL-28A to IL-10R β leads to strong angiogenic responses by exerting autocrine/paracrine actions in HUVECs.

Clinical trials of antitumor agents that suppress the function and signaling of VEGF and its receptors led to the failure of cancer therapy due to resistance to anti-angiogenic drugs [6,66,67]. Therefore, other regulators that cooperate with VEGF-independent angiogenesis need to be clarified. Although many studies have suggested FGF, IL-32, and IL-33 as VEGF-independent angiogenesis regulators, their exact molecular mechanisms have not yet been determined [28,29,68]. We recently reported that HSP70-1 is involved in the VEGF-independent angiogenic effects of IL-5 [23]. In the present study, our data indicate that HSP70-1 regulated the IL-28A-induced angiogenic responses *in vitro* and *in vivo* through a VEGF-independent action. Considering the VEGF-independent angiogenic effect of IL-28A and IL-5, HSP70-1 is thought to play a vital role in the development of effective therapeutics targeting anti-angiogenesis. In light of our data, it may be worth exploring whether other types of angiogenic mitogens and cytokines could regulate angiogenesis through HSP70-1 expression.

Collectively, the present results demonstrate that the IL-28A/IL-10R β axis induced the elevation of both eNOS/AKT signaling and the AP-1/NF- κ B/MMP-2 network by regulating HSP70-1 expression, which resulted in the progression of angiogenesis. These findings might be the first direct evidence of the correlation between molecular chaperone HSP70-1 and cytokine IL-28A during VEGF-independent angiogenesis. Our results suggest that IL-28A and HSP70-1 may be effective potential therapeutic targets to suppress angiogenesis-mediated diseases.

Materials and methods

Materials

Recombinant human IL-28A was purchased from R&D Systems (Minneapolis, MN). Polyclonal antibodies specific to AP-1, NF- κ B,

p-eNOS (S1177), eNOS, p-AKT, AKT, p-ERK1/2, and ERK1/2 were obtained from Cell Signaling (Danvers, MA). Polyclonal antibodies specific to MMP-2, IL-28A, HSP70-1, IL-10R β , VEGF-A, CD31, and actin were obtained from Sigma-Aldrich (St Louis, MO, USA).

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Animal Care and Use Committee of Chung-Ang University (EBOA-2017–12 and 202401030008).

Cell culture

The primary HUVECs were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in endothelial basic medium (EBM) containing components of EGM™-2 Bulletkit™ (Lonza, Walkersville, MD) on plates coated with 0.1 % gelatin (Sigma, San Diego, CA). The cells were incubated at a precise temperature of 37 °C in a humidified incubator featuring 5 % CO₂. All experimental procedures were conducted during passages 2 – 5.

Genetic mice

The HSP70-1 knockout mice were provided by Dr. Jeongseon Seo at Seoul National University, and HSP70-1-transgenic (Tg-(Hspa1a-luc,-EGFP)2Chco/J) mice were purchased from Jackson Laboratory. Methods for generating HSP70-1 knockout mice and HSP70-1-transgenic (Tg-(Hspa1a-luc,-EGFP)2Chco/J) mice were performed as previously described [23]. The mice were maintained in a regulated environment and monitored daily, including a constant humidity of 60 ± 10 %, temperature of 25 ± 2 °C, and given a standard pellet diet and access to drinking water. The mice were anesthetized intraperitoneally with ketamine and xylazine (100 mg/kg and 10 mg/kg; Yuhan Corp., Seoul, Korea) before surgery.

Immunoblot assay

Protein extraction was performed using RIPA lysis buffer, and the concentration of extracted protein was determined by the use of a BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Waltham, MA). A polyacrylamide gel containing SDS (0.1 %) was loaded with 20 µg of protein per well, followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresed proteins were moved onto a nitrocellulose membrane and then blocked with 5 % skim milk. The primary antibodies were applied for 24 h, followed by the secondary antibodies for 2 h. Protein expression was performed using an electrochemiluminescent (ECL) reagent (SuperSignal® West Pico Chemiluminescent Substrate, Thermo Fisher Scientific) and quantified utilizing ImageJ software.

Immunoprecipitation

The cell lysates (500 µg) were reacted with HSP70-1 antibody (1:200) for 18 h at 4 °C. Protein G Plus/Protein A Agarose was gently added to the immunocomplexes for 4 h at 4 °C, then centrifuged at 2000g for 2 min. The pellet was mixed with 5X protein loading buffer and immunoblot assay was performed.

Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

Total RNA extraction was performed using TRIzol (Sigma-Aldrich). The extracted RNA was measured for RNA quantity and

quality using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT), and cDNA synthesis was performed using the Bioneer cDNA synthesis kit according to the instructions. The primers used had the following sequences: IL-28A (391 bp), 5'-TCGCTTCTGCTGAAGGACTGCA-3' (sense) and 5'-CCTCCAGAACCTT CAGCGTCAG-3' (antisense); HSP70-1 (447 bp), 5'-TTTCGAGAGT GACTCCCGTT-3' (sense) and 5'-AAGGCCAGTGCTTCATGTC-3' (antisense); VEGF-A (233 bp), 5'-TGTCTATCAGCGCAGCTACTGCCAT-3' (sense) and 5'-GGAAGCTCATCTCTCCTATGTGCTG (antisense); and actin (235 bp), 5'-CCCAGATCATGTTTGAGACCT-3' (sense) and 5'-ATGTCACGCACGATTTCCT-3' (antisense).

Invasion assay

Transwell® chambers coated with BD Matrigel Basement Membrane Matrix (BD Biosciences, San Diego, CA) were used, and HUVECs were incubated for 3 h in EBM containing 1 % FBS prior to experimentation. EBM containing 0.1 % FBS was used as the medium for the upper and lower chambers, and the cells (5×10^4 cells/well) and IL-28A were inoculated into the upper chamber. After 24 h, the cells were fixed on the upper chamber membrane using a 4 % paraformaldehyde solution. The fixed cells were stained with a 0.01 % crystal violet solution. ImageJ was used to measure invading cells.

Wound healing migration assay

HUVECs were seeded at a density of 4×10^5 cells per well in a 6-well plate. Upon reaching 90 % confluence, the cells were subjected to a 3 h incubation in EBM supplemented with 1 % FBS. Subsequently, to create injury lines, the cell surface area was gently scratched using a 200 μ L pipette tip. The cells were then exposed to IL-28A in EBM containing 0.1 % FBS for a duration of 24 h. The progress of wound closure was observed under an inverted microscope at $\times 40$ magnification.

Colony tube formation assay

HUVECs were pre-incubated for 3 h in EBM containing 1 % FBS. The cells were seeded (1.2×10^5 cells/well) with EBM medium containing 0.1 % FBS in BD Matrigel matrix growth factor reduction-coated 24-well plate. After treatment with IL-28A by concentration, tube formation was observed 24 h later using a microscope ($\times 40$ magnification). The tube length was measured using ImageJ software.

Aortic ring assay

C57BL/6 mice were euthanized by using carbon dioxide inhalation. Aortas from the mice, sectioned into rings with a length of 1 – 1.5 mm, were embedded in Matrigel-coated wells. Embedded aortic rings were incubated with media containing IL-28A and VEGF (50 ng/mL) for 9 days. The overall length of microvascular germination was quantified using CS Analyzer 4 software (ATTO, Tokyo, Japan).

Plug in vivo assay

A mixture containing Matrigel, heparin (10 U/mL), IL-28A, and VEGF (50 ng/mL) was administered subcutaneously at 0.5 mL in 8-week-old C57BL/6 mice. After 7 days, the mice were euthanized by using carbon dioxide inhalation, and the Matrigel plugs were removed. Angiogenesis quantification was determined by measuring hemoglobin levels via the Drabkin method. Immunohistochemistry was performed to identify a CD-31 antibody in the infiltrating endothelial cells.

Small-interfering RNA transfection

G-Fectin transfection reagent (Genolution, Seoul, Korea) was used in accordance with the manufacturer's instructions for siRNA transfection. After 24 h of siRNA transfection, the transfected cells were harvested for subsequent analyses. The siRNA used had the following sequences: HSP70-1, 5'-GUUUGUCAGUUCU CAAUUUUU-3' (sense) and 5'-AAAUUGAGAACUGACAAACUU-3' (antisense); IL-10R β , 5'-GAAUGAAUUCUGUUAUUUUUU-3' (sense) and 5'-AAAUUAACAGAAUUAUUCUU-3' (antisense); and scramble (scrambled siRNA), 5'-CCUCGUGCCGUUCCAUCAGGUAGUU-3' (sense) and 5'-CUACCUGAUGGAACGGCAGAGGUU-3' (antisense). AP-1 (#6203) and NF- κ B (#6261) siRNAs were obtained from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis

Statistical analyses for the study were conducted using the SPSS 20.0 statistical software package (SPSS Inc., Chicago, IL). Each experiment was carried out independently at least three times. The data obtained were presented as the mean $\pm$ standard error of the mean (SEM). A significance threshold of P-value < 0.05 was employed to determine statistical significance in all tests.

CRediT authorship contribution statement

Jun-Hui Song: Methodology, Investigation, Validation, Visualization, Writing – review & editing. **Byungdoo Hwang:** Methodology, Investigation, Validation. **Sung Lyea Park:** Methodology, Investigation, Validation. **Hoon Kim:** Methodology, Investigation, Validation. **Soontag Jung:** Investigation, Validation, Visualization. **Changsun Choi:** Supervision, Validation, Visualization. **Hwan Myung Lee:** Supervision, Validation. **Seok-Joong Yun:** Supervision, Validation. **Yung Hyun Choi:** Supervision, Validation. **Eun-Jong Cha:** Supervision, Validation. **Cam Patterson:** Supervision, Validation. **Wun-Jae Kim:** Supervision, Validation. **Sung-Kwon Moon:** Conceptualization, Methodology, Validation, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1A6A1A03025159). This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2021R1A2C1013957).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2024.08.013>.

References

- [1] Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995;1:27–30. doi: <https://doi.org/10.1038/nm0195-27>.
- [2] M. Shibuya, Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis, *Angiogenesis* 9 (2006) 225,230; discussion 231. doi:10.1007/s10456-006-9055-8.

- [3] Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997;18:4–25. doi: <https://doi.org/10.1210/edrv.18.1.0287>.
- [4] Peluzzo AM, Autieri MV. Challenging the Paradigm: Anti-Inflammatory Interleukins and Angiogenesis. *Cells* 2022;11:587. doi: <https://doi.org/10.3390/cells11030587>.
- [5] Protopsaltis NJ, Liang W, Nudleman E, Ferrara N. Interleukin-22 promotes tumor angiogenesis. *Angiogenesis* 2019;22:311–23. doi: <https://doi.org/10.1007/s10456-018-9658-x>.
- [6] Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. *Cell* 2011;146:873–87. doi: <https://doi.org/10.1016/j.cell.2011.08.039>.
- [7] Kotelko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003;4:69–77. doi: <https://doi.org/10.1038/nl875>.
- [8] Koltisda O, Hausding M, Stavropoulos A, Koch S, Tzelepis G, Ubel C, et al. IL-28A (IFN- λ 2) modulates lung DC function to promote Th1 immune skewing and suppress allergic airway disease. *EMBO Mol Med* 2011;3:348–61. doi: <https://doi.org/10.1002/emmm.201100142>.
- [9] Hemann EA, Gale M, Savan R. Interferon Lambda Genetics and Biology in Regulation of Viral Control. *Front Immunol* 2017;8:1707. doi: <https://doi.org/10.3389/fimmu.2017.01707>.
- [10] Lasfar A, Lewis-Antes A, Smirnov SV, Anantha S, Abushahba W, Tian B, et al. Characterization of the mouse IFN-lambda ligand-receptor system: IFN-lambdas exhibit antitumor activity against B16 melanoma. *Cancer Res* 2006;66:4468–77. doi: <https://doi.org/10.1158/0008-5472.CAN-05-3653>.
- [11] Song B, Yang Y, Wang Y, Fan X, Huang Y, Ci H, et al. Adenovirus expressing IFN- λ (Ad/hIFN- λ) produced anti-tumor effects through inducing apoptosis in human tongue squamous cell carcinoma cell. *Int J Clin Exp Med* 2015;8:12509–18.
- [12] Sommereyns C, Paul S, Staeheli P, Michiels T. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathog* 2008;4:e1000017.
- [13] Witte K, Gruetz G, Volk H, Looman AC, Asadullah K, Sterry W, et al. Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines. *Genes Immun* 2009;10:702–14. doi: <https://doi.org/10.1038/gene.2009.72>.
- [14] Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte J, et al. IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression. *Am J Physiol Gastrointest Liver Physiol* 2005;289:960. doi: <https://doi.org/10.1152/ajpgi.00126.2005>.
- [15] Lee S, Lim J, Choi YH, Kim W, Moon S. Interleukin-28A triggers wound healing migration of bladder cancer cells via NF- κ B-mediated MMP-9 expression inducing the MAPK pathway. *Cell Signal* 2012;24:1734–42. doi: <https://doi.org/10.1016/j.cellsig.2012.04.013>.
- [16] Mucha J, Majchrzak K, Taciak B, Hellmén E, Król M. MDSCs mediate angiogenesis and predispose canine mammary tumor cells for metastasis via IL-28/IL-28RA (IFN- λ) signaling. *PLoS One* 2014;9:e103249.
- [17] Lindquist S, Craig EA. The heat-shock proteins. *Annu Rev Genet* 1988;22:631–77. doi: <https://doi.org/10.1146/annurev.ge.22.120188.003215>.
- [18] Li GC, Werb Z. Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proc Natl Acad Sci U S A* 1982;79:3218–22. doi: <https://doi.org/10.1073/pnas.79.10.3218>.
- [19] Vostakolaei MA, Hatami-Baroogh L, Babaei G, Molavi O, Kordi S, Abdolalizadeh J. Hsp70 in cancer: A double agent in the battle between survival and death. *J Cell Physiol* 2021;236:3420–44. doi: <https://doi.org/10.1002/jcp.30132>.
- [20] Walter L, Rauh F, Günther E. Comparative analysis of the three major histocompatibility complex-linked heat shock protein 70 (Hsp70) genes of the rat. *Immunogenetics* 1994;40:325–30. doi: <https://doi.org/10.1007/BF01246673>.
- [21] Dix DJ, Allen JW, Collins BW, Mori C, Nakamura N, Poorman-Allen P, et al. Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. *Proc Natl Acad Sci U S A* 1996;93:3264–8. doi: <https://doi.org/10.1073/pnas.93.8.3264>.
- [22] Ramirez VP, Stamatis M, Shmukler A, Aneskievich BJ. Basal and stress-inducible expression of HSPA6 in human keratinocytes is regulated by negative and positive promoter regions. *Cell Stress Chaperones* 2015;20:95–107. doi: <https://doi.org/10.1007/s12192-014-0529-0>.
- [23] Park SL, Chung T, Kim S, Hwang B, Kim JM, Lee HM, et al. HSP70-1 is required for interleukin-5-induced angiogenic responses through eNOS pathway. *Sci Rep* 2017;7:44687. doi: <https://doi.org/10.1038/srep44687>.
- [24] Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, et al. IL-1 is required for tumor invasiveness and angiogenesis. *Proc Natl Acad Sci U S A* 2003;100:2645–50. doi: <https://doi.org/10.1073/pnas.0437939100>.
- [25] Matsuo Y, Sawai H, Ma J, Xu D, Ochi N, Yasuda A, et al. IL-1 α secreted by colon cancer cells enhances angiogenesis: the relationship between IL-1 α release and tumor cells' potential for liver metastasis. *J Surg Oncol* 2009;99:361–7. doi: <https://doi.org/10.1002/iso.21245>.
- [26] Pan B, Shen J, Cao J, Zhou Y, Shang L, Jin S, et al. Interleukin-17 promotes angiogenesis by stimulating VEGF production of cancer cells via the STAT3/GIV signaling pathway in non-small-cell lung cancer. *Sci Rep* 2015;5:16053. doi: <https://doi.org/10.1038/srep16053>.
- [27] Park CC, Morel JC, Amin MA, Connors MA, Harlow LA, Koch AE. Evidence of IL-18 as a novel angiogenic mediator. *J Immunol* 2001;167:1644–53. doi: <https://doi.org/10.4049/jimmunol.167.3.1644>.
- [28] Nold-Petry CA, Rudloff I, Baumer Y, Ruvo M, Marasco D, Botti P, et al. IL-32 promotes angiogenesis. *J Immunol* 2014;192:589–602. doi: <https://doi.org/10.4049/jimmunol.1202802>.
- [29] Choi Y, Choi H, Min J, Pyun B, Maeng Y, Park H, et al. Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood* 2009;114:3117–26. doi: <https://doi.org/10.1182/blood-2009-02-203372>.
- [30] Yang T, Lin Q, Zhao M, Hu Y, Yu Y, Jin J, et al. IL-37 Is a Novel Proangiogenic Factor of Developmental and Pathological Angiogenesis. *Arterioscler Thromb Vasc Biol* 2015;35:2638–46. doi: <https://doi.org/10.1161/ATVBAHA.115.306543>.
- [31] Kim W, Kim E, Kim S, Kim Y, Ha Y, Jeong P, et al. Predictive value of progression-related gene classifier in primary non-muscle invasive bladder cancer. *Mol Cancer* 2010;9:3. doi: <https://doi.org/10.1186/1476-4598-9-3>.
- [32] Lee S, Lee E, Kim S, Jeong P, Cho Y, Yun SJ, et al. Identification of pro-inflammatory cytokines associated with muscle invasive bladder cancer; the roles of IL-5, IL-20, and IL-28A. *PLoS One* 2012;7:e40267.
- [33] Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, et al. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J Clin Invest* 1994;94:2036–44. doi: <https://doi.org/10.1172/JCI117557>.
- [34] Bu S, Nguyen HC, Nikfarjam S, Michels DCR, Rasheed B, Maheshkumar S, et al. Endothelial cell-specific loss of eNOS differentially affects endothelial function. *PLoS One* 2022;17:e0274487.
- [35] Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest* 1998;101:731–6. doi: <https://doi.org/10.1172/JCI1699>.
- [36] Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, V., Dixit et al, Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 1998;273:30336–43. doi: <https://doi.org/10.1074/jbc.273.46.30336>.
- [37] Sun C, Feng S, Cao Z, Bei J, Chen Q, Zhao W, et al. Up-Regulated Expression of Matrix Metalloproteinases in Endothelial Cells Mediates Platelet Microvesicle-Induced Angiogenesis. *Cell Physiol Biochem* 2017;41:2319–32. doi: <https://doi.org/10.1159/000475651>.
- [38] Lee S, Cho S, Lee E, Kim S, Lee S, Lim J, et al. Interleukin-20 promotes migration of bladder cancer cells through extracellular signal-regulated kinase (ERK)-mediated MMP-9 protein expression leading to nuclear factor (NF- κ B) activation by inducing the up-regulation of p21(WAF1) protein expression. *J Biol Chem* 2013;288:5539–52. doi: <https://doi.org/10.1074/jbc.M112.410233>.
- [39] Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, et al. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem* 1998;273:4220–6. doi: <https://doi.org/10.1074/jbc.273.7.4220>.
- [40] Ziche M, Morbidelli L. Nitric oxide and angiogenesis. *J Neurooncol* 2000;50:139–48. doi: <https://doi.org/10.1023/a:1006431309841>.
- [41] Perez-Zoghbi JF, Bai Y, Sanderson MJ. Nitric oxide induces airway smooth muscle cell relaxation by decreasing the frequency of agonist-induced Ca²⁺ oscillations. *J Gen Physiol* 2010;135:247–59. doi: <https://doi.org/10.1085/jgp.200910365>.
- [42] Genis L, Gonzalo P, Tutor AS, Gálvez BG, Martínez-Ruiz A, Zaragoza C, et al. Functional interplay between endothelial nitric oxide synthase and membrane type 1 matrix metalloproteinase in migrating endothelial cells. *Blood* 2007;110:2916–23. doi: <https://doi.org/10.1182/blood-2007-01-068080>.
- [43] López-Rivera E, Lizarbe TR, Martínez-Moreno M, López-Novoa JM, Rodríguez-Barbero A, Rodrigo J, et al. Matrix metalloproteinase 13 mediates nitric oxide activation of endothelial cell migration. *Proc Natl Acad Sci U S A* 2005;102:3685–90. doi: <https://doi.org/10.1073/pnas.0408217102>.
- [44] Guo H, Deng Q, Wu C, Hu L, Wei S, P., Xu et al, Variations in HSPA1B at 6p21.3 are associated with lung cancer risk and prognosis in Chinese populations. *Cancer Res* 2011;71:7576–86. doi: <https://doi.org/10.1158/0008-5472.CAN-11-1409>.
- [45] Juhasz K, Lipp A, Nimmervoll B, Sonnleitner A, Hesse J, Haselgruebler T, et al. The complex function of hsp70 in metastatic cancer. *Cancers (Basel)* 2013;6:42–66. doi: <https://doi.org/10.3390/cancers6010042>.
- [46] Jegu G, Hazoumé A, Seigneure R, Garrido C. Targeting heat shock proteins in cancer. *Cancer Lett* 2013;332:275–85. doi: <https://doi.org/10.1016/j.canlet.2010.10.014>.
- [47] Lang BJ, Prince TL, Okusha Y, Bunch H, Calderwood SK. Heat shock proteins in cell signalling and cancer. *Biochim Biophys Acta Mol Cell Res* 2022;1869. doi: <https://doi.org/10.1016/j.bbamcr.2021.119187>.
- [48] Lee SH, Kim M, Yoon BW, Kim YJ, Ma SJ, J.K., Roh et al, Targeted hsp70.1 disruption increases infarction volume after focal cerebral ischemia in mice. *Stroke* 2001;32:2905–12. doi: <https://doi.org/10.1161/hs1201.099604>.
- [49] Shim E, Kim J, Bang E, Heo J, Lee J, E., Kim et al, Targeted disruption of hsp70.1 sensitizes to osmotic stress. *EMBO Rep* 3 2002:857–61. doi: <https://doi.org/10.1093/embo-reports/kvf175>.

- [50] Hampton CR, Shimamoto A, Rothnie CL, Griscavage-Ennis J, Chong A, D.J. Dix et al. HSP70.1 and -70.3 are required for late-phase protection induced by ischemic preconditioning of mouse hearts. *Am J Physiol Heart Circ Physiol* 285 2003;866. doi: <https://doi.org/10.1152/ajpheart.00596.2002>.
- [51] Hunt CR, Dix DJ, Sharma GG, Pandita RK, Gupta A, Funk M, et al. Genomic instability and enhanced radiosensitivity in Hsp70.1- and Hsp70.3-deficient mice. *Mol Cell Biol* 2004;24:899–911. doi: <https://doi.org/10.1128/MCB.24.2.899-911.2004>.
- [52] Park KC, Kim DS, Choi HO, Kim KH, Chung JH, Eun HC, et al. Overexpression of HSP70 prevents ultraviolet B-induced apoptosis of a human melanoma cell line. *Arch Dermatol Res* 2000;292:482–7. doi: <https://doi.org/10.1007/s004030000173>.
- [53] Mestrlil R, Giordano FJ, Conde AG, Dillmann WH. Adenovirus-mediated gene transfer of a heat shock protein 70 (hsp 70i) protects against simulated ischemia. *J Mol Cell Cardiol* 1996;28:2351–8. doi: <https://doi.org/10.1006/jmcc.1996.0228>.
- [54] Kiang JG, Tsokos GC. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther* 1998;80:183–201. doi: [https://doi.org/10.1016/s0163-7258\(98\)00028-x](https://doi.org/10.1016/s0163-7258(98)00028-x).
- [55] Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 2002;295:1852–8. doi: <https://doi.org/10.1126/science.1068408>.
- [56] Ding Y, Song N, Liu C, He T, Zhuo W, He X, et al. Heat shock cognate 70 regulates the translocation and angiogenic function of nucleolin. *Arterioscler Thromb Vasc Biol* 2012;32:126. doi: <https://doi.org/10.1161/ATVBAHA.112.247502>.
- [57] Li J, Sun X, Wang Z, Chen L, Li D, Zhou J, et al. Regulation of vascular endothelial cell polarization and migration by Hsp70/Hsp90-organizing protein. *PLoS One* 2012;7:e36389.
- [58] Kim H, Skurk C, Maatz H, Shiojima I, Ivashchenko Y, Yoon S, et al. Akt/FOXO3a signaling modulates the endothelial stress response through regulation of heat shock protein 70 expression. *FASEB J* 2005;19:1042–4. doi: <https://doi.org/10.1096/fj.04-2841fie>.
- [59] Sun J, Liao JK. Induction of angiogenesis by heat shock protein 90 mediated by protein kinase Akt and endothelial nitric oxide synthase. *Arterioscler Thromb Vasc Biol* 2004;24:2238–44. doi: <https://doi.org/10.1161/01.ATV.0000147894.22300.4c>.
- [60] Shiota M, Kusakabe H, Izumi Y, Hikita Y, Nakao T, Funae Y, et al. Heat shock cognate protein 70 is essential for Akt signaling in endothelial function. *Arterioscler Thromb Vasc Biol* 2010;30:491–7. doi: <https://doi.org/10.1161/ATVBAHA.109.193631>.
- [61] Kim T, Na HJ, Lee WR, Jeoung MH, Lee S. Heat shock protein 70–1A is a novel angiogenic regulator. *Biochem Biophys Res Commun* 2016;469:222–8. doi: <https://doi.org/10.1016/j.bbrc.2015.11.125>.
- [62] Jang J, Kim MR, Kim T, Lee WR, Kim JH, Heo K, et al. CLEC14a-HSP70-1A interaction regulates HSP70-1A-induced angiogenesis. *Sci Rep* 2017;7:10666. doi: <https://doi.org/10.1038/s41598-017-11118-y>.
- [63] Sun C, Li H, Chen H, Shi M, Liu Q, Pan Z, et al. Decreased expression of CHIP leads to increased angiogenesis via VEGF-VEGFR2 pathway and poor prognosis in human renal cell carcinoma. *Sci Rep* 2015;5:9774. doi: <https://doi.org/10.1038/srep09774>.
- [64] M.d.C. Escobar, V. Souza, L. Bucio, E. Hernández, L.E. Gómez-Quiroz, M.C. Gutiérrez Ruiz,. MAPK activation is involved in cadmium-induced Hsp70 expression in HepG2 cells, *Toxicol Mech. Methods* 2009;19:503–9. doi: <https://doi.org/10.3109/15376510903325670>.
- [65] Honzawa Y, Nakase H, Shiokawa M, Yoshino T, Imaeda H, Matsuura M, et al. Involvement of interleukin-17A-induced expression of heat shock protein 47 in intestinal fibrosis in Crohn's disease. *Gut* 2014;63:1902–12. doi: <https://doi.org/10.1136/gutjnl-2013-305632>.
- [66] Brauer MJ, Zhuang G, Schmidt M, Yao J, Wu X, Kaminker JS, et al. Identification and analysis of in vivo VEGF downstream markers link VEGF pathway activity with efficacy of anti-VEGF therapies. *Clin Cancer Res* 2013;19:3681–92. doi: <https://doi.org/10.1158/1078-0432.CCR-12-3635>.
- [67] Kamba T, McDonald DM. Mechanisms of adverse effects of anti-VEGF therapy for cancer. *Br J Cancer* 2007;96:1788–95. doi: <https://doi.org/10.1038/sj.bjc.6603813>.
- [68] Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov* 2009;8:235–53. doi: <https://doi.org/10.1038/nrd2792>.