Neuropilin-1 Identifies Endothelial Precursors in Human and Murine Embryonic Stem Cells Before CD34 Expression

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Background—In murine embryonic stem cells, the onset of vascular endothelial growth factor receptor 2 (VEGFR-2) expression identifies endothelial precursors. Undifferentiated human embryonic stem cells express VEGFR-2, and VEGFR-2 expression persists on differentiation. The objective of our study was to identify a single population of endothelial precursors with common identifying features from both human and murine embryonic stem cells.

Methods and Results—We report that expression of the VEGF coreceptor neuropilin-1 (NRP-1) coincides with expression of Brachyury and VEGFR-2 and identifies endothelial precursors in murine and human embryonic stem cells before CD31 or CD34 expression. When sorted and differentiated, VEGFR-2"NRP-1" cells form endothelial-like colonies that express CD31 and CD34 7-fold more efficiently than NRP-1 cells. Finally, antagonism of both the VEGF and Semaphorin binding functions of NRP-1 impairs the differentiation of vascular precursors to endothelial cells.

Conclusions—The onset of NRP-1 expression identifies endothelial precursors in murine and human stem cells. The findings define the origin of a single population of endothelial precursors from human and murine stem cells to endothelial cells. Additionally, the function of both the VEGF and Semaphorin binding activities of NRP-1 has important roles in the differentiation of stem cells to endothelial cells, providing novel insights into the role of NRP-1 in a model of vasculogenesis. (Circulation. 2009;119:2170-2178.)

Key Words: endothelium ■ stem cells ■ vascular biology ■ vasculogenesis

In development and in adults, blood vessel formation occurs via 2 mechanisms: vasculogenesis (de novo blood vessel formation) or angiogenesis (proliferation and extension of existing vascular tissues). Although many experimental models to study the impact of therapies on angiogenesis exist, very few model systems are available to study vasculogenesis. Despite similarities in vasculogenesis between species, often preclinical testing of new therapies relies on animal models. A human model of vasculogenesis is required to identify the mechanisms controlling differentiation of human stem cells to endothelial cells and to understand the mechanisms that control vessel formation.

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In murine embryonic stem cells (ESCs), the onset of expression of vascular endothelial growth factor receptor 2 (VEGFR-2) identifies endothelial precursors that form functional vessels in vivo, and VEGFR-2 expression coincides with the mesoderm defining transcription factor Brachyury (Bry). However, undifferentiated human ESCs express VEGFR-2, and its expression persists after differentiation, indicating that additional insights are required to identify human endothelial precursors. Several authors have demonstrated isolation of functional endothelial cells from human ESCs via differentiation on stroma or as embryoid bodies in serum-containing medium using endothelial cell markers including CD31 and CD34. The origin and pathway for derivation of endothelial cells from human ESCs remain unknown.

The finding that VEGFR-2 identifies vascular precursors in murine ESCs suggests an important role for VEGF in vasculogenesis. VEGFR-2–null mice die in utero after 8.5 to 9.5 days with an absence of organized yolk sac vessels. Neuropilin-1 and -2 (NRP-1 and -2) are multifunctional proteins that bind Semaphorins as well as function as coreceptors for VEGF. In adults, both NRP-1 and NRP-2 are expressed in multiple tissue types including endothelial and vascular smooth muscle cells, as well as lymphocytes; however, the role of NRP-1 in vasculogenesis is unknown. Knockout of both NRP-1 and -2 has a phenotype similar to that of the VEGF-2 knockout, resulting in embryonic lethality at embryonic day 8.5 with an avascular yolk sac. The NRP-1/2 double knockout mouse phenotype suggests that the onset of NRP-1 expression occurs very early in development.
development, during mesoderm formation, and suggests that neuropilins have an essential function in vasculogenesis.

We hypothesized that the VEGF coreceptor NRP-1 would display similar timing of expression in differentiating human and murine ESCs and facilitate identification of endothelial precursor cells. In murine ESCs, we find that NRP-1 is coexpressed with Bry and VEGFR-2, and VEGFR-2 versus NRP-1 cells form endothelium with equivalent efficiency. In human ESCs, the onset of NRP-1 expression identifies endothelial precursors before expression of the endothelial markers CD31 and CD34. Finally, antagonism of both the VEGF binding and the Semaphorin binding functions of NRP-1 impairs the differentiation of vascular precursors to endothelial cells in vitro, indicating a functional requirement for NRP-1 in vasculogenesis. Our findings highlight the utility of the stem cell model to understand the mechanisms controlling vasculogenesis in mice and in humans.

Methods

Murine ESC Lines

Murine ESC lines were maintained on embryonic fibroblast feeders with standard methods. Murine ESC lines were adapted to serum-free, feeder-free conditions as described. Brachyury GFP murine ESCs were provided as a gift from Gordon Keller (Ontario Cancer Institute, Toronto, Ontario). Rosa 26 murine ESCs were a gift from Phillipe Soriano (Fred Hutchinson Cancer Institute, Seattle, Wash).

Human ESC Lines

Human ESCs were cultured under conditions defined previously. See Methods in the online-only Data Supplement for additional details.

Differentiation of Human and Murine ESC Lines as Embryoid Bodies

Both human and murine ESCs were differentiated as embryoid bodies by methods described previously. See Methods in the online-only Data Supplement for additional details.

Differentiation of Sorted Cells to Endothelium

Murine and human ESCs were differentiated as embryoid bodies and stained for flow cytometry. Cells were sorted on the basis of expression of VEGFR-2 or NRP-1 (murine ESCs) or VEGFR-2/NRP-1 cells (human ESCs). In some cases, human ESC-derived cells were sorted as NRP-1/CD34 cells. Murine ESCs were plated to 1% gelatin-coated dishes in Alpha minimum essential medium with 10% fetal calf serum and 10 ng/mL VEGF as described. Human ESC-derived cells were plated to 1% gelatin-coated culture dishes at a density of 1×10^5 cells/cm² in EBM2 medium (Lonza) supplemented with 50 ng/mL human VEGF and cultured for 5 to 10 days.

Matrigel In Vitro Angiogenesis Assays

Undiluted Matrigel (BD Biosciences, San Jose, Calif) was added to each well of a 24-well tissue culture dish and grown in EGM2 with 50 ng/mL VEGF for 24 hours to 7 days. Cultures were examined for cord formation with the use of a phase contrast microscope.

Western Blot Analysis

Cells were harvested in SDS-PAGE sample buffer in the absence of reducing agents, and total protein content was equalized between samples. Samples were separated on 4% to 12% SDS-PAGE gels under nonreducing conditions and transferred to polyvinylidene fluoride membranes. Blots were probed with primary antibodies (see Methods in the online-only Data Supplement), followed by species-appropriate horseradish peroxidase–conjugated secondary antibodies (WestPico kit, Pierce Biotechnology). Protein bands were visualized by exposure to x-ray film.

Polymerase Chain Reaction Analysis

Total RNA was extracted with the use of a commercial kit (RNeasy mini kit, Qiagen). Total mRNA was amplified with the use of Superscript reverse transcriptase ( Gibco) and oligo(dt) primers. Reverse transcription polymerase chain reaction (RT-PCR) was performed with the use of Taq DNA polymerase ( Gibco) with the use of primers indicated in Methods in the online-only Data Supplement. RT-PCR products were analyzed on ethidium bromide–stained agarose gels.

Inhibition of NRP-1 With Function-Blocking Antibodies

NRP-1A, NRP-1B, and mouse IgG isotype control antisera were generously provided by Genentech. Murine ESCs were differentiated as embryoid bodies, and Bry/VEGFR-2 cells were sorted and differentiated to endothelial cells as described above. Sorted cells were incubated in VEGF (10 ng/mL) with no additional treatment, mouse IgG (10 ng/mL), NRP-1A antibody (10 ng/mL), or NRP-1B antibody (10 ng/mL) for 7 days. The quantity of VE-cadherin and CD31 cells as a percentage of total cells was enumerated with the use of immunofluorescence microscopy.

Statistical Analysis

Data are expressed as mean±SEM unless specified otherwise. Statistical comparisons between 2 groups were performed with a t test. Comparisons between >2 groups at 1 time point were performed with a 1-way ANOVA with a Holm-Sidak post hoc test. Comparisons between 2 groups over a time course were compared with a 2-way ANOVA with a Holm-Sidak post hoc test.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

VEGFR-2 and NRP-1 Are Expressed in Bry⁺ Differentiating Murine ESCs Before CD34 Expression

Human ESCs are different from murine ESCs in both their marker expression and growth factor (GF) requirements. Undifferentiated human ESCs express the endothelial and progenitor cell surface proteins VEGFR-2, CD133, and CD146 but not NRP-1 (Figure 1 in the online-only Data Supplement). To test the hypothesis that the onset of NRP-1 expression identified VEGFR-2 embryonic vascular precursors, murine ESCs expressing GFP under control of the Bry locus were differentiated as embryoid bodies under serum-free conditions. To determine whether the differentiation conditions used were adequate to stimulate differentiation to mesoderm, we surveyed differentiating Bry-GFP murine ESCs for the expression transcripts encoding cell surface molecules and transcription factors that are required for vasculogenesis by PCR. This included the transcription factors SCL/Tal-1, CDX4, and LMO2 because of their roles in the formation of endothelial cells. Bry mRNA was expressed at embryoid body day 3 when treated with BMP4 and basic fibroblast growth factor (bFGF) and decreased by day 6 (Figure 1A). In the absence of GF, the onset of Bry expression occurs on day 6 embryoid bodies. VEGFR-2, NRP-1, Tie2, SCL/Tal-1, CDX4, and LMO2 were expressed...
at low levels in untreated embryoid bodies and were robustly increased when differentiated in the presence of BMP4 and bFGF. Time course experiments revealed that Bry-GFP+ cells emerged on day 3 embryoid bodies and peaked on day 4. Bry+VEGFR-2+ and NRP-1+ cells represented a subpopulation of Bry-E-cadherin− cells, consistent with observations of VEGFR-2+ cells derived from murine ESCs in the literature.3

We found that NRP-1 was expressed in Bry+ cells from day 4 embryoid bodies and correlated quantitatively with Bry+VEGFR-2+ cells (Figure 1B). However, other markers of endothelial cells were not present in the Bry+ cell population, including CD34 (Figure 1C) and VE-cadherin (not shown). In separate experiments with Rosa 26 murine ESCs, we observed cells that were double positive for VEGFR-2 and NRP-1, and NRP-1+ cells were E-cadherin− in day 4 embryoid bodies (Figure 1C) but did not express CD34 or VE-cadherin (not shown). Collectively, the findings indicate that NRP-1 is coexpressed in Bry−, VEGFR-2−, and E-cadherin− cells.

Bry+VEGFR-2+ and Bry+NRP-1+ Cells Derived From Murine ESCs Differentiate to Endothelial-Like Cells With Equal Efficiency

We found that VEGFR-2 and NRP-1 were induced by identical growth conditions (Figure II in the online-only Data Supplement) and that VEGFR-2 and NRP-1 were coexpressed in differentiating embryoid bodies. To determine whether NRP-1 expression identifies endothelial precursors derived from murine ESCs, we performed flow cytometry–based sorting of Bry+VEGFR-2+ and Bry+NRP-1+ cells from day 4 murine ESC embryoid bodies. Sorted cells were then grown under conditions that support differentiation to endothelial cells. Both Bry+VEGFR-2+ and Bry+NRP-1+ cells differentiated to endothelial cells. The differentiated cells grew as colonies and expressed CD31, CD34, VE-cadherin, and endothelial nitric oxide synthase (eNOS) and absorbed acetylated low-density lipoprotein (Figure 2). To determine whether Bry+VEGFR-2+ and Bry+NRP-1+ cells formed endothelium with equivalent efficiency, the quantity of CD34+ cells was determined after differentiation. In Bry+VEGFR-2+ cells, 55.5±4.6% of total cells cultured were CD34+ versus 57.7±5.7% of total Bry+NRP-1+ cells (Figure 2). Collectively, these findings indicate that NRP-1+ cells differentiate to endothelial-like cells with efficiency equivalent to that of VEGFR-2+ cells.

NRP-1 Is Coexpressed With VEGFR-2 in Differentiating Human ESCs Grown in Serum-Free Differentiation Conditions

Undifferentiated human ESCs express VEGFR-2 protein but not NRP-1 protein (Figure I in the online-only Data Supplement). We hypothesized that NRP-1 would identify endothelial precursors derived from human ESCs before expression of other endothelial cell markers (CD31 and CD34). Additionally, we hypothesized that endothelial precursors could be obtained from human ESC embryoid body cultures differentiated under serum-free conditions. We performed experiments to determine whether VEGFR-2 expression persisted on differentiation in mesoderm-forming conditions and to determine the time dependence of NRP-1 cell surface protein expression in human ESCs. Embryoid bodies were cultured in suspension in the absence (−GF) or presence of BMP4 (10 ng/mL), bFGF (10 ng/mL), and VEGF165 (10 ng/mL) (+GF). The quantity of VEGFR-2+ and NRP-1+ cells was determined on days 3, 4, 5, and 7 after differentiation by flow
cytometry analysis. Representative flow cytometry plots of human ESC embryoid bodies at day 5 +/− GF are shown (Figure 3A). The number of VEGFR-2+ cells decreases to 32.8±7.3% of total cells grown in the absence of GF at day 3 (Figure 3B). NRP-1+ cells were not detectable in the absence of GF until day 5 (8±2.0% of total cells) and increased further on day 7 (20.7±4.1% of total cells). In the presence of GF, VEGFR-2 expression persisted in 74% of total cells throughout the time course. NRP-1 expression appeared to be biphasic, with an initial peak of expression at day 3. However, the quantity of NRP-1+ cells obtained from day 3 embryoid bodies was not significantly different from untreated cells (11.2±4.2% +GF versus 0.63±0.17% −GF; P=0.21). A second peak of NRP-1 expression is found in day 5 embryoid bodies in the presence of GF (34.2±6.2% of total cells), and NRP-1 expression persists to day 7.

To determine whether the GF conditions used in the embryoid bodies resulted in induction of mesoderm and endothelial cell markers, we performed RT-PCR analysis (Figure 3C). Undifferentiated human ESCs express low levels of Bry transcripts, and Bry expression peaks in day 7 embryoid bodies grown in the absence of GF. Bry transcript expression was augmented in the presence of GF, resulting in earlier expression in day 3 and 5 embryoid bodies. Transcripts, but not protein (Figure IB and ID in the online-only Data Supplement), encoding NRP-1 are found in undifferentiated human ESCs and showed rapid induction in the presence of GF. We detected expression of CD31, LMO2, and FLI1 transcripts at low levels in undifferentiated human ESCs. On differentiation, human ESCs treated with GF expressed CD31, CD34, and VE-cadherin earlier than in the absence of GF.

To verify that Bry, VEGFR-2, and NRP-1 protein expression was induced in embryoid bodies grown in the absence or presence of GF, Western blot analysis was performed on day 5 embryoid bodies (Figure 3D). Bry protein was expressed at low levels in undifferentiated human ESCs, and Bry protein levels increased on differentiation, consistent with our PCR results. VEGFR-2 protein expression was observed at low levels in undifferentiated human ESCs and embryoid bodies without GF but was augmented in day 5 embryoid bodies grown in the presence of GF. NRP-1 protein expression was detected only in human ESC-derived embryoid bodies in the presence of GF. Importantly, the protein bands detected with Bry, VEGFR-2, and NRP-1 antibodies migrated at the appropriate molecular weight for each protein (Bry, 49 kDa; VEGFR-2, 200 kDa; NRP-1, 130 kDa).

Collectively, these findings indicate that VEGFR-2 expression persists in the majority of human ESC-derived embryoid bodies grown in serum-free mesoderm-inducing conditions. Additionally, the VEGF coreceptor NRP-1 protein displays a distinct time course of expression, peaking in day 5 embryoid bodies grown under mesoderm-inducing conditions. In the presence of GF, expression of Bry transcript and protein, transcripts encoding the endothelial markers CD31, CD34, and VE-cadherin, and the transcription factors LMO2 and FLI1 are induced earlier than in cells grown in the absence of GF.

### Onset of NRP-1 Protein Expression in Differentiating Human ESCs Occurs Before CD31 and CD34 Expression in Multiple Human ESC Lines

Our time course analyses indicated that VEGFR-2 expression was persistent in human ESC-derived embryoid bodies grown in the presence of GF. Our PCR analysis indicated that induction of the endothelial transcripts CD31, CD34, and VE-cadherin occurred before or during the peak of NRP-1 cell surface protein expression. We hypothesize that NRP-1 identifies endothelial precursors in human ESCs. Therefore, we wished to determine whether NRP-1+ cells expressed the endothelial cell proteins CD31 and CD34 in day 5 embryoid bodies. Additionally, we wanted to test whether the temporal pattern of NRP-1 expression could be observed in multiple different human ESC lines.

We differentiated human ESCs under serum-free conditions in the absence or presence of GF. We performed flow cytometry analysis on live cells derived from day 5 human ESC embryoid bodies to determine the quantity of VEGFR-2, NRP-1, CD31, and CD34+ cells in 7 different human ESC lines.
lines, and the data were pooled (Figure 4). VEGFR-2<sup>−</sup> cells are present in 57.6±10.5% of human ESC-derived embryoid bodies grown in the presence of GF, whereas 19.8±7.3% of cells express VEGFR-2 in the absence of GF. NRP-1 was expressed in 27.2±7.6% of human ESCs with GF versus 6.1±1.9% without GF. Both CD31 (7.5±1.6% with GF versus 2.8±0.8% without GF) and CD34<sup>−</sup> (5.9±1% with GF versus 3±0.9% without GF) cells were increased by differentiation with GF; however, the overall quantity of these markers was significantly less than the number of NRP-1<sup>+</sup> cells obtained. These findings indicate that NRP-1 is expressed before the onset of CD31 and CD34 expression in human ESCs and is consistent with the onset of NRP-1 expression as an identifying feature of endothelial precursors from human ESCs. Additionally, the findings indicate that NRP-1 expression is reproducible in 7 different human ESC lines, indicating that the findings apply to human ESCs from multiple providers.

**VEGFR-2<sup>−</sup>NRP-1<sup>+</sup> Cells Derived From Human ESCs Are Endothelial Precursors In Vitro**

We hypothesized that the onset of expression of NRP-1 identifies a population of endothelial precursors derived from human ESCs. To test this hypothesis, we differentiated human ESCs as embryoid bodies under serum-free conditions in the presence of GF. We sorted VEGFR-2<sup>−</sup>NRP-1<sup>+</sup> and

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**Figure 3.** Time dependence of VEGFR-2 and NRP-1 expression in human ESCs differentiated in serum-free conditions. A, Representative flow cytometry analysis of day 5 human ESC embryoid bodies differentiated in the absence or presence of GF (BMP4 [10 ng/mL], VEGF [10 ng/mL], and bFGF [10 ng/mL]). Quadrants are set to mouse (Ms) IgG controls. B, Time course analysis of VEGFR-2 and NRP-1 cell surface protein expression measured by flow cytometry analysis. Human ESCs (National Institutes of Health code UC06) were differentiated as embryoid bodies in the presence or absence of GF as described in Methods. Quantity of VEGFR-2<sup>−</sup> and NRP-1<sup>+</sup> cells was determined by flow cytometry and presented as percent positive cells. Significant differences between untreated and GF-treated cells were assessed by a 2-way ANOVA with Holm-Sidak post hoc test and are indicated with an asterisk for each time point. Mean±SEM values of 4 independent experiments are shown. C, PCR time course analysis of mesoderm and vascular markers in differentiating human ESCs in the absence or presence of GF. Human umbilical vein endothelial cells (HUVEC) were used as a positive control for vascular markers. Results are representative of >3 experiments. D, Western blot analysis of VEGFR-2 and NRP-1 expression in day 5 embryoid bodies (EB) derived from human ESCs (hESC). Results are representative of 4 experiments. Undiff indicates undifferentiated; MW, molecular weight.
VEGFR-2^NRP-1^ cells from day 5 embryoid bodies by flow cytometry methods. PCR analysis of the sorted cell populations revealed that VEGFR-2^NRP-1^ cells were enriched for transcripts encoding VE-cadherin, CD31, CD34, LMO2, and FLI1 versus VEGFR-2^NRP-1^ cells (Figure 5A). VEGFR-2^NRP-1^ and VEGFR-2^NRP-1^ cells were differentiated in endothelial growth conditions. On differentiation, there was a significant difference in the morphology of VEGFR-2^NRP-1^ versus VEGFR-2^NRP-1^ cells (Figure 5B). Both VEGFR-2^NRP-1^ and VEGFR-2^NRP-1^ cells differentiated to CD31, CD34, VE-cadherin, and endoglin expressing cells that also absorbed acetylated low-density lipoprotein (Figure 5B). The quantity of CD31^ or CD34^ cells derived from VEGFR-2^NRP-1^ versus VEGFR-2^NRP-1^ cells was determined by quantitative microscopy. VEGFR-2^NRP-1^ cells versus VEGFR-2^NRP-1^ cells differentiate to CD31^ cells (59.2±8% versus 5.2±1.7% of total cells) and CD34^ cells (48.7±6.5% versus 7±1.2% total cells) more efficiently than VEGFR-2^NRP-1^ cells (Figure 5C). As a model of in vitro angiogenesis, VEGFR-2^NRP-1^ and VEGFR-2^NRP-1^ cells were plated to Matrigel and cultured for up to 14 days to determine whether either cell type formed vessel-like structures. VEGFR-2^NRP-1^ cells formed elongated cord-like structures in Matrigel, whereas VEGFR-2^NRP-1^ cells remained tightly clustered and did not appear to elongate or form networks with each other (Figure 5D). To exclude the possibility that CD31^ or CD34^ cells were included in our VEGFR-2^NRP-1^ population, we performed flow cytometry–based sorting of NRP-1^CD34^ cells versus NRP-1^CD34^ cells differentiated with GF. We found that NRP-1^CD34^ cells differentiate to endothelial-like cells, whereas NRP-1^CD34^ cells do not (not shown). Collectively, the findings indicate that the onset of NRP-1 expression identifies endothelial precursors derived from human ESCs.

VEGF and Semaphorin Binding Activities of NRP-1 Are Required for Differentiation of Stem Cells to Endothelial Cells

We hypothesized that the function of NRP-1 in the differentiation of stem cells to endothelial cells was to facilitate activation of VEGF signaling, promoting endothelial cell differentiation and growth in our model of vasculogenesis.
However, NRP-1 has multiple functions, including roles in binding of Semaphorins in neuronal guidance as well as binding additional GFs, including hepatocyte GF. Therefore, an alternative hypothesis would be that NRP-1 has additional activities that are unrelated to VEGF signaling. To determine whether the non-VEGF function of NRP-1 is involved in the differentiation of stem cells to endothelial cells, we used 2 function-blocking antibodies previously shown to bind and inhibit the binding of NRP-1 ligands VEGF (NRP-1B Ab) and Semaphorins (NRP-1A). In this experiment, we differentiated murine ESCs and sorted VEGFR-2+/H11001 vascular precursors 3.5 days after differentiation as embryoid bodies. VEGFR-2+/H11001 vascular precursors were then isolated from other cells with the use of flow cytometry–based cell sorting and differentiated to endothelial cells for 7 days in the presence of VEGF-A and either mouse IgG (10 ng/mL), NRP-1A Ab (10 ng/mL), or NRP-1B (10 ng/mL). After 7 days, cultures were assessed for the number of VE-cadherin and CD31+ cells as a percentage of total cells in culture with the use of immunofluorescence microscopy. We found that in the presence of VEGF-A alone or in combination with mouse IgG, 39±2% express VE-cadherin, consistent with differentiation to endothelial cells. The addition of either NRP-1A or NRP-1B function-blocking antibodies inhibited the differentiation of VE-cadherin+ endothelial cells by 57±8% and 49±7%, respectively, and inhibited the differentiation of CD31+ endothelial cells by 62±17% and 54±3%, respectively (Figure 6). The findings are consistent with a role for both the VEGF and Semaphorin binding functions of NRP-1 as an essential component of the differentiation of stem cells to endothelial cells.

**Discussion**

Our work establishes 2 major findings: (1) NRP-1 expression occurs early in the differentiation of both murine and human stem cells to endothelial cells; and (2) both the Semaphorin...
and VEGF binding functions of NRP-1 are required for the differentiation of stem cells to ECs in the vasculogenesis model system. The findings illustrate the utility of the stem cell model for testing effects on vasculogenesis and indicate a surprising role for both the Semaphorin and VEGF binding activities of NRP-1 in vasculogenesis.

Our results show that NRP-1 is expressed early in the differentiation of stem cells, in cells that have begun the process of forming vascular, hematopoietic, and cardiac precursors (mesodermal cell types). Prior findings in the literature suggested that NRP-1 expression occurs early in development, as the NRP-1 and -2 double knockout mouse is lethal after 8.5 days of development because of failure of mesoderm formation, which is a comparable time point to our ESC expression occurs, which is a comparable time point to our ESC model. Additionally, our experiments show that NRP-1 expression occurs early in both murine and human stem cells, illustrating that this event is conserved across species.

A secondary finding of our results was that the GFs BMP4 and bFGF are sufficient for expression of NRP-1, whereas the BMP antagonist Noggin inhibits NRP-1 expression. These findings are not surprising in that the same conditions are sufficient to drive expression of VEGFR-2 from murine and human stem cells. However, the stem cell model of vasculogenesis may facilitate further investigation of the mechanisms controlling NRP-1 expression. This is of clinical relevance because NRP-1 expression has been documented in several types of cancer, it is associated with worse outcomes in cases of cancer, and the regulation of NRP-1 expression in cancer may represent a therapeutic target for cancer therapy.

NRP-1 function-blocking antibodies significantly decreased the number of endothelial cells derived from differentiating stem cells (Figure 6), and antagonism of both the Semaphorin and VEGF binding activities resulted in decreased endothelial cell formation, a novel finding. Prior work in several developmental and tumor angiogenesis models indicated that antagonism of the VEGF binding function of NRP-1 primarily blocked vessel formation in these contexts. Our results are novel in that they indicate that both the VEGF and Semaphorin binding domains of NRP-1 appear to be required for differentiation of endothelial cells from stem cells. Our findings are somewhat limited by the fact that the NRP-1 function-blocking antibodies did not completely inhibit differentiation of endothelial cells. The reason for this is not known; however, it is possible that NRP-2 expression is also present in a portion of the cells and that NRP-2 may have an overlapping function with NRP-1 in the differentiation of stem cells to endothelial cells. The NRP-1 function-blocking antibodies used in these experiments have no effect on NRP-2 function.

Prior results have indicated that antagonism of the VEGF binding activity of NRP-1 only modestly impaired VEGFR-2 activation. Work by Pan and coworkers indicates that the Semaphorin and VEGF domain binding antibodies disrupt formation of the VEGF/NRP-1/VEGFR-2 signaling complex. Therefore, both VEGF and Semaphorin binding function-blocking NRP-1 antibodies are likely acting to inhibit signaling events that are coupled to NRP-1 and VEGFR-2 complex formation or events downstream of this complex, but not simply by impairing VEGFR-2 activity alone. These findings suggest an important function of the VEGF-2/NRP-1 molecular complex in the initial differentiation of stem cells to endothelial cells and suggest a novel mechanism of this pathway, as known components of VEGF signaling downstream of VEGFR2 (ERK, p38, and AKT) are unaffected by either of the blocking antibodies in proliferating endothelial cell cultures. One likely candidate for the downstream effects of the VEGF/VEGFR-2/NRP-1 signaling complex is the PDZ domain–containing protein that interacts directly with NRP-1. However, the interaction of GIPC with the VEGF/VEGFR-2/NRP-1 complex has not been extensively explored to identify a precise mechanism, and the role of this signaling complex in vasculogenesis remains open to further investigation. Greater understanding of the VEGF/VEGFR-2/NRP-1 signaling complex and events outside of canonical VEGF signaling represents the next advance in our understanding of VEGF signaling and further development of therapeutics in this field. Additionally, extension of our finding that NRP-1 expression and function are critical events in de novo endothelial cell formation to adult stem cell models, including tissue-specific cancer stem cells and cardiac stem cells, may
represent a therapeutic target in ischemic and malignant vasculogenesis.

In conclusion, our results indicate that NRP-1 is expressed early in the differentiation of stem cells to endothelial cells and that NRP-1 function is required for the differentiation of endothelial cells from stem cells. Surprisingly, both the Semaphorin and VEGF binding functions of NRP-1 are required for endothelial cell formation, suggesting that events downstream of VEGF/VEGFR-2/NRP-1 complex formation, and not canonical VEGF signaling alone, are required for this process.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Our understanding of the manner in which endothelial cells develop from embryonic stem cells is still incomplete. An important question is whether endothelial precursor cells have common identifying features that serve as “biomarkers” for tracking their development into mature endothelial cells. Here we report that expressions of 3 markers—(1) neuropilin-1 (a vascular endothelial growth factor coreceptor); (2) Brachyury; and (3) vascular endothelial growth factor receptor 2—identify human and mouse embryonic stem cells that developmentally become endothelial cells. Neuropilin-1 expression occurs very early and defines the origin of a single population of human and mouse stem cells that progress toward endothelial cells. Use of these biomarkers may facilitate marking and tracking of embryonic stem cells that may eventually be used for therapeutic purposes.
Neuropilin-1 Identifies Endothelial Precursors in Human and Murine Embryonic Stem Cells Before CD34 Expression
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**Human Embryonic Stem Cell Lines:** Human embryonic stem cells (NIH Codes: BG02, TE03, TE06, UC01, UC06, WA07, WA13b, WA14) were obtained by the NIH Stem Cell Facility under material transfer agreements from the respective providers. All human ESC experiments were performed in the NIH Stem Cell Facility. Human ESCs were grown on irradiated MEF feeders (1.125 x 10^6 cells per 6 well dish) in DMEM/F12 medium supplemented with 20% Knockout Serum Replacer (Gibco) and 4 ng/mL human basic fibroblast growth factor. Human ESCs were passaged by incubation with collagenase IV for 20-30 minutes, and replated at a 1:3 subculture ratio. Human ESCs were karyotyped and genotyped every 40 passages (Cell Line Genetics, Madison WI). All human ESCs maintained normal cell surface markers and karyotypes over greater than 50 passages (NIH Stem Cell Facility Website, http://stemcells.nih.gov/research/nihresearch/scunit/).

**Differentiation of Human ESCs as Embryoid Bodies:** Human ESCs were passaged from their MEF feeders by incubation with collagenase IV (Gibco), and washed free of the MEFs by pelleting cell clumps at 1 x g. Human ESC colonies were then gently triturated to small aggregates (~50-100 cells) and plated at a density of approximately 3 x 10^5 cells/60mm dish on suspension culture dishes (Corning) in in Neurobasal/DMEM-F12 medium supplemented with 1x N2 and B27 supplements (Gibco), and 0.1mM beta-mercaptoethanol. Growth factors were added at the time of plating: BMP4, basic FGF, Activin A, and Noggin (all from R&D Systems, Minneapolis MN).

**Flow cytometry analysis and cell sorting:** Cells were incubated with primary antibody at for 1 h at 4°C. Cells were dissociated to a single cell suspension by incubation with 0.05% Trypsin-EDTA and resuspended in DMEM/F12 with HEPES (Gibco) supplemented with 10% FBS (FACS Staining Medium) and pelleted by centrifugation. 50-100,000 cells were plated to each well of a 96 well round bottom culture dish for staining. FACS analysis was performed on a BD FACSCalibur equipped with 488-nm and 635-nm lasers, and high throughput sampling unit. Live cells were distinguished from dead cells by FSC vs. SSC plots, and were gated based on FSC vs. SSC parameters. The staining method described results in >75-80% viable cells by these parameters. 50-100,000 live gated cells were used for analysis. Cytometry data were analyzed using FCS Express (DeNovo Software). Cells were sorted on BD FACSria and Cytomation MoFlo cytometers directly into culture medium in the NHLBI Flow Cytometry facility.

**Antibodies used for flow cytometry analysis on human cells:** Mouse IgG1-conjugates (R&D Systems), VEGFR-2-APC (Clone 89106, R&D Systems), NRP-1-APC (Clone AD5-17F6, Miltenyi Biotec), CD31/PECAM-FITC (Clone WM59,
BD Biosciences), CD34-FITC/BD Biosciences, CD133-APC-Miltenyi Biotec, CD146-PE (Clone P1H12, Santa Cruz Biotechnology), E-Cadherin (Clone 67A4, Santa Cruz Biotechnology). Conjugated antibodies were incubated at 1:10 dilution.

**Antibodies for Flow Cytometry Analysis on Murine Cells:** VEGFR-2 (Clone Avas12a1), PECAM (Clone 390), CD34 (Clone RAM34), Endoglin (Clone MJ7/18), PDGFRα (Clone APA5), PDGFRβ (APB5), and IgG isotype control rat monoclonal antibodies were from eBioscience, San Diego CA. NRP-1 (Goat polyclonal IgG) and Goat IgG were R&D Systems. E-Cadherin Rat Monoclonal antibody (Clone ECCD2) was from Zymed. Unconjugated anti-mouse antibodies were incubated at 1:50-1:100 dilution.

**Immunofluorescence Microscopy:** Cells were blocked in 5% normal donkey serum (Chemicon) for 1 hour. Primary antibody was added (1:100-200 dilution) in PBS, and incubated with primary antibodies 3 hours to overnight at room temperature. Alexa Fluor conjugated 488 or 555 donkey anti-mouse or rabbit secondary antibody (Molecular Probes) was added at a dilution of 1:500 for 1 hour at room temperature. Nuclei were stained with bis-benzamide.

**Antibodies for Microscopy:** Goat, Mouse, and Rabbit IgG (R&D Systems), VEGFR-2 (mouse monoclonal, Santa Cruz Biotechnology Catalog # SC-6251), Endoglin (mouse monoclonal, clone SN6, Dako and eBioscience), CD146 (mouse monoclonal, clone P1H12, Santa Cruz Biotechnology and Chemicon), PECAM (mouse monoclonal, clones P2B1 and WM59, Santa Cruz Biotechnology), CD34 (mouse monoclonal clone QBend/10, Dako Biotechnology).

**Immunofluorescence Microscopy:** Cultured cells were grown on 24 well tissue culture plates to 80% confluency, fixed with 4% paraformaldehyde (Electron Microscopy Services Inc.). Additional details in supplementary methods and materials. Images were collected using a Zeiss Axiovert 200 inverted microscope. Images were captured with a Qimaging Retiga EXi camera, and color channels were merged in Adobe Photoshop. All immunofluorescent images shown were taken using a Zeiss LD Achromplan 4x, 10x, and 20x objectives.

**Antibodies used for western blotting:** Brachyury (goat polyclonal antibody, Santa Cruz Biotechnology Catalog #17743), VEGFR-2 (mouse monoclonal, Santa Cruz Biotechnology Catalog # SC-6251), NRP-1 (goat polyclonal antibody (Santa Cruz Biotechnology Catalog # SC-7239), β-tubulin (Santa Cruz Biotechnology) or mouse IgG (R&D Systems).

**Acetylated LDL Uptake Assay:** Cells incubated with 10μg/mL Alexa fluor 488 or 594 conjugated acetylated LDL (Molecular Probes) for 4 hours, washed in PBS, and imaged live. For microscopy, stained cell cultures were imaged using a Zeiss
Axiovert 200 inverted microscope. Images were captured with a Qimaging Retiga EXi camera, and color channels were merged in Adobe Photoshop.

**PCR Primers:**

**Murine Primers:**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size BP</th>
<th>Annealing T(°C)</th>
</tr>
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<tr>
<td>Bry</td>
<td>F-AGGATGTTCCCGGTCTGAAGG R-ATTTGGGCGAGTCTGGTGGATG</td>
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<td>57</td>
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<td>NRP-1</td>
<td>F-CTTTCCGCAGCGAACAATG R-GCAGTCCTGTCTCGAATTC</td>
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<td>SCL/Tal1</td>
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<tr>
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<tr>
<td>β-actin</td>
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**Human Primers:**

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<th>Sequence</th>
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<th>Annealing T(°C)</th>
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**Murine and Human Endothelial Cell Lines:** Murine endothelial cell lines bEndo3 and EOMA were obtained from ATCC and grown as specified. Human endothelial cells (umbilical vein and aortic) were obtained from Lonza and grown per the manufacturers instructions.
Supplemental Results:

Undifferentiated Human Embryonic Stem Cells Express Endothelial Cell Markers VEGFR-2, CD133 and CD146

To determine if the proteins encoded by the identified transcripts were expressed in undifferentiated human ESCs, we assessed their expression by immunohistochemistry (Supplemental Figure 1A) and flow cytometry analysis of live undifferentiated human ESCs (Supplemental Figure 1B). VEGFR-2 is expressed in undifferentiated human ESCs that co-express the pluripotency maintaining transcription factors Nanog and Sox2 (Supplemental Figure 1A). The endothelial cell protein CD146 was also noted in undifferentiated human ESC colonies (Supplemental Figure 1A). To determine if VEGFR-2, CD133 and CD146 were expressed on the cell surface of undifferentiated human ESCs, we performed flow cytometry analysis on live cells. VEGFR-2, CD133 and CD146 are co-expressed with markers found in undifferentiated human ESCs including E-Cadherin (Supplemental Figure 1B), SSEA-3 and SSEA-4 (not shown). To test whether VEGFR-2, CD133 and CD146 cell surface proteins are expressed in undifferentiated human ESCs, four independent assays of undifferentiated human ESCs were performed by flow cytometry. Under the growth conditions utilized, our undifferentiated human ESCs express E-Cadherin (97.1±0.52%), SSEA-3 (90.9±3.8%), SSEA-4 (92±4.3%), VEGFR-2 (69.6±4.6%), CD133 (74.1±10.3%) and CD146 (96.3±0.9%) (Supplemental Figure 1C). Additionally, the VEGF co-receptor NRP-1 was found in a minority of live human ESCs by flow cytometry 9.8±2.2% (Supplemental Figure 1B and C). We verified that VEGFR-2, but not NRP-1 protein, is expressed in undifferentiated human ESCs by western blot analysis (Supplemental Figure 1D). These data indicate that undifferentiated human ESCs are different from murine ESCs in that they express the endothelial and progenitor cell surface proteins VEGFR-2, CD133 and CD146, but not NRP-1. The findings determine the starting point for undifferentiated human ESCs as they differentiate to endothelial precursors.

BMP4 and bFGF Are Required For Serum Free Differentiation of Bry+VEGFR-2+ and Bry+NRP-1+ Cells In Vitro

We determined that BMP4 and bFGF stimulated the expression of both VEGFR-2 and NRP-1. Prior studies have indicated that BMP4 is required for differentiation of mESCs to hemangioblasts, and for expression of SCL/Tal-1. We wanted to determine if the growth conditions required to induce NRP-1 were similar to those required for VEGFR-2. Bry-GFP murine ESCs were cultured as embryoid bodies in serum free conditions with BMP4 (5 ng/mL) and bFGF (10ng/mL), or bFGF (10ng/mL) alone. As a positive control, medium containing 15% FCS was used as a positive control. In four separate trials, we find that
BMP4 and bFGF induces expression of Bry+ (68±3.4% total cells), Bry+VEGFR-2+ (26.8±3.8%) and Bry+NRP-1+ (21.9±5.8%) from mESCs (Supplemental Figure 2). The concentration of BMP4 (1ng/mL up to 50 ng/mL) did not significantly change the quantity of Bry+VEGFR-2+ or Bry+NRP-1+ cells (not shown). Activin A, an inducer of mesendoderm, alone or with bFGF induced expression of Bry but not VEGFR-2 or NRP-1 (not shown). bFGF alone did augment VEGFR-2 (8.2±3.3%+FGF vs. 2.8±0.99% no treatment), and NRP-1 (0.9±0.1% +FGF vs. 2.1±0.6% no treatment) expression, but the difference was not significant versus untreated cells (Supplemental Figure 2). Noggin is an antagonist of BMP4. Competition of BMP4 by co-incubation with Noggin (100ng/mL) antagonized VEGFR-2 and NRP-1 expression (Supplemental Figure 2). The data indicate that the combination of BMP4 and bFGF is most efficient under serum free conditions. Additionally, the results indicate that BMP4 is required for expression of VEGFR-2 and NRP-1 as antagonism of BMP4 with Noggin blocks their expression. The results define the conditions required for serum free differentiation of murine ESCs, indicate the same conditions induce VEGFR-2 and NRP-1, and show that VEGFR-2 and NRP-1 expression can be antagonized by Noggin.

Supplemental Figure Legends:

Supplemental Figure 1: Undifferentiated Human ESCs Express VEGFR-2, CD133 and CD146

Panel A- Immunofluorescent images of human ESC colonies on MEF feeders. Nuclei were counterstained with bis-benzamide in all photos. Results are representative of >3 experiments. Mouse IgG, VEGFR 2/Nanog, VEGFR 2/Sox 2 images shown at 10x magnification. CD 146 image shown at 15x magnification. Scale bar=20 microns.

Panel B: Flow cytometry analysis of undifferentiated human ESCs. Isotype controls are shown in the left-most panel. Results are representative of >4 experiments in multiple human ESC lines.

Panel C: Single marker flow cytometry analysis of undifferentiated human ESC lines. E-Cadherin (*) p<0.002, n=3, SSEA 4 (**) p<0.0004, n=5, VEGFR-2 (*** p<0.008, n=4, CD133 (†) p<0.02 n=3, CD146 (‡) p<0.00002 n=4, NRP-1 p=0.15, n=4 versus Mouse IgG control. Data are pooled from replicate observations in multiple human ESC lines. Data presented are means ± SEM.

Panel D: Western blot analysis of VEGFR-2 and NRP-1 in undifferentiated human ESCs. Human umbilical vein endothelial cells (HUVECs) shown as a positive control. β-tubulin shown as a loading control. Molecular weight (MW) in kD.
Supplemental Figure 2: Growth Factor Dependence of Bry, VEGFR-2 and NRP-1 in Murine Embryonic Stem Cells. Bry GFP murine ESCs were differentiated for four days in the presence of the indicated growth factors. Quantities of Bry+VEGFR-2+, Bry+NRP-1+, and total Bry+ cells were determined by flow cytometry. p-values: < 0.05 for Bry+VEGFR-2+ cells (*), Bry+NRP-1+ cells (**), Bry+ alone (***)) versus no treatment control, <0.05 Noggin (100ng/mL) BMP4 and bFGF versus BMP4 and bFGF (†-Bry+VEGFR-2+, ††-Bry+NRP-1+, †††-Bry+ alone). N=5 experiments.
Supplemental Figure 1:

A

B

C

D

Mouse IgG
Goat IgG
VEGFR-2
Nanog
VEGFR-2
Sox2
CD146

Ms IgG APC
VEGFR-2 APC
Ms IgG PE
CD146 PE
Ms IgG 647
E-Cadherin
E-Cadherin
NRP-1 APC

% Positive Cells

Mouse IgG
E-Cadherin
SSEA3
SSEA4
VEGFR-2
CD133
CD146
NRP-1

175-
83-
90-
Blot-VEGFR-2
NRP-1
Tubulin
Supplemental Figure 2: