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BDNF (Brain-Derived Neurotrophic Factor) Promotes Embryonic Stem Cells Differentiation to Endothelial Cells Via a Molecular Pathway, Including MicroRNA-214, EZH2 (Enhancer of Zeste Homolog 2), and eNOS (Endothelial Nitric Oxide Synthase)

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Objective—The NTs (neurotrophins), BDNF (brain-derived neurotrophic factor) and NT-3 promote vascular development and angiogenesis. This study investigated the contribution of endogenous NTs in embryonic stem cell (ESC) vascular differentiation and the potential of exogenous BDNF to improve the process of ESC differentiation to endothelial cells (ECs).

Approach and Results—Mouse ESCs were differentiated into vascular cells using a 2-dimensional embryoid body (EB) model. Supplementation of either BDNF or NT-3 increased EC progenitors' abundance at day 7 and enlarged the peripheral vascular plexus with ECs and SM22α^+ (smooth muscle 22 alpha-positive) smooth muscle cells by day 13. Conversely, inhibition of either BDNF or NT-3 receptor signaling reduced ECs, without affecting smooth muscle cells spread. This suggests that during vascular development, endogenous NTs are especially relevant for endothelial differentiation. At mechanistic level, we have identified that BDNF-driven ESC-endothelial differentiation is mediated by a pathway encompassing the transcriptional repressor EZH2 (enhancer of zeste homolog 2), microRNA-214 (miR-214), and eNOS (endothelial nitric oxide synthase). It was known that eNOS, which is needed for endothelial differentiation, can be transcriptionally repressed by EZH2. In turn, miR-214 targets EZH2 for inhibition. We newly found that in ESC-ECs, BDNF increases miR-214 expression, reduces EZH2 occupancy of the *eNOS* promoter, and increases eNOS expression. Moreover, we found that NRP-1 (neuropilin 1), KDR (kinase insert domain receptor), and pCas^{130} (p130 Crk-associated substrate kinase), which reportedly induce definitive endothelial differentiation of pluripotent cells, were increased in BDNF-conditioned ESC-EC. Mechanistically, miR-214 mediated the BDNF-induced expressional changes, contributing to BDNF-driven endothelial differentiation. Finally, BDNF-conditioned ESC-ECs promoted angiogenesis in vitro and in vivo.

Conclusions—BDNF promotes ESC-endothelial differentiation acting via miR-214.

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Key Words: angiogenesis ■ brain-derived neurotrophic factor ■ embryonic stem cells ■ endothelial differentiation ■ microRNAs

NTs (neurotrophins) are a family of protein ligands, including NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), NT-3, and NT-4/5. They act on 2 distinct receptor types: the TRK (tropomyosin kinase receptors) A, B, and C and the neurotrophin receptor p75^{NTR}, which belongs to the TNF-α (tumor necrosis factor) receptor family[1] (Figure IA in the online-only Data Supplement). NTs were initially identified as regulators of neuronal prosurvival and regenerative actions. We and others have later shown that NTs also promote cardiovascular development, protection, and regeneration, including by stimulating postischemic angiogenesis (reviewed in Caporali and Emanueli[1]). Additional evidence suggests a regulatory role of BDNF on stem cells (SCs) and vascular progenitor cells.[2–8] In fact, pluripotent SCs have been reported to express TRKB and TRKC and to secrete BDNF, NT-3, and NT-4/5, which serve as autocrine prosurvival

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Nonstandard Abbreviations and Acronyms

BDNF	brain-derived neurotrophic factor
EB	embryoid body
EC	endothelial cell
eNOS	endothelial nitric oxide synthase
EZH2	enhancer of zeste homolog 2
mESC	mouse embryonic stem cell
miR	microRNA
NGF	nerve growth factor
NT	neurotrophin
PcG	polycomb group
PRC2	polycomb Repressive Complex 2
TNF-α	tumor necrosis factor α
TRKB/C	tropomyosin receptor kinase B/C
VEGF	vascular endothelial growth factor

factors.[2] Moreover, BDNF reportedly induced the endothelial differentiation of human fetal CD133$^+$ SCs and mesenchymal SCs.[3,9]Studies on global knockout mouse models revealed the importance of NT-3 and BDNF for vascular development.[10,11] In particular, BDNF deficiency reduced endothelial cell-cell contact in the mouse embryonic heart, leading to intraventricular wall hemorrhage.[11] Similarly, mice with gene knockout for the BDNF receptor TRKB showed a decreased blood vessel density and increased number of TUNEL-positive apoptotic endothelial cells (EC).[12] NT-3 knockout mice developed abnormalities of the great vessels.[10,13,14] Taken together the NT family have important roles in vascular morphology and function.

Research in regenerative medicine is currently focussing extensively on tissue engineering. These applications require large numbers of well-characterised cells for larger scale fabrication of biomaterials.[15] Simultaneously, the interest in the use of SCs for disease modeling and drug testing in the dish is growing. For the aforementioned areas of translational research, the use of naive or genetically engineered embryonic stem cell (ESC) lines offers clear advantages in comparisons to patient-derived adult progenitor cells, which had limited plasticity and capacity of growth and differentiation, epigenetic defects, and difficulties in harvesting from patients.[15,16] Within vascular biomedicine the protocol for production of ESC-derived ECs are still being optimized to meet the criteria of first-in-man trials and clinical translation.[17–20] The precise growth factor and cytokine cocktail required for optimal differentiation of cultured ESCs into ECs remain to be accurately determined, in part, because the mediators of the endothelial differentiation process are only partially explored. Therefore, further research is needed to understand the mechanisms required for generation of robust and highly angiogenic ECs.

PcG (Polycomb group) proteins play a key role in ESC commitment and differentiation by repressing transcription of developmental regulators.[21–25] EZH2 (enhancer of zeste homolog 2), the PcG catalytic subunit, is part of the PRC2 (polycomb repressive complex 2) and mediates the trimethylation of histone H3 on lysine 27 (H3K27m3) in target gene promoters leading to epigenetic silencing.[26] EZH2 was previously suggested to regulate both maintenance of pluripotency and vascular commitment[4,27–31] and PRC2 has been shown to silence cohorts of developmental genes in murine and human ESCs.[21,32–34] We have previously reported that EZH2 represses eNOS (endothelial nitric oxide synthase) transcription in mature ECs.[4] NO is essential for the mesodermic differentiation of mouse ESC (mESC) and to produce proangiogenic mESC-derived ECs.[35] Moreover, mESCs respond to increased levels of either intracellular (produced by eNOS) or extracellular NO (NO donors) by upregulating expression of vascular lineage–associated genes, including CD31, VEGF (vascular endothelial growth factor) receptor 2 (VEGF-R^2, also known as Flk1 [fetal liver kinase 1 receptor] in mouse and KDR [kinase insert domain receptor] in human), smooth muscle actin, and the α-sarcomeric actin (reviewed in Wu and Zhang[36]). MicroRNAs (miRs) have been shown to contribute to EZH2 regulation.[31,37] miRs are a class of endogenous small noncoding RNAs that control post-transcriptional gene expression, thus influencing vascular cell growth, differentiation, and function[38–41] and are expressed in ESCs[42] and vascular cells.[43] EZH2 has been shown to inhibit the transcription of miR-214 during skeletal muscle cell differentiation from ESC[44,45] and as a feedback mechanism, miR-214 inhibits EZH2 at the post-transcriptional level.[44] miR-214 has been shown to regulate apoptosis, proliferation, cancer angiogenesis,[46,47] skeleton formation,[48] and heart hypertrophy.[37] However, the function of miR-214 in vascular differentiation and the possible link between miR-214 and EZH2 in the context of ESC-endothelial differentiation have not been reported.

An important objective for endothelial differentiation of pluripotent SCs is the stability of the differentiation. NRP-1 (neuropilin 1) is a nontyrosine kinase receptor for VEGF and other growth factors or morphogens. In EC, p130(Cas) tyrosine phosphorylation is stimulated by VEGF and is dependent on the NRP-1 intracellular domain. In fact, NRP-1 silencing reduces VEGF-R^2 in ECs.[49,50] Importantly, NRP-1-mediated enhancement of VEGF-R^2 signaling through VEGF was shown to produce a stable endothelial phenotype with high clonal proliferative potential and functional neovascularization capacity in vivo.[51]

Therefore, understanding the transcriptional and post-transcriptional mechanisms controlling ESC-EC differentiation in response to growth factors might be key to developing better ESC-derived approaches to treating vascular diseases. Here, we have mechanistically investigated the potential of NTs to contribute to ESC differentiation in ECs.

Materials and Methods

The authors declare that all supporting data are available within the article (and its online-only Data Supplement files). A comprehensive version of the Materials and Methods has been included in the online-only Data Supplement.

Western Blot

Western blot was performed as previously described.[52] Please refer Materials and Methods section in the online-only Data Supplement for further details.

Three-Dimensional–Fibrin Gel Bead Assay

CD31+ cells (ESC-EC) were isolated from differentiated cells using MACS (magnetic-activated cell sorting) columns (as described above). Passage 3 cells were used for Fibrin gel bead assay and Matrigel plug assay (vide infra). Fibrin gel bead assay was performed as previously described.[53] Please refer Materials and Methods section in the online-only Data Supplement for further details.

Matrigel Plug Assay

Cells were implanted in Matrigel plugs and injected subcutaneously to evaluate their angiogenic effect in vivo as previously described.[54,55] Please refer Materials and Methods section in the online-only Data Supplement for further details.

Results

Expression of NT Ligands and Receptors During Vascular Embryoid Body Differentiation

We measured expression of the NT system components (Figure IA in the online-only Data Supplement). BDNF and NT-3 (Figure IB in the online-only Data Supplement) together with their respective preferential receptors TRKB and TRKC (Figure IC in the online-only Data Supplement) appeared expressionally regulated during the process of ESC differentiation in ECs were, suggesting functional roles for both BDNF/TRKB and NT-3/TRKC in the endothelial

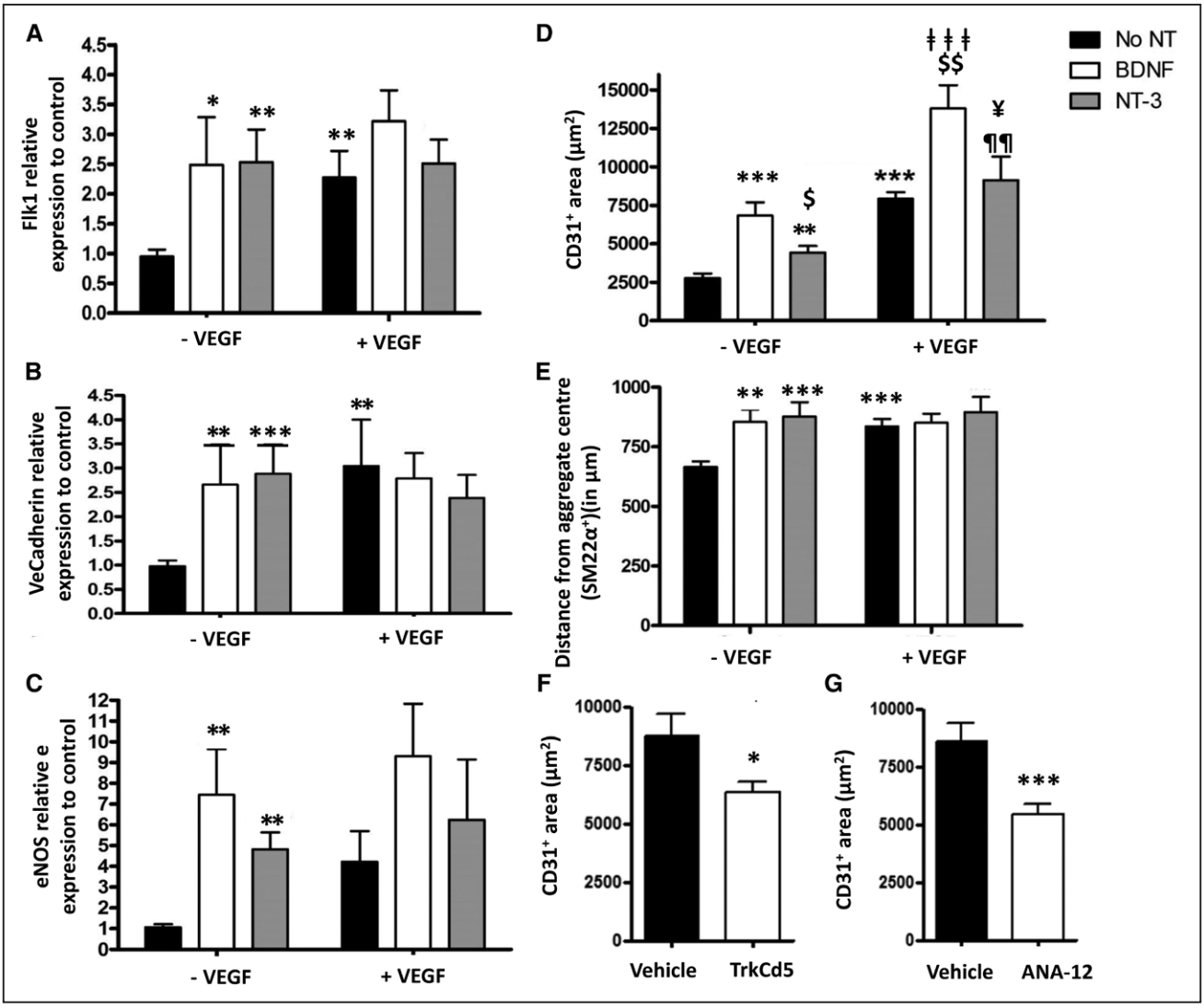

Figure 1. NTs (neurotrophins) promotes endothelial differentiation, and the differentiation process is impaired by NT inhibition. Mouse embryonic stem cells were differentiated using an embryoid body (EB) hanging drop protocol. After 4 d, EBs were seeded on 8-well glass culture slides for immunocytochemistry or 12 well-plate for RNA and protein analysis. Cells were treated with BDNF (brain-derived neurotrophic factor) or NT-3 (control: vehicle) and without VEGF-A (vascular endothelial growth factor A). **A**–**F**, mRNA expression (measured by quantitative real-time polymerase chain reaction) level (expressed as relative to vehicle control) of Flk1(fetal liver kinase 1 receptor; n=9; **A**), Ve-Cadherin (vascular endothelial cadherin; n=8; **B**) and eNOS (endothelial nitric oxide synthase; n=7; **C**) at day 7 of EB vascular development. **D**, Quantification of CD31+ area in automatic confocal microscopy images (µm²; n=11). **E**, Quantification of smooth muscle cells spread at day 13 NT (n=11); **F** and **G**, Quantification at day 13 of CD31+ (red) vascular structures in EB treated with ANA-12 (*N*-[2-[[(hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]-benzo[b]thiophene-2-carboxamide) and TrkCd5 (tropomyosin kinase receptor C domain 5) inhibitors (n=11). **A**–**E**, Comparisons across groups performed both for –VEGF and +VEGF scenarios using Kruskal-Wallis test with Dunn test for post hoc testing; the effect of –/+VEGF was tested for each subgroup with Mann-Whitney *U* test. **F**–**G**, Comparisons between presence/absence of inhibitors performed with Mann-Whitney *U* test, **P*<0.05, ***P*<0.01, ****P*<0.001 vs control (No VEGF, No NT), $*P*<0.05, $$*P*<0.01 vs –VEGF+BDNF, ǂǂǂ*P*<0.001 vs VEGF only (without NTs), ¶¶*P*<0.01 vs –VEGF+NT-3, γ*P*<0.05 vs +VEGF+BDNF. SM22α indicates smooth muscle 22 alpha.

differentiation process. Therefore, we decided to investigate those further.

BDNF and NT-3 Promote the Differentiation of ESC in Vascular Progenitor Cells

As shown in Figure 1A–1C, either BDNF or NT-3 increased the mRNA expression of endothelial markers Flk1, Ve-Cadherin (vascular endothelial cadherin), and eNOS in embryoid bodies (EBs). Such changes were similar to the ones observed after VEGF-A (used as positive control[56]) and were not further increased by combining NT and VEGF treatment. Flow cytometry analysis confirmed these results, showing that supplementation of either BDNF or NT-3 increased the number of Flk1$^+$ vascular progenitor cells at day 7 (Figure IIA in the online-only Data Supplement). A similar trend was seen with CD31$^+$ EC; however, it was not statistically significant (Figure IIB in the online-only Data Supplement). To further study the impact of the NTs on vascular differentiation, we analyzed the EBs at day 13 of differentiation using automatic confocal microscopy mosaic acquisition (Figure IVA and IVB in the online-only Data Supplement). As shown in Figure 1D and 1E, under basal culture conditions, EBs developed a peripheral vascular plexus composed of CD31$^+$ and SM22α$^+$ (smooth muscle protein 22-alpha positive) vascular structures. Exogenous BDNF and NT-3 were equally powerful as VEGF to improve the formation of an organized vascular structure composed of mural cells surrounding endothelial tubes. Moreover, endogenous NTs appeared important for the native vascular development process. In fact, as shown in Figure 1F and 1G and Figure IVC and IVD in the online-only Data Supplement, inhibition of endogenous NT/receptor signaling by blocking the function of either TRKB (via a small non peptidic compound ANA-12 (*N*-[2-[[(hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl] phenyl]-benzo[b]thiophene-2-carboxamide), 25 mmol/L) or TRKC (via soluble TrkC receptor domain TrkCd5 neutralizing NT-3, 2μg/mL) led to the formation of a less structured endothelial plexus, with the loss of 38±9% or 28±8% of CD31$^+$ cells, respectively. However, the inhibitors did not affect the smooth muscle cells expansion (Figure V in the online-only Data Supplement). Therefore, we restricted our research focus to investigate the impact of NTs on endothelial differentiation.

BDNF Promotes Endothelial Differentiation Generating Functional ECs

Based on the expressional trends of the NT family components observed during mESC-EC differentiation (Figure I in the online-only Data Supplement), we decided to focus on BDNF treatment to investigate how NTs impact on the proangiogenic capacity of the mESC-derived ECs derived from the differentiation process. To this aim, CD31$^+$ cells were sorted from EBs at day 10 and plated onto 0.1% gelatin in a differentiation medium supplemented or not with VEGF and BDNF (Figure III in the online-only Data Supplement). The CD31$^+$ EC progenitors were cultured for up to 6 passages. Similar to differentiated ECs, mESC-derived CD31$^+$ cells were able to uptake acetylated-LDL (low-density lipoprotein; Figure 2A), showed typical endothelial cobblestone morphology, and expressed the CD31 endothelial marker(Figure 2B) and were capable of forming vascular tube-like structures in a 2-dimensional Matrigel assay (Figure 2C). Untreated ESC-CD31$^+$ cells showed a proangiogenic capacity from passage 3 (Figure VI in the online-only Data Supplement), whereas both individual and combined BDNF or VEGF treatment accelerated the proangiogenic capacity of the mESC-derived CD31$^+$ cells to passage 2 and 1, respectively (Figure 2C), revealing the capacity of BDNF to accelerate endothelial specification and improve functionality of derived ECs. By contrast, when compared with VEGF-, BDNF-, or BDNF/VEGF-treated ESC-derived cells, at passage 3, the cobblestone morphology of untreated ESC-CD31$^+$ cells was not homogenous and rather composed of a mix of different cells (Figure 2B). Moreover, immunostaining highlighted lower expression of endothelial markers, such as CD31 and vWF (von Willebrand factor) in control cells, revealing that endothelial differentiation was not fully achieved in untreated cells (Figure 2B). Importantly, the angiogenic capacity of BDNF-induced EC (ESC-EC) was confirmed in both the 3-dimensional in vitro angiogenesis model and in vivo. In both the fibrin gel bead assay (Figure 2D–2F) and the in vivo Matrigel implant assay (Figure 2G and 2H), BDNF-induced EC (ESC-EC) showed increased tube length and sprout formation when compared with ESC.

BDNF Induces Stable Endothelial Differentiation Through miR-214-Dependent Inhibition of EZH2

During ESC-EC differentiation, exogenous BDNF increased eNOS mRNA expression (Figure 1C) and decreased EZH2 expression (Figure 3A). These 2 responses are possibly correlated because a CHIP (chromatin immunoprecipitation) for EZH2 followed by polymerase chain reaction for the TSS (transcription start site) regulatory region of *eNOS* promoter showed that BDNF treatment reduces the promoter (TSS –425/–233) occupancy by EZH2 in ESC-ECs (Figure 3B). In a different cell type, EZH2 was inhibited by miR-214 at post-transcriptional level.[44] In line with that, BDNF treatment increased miR-214 expression in ESC-ECs (Figure 3C). To understand if these events downstream to BDNF are consequential, we successfully inhibited miR-214 expression using an anti-miR-214 (Figure 3D). In the presence of BDNF, miR-214 inhibition increased EZH2 expression (Figure 3E) and decreased eNOS expression (Figure 3F), suggesting that miR-214 mediated the BDNF-induced expressional responses. To better understand the stability of endothelial differentiation in our system, we next analysed the expression of NRP-1, KDR, and p130^{Cas}. BDNF increased the expression of NRP-1 (Figure 4A), VEGF-R^2 (Figure 4B), and p130^{Cas} (Figure 4C and 4D) and all these BDNF-induced changes were inhibited by anti-miR-214 (Figure 4A–4D).

We finally assessed if BDNF could regulate miR-126, which has been described to be important for ESC differentiation to EC and for in vivo vascular development.[57] Figure VIII in the online-only Data Supplement shows that BDNF does not affect miR-126 expression, providing further evidence in support of the hypothesis that miR-214 is an important mediator of the BDNF action on mESC-EC. Finally, we investigated if miR-214 mediates the action of BDNF in mature EC. Interestingly, in human umbilical vein ECs, miR-214 inhibition did not prevent the BDNF-induced increase in eNOS

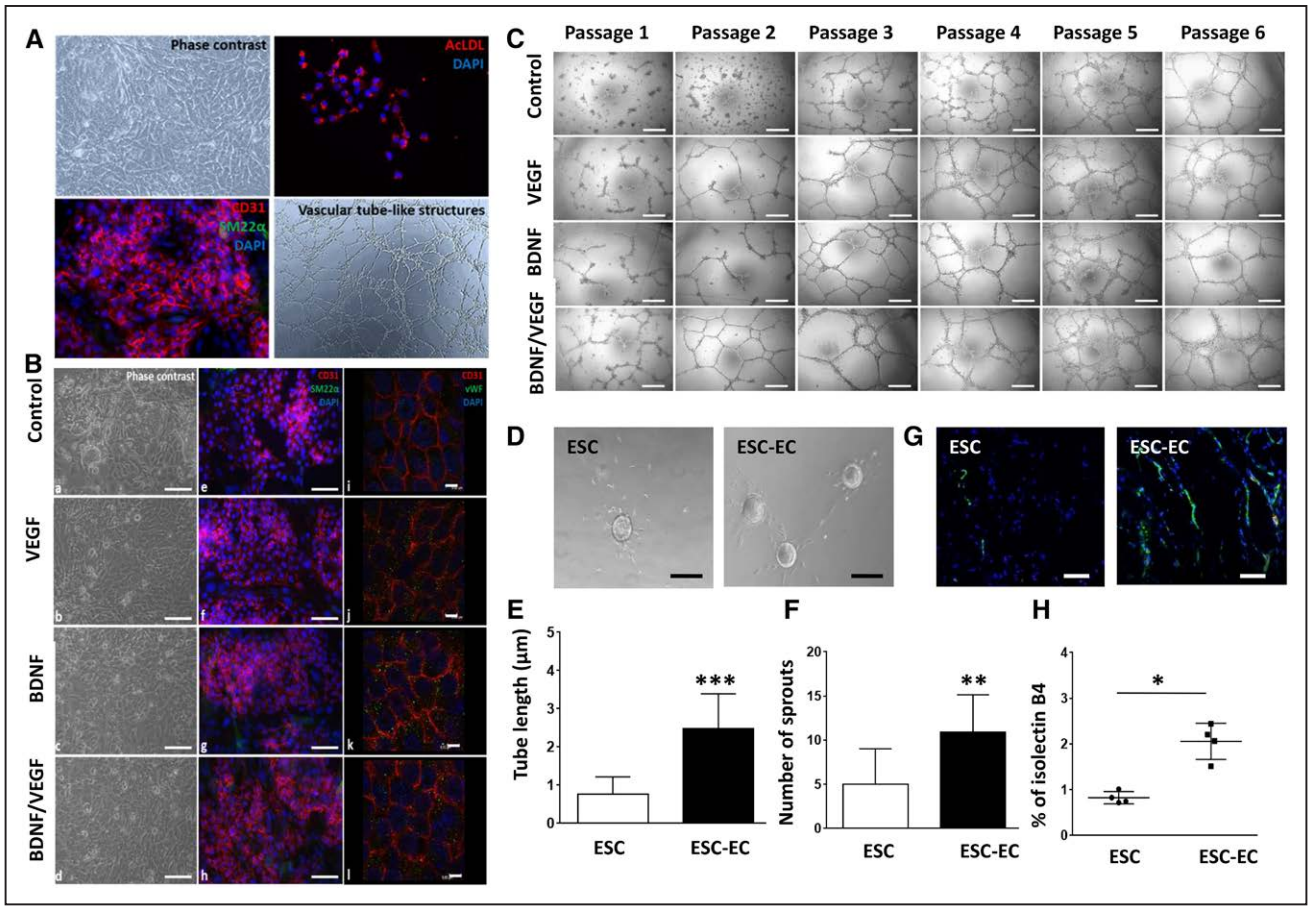

Figure 2. BDNF (brain-derived neurotrophic factor) promotes endothelial differentiation and formation of functional endothelial cells (ECs). To investigate the impact of BDNF on the proangiogenic capacity of mouse embryonic stem cell (mESC)-derived ECs, CD31⁺ cells were sorted from embryoid bodies at day 10 and plated onto 0.1% gelatin in a differentiation medium supplemented or not with VEGF-A (vascular endothelial growth factor A) and BDNF. **A**, Sorted CD31⁺ cells were cultured and characterized at passage 3 (n=4).Sorted CD31⁺ cells were cultured and characterized at passage 3 (n=4). **A**, Cells present cobblestone morphology (scale bar 200 µm) and express CD31 endothelial marker (scale bar 50 µm). They uptake ac-LDL (low-density lipoprotein; n=4; scale bar 50 µm) and **B**, Characterization (cobblestone morphology [**A**–**D**; scale bar 200 µm] and immunostaining for CD31/SM22α [smooth muscle 22 alpha; **E**–**H**; scale bar 50 µm] and CD31/vWF [von Willebrand factor; **I**–**L**; scale bar 5.5 µm] marker expression) of EC-ESCs treated with no cytokine (**A, E,** and **I**), VEGF (**D, F,** and **J**), BDNF (**C, G,** and **K**) or both growth factors (**D, H,** and **L**) at passage 3. **C**, Representative images of tube length relative to control formed on Matrigel (24 h) at passage 3 with different cytokine treatments. (scale bar 500 µm; n=4). **D**–**F**, Representative images (**D**) and quantification of tube length (n=4; **E**) and sprout formation (n=4; **F**) in cytobeads by pluripotent ESC and ESC-EC (scale bar 50 µm). **G**–**H**, Representative images (**G**) and quantification (**H**) of percentage of isolectin B4 (n=4) comparing ESC and ESC-EC in in vivo Matrigel plug assay. All data are expressed as Mean±SEM. Comparisons between groups performed with Mann-Whitney *U* test. **P*<0.05, ***P*<0.01, ****P*<0.001 vs pluripotent ESC.

expression (Figure VIIB in the online-only Data Supplement) or the BDNF-induced decrease in EZH2 expression (Figure VIIC in the online-only Data Supplement). Taken together, the data suggest that the BDNF-miR-214 pathway is important for endothelial differentiation rather than for postnatal angiogenesis. Figure IX in the online-only Data Supplement illustrates our proposed model with the cascade of molecular events activated by BDNF/TRKB.

Discussion

Our study has provided novel evidence for a role for the NT system in stimulating vascular differentiation of ESCs. We have shown that BDNF induces endothelial commitment and improves the production of ECs, which are proangiogenic in vitro and in vivo. We have characterized the molecular changes associated with the endothelial differentiation of BDNF-treated cells and demonstrated that they are mediated by miR-214. In the presence of BDNF, miR-214 expression is increased, thus, leading to reduced EZH2 expression. In line with our data, miR-214 was already known to target EZH2 mRNA for repression in a different cell context.[44] Expression of eNOS, a well-characterized example of an endothelial gene, is regulated by its chromatin accessibility, and is required for ESC-EC differentiation.[35,36] The murine eNOS promoter has been previously characterized.[36,58] Here, we have shown that BDNF increases eNOS in a miR-214-dependent manner. We have also demonstrated that the presence of BDNF is associated with a reduced EZH2 occupancy of the *eNOS* promoter and ,therefore, removal of repressive regulation and an associated increase in eNOS expression. Specifically, we have demonstrated that BDNF treatment reduced EZH2 occupancy in the *eNOS* promoter regulatory sequence between −425/−233 bp upstream to the TSS. This region is characterized by the presence of 2 Ets binding sites. Ets transcription factors regulate important signaling pathways involved in cell differentiation and development in many tissues and are critical for driving endothelial gene expression.[59]

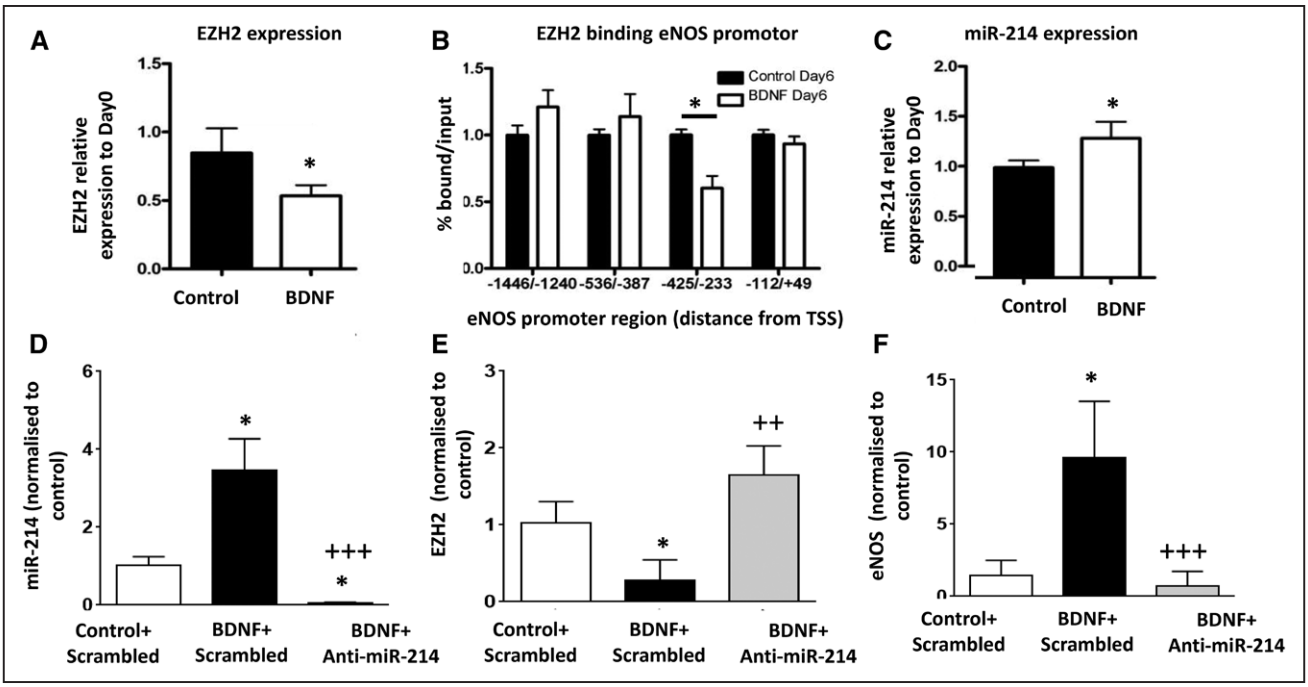

Figure 3. BDNF (brain-derived neurotrophic factor) induced functional endothelial differentiation through activation of micro RNA (miR)-214, leading to EZH2 (enhancer of zeste homolog 2) inhibition and promotion of eNOS (endothelial nitric oxide synthase) transcription. Gene expression was evaluated from undifferentiated or differentiated embryoid bodies (EBs) treated or not with BDNF during vascular EB differentiation. **A**, EZH2 mRNA expression (n=3) is modulated by BDNF during EB vascular differentiation. **B**, EZH2 occupancy on transcriptional regulatory region (−425/−233 bp from TSS [transcription start site]) of the *eNOS* promoter was reduced by BDNF (n=3). IgG was used as a control in the EZH2-CHIP (chromatin immunoprecipitation). Data were calculated as % bound/input. **C,** miR-214 expression (n=3) is modulated by BDNF during EB vascular differentiation. mRNA expression of miR-214 (**D**), EZH2 (**E**), and eNOS (**F**) after treatment of EB with anti-miR-214 50 nM (n=3). **A** and **C**, Comparison across subgroups performed with Kruskal-Wallis test with Dunn test for post hoc testing; comparison of the effect of BDNF vs control at day 6 performed with Mann-Whitney *U* test. **D**–**F**, Comparisons across groups performed with Kruskal-Wallis test with Dunn test for post hoc testing. **P*<0.05 vs control of BDNF+scrambled control of anti-miR-214 for **D**, **E**, and **F**), +*P*<0.05, ++*P*<0.01, +++*P*<0.001 vs BDNF+scrambled.

Previous studies using genetic mouse models reported that NTs regulate cardiovascular development[10–12,14] (reviewed by Caporali and Emanueli[13] and Tessarollo[1]). Despite the importance of the NT ligand-receptor systems during development and in angiogenesis, the NTs implication in the process of ESC differentiation to vascular cells was unaddressed. Here, we show for the first time that endogenous expression of BDNF and NT-3 and their respective receptors increased throughout EB differentiation to vascular cells and that exogenous addition of BDNF or NT-3 increased the expression of VEGF-R^2, one of the earliest genes expressed during endothelial lineage development.[60] Ve-Cadherin, a marker of mature ECs,[61] also increased in differentiating cells treated with NTs. The primary vascular plexus in the EB is remodeled from day 6 onwards by sprouting angiogenesis. The morphology of the vascular plexus formed in 2-dimensional EB cultures is dependent on the growth factors present in the culture.[56] VEGF is the prototypic angiogenic regulator and, therefore, is the fundamental factor for vascular development and endothelial differentiation,[62] and it is commonly used in protocols aiming at inducing the endothelial differentiation of pluripotent SCs. VEGF induces the formation of a peripheral vascular plexus,[56] which we have confirmed in this study. However, BDNF or NT-3 also induced the formation of an organized vascular structure with mural cells surrounding endothelial tubules. The endothelial pattern formed after treatment with NTs look different from VEGF induced patterning though. VEGF induced a peripheral endothelial plexus, whereas BDNF or NT-3 created a more central endothelial structure. Interestingly, combining VEGF with BDNF resulted in higher and thicker vascular structures with the formation of both central and peripheral endothelial plexus suggesting complementarity between VEGF and BDNF, later during EB vascular development.[63] Inhibition of TRKB or TRKC resulted in a less structured endothelial plexus with a significant loss of $CD31^+$ cells. NTs increased the smooth muscle cell spread, but interestingly, their inhibition did not reduce smooth muscle cell spread suggesting other possible mechanisms are involved.

BDNF and VEGF share common molecular targets and work co-ordinately in some scenarios.[64] In 2014, Prasain et al[51] reported that NRP-1-mediated enhancement of VEGF-R^2 signaling through VEGF is crucial for VEGF mediated endothelial differentiation. Here, we have newly demonstrated that BDNF, via miR-214, increases NRP-1, VEGF-R^2, and $p130^{Cas}$. This suggests that BDNF might support the production of cells with a stable arterial endothelial phenotype[51] and which are highly responsive to hemodynamic flow.[65]

We have provided evidence that in mESC-ECs, miR-214 is increased by BDNF and is fundamental to mediate endothelial differentiation. In this context, miR-214 inhibition prevented the BDNF-induced decrease of EZH2 and increase of eNOS in mESC-ECs. However and interestingly, in mature

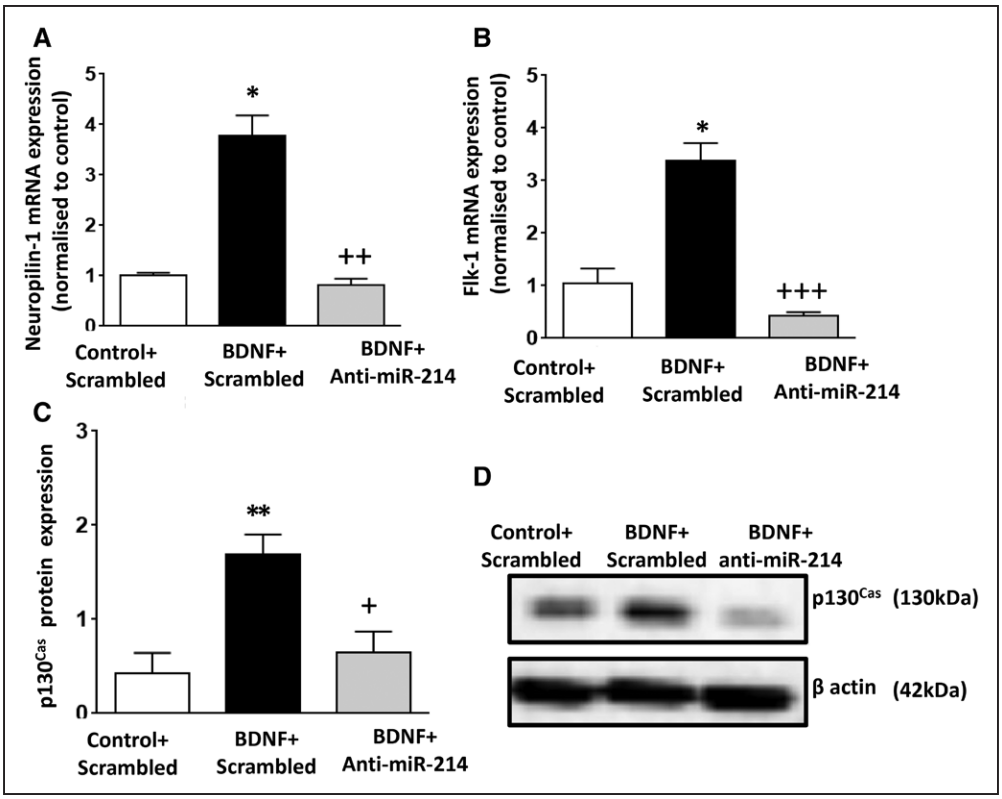

Figure 4. BDNF (brain-derived neurotrophic factor) induced stable endothelial differentiation through activation of NRP-1 (neuropilin 1), flk-1 (fetal liver kinase 1 receptor), and $p130^{Cas}$ (p130 Crk-associated substrate kinase) expression. Expression levels of NRP-1, flk-1, and $p130^{Cas}$ were analysed in cells treated BDNF with or without anti-micro RNA (miR)-214. mRNA expression levels of NRP-1 (**A**) and flk-1 (**B**; n=4) are shown**. C** and **D,** Quantification of protein expression levels of $p130^{Cas}$ (n=3) by Western blot. **A**–**C,** Data are expressed as mean±SEM; comparisons across groups performed with Kruskal-Wallis test with Dunn test for post hoc testing. *P<0.05, ** P<0.01 vs control of BDNF+scrambled control of anti-miR-214, +P<0.05, ++P<0.01, +++P<0.001 vs BDNF+scrambled.

ECs, miR-214 inhibition did not prevent the EZH2 and eNOS response to BDNF. We have also investigated if other miRs could regulate the action of BDNF in mESC-ECs. In particular, miR-126, which is enriched in ECs and mediates their function during ESC-EC differentiation and in vivo developmental angiogenesis.[57] However, miR-126 expression did not seem to be regulated by BDNF. Taken together, the above results reinforce the evidence in support of the hypothesis that miR-214 is a key mediator of the BDNF actions leading to SCs endothelial differentiation.

This basic study has been developed using a murine cell line and a well-established EB model of ESC differentiation. Future studies are deemed necessary to validate the importance of BDNF for human ESC-EC-based therapies. Human ESC offer obvious translational advantages, especially when human ESC-EC are derived and banked under conditions that respect the European Tissue and Cells Directives or equivalent extra-European regulatory authorities. For translational studies aimed at the production of cells for clinical trials, alternative differentiation protocols, ideally compatible with automated culturing of adherent cells in bioreactors, might be favored to the use of EBs.

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Disclosures

None.

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Highlights

- Endogenous neutrotrophins promote endothelial differentiation of embryonic stem cells (ESCs).
- Exogenous BDNF (brain-derived neurotrophic factor) supplementation improves the production of functional endothelial cells from differentiating ESC.
- BDNF acts via a microRNA-214, which regulates EZH2 (enhancer of zeste homolog 2) and eNOS (endothelial nitric oxide synthase).
- BDNF-conditioned ESC-endothelial cells express higher NRP-1 (neuropilin 1) level, which suggests their stable arterial endothelial phenotype and shown functionality in vitro and in vivo.
- Use of BDNF in ESC differentiation protocols might increase the availability of proangiogenic arterial ESC-endothelial cells for therapeutic applications.