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Short Communication

Comparison of two *in vitro* angiogenesis assays for evaluating the effects of netrin-1 on tube formation

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Netrin-1 is a neural guidance cue that also regulates vascular development. Controversial results, however, have been obtained concerning the roles of netrin-1 in vascular development both *in vivo* and *in vitro*. In the present study, two *in vitro* angiogenesis assays were compared to evaluate the effects of netrin-1 secreted by retrovirally transduced melanoma cells (Mel2a-netrin1) on tube formation. The results showed that there was no obvious difference in tube formation induced by conditioned media (CM) from the control, Mel2a-netrin1 and Mel2a cells in a matrigel assay. The results of another *in vitro* assay, in which endothelial cells were co-cultured with human fibroblasts, however, showed that Mel2a-netrin1 CM inhibited the tube formation, supposedly through blocking the elongation and coalescence of human umbilical vein endothelial cells (HUVECs). These results confirmed that the matrigel assay is not able to demonstrate the anti-angiogenic roles of netrin-1.

Keywords netrin-1; co-culture; matrigel; tube formation; human umbilical vein endothelial cells (HUVECs)

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Introduction

Blood vessels and nerves behave very similarly in physiological activities such as supplying two-way flow of nerve conduction and blood, which are mediated by sensory and motor nerves, arteries and veins, respectively. Moreover, blood vessels and nerves are complex and branched networks that travel parallelly, indicating that common regulatory mechanisms might function in the two systems.

Netrin-1 is a laminin-related diffusible protein that was first discovered as a guidance cue during embryo neurogenesis [1–3]. Recent studies have demonstrated that netrin-1 has regulatory function not only in axon guidance, but also in vascular development [4–7]. Interestingly, several research groups have reached opposite conclusions on the effect of netrin-1 in vascular development. In 2004, Lu *et al.* [4] showed that *netrin-1a* (a netrin-1 ortholog) silencing in zebrafish using translational blocking morpholino leads to excessive sprouting of intersegmental vessels, suggesting that netrin-1a is anti-angiogenic. On the contrary, Wilson *et al.* [7] found that *netrin-1a* silencing using splice-blocking morpholino strongly inhibits the formation of parachordal vessels (PAVs). This was interpreted as evidence for the pro-angiogenic role of netrin-1a. The controversial results were also shown in *in vitro* experiments. For example, Wilson *et al.* [7] showed that netrin-1 stimulated the migration and proliferation and tube formation of several human endothelial cell lines on matrigel, whereas Lu *et al.* [4] showed that it inhibited the migration of human umbilical artery endothelial cells (HUAECs) and induced retraction of the tip cells of filopodia sprouting from rat aortic ring.

Due to its rapidness and easiness to perform, matrigel assay is the most commonly used *in vitro* angiogenesis assay. However, matrigel assay for tube formation has three obvious defects. First, matrigel is a strong promoter of differentiation for many different cell types. Not only endothelial cells (ECs), but also non-ECs could form tubule-like structures in matrigel [8], though the formed structures were supposed to be different from those from ECs [9]. Therefore, the stimulatory effects of matrigel on the tube formation might mask the delicate effects of various pro- or anti-angiogenic regulators, making it difficult to define the exact functions exerted by these regulators. Secondly, the tube formation on matrigel is so quick that a certain step of angiogenesis, such as proliferation, or long distance migration might be skipped or bypassed too quickly to provide clear insights into the mechanisms underlying the process of angiogenesis. Thirdly, matrigel is an extracellular matrix (ECM) purified from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, and may not be able to fully mimic the *in vivo* microenvironment.

Co-culture of ECs with fibroblasts to induce the formation of tubule-like structure provides a new and more reliable *in vitro* angiogenesis assay, where various essential components of the ECM are released by the fibroblasts [8,10]. The aim of the present study was to compare the tube formation

induced by netrin-1 secreted by retrovirally transduced melanoma cells using matrigel and co-culture assays. We showed here that the tube formation in the co-culture assay was inhibited by netrin-1, at least in part through blocking the elongation and coalescence of HUVECs, a phenomenon that cannot be demonstrated by the matrigel assay, which confirms that netrin-1 is an anti-angiogenic regulator.

Materials and Methods

Cells

HUVECs were purchased from ScienCell (Carlsbad, USA) and cultured in Lonza endothelial cell growth medium-2 (EGM-2) (Lonza, Allendale, USA). Human diploid embryonic lung fibroblasts (HFL-1) were obtained from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Human melanoma Mel2a cells retrovirally transduced with vehicle (Mel2a) or human *netrin-1* (Mel2a-netrin1) were a kind gift from Dr A. Eichmann [5]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS) from Hyclone. All the cells were incubated in a 5% CO_2 under humidified atmosphere at 37°C.

Preparation of tumor-conditioned medium

Mel2a and Mel2a-netrin1 cells were seeded at 2×10^6 cells/dish in 60 mm dishes with DMEM containing 10% FBS and cultured overnight. The medium was replaced with 2 ml serum-free DMEM and incubated for 24 h. The conditioned medium (CM) was collected and filtered through a filter with 0.22-μm pore size. The aliquots were stored in a −80°C freezer. The control medium is freshly prepared serum-free DMEM.

Preparation of GFP-labeled HUVECs

HUVECs (passage 3) were stably transfected with lentiviruses pBobi expressing GFP, and selected with 0.5 μg/ml puromycin. The selected GFP-labeled HUVECs at passage 4 to 6 were used for the subsequent assays.

Tube formation with matrigel assay

First, the 96-well microplates (Nunc, Waltham, USA) were coated with matrigel (40 μl/well) (Cat. 354234; Becton Dickinson Company, Piscataway, USA), allowed to polymerize for 30 min at 37°C. HUVECs were added at 2×10^4 cells/well into each well covered with matrigel in 100 μl of a mixture of CM and EGM-2 (2 : 1, v/v). Tube formation was recorded at different time points with an IX71 inverted fluorescent microscope (Olympus, Tokyo, Japan) at low magnification (40×), which covers the whole field of one well. Four wells were photographed for each treatment. The images were saved as TIFF files and analyzed using TCS Cellworks AngioSys 1.0 software (Botolph Claydon, Buckingham, UK) to quantify angiogenesis. Data were analyzed by unpaired Student's *t*-test using Graphpad Prism 5.0 software.

Tube formation with co-culture assay

The GFP-labeled HUVECs were mixed with HFL-1 cells in a ratio of 1 : 2, seeded in a 24-well plate, and co-cultured in EGM-2 medium overnight. The medium was removed and replaced with the mixture of CM and EGM-2 (2 : 1, v/v), and changed every other day. The formed tubules were monitored every other day as described for matrigel assay. On day 11, the cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI) (sigma), and pictured at a higher magnification (200×). Four fields of view (three quadrants and the center) were photographed for each treatment in triplicates. The images were analyzed as described for the matrigel assay.

Results and Discussion

The role of netrin-1 in vascular development was first revealed in 2004 [4]. Noteworthily, using the same animal models, zebrafish and mouse, and the same genetic inactivation strategy by netrin-1, two research groups have reported opposite conclusions about the effect of netrin-1 on vascular development. The focus of controversy is whether netrin-1 is a pro- or anti-angiogenic factor. Lu *et al.* [4] showed that knockdown of *netrin-1a* resulted in aberrant vessel branching, suggesting that netrin-1 is anti-angiogenic. Wilson *et al.* [7] however showed that knockdown of *netrin-1a* led to loss of vessels, suggesting that netrin-1 is pro-angiogenic. The controversy was also shown in the genetic inactivation of *Unc5B* which is the only known netrin-1 receptor with prominent expression in endothelial cells [4]. Lu *et al.* [4] reported that the disruption of *Unc5b* resulted in excessive vessels branching which is consistent the results from netrin-1 inactivation, whereas Navankasattusas [11] reported opposite results. The contradictory results also extended to *ex vivo* assays, including rat aortic ring assay [4,5,12] and CAM assay [6,13]. The controversy reflected the complexity of the roles of netrin-1 revealed in *in vivo* assays.

Although *in vitro* assay may not fully mimic the *in vivo* environment, it offers opportunity to explore the cellular mechanisms by reducing the influence of microenvironmental factors from the *in vivo* study. However, our data showed that the formation of tubule-like structures was different in matrigel and co-culture assays.

We first examined the effects of Mel2a-netrin1 CM on the tube formation in matrigel assay. To study the kinetics of the network formation induced by netrin-1, time lapse experiments were performed for 20 h, during which morphological changes of HUVECs were captured with an inverted fluorescent microscope every 2 h after plating. As shown in **Fig. 1**,

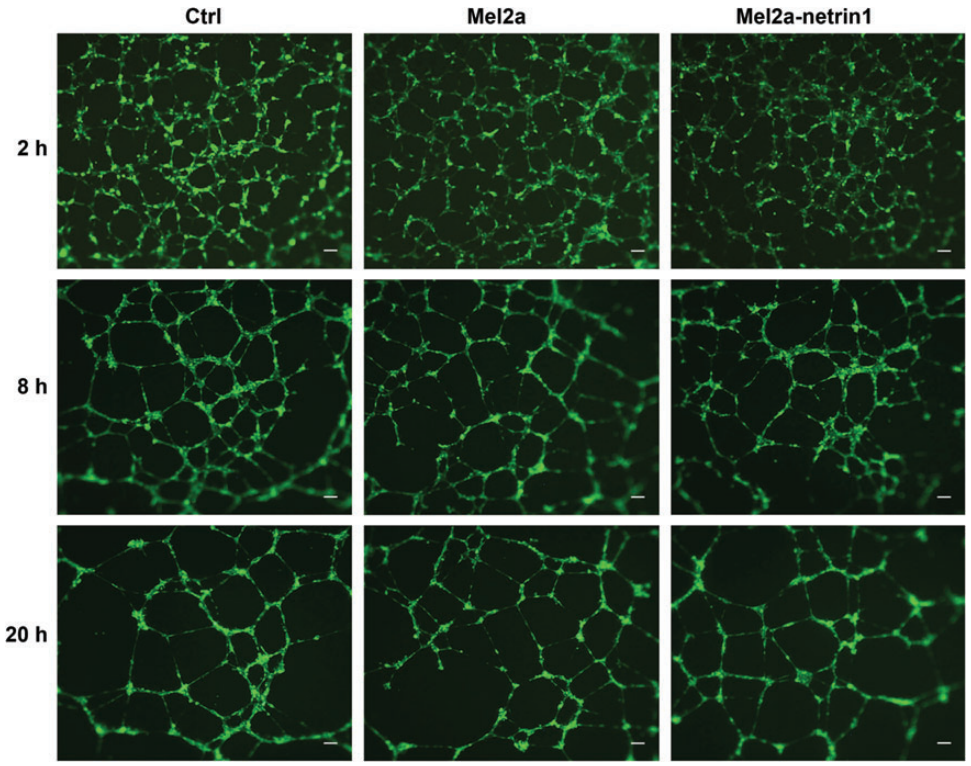

Figure 1. The time course of tube formation in matrigel assay stimulated by CM GFP-labeled HUVECs were seeded in 96-well microplates at 2×10^4 cells/well in 100 μl of mixture of tumor cell CM and EGM-2 (2 : 1, v/v). Images of approximate same fields were taken at 2, 8, and 20 h after seeding, respectively. Representative images of four repeats were shown ($n = 4$, 40 ×). Scale bar, 200 μm.

HUVECs aggregated and elongated into tube-like structures within 2 h, and then the formed mesh of tube-like structures become sparse and wide during the 20 h period. No obvious morphological difference was observed in matrigel assay for the tube formation induced by the control, Mel2a-netrin1 CM, and Mel2a CM. To quantify angiogenesis, the tube formation was analyzed statistically at 2, 8, and 20 h after plating. The angiogenesis parameters analyzed were the number of vessel junctions, vessel area, length, and number of tubules using an image analysis software (Aiosys1.0). Although the seeded ECs were found to differentiate into tubule-like structures in matrigel assay within 2 h, no statistical differences of the tube formation were observed among three different conditioned media used (control, Mel2a CM, and Mel2a-netrin1 CM) at any observing time-point (**Supplementary Fig. S1**), indicating that effects of netrin-1 on the tube formation was not evident in matrigel assay.

We then used the co-culture assay to examine the effects of Mel2a-netrin1 and Mel2a CM on the tube formation. GFP-labeling facilitated the observation of morphological changes in HUVECs during 11 days of co-incubation period with human fibroblasts. The formation of tubule-like structures was stimulated by growth factors present in EGM-2 and Mel2a CM. In contrast to matrigel assay, it took 3 days for the HUVECs to form tubule-like structures in the co-culture assay (**Fig. 2**), and tubules formed during the 11 days co-culture period showed remarkable morphological changes in the three media, especially in Mel2a CM (**Fig. 3B**) and Mel2a-netrin1 CM (**Fig. 3C**).

When co-cultured with fibroblasts for 1 day, single or small clusters of the GFP-labeling HUVECs were seen dispersed evenly on the fibroblasts. As incubation continued with time, drastic morphological changes of HUVECs, such as cell elongation and cell−cell coalescence were observed. The process continued till day 3, resulting in the formation of tubule-like structures. The time courses of tube formation on day 1, 3, 5, 7, and 9 are shown in **Fig. 2**, where only the GFP-labeled HUVECs were visible under fluorescent microscope, while the co-cultured human fibroblasts were invisible. After the formation of tube on day 3, small morphological changes continued to occur, such as remodeling or elongation of the formed tubule-like structures till day 11. Both the control and Mel2a CM induced elongation and coalescence of HUVECs dispersed on the fibroblasts (**Fig. 3A,B**, GFP at 200×) and finally formed the tubule-like structures, whereas most of the HUVECs treated with Mel2a-netrin1 CM were relatively shorter and aggregated together without coalescence (**Fig. 3C**, GFP at 200×), indicating that the inhibition of coalescence of HUVECs may be the reason that Mel2a-netrin1 CM induced fewer tubule-like structures in HUVECs.

To quantify the effects of CM on angiogenesis, the tube formation on day 11 was analyzed statistically. It was shown that all angiogenesis parameters, except vessel area, were

significantly less in Mel2a-netrin1 CM as compared with Mel2a CM (**Fig. 4**). The vessel areas were 1176592 ± 142766, 1510692 ± 214618 and 1180217 ± 268574 μm^2 in the control, Mel2a CM, and Mel2a-netrin1 CM, respectively (**Fig. 4A**). The vessel areas were measured by adding up the surface areas of all GFP-labeled HUVECs, including aggregated clusters that failed to form tubules and HUVECs that formed tubules, whereas the other parameters were measured by excluding the aggregated clusters. This may be the reason why no difference in the vessel areas was seen between the treatments. The number of junctions reflects the extent of vessel branch. Our analysis showed that the number of junctions (73.3 ± 12.1) in Mel2a CM treatment was the highest, and was significantly ($P < 0.01$) greater than that of Mel2a-netrin1 CM (7.6 ± 3.5) (**Fig. 4B**). Mel2a CM also induced the highest number of tubules (169.3 ± 17.3), and longest tubule (31680 ± 7029 μm), both of them were significantly ($P < 0.05$) greater than those of Mel2a-netrin1 CM (47.33 ± 17.9 and 7720 ± 1161 μm, respectively) (**Fig. 4C**).

Several steps including adhesion, migration, alignment, elongation, coalescence, and tube formation are involved in tube formation. In the co-culture assay, the length of elongation in HUVECs treated with Mel2a-netrin1 CM was

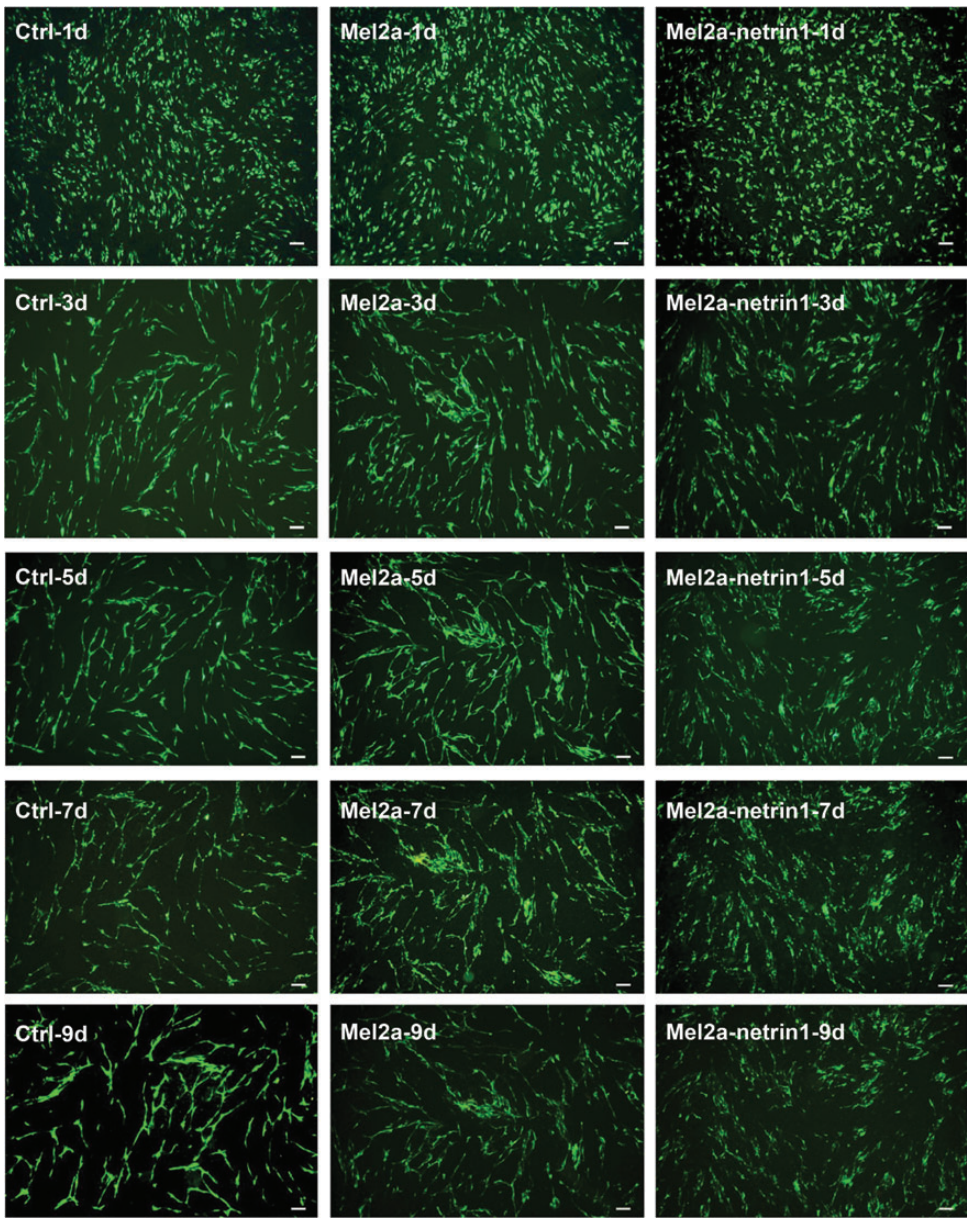

Figure 2. The time course of the tubule formation in co-culture assay induced by CM GFP-labeled HUVECs (4 × 10^4 cells/well) mixed with human fibroblasts (8 × 10^4 cells/well) were seeded in wells of 24-well microplate in 500 μl of mixture of tumor cells CM and EGM-2 (2 : 1, v/v). The mixed medium was changed every other day. Images of approximate same field were taken on days 1, 3, 5, 7, and 9 after seeding, respectively. Representative images of three fields were shown ($n = 3$, 40×), Scale bar, 200 μm.

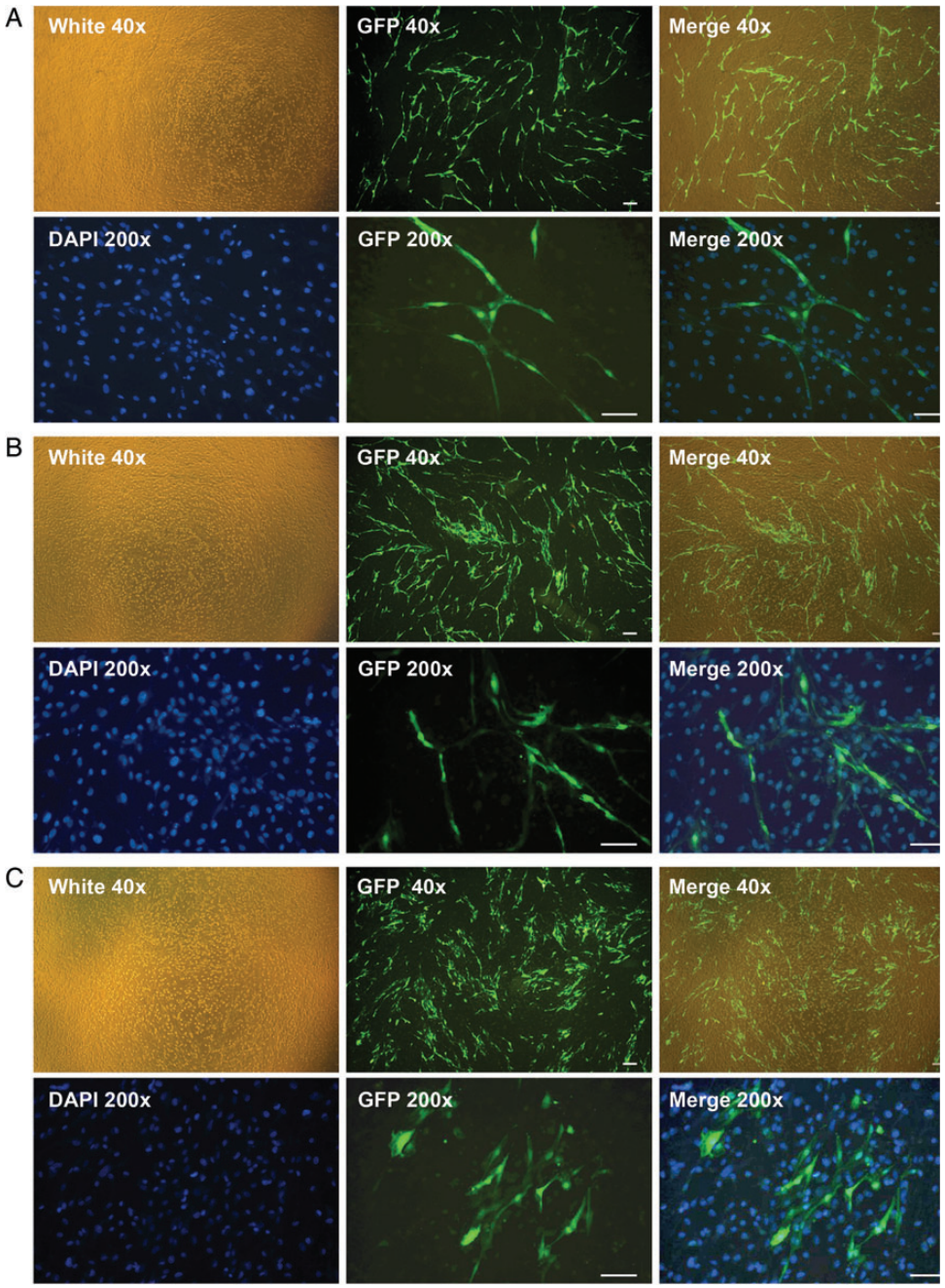

Figure 3. The effects of CM on tube formation in co-culture assay on day 11 On day 11, cells were stained with DAPI, images of approximate same field were taken at both low (40×) and high magnifications (200×). (A) Control CM. (B) Mel2a CM. (C) Mel2a-netrin1 CM. Representative images of four fields were shown ($n = 3$, 40×). Scale bar, 200 μm (40×), 100 μm (200×).

significantly shorter than that of control and Mel2a CM (**Fig. 4D**). The coalescence of HUVECs treated with Mel2a-netrin1 CM was also obviously reduced, and most of HUVECs maintained as single cells without coalescence with others (**Fig. 3C**, GFP 200×). Thus, the inhibition of tube formation by Mel2a-netrin1 CM may be attributed to the blockage of elongation and coalescence of HUVECs, which was not clearly detectable with the matrigel assay.

In the nervous system, netrin-1 is a bifunctional guidance cue that attracts some axons while repels others [14], depending on which receptors it binds to [15]. In blood vessels, netrin-1 has been reported to mediate repulsive effects through Unc5B receptor whose expression in HUVECs has been confirmed [5], thus the blockage of elongation and coalescence of HUVECs by Mel2a-netrin1 CM might be mediated by Unc5B receptor.

The concentration of netrin-1 has also been shown to affect the tube formation. Wilson *et al.* [7] showed that netrin-1 displayed the pro-angiogenic activity at 200 ng/ml on that tube formation using the matrigel assay. However,

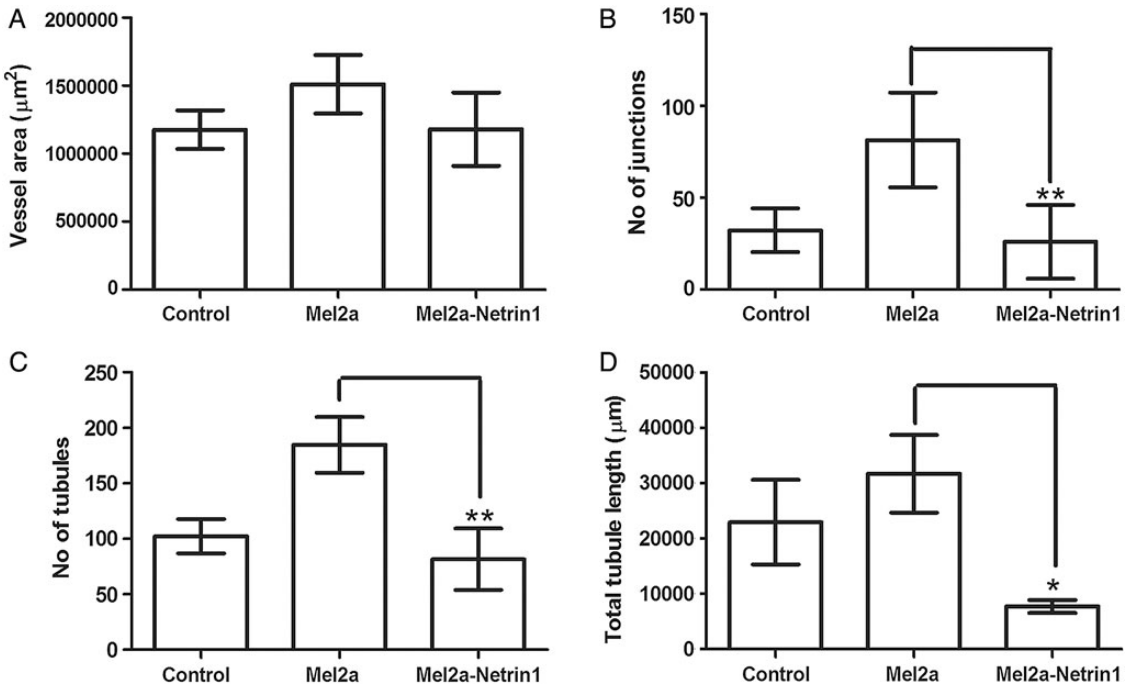

Figure 4. Quantification of tube formation in co-culture assay on day 11 Parameters including vessel area (A), number of junctions (B), number of tubules (C) and total tubule length (D) were analyzed. Four fields per well were selected. Data were expressed as mean ± SD, $n = 3$. Significant differences ($^*P < 0.05$, $^{**}P < 0.01$) were determined by unpaired Student's *t*-test using Graphpad prism 5.0 software.

Yang [16] found that netrin-1 at 100 ng/ml had no effect on the tube formation, whereas at high concentration (1000 ng/ml) it inhibited the tube formation, indicating that the protein is cytotoxic at high concentration. The physiological concentration of netrin-1 is between 50 and 150 ng/ml [2,17]. The level of netrin-1 in the Mel2a-netrin1 CM group was ~98 ± 20 ng/ml [5], and the Mel2a CM and Mel2a-netrin1 CM were prepared under the same conditions. Therefore, the inhibitory effects of netrin-1 on the tube formation in the co-culture assay could not be ascribed to cytotoxic effects. It took more time for HUVECs to form tubule-like structures in the co-culture assay than in the matrigel assay. We cannot exclude the possibility that netrin-1 has long-term effects on the tube formation in the co-culture assay which cannot be evidenced in the matrigel assay.

In summary, our findings showed that although matrigel has been widely used as a screen assay for angiogenic and anti-angiogenic factors, the fact that matrigel is a strong promoter for EC differentiation into tubule-like structure might mask the key steps during the tube formation stimulated by the agents studied. The co-culture assay is more sensitive as an *in vitro* assay for angiogenesis, because it can show delicate effects which cannot be observed on the matrigel assay, though it takes longer time to complete. These results confirmed that netrin-1 is anti-angiogenic *in vivo* [4], although further studies are needed to investigate the mechanisms underlying the inhibition effect on netrin-1 on tube formation.

Supplementary Data

Supplementary Data is available at *ABBS* online.

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References

1. Kennedy E, Serafini T, de la Torre JR and Tessier-Lavigne M. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell 1994, 78: 425–435.
2. Serafini T, Kennedy TE, Galko MJ, Mirzayan C, Jessell TM and Tessier-Lavigne M. The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. Cell 1994, 78: 409–424.
3. Cohen-Cory S. The double life of netrin. Nature Neurosci 2002, 5: 926–928.
4. Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D and Bréant C, *et al*. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature 2004, 432: 179–186.
5. Larrivée B, Freitas C, Trombe M, Lv X, Delafarge B, Yuan L and Bouvrée K, *et al*. Activation of the UNC5B receptor by netrin-1 inhibits sprouting angiogenesis. Genes Dev 2007, 21: 2433–2447.
6. Park KW, Crouse D, Lee M, Karnik SK, Sorensen LK, Murphy KJ and Kuo CJ, *et al*. The axonal attractant netrin-1 is an angiogenic factor. Proc Natl Acad Sci USA 2004, 101: 16210–16215.
7. Wilson BD, Ii M, Park KW, Suli A, Sorensen LK, Larrieu-Lahargue F and Urness LD, *et al*. Netrins promote developmental and therapeutic angiogenesis. Science 2006, 313: 640–644.

8. Donovan D, Brown NJ, Bishop ET and Lewis CE. Comparison of three *in vitro* human angiogenesis assays with capillaries formed *in vivo*. Angiogenesis 2001, 4: 113–121.
9. Kleinman HK and Martin GR. Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol 2005, 15: 378–386.
10. Bishop ET, Bell GT, Bloor S, Broom IJ, Hendry NF and Wheatley DN. An *in vitro* model of angiogenesis: basic features. Angiogenesis 1999, 3: 335–344.
11. Navankasattusas S, Whitehead KJ, Suli A, Sorensen LK, Lim AH, Zhao J and Park KW, *et al*. The netrin receptor UNC5B promotes angiogenesis in specific vascular beds. Development 2008, 135: 659–667.
12. Wang Q, Zhu J, Zou L and Yang Y. Role of axonal guidance factor netrin-1 in human placental vascular growth. J Huazhong Univ Sci Technol Med Sci 2011, 31: 246–250.
13. Bouvrée K, Larrivée B, Lv X, Yuan L, DeLafarge B, Freitas C and Mathivet T, *et al*. Netrin-1 inhibits sprouting angiogenesis in developing avian embryos. Dev Biol 2008, 318: 172–183.
14. Dickson BJ. Molecular mechanisms of axon guidance. Science 2002, 298: 1959–1964.
15. Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M and Stein E. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell 1999, 97: 927–941.
16. Yang Y, Zou L, Wang Y, Xu KS, Zhang JX and Zhang JH. Axon guidance cue netrin-1 has dual function in angiogenesis. Cancer Biol Ther 2007, 6: 743–748.
17. Serafini T, Colamarino SA, Leonardo ED, Wang H, Bedington R, Skarnes WC and Tessier-Lavigne M. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 1996, 87: 1001–1014.