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Targeting CD9 produces stimulus-independent antiangiogenic effects predominantly in activated endothelial cells during angiogenesis: A novel antiangiogenic therapy

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ABSTRACT

The precise roles of tetraspanin CD9 are unclear. Here we show that CD9 plays a stimulus-independent role in angiogenesis and that inhibiting CD9 expression or function is a potential antiangiogenic therapy. Knocking down CD9 expression significantly inhibited *in vitro* endothelial cell migration and invasion induced by vascular endothelial growth factor (VEGF) or hepatocyte growth factor (HGF). Injecting CD9-specific small interfering RNA (siRNA-CD9) markedly inhibited HGF- or VEGF-induced subconjunctival angiogenesis *in vivo*. Both results revealed potent and stimulus-independent antiangiogenic effects of targeting CD9. Furthermore, intravitreous injections of siRNA-CD9 or anti-CD9 antibodies were therapeutically effective for laser-induced retinal and choroidal neovascularization in mice, a representative ocular angiogenic disease model. In terms of the mechanism, growth factor receptor and downstream signaling activation were not affected, whereas abnormal localization of integrins and membrane type-1 matrix metalloproteinase was observed during angiogenesis, by knocking down CD9 expression. Notably, knocking down CD9 expression did not induce death and mildly inhibited proliferation of quiescent endothelial cells under conditions without an angiogenic stimulus. Thus, CD9 does not directly affect growth factor-induced signal transduction, which is required in angiogenesis and normal vasculature, but is part of the angiogenesis machinery in endothelial cells during angiogenesis. In conclusion, targeting CD9 produced stimulus-independent antiangiogenic effects predominantly in activated endothelial cells during angiogenesis, and appears to be an effective and safe antiangiogenic approach. These results shed light on the biological roles of CD9 and may lead to novel antiangiogenic therapies.

1. Introduction

Angiogenesis is essential for not only organ growth and development in fetuses but also such physiologic processes as tissue homeostasis in adults [1]. Dysregulated angiogenesis contributes to the pathogenesis of many diseases, including several ocular disorders (e.g., diabetic retinopathy, retinopathy of prematurity, age-related macular degeneration), solid tumor growth and/or metastasis, rheumatoid arthritis, and psoriasis [1]. Angiogenesis requires endothelial cells to sprout, migrate, and proliferate and to degrade the extracellular matrix and basement membrane before invasion [2].

Although a variety of molecules are involved in these complex multistep processes, angiogenic growth factors have been implicated as the predominant determinants of angiogenic phenotypes [3]. Particularly, the potent angiogenic activities of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and heparin-binding epidermal growth factor-like growth factor (HB-EGF) have been extensively studied [3–5]. Moreover, the therapeutic effectiveness of anti-VEGF agents for cancer and ocular diseases has been recently shown in experiments and clinical studies, highlighting the importance of angiogenesis and the promise of antiangiogenic therapy [6]. For instance, clinical studies examining neovascular age-related

Abbreviations: VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; siRNA-CD9, CD9-specific small interfering RNA; bFGF, basic fibroblast growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HMVECs, normal adult human dermal microvascular endothelial cells; siRNA-LaminA/C, lamin A/C-specific siRNA; siRNA-Random, control siRNA; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; FAK, focal adhesion kinase; PBS, phosphate-buffered saline; CNV, choroidal neovascularization.

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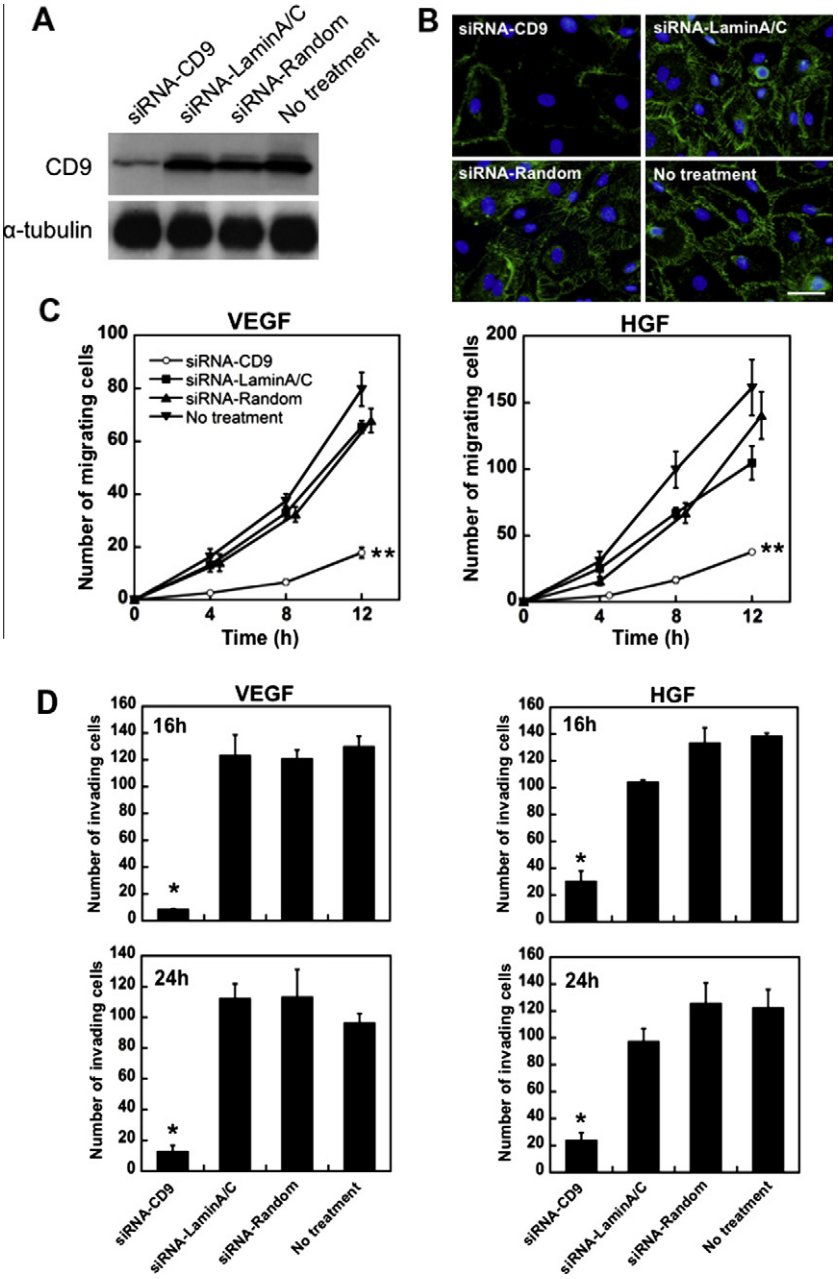

Fig. 1. Knocking down CD9 expression inhibited endothelial cell migration and invasion *in vitro*. (A) Western blot analysis. (B) Immunocytochemical analysis. CD9 protein expression was inhibited by siRNA-CD9. Scale bar, 50 μm. (C, D) Migration (C) and invasion (D) of siRNA-transfected HMVECs through chamber filters and Matrigel-coated membrane filters, respectively, at the indicated time points after treatment with VEGF or HGF. Transfection with siRNA-CD9 significantly inhibited migration and invasion of HMVECs. Results are shown as means ± SEM. $n = 5$ (C) and $n = 3$ in three experiments (D); $^{*}P < 0.05$ and $^{**}P < 0.005$ compared with siRNA-Random (Student's *t*-test).

macular degeneration revealed more meaningful improvements with intravitreous injections of ranibizumab (anti-VEGF-A antibody) than that with conventional photodynamic therapy [6]. Despite the promising clinical results, anti-VEGF agents do have some limitations as antiangiogenic therapies. Anti-VEGF antibodies, for example, are unlikely to inhibit all VEGF subtypes and receptor families, and clearly do not directly address angiogenesis that is induced by other growth factors, such as HGF [4,5]. Moreover, clinical studies showed that the effectiveness of anti-VEGF antibodies diminished during long-term treatment, and anti-VEGF pharmacotherapy was associated with significant adverse effects, likely owing to responses in normal vessels [7,8]. Rather, an ideal antiangiogenic agent would be easily administered, inhibitory to angiogenesis induced by a range of stimuli, and free of significant safety concerns.

CD9 is a tetraspanin that forms multimolecular complexes via lateral associations with various membrane proteins [9]. Although CD9 has been implicated in cell morphology, motility, and fusion, the precise *in vivo* functions of CD9 have yet to be elucidated [9]. In fact, recent studies of CD9 knockout mice, including experiments performed in several laboratories, identified only an essential role in gamete membrane fusion as a nonredundant function of CD9, even though CD9 is expressed in a range of tissues, including Schwann cells, platelets, smooth muscle cells, and endothelial cells [10,11]. In addition, CD9 was independently cloned as a motility-related protein that may inhibit cancer cell metastasis [12]; the antimetastatic activity remains controversial, however, owing to inconsistent and at times contradicting results, which may reflect differences in the examined cancer types [13,14].

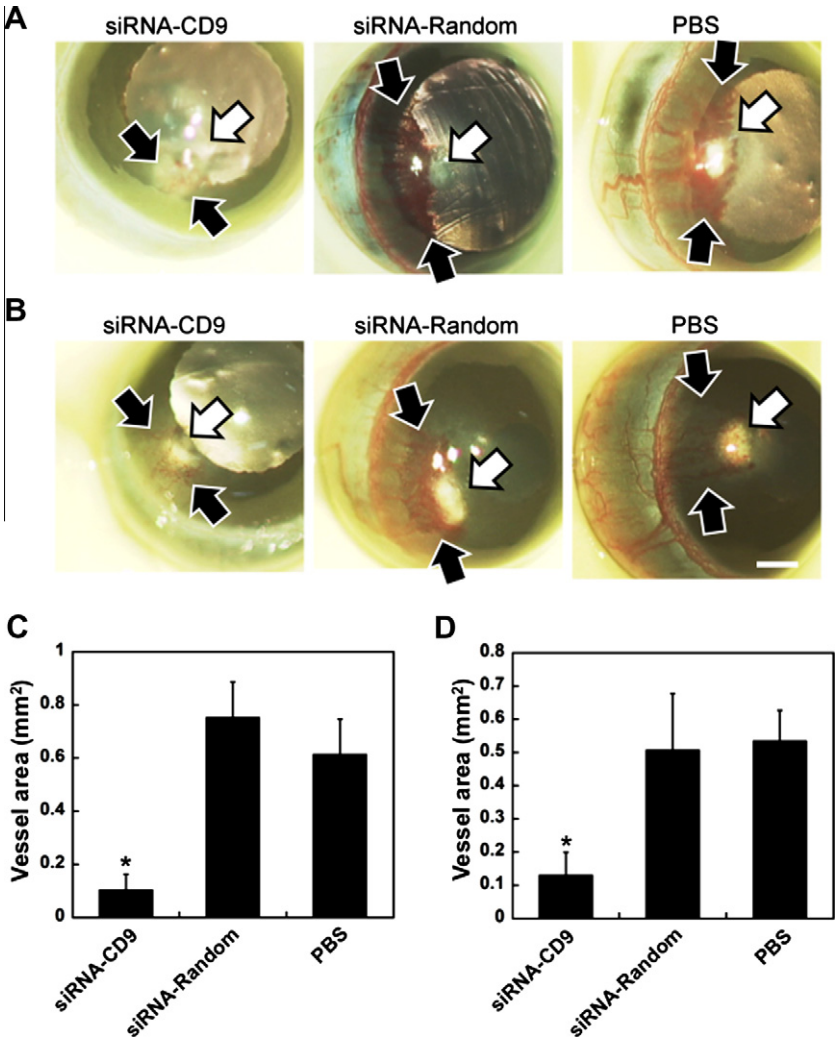

Fig. 2. Injections of siRNA-CD9 efficiently inhibited *in vivo* angiogenesis induced by various stimuli in rat cornea. (A, B) Macroscopic images of neovascularization in rat cornea 7 days after insertion of slow-releasing VEGF (A) or HGF (B) pellets into rat corneal micropockets and subsequent subconjunctival injections of siRNA at 0 and 24 h. White and black arrows indicate inserted pellets and neovascularization, respectively. Scale bar, 1 mm. (C, D) Vessel lengths (C; VEGF, D; HGF) were measured on days 4 and 7, and angiogenic areas were calculated as described in the methods. Results are shown as means ± SEM. $n = 7–10$ in each group. $^{*}P < 0.05$ versus siRNA-Random (Student's *t*-test).

We hypothesized that downregulating CD9 expression in endothelial cells during active angiogenesis would reveal the role of CD9, which would be inconspicuous in relatively quiescent endothelial cells. Here, we show that CD9 is stimulus-independently required for angiogenesis *in vitro* and *in vivo*, and demonstrate that a relatively simple injection of CD9-specific small-interfering RNA (siRNA-CD9) was beneficial in a representative ocular angiogenesis disease model.

2. Materials and methods

2.1. Cell culture

Normal adult human dermal microvascular endothelial cells (HMVECs) (Cambrex, East Rutherford, NJ) were cultured in EGM®-2-MV BulletKit® media (Takara Bio, Otsu, Shiga, Japan) according to the manufacturer's protocol except that serum concentrations were reduced from the standard 5% to 2%.

2.2. siRNA transfection

HMVECs were transfected with siRNA-CD9 (the sequence for silencing CD9; sense: 5′-GAGCAUCUUCGAGCAAGAAtt-3′; antisense: 5′-UUCUUGCUCGAAGAUGCUCtt-3′) [15]; lamin A/C-specific siRNA (siRNA-LaminA/C) (sense: 5′-CUGGACUUCCAGAAGAACAtt-3′; anti-sense: 5′-UGUUCUUCUGGAAGUCCAGtt-3′) [16]; or control siRNA (siRNA-Random) (sense: 5′-UCUUAAUCGCGUAUAAGGCtt-3′; antisense: 5′-GCCUUAUACGCGAUUAAGAtt-3′) using Lipofectamine RNAiMAX® and Opti-MEM® (Invitrogen, Carlsbad, CA).

2.3. Migration and invasion assays

For migration and invasion assays, siRNA-transfected HMVECs were plated in media on the upper chamber containing uncoated or Matrigel-coated membrane filters (8.0-μm pore size; Becton Dickinson, Tokyo, Japan). Recombinant VEGF, or HGF (final concentration, 20 ng/mL) was added to the lower chamber. After incubation for the indicated period, the numbers of migrating and invading cells were counted as described previously [17].

2.4. Viability assays

Cell viability was determined using a WST-8 assay as described previously [18,19].

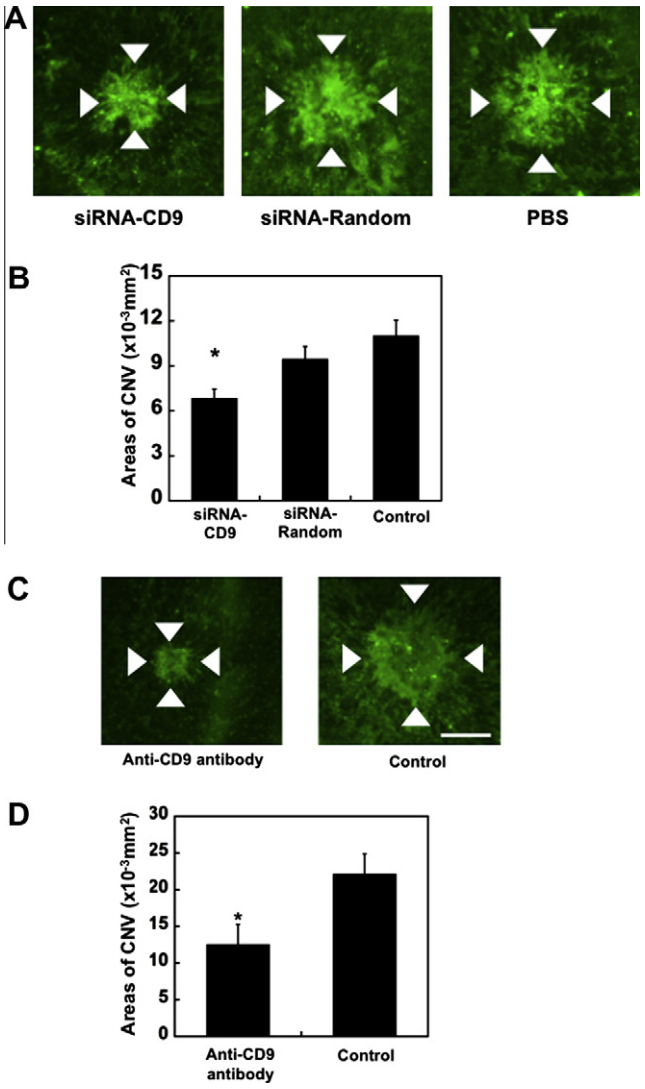

Fig. 3. Intravitreous injections of siRNA-CD9 or anti-CD9 antibodies effectively inhibited CNV. (A, C) Fluorescence images after laser photocoagulation and subsequent siRNA injections (A) or anti-CD9 antibodies (C). Ruptures in Bruch's membrane were induced at 4–5 locations in each eye on day 1. (A) On days 1 and 7, the mice received 3 μL (20 pmol/μL) intravitreous injections of siRNA or PBS in the eyes. On day 14, the mice were perfused with fluorescein-labeled dextran and choroidal flat mounts were examined using fluorescence microscopy. Original magnification, 200×. (C) The same experiment was performed using 5 μL (10 ng) of anti-CD9 antibodies instead of siRNA. Scale bar, 100 μm. Arrowheads in (A, C) indicate areas of CNV. (B, D) Areas of CNV on day 14 as shown in (A, C) were measured. Injections of siRNA-CD9 or anti-CD9 antibodies resulted in significantly smaller areas of CNV than those observed in control samples. Results are shown as means ± SEM. $n = 14–17$ in each group. $^*P < 0.05$ siRNA-CD9 versus siRNA-Random, and anti-CD9 antibodies versus control (Student's *t*-test).

2.5. Immunocytochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Immunocytochemical analysis was performed as described previously [20,21] using either primary anti-CD9 (ALB6, Immunotech by Beckman Coulter, Brea, CA) or anti-Ki-67 (Abcam, Tokyo, Japan) antibodies, fluorescent secondary antibodies (Alexa 488 and/or 594, Invitrogen) and Hoechst 33342 (Invitrogen). TUNEL staining was performed as described previously [19,21].

2.6. In vitro wound healing assay

Wound healing assays were performed as described previously with some modifications [22]. Briefly, monolayers of siRNA-transfected HMVECs on fibronectin-coated slides were disrupted with a pipette tip and subsequently cultured in media containing 20 ng/mL VEGF for 24 h. Lesions were fixed and immunocytochemically labeled with rabbit anti-human MMP14 (MT1-MMP) (Abcam) or rabbit anti-human integrin β1 (Millipore, Tokyo, Japan) antibodies. Secondary anti-rabbit fluorescent antibodies were used for visualization.

2.7. Immunoblotting

Western blot analysis was performed as described previously [19,20] using the following antibodies: anti-CD9 (MM2/57, Southern Biotech, Birmingham, AL), anti-α-tubulin (Sigma–Aldrich, Tokyo, Japan), anti-Flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Met (Santa Cruz Biotechnology), anti-FAK (Santa Cruz Biotechnology), anti-phospho-Flk-1 (Tyr951 and Tyr1175, Cell Signaling Technology, Tokyo, Japan), anti-phospho-Met (Tyr1234/1235 and Tyr1349, Cell Signaling Technology; Tyr1356, Abcam), and anti-phospho-FAK (Tyr397, Invitrogen; Tyr925, Santa Cruz Biotechnology).

2.8. Animal experiments

To analyze *in vivo* angiogenic activities, a rat corneal micropocket assay was performed as described previously [23]. Briefly, pellets containing VEGF or HGF (160 ng per pellet) were inserted into surgically created superficial corneal micropockets of 9- to 10-week-old male brown Norway rats (Kyudo, Fukuoka, Japan) on day 1. siRNA in phosphate-buffered saline (PBS; 120 pmol/10 μL per eye) was subconjunctivally injected 1 mm behind the limbus 6 h and 24 h after VEGF or HGF pellet implantation. On days 4 and 7, the lengths of the vessels were measured, and the angiogenic area (mm^2) was calculated according to the following formula: $0.02\pi \times$ vessel length (mm) $\times$ hour.

To assess the therapeutic potential of this approach in angiogenic diseases, a choroidal neovascularization (CNV) model was employed as described previously [24]. Briefly, each retina of 7- to 8-week-old male C57BL/6J mice was subjected to 659-nm diode laser photocoagulation to generate burns. Later on the same day (day 1) and on day 7, mice were given intravitreous injections of 2 μL (20 pmol/μL) of siRNA-CD9, siRNA-Random, or PBS alone. Mice were perfused with fluorescein-labeled dextran (Sigma, Aldrich) and sacrificed 14 days after laser photocoagulation. The eyes were removed and flat mounts were examined under a fluorescence microscope. The strongly labeled area associated with each burn, which represented the total fibrovascular scar, was measured.

The protocol for the animal experiments was approved by the Animal Research Committee of Kagoshima University and animal experiments were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

2.9. Statistical analysis

Data are represented as means ± standard errors. Statistical analysis between test and control groups was performed using Student's *t*-tests, and $P < 0.05$ was defined as statistical significance.

3. Results

3.1. Migration-inducing activities of angiogenic factors and adenoviral CD9 transduction

After screening four representative angiogenic growth factors—i.e., VEGF, HGF, bFGF and HB-EGF—we chose VEGF and HGF as

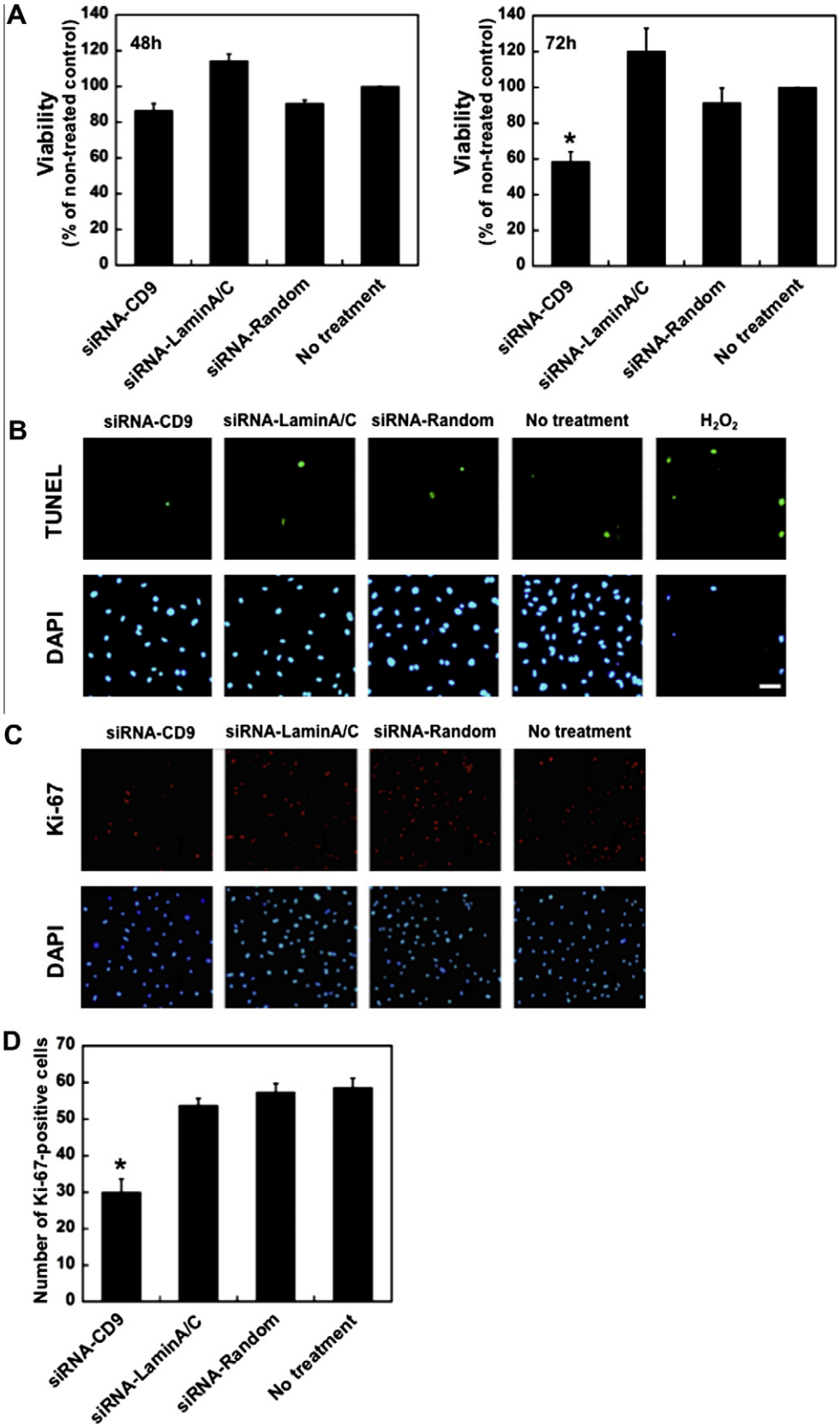

Fig. 4. CD9 is involved in endothelial cell proliferation under physiologic conditions. (A) HMVECs were counted in a WST-8 assay 48 and 72 h after siRNA transfection and subsequent culture without angiogenic stimuli. Knocking down CD9 expression resulted in a significant decrease in the cell number at 72 h but not at 48 h. Results are shown as means ± SEM. $n = 6$ in each group. $^{*}P < 0.05$ versus siRNA-Random (Student's t-test). (B) TUNEL-staining at 72 h using the same experimental conditions described in (A). The number of TUNEL-positive, apoptotic HMVECs did not significantly differ among the treatment groups. Hydrogen peroxide (H_2O_2) was used as a positive control to produce TUNEL-positive apoptotic cells. Scale bar, 100 μm. (C) Fluorescence images of Ki-67-immunocytochemical staining at 72 h using the same experimental condition shown in (A). (D) Ki-67-positive cells were counted in 60 fields under 200× magnification (as shown in (C)). Results are shown as means ± SEM. $n = 12$ for each group. $^{*}P < 0.05$ versus siRNA-Random (Student's t-test).

diverse angiogenic stimuli and used them in the following experiments (Supplementary Fig. S1).

We detected high levels of endogenous CD9 protein in HMVECs, and markedly increased expression was not observed following adenoviral CD9 gene transduction (Supplementary Fig. S2).

3.2. Stimulus-independent inhibition of in vitro migration and invasion by knocking down CD9 expression

We examined whether knockdown of CD9 expression affected *in vitro* HMVEC migration and invasion of using CD9-specific siRNA sequence. Western blotting and immunocytochemical analyses demonstrated that transduction of siRNA-CD9 potently inhibited CD9 expression (Fig. 1A and B). Transduction of siRNA-CD9 significantly inhibited both VEGF- and HGF-induced migration and invasion of endothelial cells (Fig. 1C and D).

3.3. Stimulus-independent inhibition of in vivo angiogenesis by injecting siRNA-CD9

We then examined whether the stimulus-independent antiangiogenic effects of reduced CD9 expression manifest *in vivo* using a rat cornea micropocket angiogenesis assay. With either control injection, both VEGF and HGF induced prominent neovascularization in the avascular corneas after 7 days (Fig. 2A and B). The average neovascular areas in rats administered VEGF or HGF followed by siRNA-CD9 injections were >70% smaller compared with results obtained with control injections (Fig. 2C and D). Our *in vivo* and *in vitro* results demonstrated that knocking down CD9 expression had potent antiangiogenic effects, which were not specific to the stimulus that was used to induce angiogenesis.

3.4. Therapeutic effect of injection of siRNA-CD9 or anti-CD9 antibodies in an eye disease model

We assessed the therapeutic potential of intravitreous injections of siRNA-CD9 using a laser-induced CNV model. Advantages of this animal model include the involvement of a number of factors and a location at which many human ocular angiogenic diseases occur [24]. On day 14, the areas of CNV were 28% and 38% smaller in eyes treated with siRNA-CD9 than in eyes treated with siRNA-Random and vehicle, respectively; these differences were statistically significant (Fig. 3A and B). To further verify the biological finding and to examine the possibility of other therapeutic options, the same experiment was performed using anti-CD9 antibodies rather than siRNA-CD9. The antiangiogenic effects of injections of anti-CD9 antibodies were significant (Fig. 3C and D). Thus, relatively simple interventions, regardless of inhibiting CD9 expression or function, resulted in significant therapeutic effects in an animal disease model. These results further support the crucial role of CD9 in angiogenesis and suggest that various anti-CD9 agents may be clinically effective as antiangiogenic pharmacotherapies.

3.5. Effects of CD9 expression knockdown on activation of growth factor receptors

We examined two possible mechanisms of antiangiogenic effects of targeting CD9. We first analyzed a speculation, whether knocking down CD9 expression affected the activation of growth factor receptors or intercellular signal transduction *in vitro*. Following VEGF stimulation, no significant differences were detected among the groups in VEGF receptor-2 expression levels or activation of this receptor at two well-known phosphorylation sites (Tyr951 and Tyr1175) [4] (Supplementary Fig. 3A). Similarly, expression levels and activation of c-Met/HGF receptors (Tyr1234/1235, Tyr1349 and Tyr1356) [5] following HGF stimulation were not affected by knocking down CD9 expression (Supplementary Fig. 3B). In addition, expression and activation of focal adhesion kinase (FAK), a key mediator of crosstalk between integrin and growth factor receptors [25], were not affected by knocking down CD9 expression (Supplementary Fig. 3A and B). These results suggest that the antiangiogenic effect of targeting CD9 expression is not caused by direct inhibition of angiogenic signaling pathways.

3.6. Abnormal localization of integrins and membrane type 1-matrix metalloproteinase by knocking down CD9 expression

To examine another speculation that CD9 actively functions in the angiogenesis machinery of endothelial cells, the expression and localization of integrin $\alpha3\beta1$ and membrane type 1-matrix metalloproteinase (MT1-MMP) were examined by a Western blotting analysis and in an *in vitro* wound healing assay. These proteins were selected because both are involved in angiogenesis and are directly associated with CD9 in some types of cancer [9]. The expression levels of integrin $\alpha3$ and $\beta1$ were not affected by knocking down CD9 expression (Supplementary Fig. 4). Reduced CD9 expression, however, resulted in abnormal localization of integrin $\beta1$ and MT1-MMP and accumulation of both proteins at membrane leading edge in migrating cells at the wound border (*Supplementary* Fig. 4B–D). Thus, CD9 appears to, at least in part, contribute to the delocalization of integrins and MT1-MMP, disruption of which may prevent angiogenesis.

3.7. The roles of CD9 in relatively quiescent endothelial cells

To examine the roles of CD9 in relatively quiescent rather than actively stimulated endothelial cells, changes in viable cell numbers in response to reduced CD9 expression without an angiogenic stimulus were examined *in vtiro*. Significantly fewer cells were present 72 h but not 48 h after transduction of siRNA-CD9 compared with results obtained with any of the control samples (siRNA-LaminA/C, siRNA-random, and no siRNA) (Fig. 4A). Because these results may have reflected induction of cell death or inhibition of cell growth, we assessed the samples using TUNEL and immunocytochemistry for Ki-67. The number of TUNEL-positive apoptotic cells was not affected by knocking down CD9 expression, suggesting that CD9 is not an angiotrophic factor that is essential for endothelial cell survival (Fig. 4B). In contrast, the number of Ki-67-positive proliferating cells decreased in response to siRNA-CD9 (Fig. 4C and D). Thus, CD9 is involved in the proliferation but not survival of relatively quiescent endothelial cells, although knocking down CD9 expression under these conditions produced a phenotype that was less severe than that detected during angiogenesis.

4. Discussion

This is the first study to elucidate that targeting CD9 can be an effective and stimulus-independent antiangiogenic medicine, at least for ocular diseases, and has provided clinically and biologically meaningful information. Interestingly, CD9 plays a critical stimulus-independent and predominant role in activated rather than quiescent endothelial cells. In contrast to its significant contribution to angiogenesis, CD9 showed a lesser role in the proliferation but not survival of endothelial cells under quiescent conditions. Although a previous study showed an association between CD9 and growth factor receptors in cancer [26], the phenotype observed in our study suggested that CD9 does not interact directly with growth factor receptors to transduce angiogenic

signals in endothelial cells. In this regard, it should be noted that both integrin β1 and MT1-MMP were abnormally localized at the leading edge of the lesion following knock down of CD9 expression. CD9 colocalizes and/or associates on membranes with integrins, particularly integrin α3β1 in endothelial cells, and integrins themselves are critically involved in angiogenesis [27]. Similarly, MT1-MMP contributes to angiogenesis by associating with actin fibers in the lamellipodia of migrating cells where it performs proteolytic functions and by internalization of MT1-MMP via caveolae [28]. On the other hand, cellular delocalization of integrins and MT1-MMP is essential for cell migration and/or invasion [29]. In this respect, our findings suggest that CD9, unlike VEGF, is not an angiotrophic factor that is required for endothelial cell survival, but is instead part of the angiogenesis machinery in endothelial cells, mediating migration, proliferation, and degradation of the extracellular matrix during invasion, probably by interacting with other membrane-associated partner proteins, e.g., the delocalization of integrins and MT1-MMP [2,9]. Elucidating the precise underlying molecular mechanisms should be examined in future studies focusing on a number of candidate and/or yet to be identified partner proteins [9].

Clinically, targeting CD9—possibly via simple intravitreous injections of siRNA-CD9 or anti-CD9 antibodies—appears to be an effective, stimulus-independent, and safe antiangiogenic approach. Importantly, stimulus-independent therapy may be superior to specific anti-VEGF agents. Moreover, this method also benefits from the predominant effects during angiogenesis rather than on existing vasculature. Deficiencies identified in clinical applications of bevacizumab (anti-VEGF antibody) include macular ischemia and sustained elevation in intraocular pressure after local intravitreous injections for ocular diseases, and more widespread side effects after systemic injections of bevacizumab for cancer [7,8,30]. These effects are likely caused, at least in part, by roles of VEGF in both angiogenesis and the normal vasculature. On the other hand, siRNA-CD9 and anti-CD9 antibodies may have fewer side effects for a number of reasons. First, unlike anti-VEGF agents, knocking down CD9 expression did not directly inhibit growth factor-induced signal transduction, which is required in normal vasculature and angiogenesis. Second, knocking down of CD9 expression did not induce cell death under conditions in which the cells were relatively quiescent. Third, adult CD9 knockout mice in previous studies did not show marked abnormalities in vasculogenesis or vasculature [10]. These results support our hypothesis that targeting CD9 may have fewer side effects, although this must be carefully studied in future preclinical studies.

In conclusion, the present study revealed that CD9 plays a stimulus-independent role in the angiogenic activity of endothelial cells, and inhibition of CD9 expression or function via intravitreous injections of siRNA-CD9 or anti-CD9 antibodies may provide a new therapeutic strategy for angiogenesis such as that observed in certain ocular diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.08.068.

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