Tie2 Receptor Ligands, Angiopoietin-1 and Angiopoietin-2, Modulate VEGF-Induced Postnatal Neovascularization

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Abstract—Angiopoietin-1 (Ang1) has been recently identified as the major physiological ligand for the tyrosine kinase receptor Tie2 and assigned responsibility for recruiting and sustaining periendothelial support cells. Angiopoietin-2 (Ang2) was found to disrupt blood vessel formation in the developing embryo by antagonizing the effects of Ang1 and Tie2 and was thus considered to represent a natural Ang1/Tie2 inhibitor. In vivo effects of either angiopoietin on postnatal neovascularization, however, have not been previously described. Accordingly, we used the cornea micropocket assay of neovascularization to investigate the impact of angiopoietins on neovascularization in vivo. Neither Ang1 nor Ang2 alone promoted neovascularization. Pellets containing vascular endothelial growth factor (VEGF) alone induced corneal neovascularity extending from the limbus across the cornea. Addition of Ang1 to VEGF (Ang1 + VEGF) produced an increase in macroscopically evident perfusion of the corneal neovascularature without affecting macroscopic measurements of length (0.58 ± 0.03 mm) or circumferential neovascularity (1.36 ± 10°). In contrast, pellets containing Ang2 + VEGF promoted significantly longer (0.67 ± 0.05 mm) and more circumferential (160 ± 15°) neovascularity than VEGF alone or Ang1 + VEGF (P < 0.05). Excess soluble Tie2 receptor (sTie2-Fc) precluded modulation of VEGF-induced neovascularization by both Ang2 and Ang1. Fluorescent microscopic findings demonstrated enhanced capillary density (fluorescence intensity, 2.55 ± 0.23 e⁻¹⁹ versus 1.23 ± 0.17 e⁻¹⁹, P < 0.01) and increased luminal diameter of the basal limbus artery (39.0 ± 2.8 versus 27.9 ± 1.3 μm, P < 0.01) for Ang1 + VEGF compared with VEGF alone. In contrast to Ang1 + VEGF, Ang2 + VEGF produced longer vessels and, at the tip of the developing capillaries, frequent isolated sprouting cells. In the case of Ang2 + VEGF, however, luminal diameter of the basal limbus artery was not increased (26.7 ± 1.9 versus 27.9 ± 1.3 μm, P = NS). These findings constitute what is to our knowledge the first direct demonstration of postnatal bioactivity associated with either angiopoietin. In particular, these results indicate that angiopoietins may potentiate the effects of other angiogenic cytokines. Moreover, these findings provide in vivo evidence that Ang1 promotes vascular network maturation, whereas Ang2 works to initiate neovascularization. (Circ Res. 1998;83:233-240.)

Key Words: angiopoietin ■ Tie2 receptor ■ vascular endothelial growth factor ■ angiogenesis ■ endothelial cell

Establishment and remodeling of blood vessels is regulated by paracrine signals from the transmembrane TKRs of endothelial cells. Flk-1 and Flt-1 are 2 such TKRs, which, together with their cognate ligand VEGF, have been shown to be required for blood vessel development during embryogenesis; this receptor/ligand family also has been shown to augment postnatal neovascularization.

The Tie receptors, Tie1 and Tie2, constitute a second family of endothelial cell-specific TKRs, and the latter has been shown recently to be widely expressed in the quiescent vasculature of adult tissues. Tie2−/− mice die during embryogenesis at day 9.5 to 10.5; necropsy analyses have shown that the endothelial cells of such mice are present in normal numbers and assembled into tubes, but the vessels are immature and lack branch networks and hierarchical organization into large and small vessels. Deletion of Tie1 results in embryonic lethality at day 14.5; the finding of edema and hemorrhage in these mice has been interpreted as evidence that Tie1 signaling modulates the hemodynamics of transcapillary fluid exchange.

Observations in human subjects have revealed that deficient SM cell investment typical of venous malformations is associated with a mutation in the Tie2 TKR, suggesting that the Tie2 system may regulate the endothelial cell recruitment of stromal cells required to encase and thereby stabilize primitive endothelial tubes.

Recently, the ligands of the Tie2 receptor have been identified as Ang1 and Ang2. Ang1 was identified as the major physiological ligand for Tie2, responsible for recruiting and sustaining periendothelial support cells. Ang2 was found to disrupt blood vessel formation in the developing embryo by antagonizing the effects of Ang1 and Tie2, and it was thus concluded that Ang2 represents a natural Ang1/Tie2 inhibitor. Extrapolation of these developmental findings to postna-
neovascularization has led to the dual inferences that Ang1 may induce maturation and stabilization of developing neo-
vasculature,\textsuperscript{13} whereas Ang2 may cause destabilization re-
quired for additional sprout formation.\textsuperscript{14} In vivo effects of
either angiopoietin on postnatal neovascularization, however,
have not been previously described.

Accordingly, in the present series of experiments, we used
the mouse corneal micropocket assay to determine the func-
tion of these novel endothelial cell–specific cytokines on
postnatal neovascularization in vivo.

Materials and Methods

Reagents

Human Ang1, Ang2, and sTie2-Fc were kindly supplied by Regen-
eron Pharmaceuticals, Inc. The recombinant protein was formulated
in buffer solution consisting of 0.05 mol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.05% Chaps. Ang1 is a genetically engi-
neered variant of naturally occurring Ang1 that retains similar
properties in all assays. Soluble Tie2-Fc is a recombinant fusion
protein consisting of the ectodomain of the Tie2 receptor fused to the
Fc portion of human IgG1. Previous studies have shown that
sTie2-Fc binds to Ang1, Ang2, and mouse angiopoietins.\textsuperscript{15} Recom-
binant human VEGF\textsubscript{165} was a generous gift of Dr Bruce Keyt
(Genentech Inc, South San Francisco, Calif).

Mouse Cornea Neovascularization Assay

Age-matched (8 weeks) C57BL/6J male mice (Jackson Labs, Bar
Harbor, Me) were used for all experiments. Preoperative anesthesia
was limited to intraperitoneal pentobarbital injection (160 mg/kg).

Preoperative anesthesia was limited to intraperitoneal pentobarbital injection (160 mg/kg). Corneal pockets were created in the eyes of each mouse using a
modified von Graefe cataract knife.\textsuperscript{15}

A 0.34×0.34-mm sucrose aluminum sulfate (Bukh Meditec)
pellet coated with hydron polymer type NCC (IFN Science) contain-
ing 1 of the agents indicated below was implanted into the corneal
pocket. Pellets were positioned 1.0 mm from the corneal limbus, and
erythromycin ophthalmic ointment (E. Fouera) was applied to each
operated eye. The corneas of all mice were routinely examined by
slit-lamp biomicroscopy on postoperative days 4 or 6 after pellet
implantation. Vessel length and the arc of corneal circumference
occupied by neovascularity (circularmerial neovascularity, in de-
grees) were measured on the sixth postoperative day when all
corneas were photographed. After these measurements were com-
pleted, mice received an intravenous injection of 500 µg of the
endothelial cell–specific marker BS-1 lectin conjugated to FITC
(Vector Laboratory). Thirty minutes later, the animals were killed.

The eyes were enucleated and fixed in 1% paraformaldehyde
solution. After fixation, the corneas were placed on glass slides and
examined by fluorescence microscopy. Several mice in each group
did not receive BS-1 lectin injection; instead, the eyes were excised
and fixed in 100% methanol solution for immunohistochemical
staining.

Study Design

Age-matched (8 weeks) C57BL/6J male mice (Jackson Labs, Bar
Harbor, Me) were divided into 9 groups. Each mouse underwent surgical implan-
tation of pellets containing 1 of the following: control buffer (0.05 mol/L
Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Chaps) alone; Ang1 alone
(300 ng/pellet); Ang2 alone (300ng/pellet); control buffer with VEGF
(control + VEGF, 300 ng/pellet); Ang1 with VEGF (Ang1 + VEGF, 300
ng/pellet); Ang2 with VEGF (Ang2 + VEGF, 300 ng/pellet); sTie2-Fc with
control buffer and VEGF (sTie2-Fc + control + VEGF, 300 ng/pellet); sTie2-Fc
with Ang1 and VEGF (sTie2-Fc + Ang1 + VEGF, 300 ng/pellet); and sTie2-Fc
with Ang2 and VEGF (sTie2-Fc + Ang2 + VEGF, 300 ng/pellet).

Quantification of Corneal Neovascularure

Integrated optical density (IOD) from the cornea-derived fluorescent
slides was measured using a Cooke SVGa cooled CCD camera
attached to a Nikon Diaphot inverted microscope. The digital images
were processed using Media Cybernetics Image Pro Plus 3.0 soft-
ware. Initially, all fluorescent images from different groups were
acquired directly from the microscope to the computer screen using
the same 2-second exposure time. The contour of the vascular area
was traced, and the IOD was automatically calculated. The IOD
equals the sum of all pixels, each of which has a value from 1 to 4096
gray levels, divided by the whole area. Results were expressed as
mean±SEM. The differences between each individual group were
evaluated by ANOVA. Differences were considered significant at
P<0.05.

Immunohistochemistry

For immunohistochemical staining, the excised eyes were fixed
immediately in 100% methanol overnight. After enucleation, the
excised corneal hemispheres prepared under the dissecting micro-
scope were embedded in paraffin. Sections of 5 µm thickness, cut
either in cross section or longitudinally, were used for immunohis-
tochemistry. Immunohistochemical staining for endothelial cells was
conducted using a rat monoclonal antibody against mouse CD-31
(Pharmingen). For detection of periendothelial cells, a mouse mono-
clonal antibody against SM α-actin conjugated with alkaline phos-
phatase (Sigma) was used. A polyclonal peroxidase–labeled anti-rat
immunoglobulin (Signet Laboratories) was used as secondary anti-
body for CD31. Vascular lumens bordered by CD31-positive cells
were counted on cross sections of neovascularized cornea.

To evaluate the frequency of periendothelial cell recruitment in corneal
neovascularization, SM α-actin–positive cells were manually
counted on tissue sections cut in cross section as well. The number
of positive lumens in each case was expressed as mean±SEM.

Group differences were analyzed by ANOVA. Differences were considered statistically significant at
P<0.05.

Results

On the sixth postoperative day after pellet implantation, corneal
neovascularization was evaluated qualitatively (Figure 1) and
quantitatively using slit-lamp biomicroscopy to measure vessel
length and circumferential neovasculature (Figure 2). Neither the
control buffer pellet nor pellets containing Ang1 or Ang2 alone
induced neovascularization. Pellets containing VEGF alone
induced corneal neovascularization extending from the limbus across
the cornea (Figure 1); these vessels constituted 120° of the
corneal circumference and measured 0.52±0.03 mm in length.

The addition of Ang1 to VEGF (Ang1 + VEGF) produced an
increase in macroscopically evident perfusion of the corneal
neovascularization (Figure 3) without affecting macroscopic mea-
surements of length (0.58±0.03 mm) or circumferential neovas-
cularity (136±10°). In contrast, pellets containing Ang2 + VEGF
promoted significantly longer (0.67±0.05 mm) and more cir-
mferential (160±15°) neovascularity than VEGF alone or
Ang1 + VEGF (P<0.05). An excess of sTie2-Fc precluded
modulation of VEGF-induced neovascularization by either an-
giopoietin (Ang2 as well as Ang1). Neovascularization induced
by control buffer + VEGF was not attenuated by sTie2-Fc,
excluding a potential contribution from endogenous angiopoietins.

Fluorescence microscopic findings resulting from premortem immunostaining with BS-1 lectin permitted more detailed histopathologic analysis. Compared with control + VEGF, Ang1+VEGF enhanced capillary density and luminal diameter of the basal limbus artery (Figure 3); this corresponded to a significant increase in fluorescence intensity (2.55±0.23 e−9 versus 1.23±0.17 e−9, P<0.01; Figure 4) and luminal diameter of the basal limbus artery (39.0±2.8 versus 27.9±1.3 μm, P<0.01; Figure 5). In contrast to Ang1+VEGF, Ang2+VEGF produced longer vessels and, at the tip of the developing capillaries, frequent isolated sprouting cells (Figure 3). These findings were not observed with Ang1+VEGF. In the case of Ang2+VEGF, however, luminal diameter of the basal limbus artery was not increased (26.7±1.9 versus 27.9±1.3, P=NS; Figure 5). The differential effects observed for Ang1 versus Ang2 in combination with VEGF were apparent at day 4 (Figure 6).

When sTie2-Fc was included with VEGF and control buffer in the implanted pellets, histopathologic vascular morphometry was unchanged. In contrast, sTie2-Fc obviated modulation of corneal neovascularization resulting from addition of Ang1 and Ang2 to the implanted pellets. These findings suggest an agonist function for both Ang1 and Ang2 in modulating VEGF-induced corneal neovascularization.

Light microscopic sections stained with hematoxylin and eosin disclosed only rare inflammatory cells associated with corneal neovascularization. A similar extent of low-level inflammation was observed after implantation of the control

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**Figure 1.** Macroscopic photographs of mouse cornea obtained by slit-lamp biomicroscopy 6 days after pellet implantation. Macroscopic evidence of corneal neovascularization was not observed with control buffer pellet or with pellets containing Ang1 or Ang2 alone. Pellets containing VEGF alone induced corneal neovascularity extending from the limbus across the cornea. The combination of Ang1 and VEGF showed enriched vascularity, although length (distance from limbus toward pellet) of the new vessels was similar to that seen with VEGF alone. Ang2+VEGF resulted in vessels with increased length (as well as circumferential extent of neovascularity; see Figure 2) compared with VEGF alone or Ang1+VEGF. Addition of sTie2-Fc did not change vascularity induced by VEGF but reduced both vascularity seen with Ang1+VEGF and increased vessel length seen with Ang2+VEGF.

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**Figure 2.** Vessel length (millimeters) and circumferential extent (degrees) of neovascularization measured macroscopically by slit-lamp biomicroscopy 6 days after surgery. Neovascularization induced by Ang2+VEGF is characterized by a statistically significant increase in both vessel length and circumferential extent of neovascularity compared with control (Cont) pellet or Ang1+VEGF. Introduction of sTie2-Fc obviates both findings (*P<0.05).
buffer pellet as well as pellets containing Ang1 or Ang2 alone, none of which induced neovascularization. This suggests that an inflammatory response is not a prerequisite for neovascularization in this cornea model, as suggested by others.\textsuperscript{16}

To investigate specific histoarchitectural differences in neovascularization resulting from Ang1 versus Ang2 in combination with VEGF, we analyzed light microscopic sections prepared for immunohistochemical staining with antibodies to CD31 and SM α-actin. Immunostaining for CD31 disclosed an increased number of vascular lumens per cross section in tissue sections retrieved from animals receiving Ang1+VEGF versus control buffer+VEGF (155.8±32.1 versus 33.0±3.9 per cross section, \( P < 0.05 \)). Sections retrieved from the Ang2+VEGF group also disclosed an increased number of vascular lumens compared with the control+VEGF group (80.6±16.2 versus 33.0±3.9 per cross section).

**Figure 3.** In situ BS-1 lectin fluorescent staining of corneal limbus vessels 6 days after pellet insertion. Limbus vessels were unchanged in response to pellets containing control buffer, Ang1 alone, and Ang2 alone. Pellet containing VEGF alone resulted in corneal neovascularization. Further enhancement of vascularity and limbus arterial diameter (arrowhead) was observed with the combination of Ang1+VEGF; note that the length of the new vascular sprouts, however, is similar to those seen with VEGF alone. In contrast, the length of the vascular sprouts was observed to be increased in response to Ang2+VEGF; moreover, frequent isolated endothelial cells (arrows) were observed at the growing edge of the capillary tips. The fluorescent photomicrographs confirm macroscopic observations regarding addition of sTie2-Fc: no change in VEGF alone but substantial attenuation of vascularity in the case of Ang1+VEGF and vessel length as well as vascularity in the case of Ang2+VEGF.

**Figure 4.** Quantitative analysis of neovasculature induced by control buffer (Cont)+VEGF, Ang1+VEGF, or Ang2+VEGF. Measurement of integrated optical density from fluorescent photomicrograph of cornea with digital image analysis was used to determine fluorescence density. Both Ang1+VEGF and Ang2+VEGF revealed significant increase in fluorescence density in comparison to VEGF alone (\( P < 0.01 \)).
section), but this failed to achieve statistical significance ($P=\text{NS}$).

While both Ang1 and Ang2 resulted in an increased number of vascular lumens, the neovasculature developing in response to each of these 2 cytokines differed in 2 respects. First, the lumens of vessels constituting the neovasculature of Ang1+VEGF group were typically larger than those of the control+VEGF group; those vessels that developed in response to Ang2+VEGF, however, were of lesser luminal diameter than those in the Ang1+VEGF group and in this respect similar to those of the control+VEGF group (Figure 7). While this feature was difficult to quantify because of the diminutive size of the lumens in general, it is illustrated qualitatively in the representative histological sections shown in Figure 7. Second, the neovasculature of these 2 groups was characterized by a marked difference in periendothelial cell frequency (Figure 8). Recruitment of periendothelial cells was more frequent in Ang1+VEGF (50.0±14.2 per cross section) than in Ang2+VEGF (11.6±1.4) or control+VEGF (5.4±1.4) groups.

Discussion

We used the cornea micropocket assay of neovascularization to investigate the impact of angiopoietins on neovascularization in vivo. This cytokine-containing sucralfate assay is unique by virtue of the fact that it yields a predictable, persistent, and aggressive neovascular response that is dependent on direct stimulation of blood vessel development rather than indirect neovascularization induced by inflammation.16 We observed only very few inflammatory cells in association

Figure 5. Luminal diameter of cornea limbus arteries treated with control buffer (Cont) +VEGF, Ang1+VEGF, or Ang2+VEGF. The luminal diameter of basal limbus artery in response to Ang1+VEGF was significantly increased in comparison with VEGF alone or Ang2+VEGF ($P<0.05$).

Figure 6. Corneal neovascularization induced by VEGF with control buffer, Ang1, or Ang2 and evaluated by in situ BS-1 lectin fluorescence staining 4 days after pellet insertion. A, Appearance of nascent vascular sprouts induced by VEGF. B, More complex vascularity induced by combination of Ang1+VEGF. C, Ang2+VEGF also results in more robust vasculature, including increased length of sprouts and plethora of isolated endothelial cells at the growing tips of the sprouts. D, Higher-power photomicrograph of Ang1+VEGF showing lumen development at 4 days. E, Higher power view of Ang2+VEGF showing isolated endothelial cells (arrows) at sprout tips.
with corneal neovascularization. The fact that a similarly limited number of inflammatory cells was observed after implantation of the control buffer pellet as well as pellets containing Ang1 or Ang2 alone—none of which induced neovascularization—suggests that an active inflammatory response is not a prerequisite for neovascularization in this cornea model, as suggested by others. Neovascularity related to pellet implantation was thus exclusively cytokine dependent.

Previous reports have suggested that angiopoietins alone were not mitogenic for endothelial cells in culture nor did they induce tube formation within a collagen substrate. In the present experiments, neither Ang1 nor Ang2 alone promoted neovascularization. In contrast, however, when coadministered with VEGF, Ang1 had an important modulatory effect on corneal neovascularization. The increase in vascular density seen with Ang1+VEGF is consistent with previous studies showing that combined administration of endothelial mitogens may have a synergistic effect on neovascularization. The increase in luminal diameter of vessels constituting the neovascularity as well as the limbus artery may represent an additional modulatory effect, although the possibility that this was due to vasodilation resulting from augmented flow into the enriched plexus of corneal vessels.

Figure 7. Immunohistochemical staining of neovasculature induced by VEGF, Ang1+VEGF, and Ang+VEGF. A through C, Representative photomicrographs of tissue sections stained with antibody to CD31. The number of vascular lumens in Ang1+VEGF was significantly (P<0.05) higher than that observed with control buffer+VEGF. Ang2 increased the number of vascular lumens compared with control buffer+VEGF but failed to achieve statistical significance (P=NS). Note that Ang1, but not Ang2, also resulted in increased size of vascular lumens. D through F, Sections stained with antibody to SM α-actin show that Ang1, but not Ang2, also increased recruitment of periendothelial cells.

Figure 8. A, Quantification of vascular lumens per cross section. B, Quantification of SM α-actin–positive cells per cross section (*P<0.05 versus control+VEGF by ANOVA).
cannot be excluded. The development of a more complex vascular network characterized by vessels of increased lumen size and more frequent recruitment of periendothelial cells may be considered evidence for a maturation effect of Ang1 on VEGF-induced neovascularization. This idea was suggested by Suri et al., who characterized the vascular network of Ang1−/− mice as less complex and comprising fewer large arteries, most of which were of lesser caliber and more poorly invested by periendothelial cells than the vasculature of wild-type mice.

The modulatory impact of Ang2 on VEGF-induced neovascularization was morphologically distinct and thereby quite different from simple enhancement of vascular density induced by VEGF. The circumferential extent and the length of vessels cooperatively induced by Ang2+VEGF were significantly increased compared with control+VEGF or Ang1+VEGF. Moreover, histological examination disclosed isolated migrating endothelial cells at the leading tip of capillaries coursing toward the pellet. Neither the size of the limbus artery nor that of vessels constituting the neovascularure, however, were enhanced in the case of Ang2+VEGF. We interpret these findings to indicate that the effect of Ang2 on VEGF-induced neovascularization is to promote vascular destabilization and sprouting required to initiate neovascularization. Consistent with this interpretation, Maisonnier et al previously suggested that Ang2 may collaborate with VEGF at the leading edge of invading vascular sprouts by blocking constitutive stabilization or the maturation function of Ang1, thus allowing vessels to revert to and remain in a more plastic state in which they may be more responsive to sprouting signals provided by VEGF.

The effect of Ang1 or Ang2 on VEGF-induced neovascularization constitutes inferential evidence for Tie2 expression in the cornea micropocket assay. Coadministration of sTie2-Fc had no inhibitory effect on angiogenesis induced by VEGF alone, indicating that endogenous angiopoietins do not contribute to corneal neovascularization. Moreover, we found very few α-SM actin-positive periendothelial cells in VEGF-induced neovascularure, whereas abundant periendothelial cells were seen in the cornea of Ang1+VEGF group. This suggests that periendothelial cell recruitment in neovascular development is dependent on Ang1 and that VEGF-induced corneal neovascularization, in the absence of endogenous angiopetin, does not include Ang1-dependent periendothelial cell recruitment for vascular maturaton, at least up to day 6.

Second, coadministration of sTie2-Fc abolished the modulating effects of Ang1 and Ang2 on VEGF-induced neovascularization. This finding is consistent with the recent demonstration by Lin et al. that a soluble form of the extracellular domain of murine Tie2 blocked tumor angiogenesis, including tumor vascular length density, and consequently tumor growth. Moreover, the fact that sTie2-Fc preempted the modulating influence of not only Ang1 but Ang2 as well suggests an agonist effect for Ang2 on neovascularization. Maisonnier et al. were unable to show evidence of Tie2 activation by Ang2 (no tyrosine phosphorylation) in human endothelial cells; in contrast, Ang2 was shown to activate the Tie2 receptor when it was ectopically expressed in nonendothelial (NIH 3T3) cells. The present findings thus constitute the initial demonstration that Ang2 may activate the Tie2 receptor in vascular cells and support the notion that Ang2 may facilitate neovascularization.31

Because Tie2 expression is restricted to endothelial lineage and certain phenotypes of immature hematopoietic cells, it is possible that the cells responding to Ang2 in the present experiment could be either activated endothelial cells or endothelial progenitor cells derived from circulating blood that have homed to the site of corneal neovascularization. We have found previously that endothelial progenitor cells express the Tie2 receptor and, more recently, shown that endothelial progenitor cells make a substantive contribution to VEGF-induced corneal neovascularur (T. Asahara, D. Chen, J.M. Isner, unpublished data, 1997). Endothelial progenitor cells, in contrast to fully differentiated endothelial cells, may constitute a state that allows signaling from the Tie2 receptor to occur after binding by Ang2. In summary, these findings constitute what is to our knowledge the first direct demonstration of angiopoietin bioactivity in postnatal animals. In particular, these results indicate that angiopoietins, as suggested by Wong et al., may potentiate the effects of other angiogenic cytokines. Moreover, these findings provide in vivo evidence that Ang1 promotes vascular network maturation, whereas Ang2 works to initiate neovascularization, concepts that were inferred from targeted genetic experiments13,14 or detection of gene expression in postnatal organs. Further studies will be required to elucidate the regulatory mechanisms by which angiopoietins modulate cells of endothelial lineage through the Tie2 receptor.

References


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