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A designed angiopoietin-2 variant, pentameric COMP-Ang2, strongly activates Tie2 receptor and stimulates angiogenesis

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ABSTRACT

Despite that angiopoietin-2 (Ang2) produces more versatile and dynamic functions than angiopoietin-1 (Ang1) in angiogenesis and inflammation, the molecular mechanism that underlies this difference is still unknown. To define the role of oligomerization of Ang2 in activation of its receptor, Tie2, we designed and generated different oligomeric forms of Ang2 by replacement of the amino-terminal domains of Ang2 with dimeric, tetrameric, and pentameric short coiled-coil domains derived from GCN4, matrillin-1, and COMP. COMP-Ang2 strongly binds and activates Tie2, whereas GCN4-Ang2 and MAT-Ang2 weakly to moderately bind and activate Tie2. Although native Ang2 strongly binds to Tie2, it does not activate Tie2. Accordingly, COMP-Ang2 strongly promotes endothelial cell survival, migration, and tube formation in a Tie2-dependent manner, and the potency of COMP-Ang2 is almost identical to that of COMP-Ang1. Furthermore, the potency of COMP-Ang2-induced enhanced angiogenesis in the wound healing region is almost identical to the potency of COMP-Ang1-induced enhanced angiogenesis. Overall, there is no obvious difference between COMP-Ang2 and COMP-Ang1 in *in vitro* and *in vivo* angiogenesis. Our results provide compelling evidence that proper oligomerization of Ang2 is a critical determinant of its binding and activation of Tie2.

1. Introduction

The angiopoietin (Ang) family of growth factors, Ang1, Ang2, Ang3 and Ang4, are secreted protein ligands of *T*yrosine kinase with *I*mmunoglobulin and *E*pidermal growth factor homology domain *2* (Tie2) [1], which is mainly expressed in vascular endothelial cells [2,3], hematopoietic stem cells [4,5] and a subset of monocytes/macrophages [6]. The structure of all angiopoietins basically consists of a carboxy-terminal fibrinogen-like domain that is responsible for receptor binding, a central coiled-coil domain that oligomerizes these fibrinogen-like domains, and a short amino-terminal domain that superclusters these oligomers into variably-sized multimers [1,7–10]. Despite the high similarity in structure among angiopoietins, they have distinct physiological functions in angiogenesis, lymphangiogenesis, and inflammation [1,11–14]. Ang1 induces well-organized angiogenesis as well as vascular stabilization by enhancing endothelial integrity [9,15], whereas Ang2 induces more versatile and dynamic functions depending on its environment [13]. For instance, Ang2 regresses and destabilizes blood vessels in the absence of VEGF, but leads to robust angiogenesis in the presence of VEGF, specifically in the context of tumor angiogenesis [16], transient vascular network formation in the pupillary membrane of the developing eye [17], and subcutaneous wound healing [14]. Moreover, Ang2 sensitizes endothelial cells to TNF-α and has a crucial role in the induction of inflammation [14,18,19]. In contrast, compelling evidence for an antagonistic role of Ang2 on Ang1/Tie2 signaling in quiescent endothelium was recently reported by the demonstration that conditional transgenic overexpression of Ang2 in vascular endothelial cells completely abolished Tie2 phosphorylation *in vivo* [20]. However, the underlying molecular and cellular mechanisms leading to these distinct roles of Ang1 and Ang2 are still poorly understood.

Oligomerization of the Tie2 receptor brings its kinase domains into close proximity, thus allowing the kinase domains to phosphorylate each other in *trans*. This process appears to be critical for Tie2 activation and Ang1/Tie2-induced endothelial integrity [21,22]. To achieve Tie2 oligomerization and activation, Ang1 is assumed to use a modular and multimeric structure unlike that of any other known growth factor [8,10,23]. In fact, our previous rotary metal-shadowing transmission electron microscopy (RMSTEM) analysis revealed that Ang1 exists as heterogeneous multimers with basic trimeric, tetrameric, and pentameric oligomers, while Ang2 exists mainly as trimeric and tetrameric oligomers [10]. These oligomers of Ang1 are able to bind and activate Tie2 properly, whereas recombinant monomeric and dimeric Ang1 bind only weakly and are not able to activate Tie2 [9], supporting the idea that proper oligomerization of Ang1 is required for Tie2 oligomerization and activation. In contrast, oligomeric native

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Ang2 binds strongly to Tie2, but activates the receptor weakly or not at all, suggesting that Ang2 may not be able to induce adequate Tie2 oligomerization. However, the molecular mechanism that underlies this difference between Ang1 and Ang2 on Tie2 oligomerization and activation is still unknown. Previously, we invented Ang1 variant, COMP-Ang1, by replacement of the amino-terminus of Ang1 with the short coiled-coil domain of cartilage oligomeric matrix protein (COMP) [24]. COMP-Ang1 is more potent than native Ang1 in phosphorylating the Tie2 receptor and signaling via Akt in primary cultured endothelial cells [9] and in *in vivo* angiogenesis [25]. During these studies, we learned that proper oligomerization of Ang1 is necessary for producing its biological effects via Tie2. However, we do not yet understand the role of proper oligomerization of Ang2 in Tie2 activation.

To define the role of oligomerization of Ang2 in Tie2 receptor oligomerization and activation, we generated dimeric, tetrameric, and pentameric Ang2 by replacement of the amino-terminal superclustering and coiled-coil domains with dimeric, tetrameric, and pentameric short coiled-coil domains. Then we characterized the activities of these constructs in binding and phosphorylating Tie2, and in endothelial survival, migration, and tube formation *in vitro*. Because pentameric Ang2 and pentameric Ang1 show similar effects *in vitro*, we compared their angiogenic effects in a wound healing model. Our results show that pentameric Ang2 is equivalent to pentameric Ang1 and shed light on the role of oligomerization of angiopoietin in the activation of Tie2.

2. Materials and methods

2.1. Generation of recombinant Ang2 proteins

The pcDNA vector (Invitrogen) containing a secretory signal sequence for hemagglutinin and a FLAG tag was used to construct the coiled-coil domains of GCN4 [26], matrilin-1 (MAT) [27], or COMP [10,24] fused to the linker and fibrinogen-like domain (amino acid 258–496) of Ang2 (GCN4-Ang2, MAT-Ang2, and COMP-Ang2, respectively). The recombinant Ang2 proteins were produced by transient expression of the gene constructs in human embryonic kidney 293 (HEK293) cells (American Type Culture Collection) using Effectene liposomal transfection according to the manufacturer's instructions (Qiagen, Inc.). The transfected HEK293 cells were maintained with Dulbecco's modified Eagle's medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum at 37 °C in 5% CO_2. The supernatant was harvested from the transfected cells after 4 days, and the recombinant proteins containing the FLAG sequence were purified using column chromatography on anti-FLAG M2 antibody agarose affinity gel (Sigma) and eluted with FLAG peptide (Sigma). After purification, the recombinant proteins were quantitated using the Bradford assay and confirmed with Coomassie blue staining of an SDS-polyacrylamide gel. These analyses showed that the yield was 400–500 μg of each recombinant protein per liter of the HEK293 cell supernatant.

2.2. Transmission electron microscopy

The Ang2 recombinant proteins were negatively stained by uranyl formate and imaged at room temperature in a Tecnai T120 microscope (FEI) operated at 120 kV at a magnification of 52,000×. Images were recorded on a 4 k×4 k UltraScan CCD camera (Gatan Inc.) with a defocus of −1.5 μm. Particles were manually selected by using a program Ximdisp, the display program associated with the MRC program suite [28]. Image processing was performed using SPIDER [29]. These particle images were first subjected to 10 rounds of standard multi-reference alignment and classification protocols specifying 40 classes [30]. Two unique averages were selected from the resulting class averages and used as references for another 10 cycles of multi-reference alignment.

2.3. Characterization of the recombinant proteins and Tie2 binding assay

SDS-PAGE analysis of proteins was performed under non-reducing and reducing (heating for 10 min in 0.435 M β-mercaptoethanol) conditions. Binding of the recombinant proteins to the soluble extracellular domain of Tie1-Fc (sTie1-Fc, T1) or Tie2-Fc (sTie2-Fc, T2) (R&D systems) was assayed using an *in vitro* binding assay. Briefly, each recombinant protein (100 ng) was mixed with 500 ng of sTie1-Fc or sTie2-Fc and incubated in 500 μl of Tris buffer solution (50 mM Tris, 100 mM NaCl, pH 7.4) containing 0.02% Triton X-100 at 4 °C for 2 h. Then, 20 μl of Protein-A agarose beads (Oncogene) was added and incubated for another 1 h. The Protein-A-conjugated samples were washed twice with 1 ml of Tris buffer containing 0.02% Triton X-100. The samples were separated further by SDS-PAGE, electroblotted onto nitrocellulose membranes, and probed with anti-FLAG M2 antibody (Sigma).

2.4. Characterization of endogenous Ang2 secreted from primary cultured HUVECs

Human umbilical vein endothelial cells (HUVECs) were prepared from human umbilical cords by collagenase digestion and maintained as described previously [31]. HUVECs were seeded at a density of 1×10^6 cells/ml on gelatin coated dish. After cells were incubated for 4 days in Endothelial Cell Basal Medium-2 (EBM-2) supplemented with 5% FBS, supernatant media (~40 ml) harvested and pre-cleared with 20 μl of Protein-A agarose beads (Oncogene) for 2 h at 4 °C. After 3 times of pre-clearing, 10 μg of sTie2-Fc was added and incubated at 4 °C for 4 h, and then 50 μl of Protein-A agarose beads added and incubated at 4 °C for 2 h. The Protein-A-conjugated samples were washed twice with 1 ml of Tris buffer containing 0.02% Triton X-100. The samples were separated further by SDS-PAGE under non-reducing and reducing (heating for 10 min in 0.435 M β-mercaptoethanol) conditions, electroblotted onto nitrocellulose membranes, and probed with anti-Ang2 antibody (R&D systems).

2.5. Tie2 and Akt phosphorylation assays

The primary cultured HUVECs used for this study were between passages 2 and 3. To generate Tie2 expressing stable cells, a cDNA fragment encoding full length Tie2 was cloned into the pCMV-dhfr2 vector, which includes the cytomegalovirus (CMV) promoter, dihydrofolate reductase (dhfr) gene, and neomycin-resistance gene. This construct was used to generate a Tie2 expressing Chinese hamster ovary cell line (CHO-Tie2) as previously described [32,33]. To generate transient Tie2 expressing embryonic kidney 293 (HEK293) cells, 0.2, 1.0 and 5.0 μg of pCMV-dhfr2-Tie2 gene constructs were transfected into HEK293 cells using Effectine (Qiagen). HUVECs and CHO-Tie2 were seeded in 100-mm dishes, whereas transient Tie2 expressing HEK293 cells were incubated in serum containing medium for 48 h after the transfection. When the cells became confluent, they were incubated with serum-free media for 12 h, and the medium was changed to fresh serum-free medium. One hour later, the cells were treated with the indicated proteins for 15 min (for Tie2 phosphorylation) or 30 min (for Akt phosphorylation). For Tie2 phosphorylation, the cell lysates were immunoprecipitated with anti-Tie2 antibody (R&D systems) and immunoblotted with anti-phosphotyrosine antibody (Upstate). Alternatively, for Akt phosphorylation, the cell lysates were harvested, and 50 μg of total protein was loaded onto SDS-PAGE and immunoblotted with anti-phospho-Akt (Ser473) antibody (Cell Signaling). The membranes were stripped and reprobed with anti-Tie2 antibody (Santa Cruz) or anti-Akt antibody (Cell Signaling) to verify equal loading of protein. All signals were visualized and analyzed by densitometric scanning (LAS-1000, Fuji Film).

2.6. Survival, wound healing cell migration, and tube formation assays

For the survival assay, confluent HUVECs were incubated with indicated proteins in serum free M-199 medium for 24 h. The cells were photographed using a phase-contrast microscope, then stained with the Annexin-V-FLUO staining kit (Roche Molecular Biochemicals, Mannheim, Germany) for 15 min at 20 °C. The stained cells were analyzed on a flow cytometer, and data were analyzed with CellQuest software (Becton Dickinson). For the wound healing cell migration assay, HUVECs were cultured to confluence, and a scratch was made using a sterile blue tip. Detached cells were removed, and dishes were treated with the indicated protein for 24 h. Cells were photographed at 0 h and 24 h, and distances of migration were measured. For the tube formation assay, 200 μl of growth factor reduced Matrigel™ (BD Biosciences) were loaded into a 24-well plate on ice and allowed to solidify for 30 min at 37 °C. HUVECs ($\sim 5\times 10^4$ cells) were resuspended in 1 ml of M-199 medium with the indicated proteins and seeded onto a Matrigel™ coated well plate. After a 24-hour incubation at 37 °C, the cells were photographed, and tube forming activities were quantified by measuring tube-like structure lengths exceeding 50 μm per field of 10 mm^2.

2.7. In vivo angiogenesis assay

Recombinant adenoviruses expressing COMP-Ang2, COMP-Ang1, Ang2, or β-galactosidase (β-gal) were constructed using the pAdEasy vector system (Qbiogene) as previously described [25]. Specific pathogen-free FVB/N mice were purchased from Jackson Laboratory (Jackson Labs, Bar Harbor, ME) and bred in our pathogen-free animal facility. Male 7–8-week old mice were used for this study. Animal care and experimental procedures were performed under approval from the Animal Care Committees of KAIST. For hole-punch assays, a 2.0-mm hole was made in the center of both ears of the mouse using a metal ear punch (Harvard Apparatus, Holliston). At 12 h after wounding, $\sim 1\times 10^9$ pfu Ade-

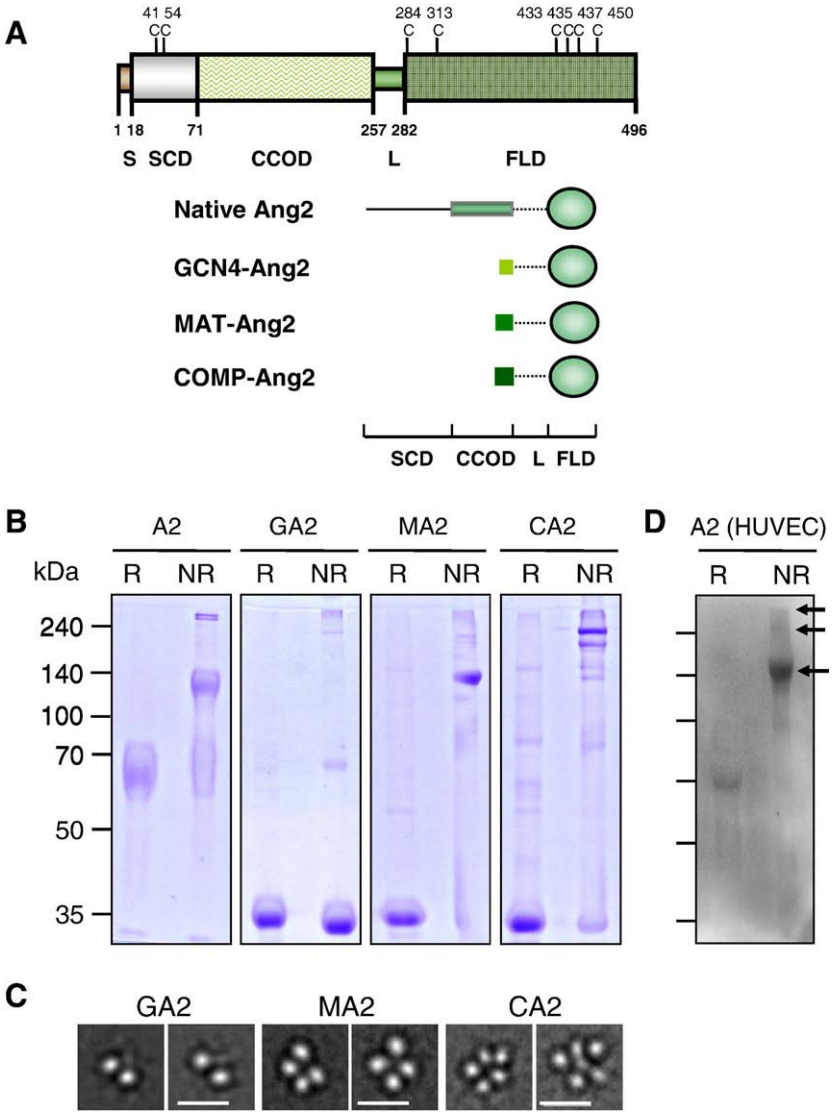

Fig. 1. Protein structure analyses of native recombinant Ang2 and engineered Ang2 variants, and secreted endogenous Ang2 from HUVECs. (A) Schematic diagrams of native Ang2 (A2), GCN4-Ang2 (GA2), MAT-Ang2 (MA2), and COMP-Ang2 (CA2). Amino acids 1–18 are the secretory signal sequence (S), amino acids 19–70 are the superclustering domain (SCD), amino acids 71–257 are the coiled-coil oligomerization domain (CCOD), amino acids 258–281 are the linker region (L), and amino acids 282–496 are the fibrinogen-like domain (FLD) of Ang2. Ang2 contains cysteines (C) at amino acids 41, 54, 284, 313, 433, 435, 437, and 450. The amino-terminal portion (amino acid 1–257) of Ang2 was replaced with the short coiled-coil domains of GCN4, MAT, and COMP to generate oligomeric Ang2 variants. (B) Reduced (R) and non-reduced (NR) proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. Molecular masses (kDa) are indicated at left. (C) The oligomeric states of recombinant proteins were imaged by the electron microscopy. Scale bars = 10 nm. GCN4-Ang2, MAT-Ang2, and COMP-Ang2 form dimeric, tetrameric, and pentameric structures, respectively. (D) Immuno-blotting analysis of secreted Ang2 from primary cultured HUVECs. Arrows indicate different oligomeric forms of Ang2. Left bars indicate molecular masses (kDa) shown in (B). Independent immuno-blotting analyses ($n = 3\sim 4$) show similar findings.

COMP-Ang2, Ade-COMP-Ang1, Ade-Ang2, or Ade-β-gal diluted in 50 μl of sterile 0.9% NaCl was injected intravenously through the tail vein. Circulating recombinant angiopoietins were measured by our established enzyme-linked immunosorbent assay as previously described [14]. At 2 weeks later, the mice were anesthetized with 80 mg/kg of ketamine hydrochloride and 12 mg/kg of xylazine, perfused with 1% paraformaldehyde in PBS, and the ear skins were harvested and immunostained as whole mounts. After blocking with 5% donkey serum in PBST (0.3% Triton X-100 in PBS) for 1 h at RT, samples were incubated with anti-mouse PECAM-1 antibody, hamster clone 2H8, 1:1000 (Chemicon International, Temecula, CA). After several washes in PBST, the samples were incubated for 2 h at RT with Cy3-conjugated anti-hamster IgG antibody, 1:1,000 (Jackson ImmunoResearch). Fluorescent signals were visualized, and digital images were obtained using a Zeiss LSM 510 confocal microscope (Carl Zeiss). For each whole mount, blood vessel densities were measured in each 0.2 mm^2 region of the marginal area and quantified using Image J software.

2.8. Statistics

Values presented are means ± standard deviation (SD). Significant differences between means were determined by Student's *t*-test or analysis of variance followed by the Student–Newman–Keuls test. Statistical significance was set at $P<0.05$.

3. Results

3.1. Generation and analyses of designed oligomeric Ang2 variant proteins

We designed and generated Ang2 oligomeric variants as shown in Fig. 1A. The corresponding variants were termed GCN4-Ang2, MAT-Ang2, and COMP-Ang2. The recombinant proteins were analyzed on SDS-PAGE under denaturing reducing conditions and non-reducing conditions (Fig. 1B). Under reducing conditions, native Ang2 formed a single band of the expected molecular size ~65 kDa, whereas GCN4-Ang2, MAT-Ang2, and COMP-Ang2 each formed a single band of ~35–40 kDa. Under non-reducing conditions, native recombinant Ang2 formed multimeric disulfide patterns, but it formed mainly a dimeric form at ~130 kDa and also higher-order oligomeric-forms with sizes over 240 kDa. MAT-Ang2 mainly formed a disulfide tetramer ~140 kDa, and COMP-Ang2 formed mainly pentamer and hexamers at ~180–210 kDa, whereas GCN4-Ang2 formed monomeric Ang2 at ~35–40 kDa. To confirm the oligomeric structures of the Ang2 variants, images for the multi-reference aligned molecules were captured using negative staining electron microscopy [30]. The most frequent images for GCN4-Ang2, MAT-Ang2, and COMP-Ang2 were dimers, tetramers, and pentamers, respectively (Fig. 1C). In comparison, endogenous Ang2 secreted from primary cultured HUVECs formed multimeric disulfide patterns, but it formed mainly a dimeric form at ~150–160 kDa and also higher-order oligomeric-forms with sizes over 240 kDa under non-reducing condition on SDS-PAGE analysis (Fig. 1D).

3.2. COMP-Ang2 binds and activates Tie2 receptor

In vitro binding analysis revealed that native Ang2 and COMP-Ang2 exhibited relatively strong binding to Tie2 (~95% and ~99% of total input), whereas GCN4-Ang2 and MAT-Ang2 exhibited relatively low binding to Tie2 (~25% and ~33% of total input) (Fig. 2A). However, none of these Ang2 variant proteins bound to Tie1. These results suggested that higher-order oligomerized forms of Ang2 have a higher affinity to Tie2, which is similar to the findings with several types of oligomeric variants of Ang1 [9]. To compare the effect of native Ang2 and COMP-Ang2 on Tie2 activation, the extent of Tie2 phosphorylation was assayed in primary cultured HUVECs and CHO-Tie2 cells (ectopically Tie2-expressing non-endothelial cells). In both cells, a relatively low concentration (0.2 μg/ml) of Ang2 slightly increased Tie2 phosphorylation, whereas the half concentration of COMP-Ang2 markedly increased Tie2 phosphorylation in both HUVECs and CHO-Tie2 cells (~3.0-fold and ~2.4-fold compared to 0.2 μg/ml of native Ang2) (Fig. 2B and C). Even with a relatively high concentrations of COMP-Ang2 (0.5 μg/ml) and Ang2 (1.0 μg/ml), COMP-Ang2-induced

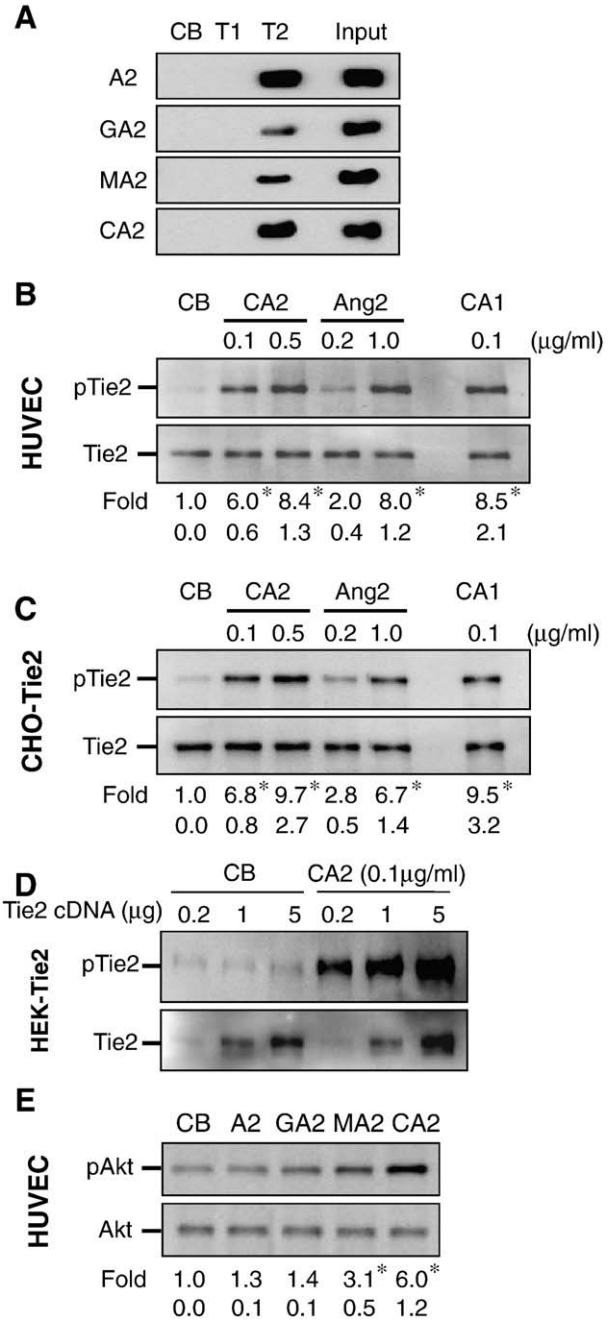

Fig. 2. COMP-Ang2 activates Tie2/Akt signaling. (A) *In vitro* binding analyses of native Ang2 (A2), GCN4-Ang2 (GA2), MAT-Ang2 (MA2), and COMP-Ang2 (CA2) with control buffer (CB), sTie1-Fc (T1) and sTie2-Fc (T2). (B–E) Tie2 and Akt phosphorylation assays. Serum-starved HUVECs CHO-Tie2 or transient Tie2 expressing HEK293 cells were treated with control buffer (CB), indicated concentrations of CA2 or A2, or COMP-Ang1 (CA1) for 15 min, and Tie2 phosphorylation was measured. Serum-starved HUVECs were treated with CB, A2 (400 ng/ml), and GA2, MA2, and CA2 (each 200 ng/ml) for 30 min, and the phosphorylation of Akt (Ser473) was measured. Fold, densitometric analyses presented as the relative ratio of phospho-Tie2 (pTie2) or phospho-Akt (pAkt) to Tie2 or Akt. The relative ratio of phospho-Tie2 to Tie2 or phospho-Akt to Akt with CB is arbitrarily presented as 1. Numbers represent the mean (upper) ± S.D. (lower) from 3–4 experiments. *$P<0.05$ *versus* CB.

Tie2 phosphorylation rates were higher (~1.4-fold and ~1.5-fold) than Ang2-induced phosphorylation rates in both HUVECs and CHO-Tie2 cells (Fig. 2B and C). In fact, expression of Tie2 at varied levels in HEK293 cells resulted in barely detectable but similar levels of Tie2 phosphorylation in the absence of COMP-Ang2 stimulation (Fig. 2D). However, COMP-Ang2 induced marked Tie2 phosphorylation proportionate to the expression level of Tie2 (Fig. 2D). Moreover, the COMP-Ang2-induced increase in Tie2 phosphorylation was comparable to the COMP-Ang1-induced increase in Tie2 phosphorylation (Fig. 2B and C). Thus, proper oligomerization of Ang2, rather than extent of Tie2 expression, is a critical determinant of Tie2 activation. Akt signaling is a major route of downstream signaling in Tie2-induced endothelial cell integrity and angiogenesis [34]. Therefore, we tested the effect of the Ang2 variant proteins on the extent of Akt (Ser473) phosphorylation in HUVECs. Akt (Ser473) phosphorylation was increased 1.3-fold by Ang2, 1.4-fold by GCN4-Ang2, 3.1-fold by MAT-Ang2, and 6.0-fold by COMP-Ang2, compared to that of control buffer (Fig. 2E). Thus, COMP-Ang2 could be an alternative to Ang1 or COMP-Ang1 at least in the activation of Tie2/Akt signaling in endothelial cells.

3.3. COMP-Ang2 promotes endothelial cell survival, migration, and tube formation in a Tie2-dependent manner

Activation of Tie2/Akt signaling is directly involved in endothelial cell survival, migration, and tube formation [34]. Phase contrast microscopic analysis revealed that, under serum starvation, a fair number of floating dead cells were detected in HUVECS treated with control buffer, Ang2, and GCN4-Ang2, whereas few floating dead cells were detected after treatment with 10% serum, COMP-Ang2, or COMP-Ang1 (Fig. 3A). In comparison, low numbers of floating dead cells were detected in HUVECS treated with MAT-Ang2 and Ang1 (Fig. 3A). The cell survival induced by COMP-Ang2 and COMP-Ang1 were largely abolished by adding an excess of

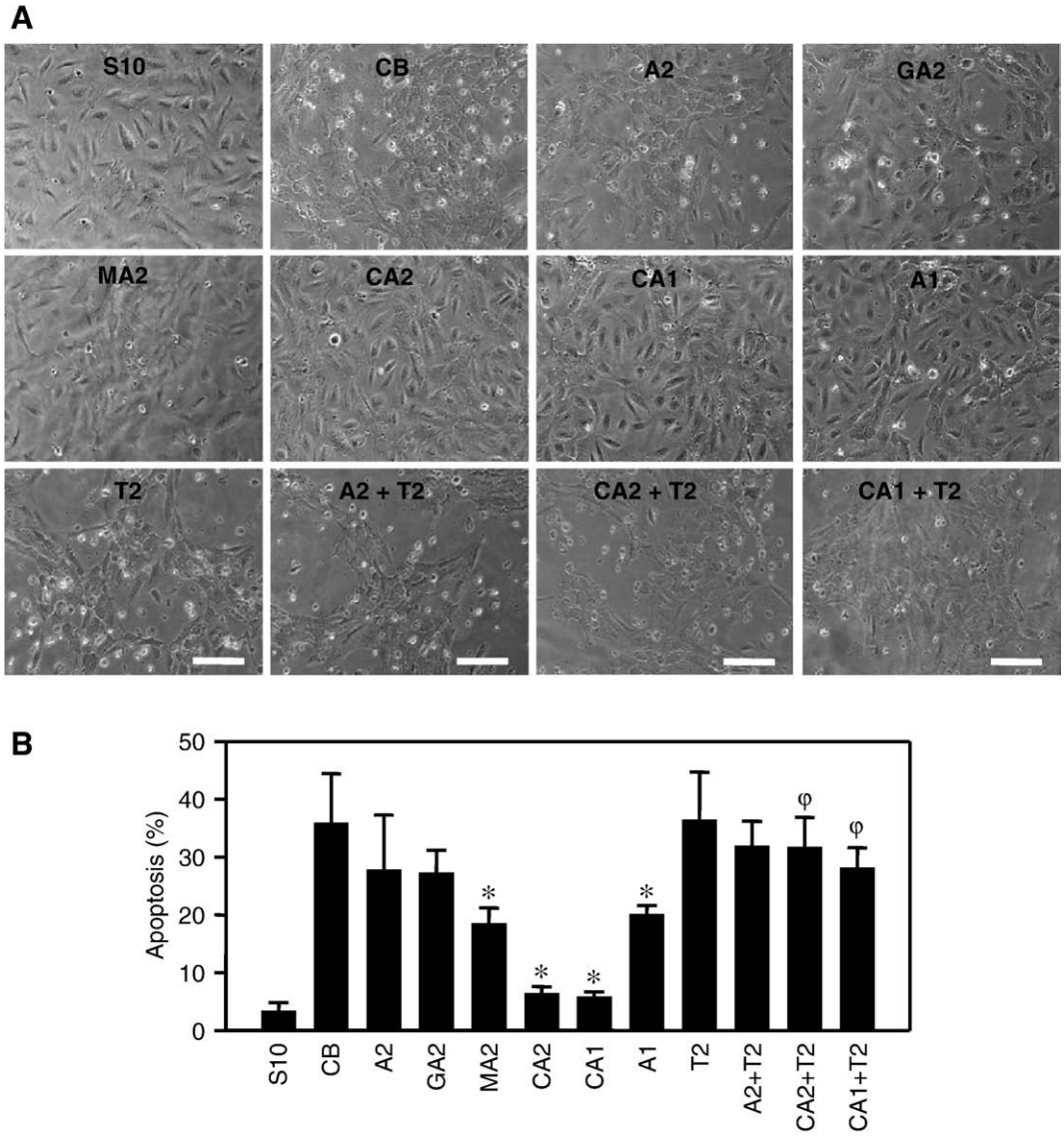

Fig. 3. COMP-Ang2 blocks endothelial cell apoptosis in a Tie2-dependent manner. (A) Representative phase-contrast photographs for effects of Ang2 and Ang1 variants on the serum deprivation-induced endothelial cell apoptosis. HUVECs were incubated with 10% serum-containing medium (S10), serum-free medium with control buffer (CB), native Ang2 (400 ng/ml, A2), each Ang2 variant (200 ng/ml, GA2, MA2 and CA2), COMP-Ang1 (200 ng/ml, CA1) and native Ang1 (400 ng/ml, A1), and serum-free medium with sTie2-Fc (2 μg/ml, T2), A2 + T2, CA2 + T2 and CA1 + T2 for 24 h. Note that the cells incubated with CA2 and CA1 are the most adherent and the least floating, whereas the cells incubated with CB and T2 are the least adherent and the most floating. Scale bars, 0.5 mm. (B) Measurement of apoptosis by Annexin-V immunostaining and flow cytometry. Bars represent the mean ± S.D. from 4 experiments. *$P<0.05$ *versus* CB; $^{\varphi}P<0.05$ *versus* CA2 or CA1.

sTie2-Fc (Fig. 3A), indicating that these actions are Tie2-dependent. Analyses for cell apoptosis/survival using an Annexin-V staining produced results (Fig. 3B and Supplementary Fig. 1) almost identical to those obtained from the phase contrast microscopy. An endothelial cell migration assay was performed by an assessment of wound healing migration after making a scratch on confluent HUVECs [35] and incubating them with each recombinant protein (Fig. 4A). The migration activity of the recombinant proteins were, in order, Ang2<GCN4-Ang2<MAT-Ang2 = Ang1<COMP-Ang2<COMP-Ang1 (Fig. 4B). The cell migration activities of COMP-Ang1 and COMP-Ang2 were largely abolished by an excess of sTie2-Fc (Fig. 4). We also stimulated HUVECs in Matrigel™ with each recombinant protein for 24 h. COMP-Ang2 potently promoted tube formation, and its potency was equivalent to COMP-Ang1, but more than Ang1, MAT-Ang2, and Ang2, and its effect was totally abolished by an excess of sTie2-Fc (Fig. 5A and B).

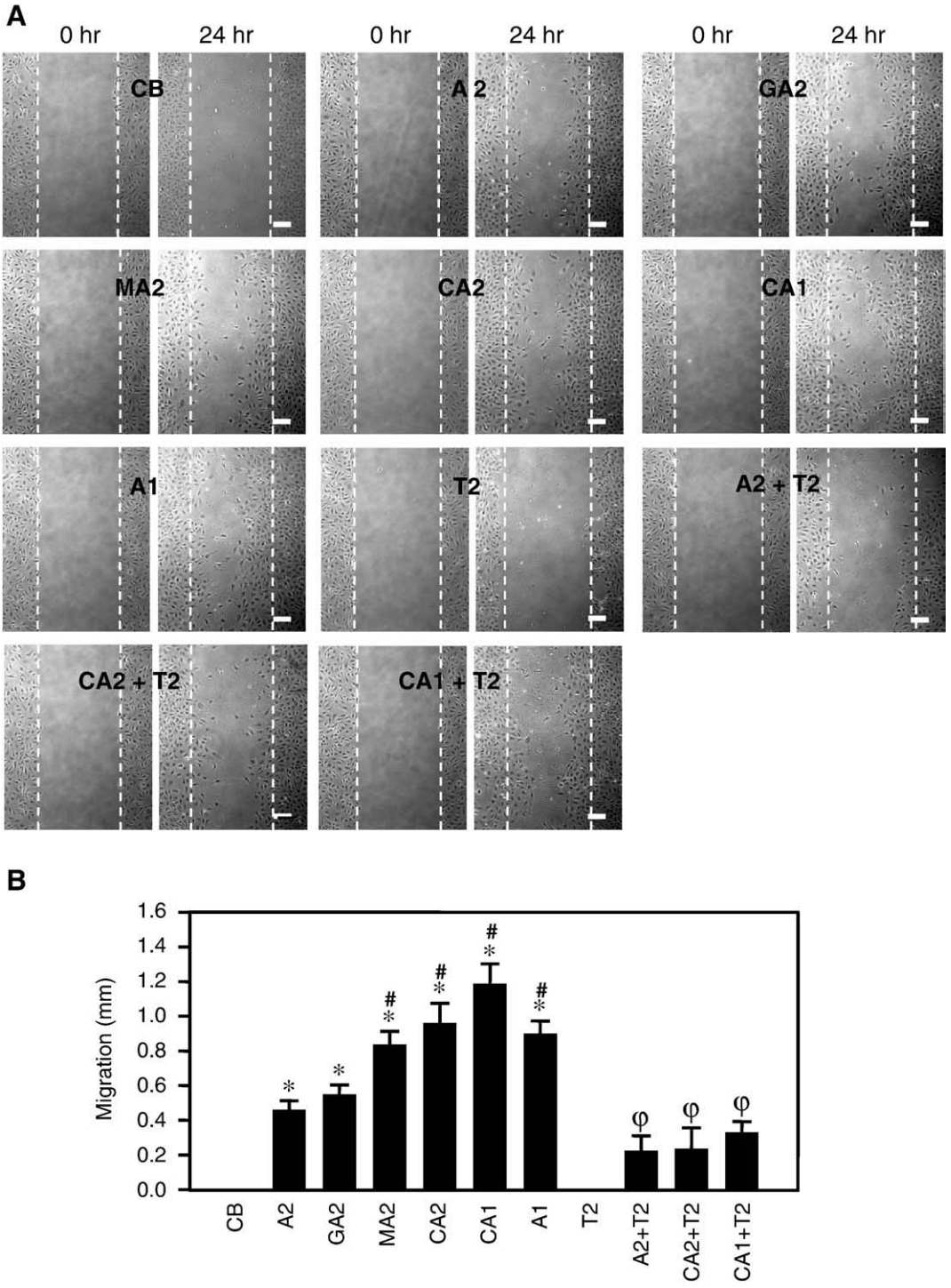

Fig. 4. COMP-Ang2 profoundly induces endothelial cell migration. (A) Representative photographs of the wound healing cell migration. HUVECs were cultured to confluence, and a scratch was made. Then the cells were treated with control buffer (CB), native Ang2 (400 ng/ml, A2), Ang2 variants (GA2, MA2 and CA2, each 200 ng/ml), COMP-Ang1 (200 ng/ml, CA1), native Ang1 (400 ng/ml, A1), sTie2-Fc (2 μg/ml, T2), A2 + T2, CA2 + T2, and CA1 + T2. Photographs were taken at 0 and 24 h after the wound scratch. Dashed lines indicate migration starting points. Scale bars, 0.5 mm. (B) The extent of migration was quantified by the distances of cell migration. Bars represent the mean ± S.D. from 4 experiments. $^{*}P<0.05$ *versus* CB; $^{\#}P<0.05$ *versus* A2; $^{\varphi}P<0.05$ *versus* A2, CA2 or CA1.

3.4. COMP-Ang2 promotes angiogenesis in wound healing

To examine the effect of COMP-Ang2 in angiogenesis *in vivo*, we made a hole-punch injury in the ear of adult mice and treated them with 1×10^9 pfu of Ade-β-gal (control), Ade-COMP-Ang2, Ade-Ang2, or Ade-COMP-Ang1. The circulating plasma level of each recombinant protein was measured at several time points over 2 weeks (Supplementary Fig. 2). All circulating recombinant angiopoietins increased similarly as early as 1 day after treatment, peaked at 3 to 5 days, and declined gradually thereafter (Supplementary Fig. 2). The peak concentrations of all 3 circulating angiopoietins were ~3.0 to 3.5 μg/ml. All mice treated with any of the adenoviral constructs appeared generally healthy and gained weight normally. The mice treated with Ade-COMP-Ang2 exhibited profound increases in vessel diameter and density in the wound healing margin of the ear skin, and its effects were higher (~1.3 fold) than Ade-Ang2 but were equivalent to Ade-COMP-Ang1 (Fig. 5C and D).

4. Discussion

Structure–function studies on oligomeric and multimeric ligands provide an important clue about how they activate their receptors and point to new directions to improve their activity by protein engineering. Here, we have designed and generated oligomeric Ang2 variant proteins of GCN4-Ang2, MAT-Ang2 and COMP-Ang2 using a mammalian cell expression system. Using SDS-PAGE, TEM, *in vitro* binding assays, Tie2 phosphorylation assays, and *in vitro* and *in vivo* biological assays, we provide a critical clue about the role of oligomerization and function of Ang2.

The extracellular region of Tie2 contains two immunoglobulin-like domains (Ig1 and Ig2), three epidermal growth factor-like modules (3EGF), one immunoglobulin-like domain (Ig3), and three fibronectin type III (FNIII) repeats, in order from the amino-terminus to the carboxy-terminus. Although the Ig2 of Tie2 has a sole binding site to Ang1 and Ang2, a compact globular structure

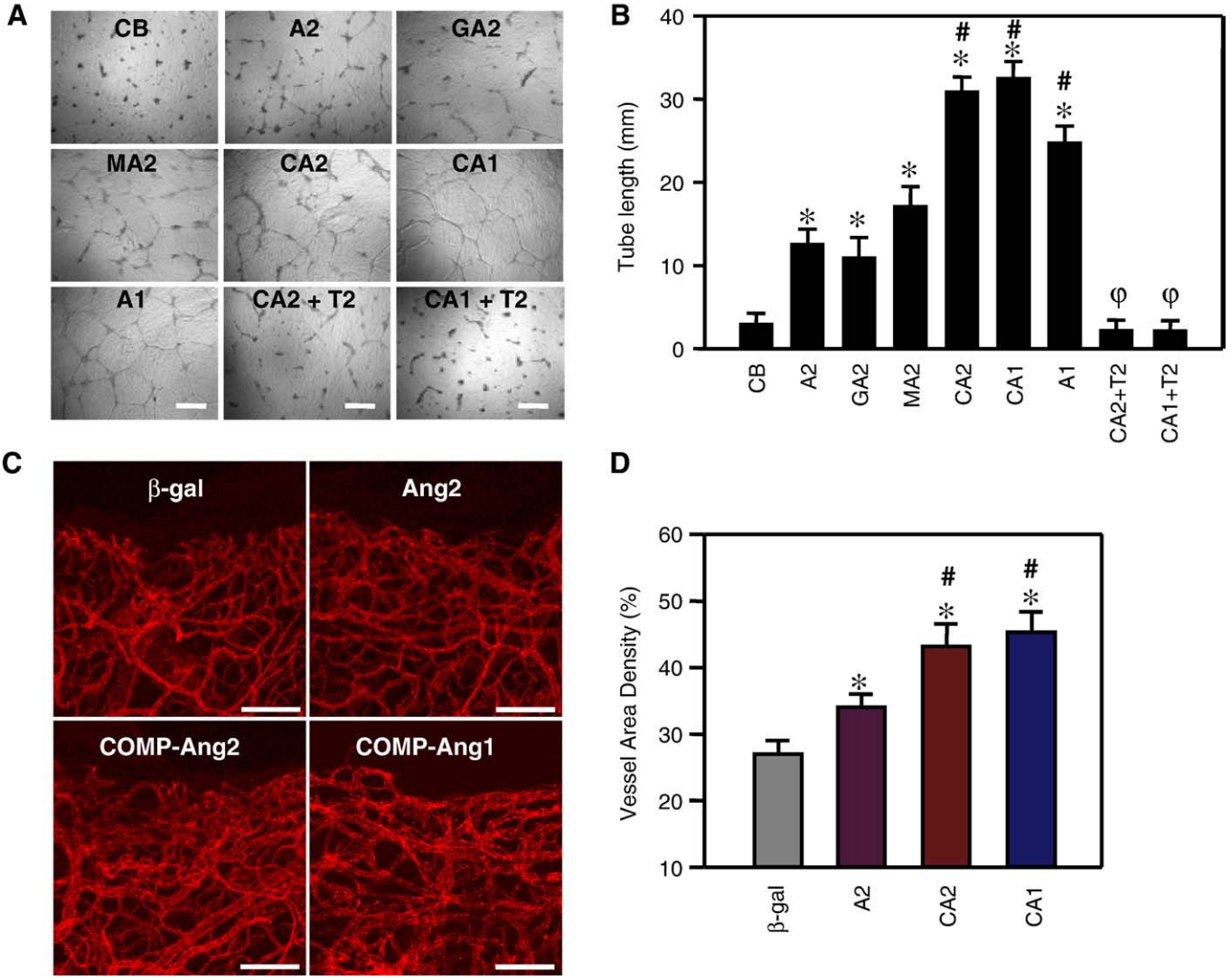

Fig. 5. COMP-Ang2 stimulates tube formation of endothelial cells *in vitro* and promotes *in vivo* angiogenesis in wound healing ear skin. (A) Representative photographs of the tube formation. HUVECs were seeded on Matrigel™ coated wells and treated with control buffer (CB), native Ang2 (400 ng/ml, A2), Ang2 variants (GA2, MA2 and CA2, each 200 ng/ml), COMP-Ang1 (200 ng/ml, CA1), native Ang1 (400 ng/ml, A1), CA2 + T2 and CA1 + T2 for 24 h. Scale bars, 2 mm. (B) Tube formation activities were quantified by measurement of total tube length exceeding 50 μm per field (10 mm^2). Bars represent the mean ± S.D. from 3–4 experiments. $^*P<0.05$ *versus* CB; $^{\#}P<0.05$ *versus* A2; $^{\varphi}P<0.05$ *versus* CA2 or CA1. (C) *In vivo* angiogenesis induced by adenoviral COMP-Ang2 in wound healing ear skin. FVB/N mice were treated with 1×10^9 pfu of Ade-β-gal (control), Ade-COMP-Ang2, Ade-Ang2, and Ade-COMP-Ang1. Two weeks later, blood vessels at the healing margins were visualized with PECAM-1 (red) immunostaining. Scale bars, 100 μm. Note that the mice treated with Ade-COMP-Ang2 and Ade-COMP-Ang1 show increased vessel diameters and densities in ear skin. (D) Area densities of blood vessels were measured in each 0.2 mm^2 in the marginal area and presented as a percentage of total blood vessels in the total area. Bars represent the mean ± S.D. from 4 experiments. $^*P<0.05$ *versus* Ade-β-gal. $^{\#}P<0.05$ *versus* Ade-Ang2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

consisting of Ig1–Ig2–3EGF–Ig3 of Tie2 is necessary and sufficient to bind both Ang1 and Ang2 [36]. Recently, Barton et al. [37] showed that the structure of the Ang2-Tie2 complex reveals an unusual mode of interaction of receptor without major conformational changes or domain rearrangements on its extracellular domain. Thus, recognition of angiopoietin by Tie2 utilizes a lock-and-key mechanism, in which they bind each other without marked rearrangements. Likewise, the binding of COMP-Ang2 and MAT-Ang2 to Tie2 may utilize a lock-and-key mechanism, in which they bind each other without marked rearrangements in either binding partner. Questions remain about how Ang1, COMP-Ang1, COMP-Ang2, and MAT-Ang2 activate Tie2, while Ang2 does not activate Tie2. The mechanistic basis of Tie2 phosphorylation upon ligand activation is still not exactly known. One would imagine that Ang1–Tie2 and Ang2–Tie2 interactions could produce different oligomerization/multimerization states of Tie2, which might activate different downstream signaling pathways for cellular responses. Our results support why Ang1 and Ang2 produce different, even opposing but sometimes similar, functions. The designed pentameric Ang2, COMP-Ang2, produces almost identical effects, compared to Ang1 and COMP-Ang1, on Tie2 phosphorylation, and on *in vitro* and *in vivo* angiogenesis. Previously, we provided critical clues about the structure–function relationship of Ang1 and Ang2. In order to activate Tie2, oligomerization and multimerization of at least 4 subunits of Ang1 ligand are required [8,10]. Thus, the proper oligomerization of Ang2 may be required to produce consistent agonistic effects on Tie2 by promoting Tie2 clustering/oligomerization, whereas improper oligomerization of Ang2 may act as an antagonist on Tie2 by preoccupation to Tie2 without Tie2 clustering/oligomerization through inhibition of further binding of properly oligomerized forms of Ang2 and Ang1.

Nevertheless, we still do not know about exact oligomerization status of endogenous Ang2 in different tissues and under different conditions. Based on SDS-PAGE analysis, previous studies have shown that recombinant Ang2 is present as disulfide-linked dimers [1,10,23,38]. However, our previous RMSTEM analysis indicated that recombinant Ang2 was present mainly as trimers and tetramers, plus a small fraction of higher order multimers [38]. Therefore, we believe that endogenous Ang2 exists as several types of oligomers rather than simply as a dimer. Our present results show that HUVECs secrete variable oligomeric forms of Ang2, suggesting that endogenous Ang2 could consist of variable oligomeric forms. Therefore, the oligomerization status of the endogenous secretory form of Ang2 needs to be elucidated by future studies in order to distinguish the agonistic and antagonistic roles of Ang2 *in vivo*.

Ang1- or COMP-Ang1-induced activation of Tie2/Akt is a main signaling pathway for survival, migration, and tube formation in vascular endothelial cells [34]. The COMP-Ang2-induced promotion of these processes could mainly be mediated through Tie2/Akt because all these cellular effects are Tie2 dependent, and the effects of COMP-Ang2 were identical to the effects of COMP-Ang1. Furthermore, the COMP-Ang2-induced angiogenesis in the wound healing region is almost identical to COMP-Ang1-induced promoted angiogenesis. Thus, any obvious differences between COMP-Ang2 and COMP-Ang1 are not found in *in vitro* or *in vivo* angiogenesis, supporting our mechanistic interpretation that the oligomerization status of Ang2 is the critical determinant of the binding and activation of Tie2.

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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.bbamcr.2009.01.018.

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