Expression of the Growth Factor Progranulin in Endothelial Cells Influences Growth and Development of Blood Vessels: A Novel Mouse Model

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Abstract

Progranulin is a secreted glycoprotein that regulates cell proliferation, migration and survival. It has roles in development, tumorigenesis, wound healing, neurodegeneration and inflammation. Endothelia in tumors, wounds and placenta express elevated levels of progranulin. In culture, progranulin activates endothelial proliferation and migration. This suggested that progranulin might regulate angiogenesis. It was, however, unclear how elevated endothelial progranulin levels influence vascular growth in vivo. To address this issue, we generated mice with progranulin expression targeted specifically to developing endothelial cells using a Tie2-promoter/enhancer construct. Three Tie2-Grn mouse lines were generated with varying Tie2-Grn copy number, and were called GrnLo, GrnMid, and GrnHi. All three lines showed increased mortality that correlates with Tie2-Grn copy number, with greatest mortality and lowest germine transmission in the GrnHi line. Death of the transgenic animals occurred around birth, and continued for three days after birth. Those that survived beyond day 3 survived into adulthood. Transgenic neonates that died showed vascular abnormalities of varying severity. Some exhibited bleeding into body cavities such as the pericardial space. Smaller localized hemorrhages were seen in many organs. Blood vessels were often dilated and thin-walled. To establish the development of these abnormalities, we examined mice at early (E10.5–14.5) and later (E15.5–17.5) developmental phases. Early events during vasculogenesis appear unaffected by Tie2-Grn as apparently normal primary vasculature had been established at E10.5. The earliest onset of vascular abnormality was at E15.5, with focal cerebral hemorrhage and enlarged vessels in various organs. Aberrant Tie2-Grn positive vessels showed thinning of the basement membrane and reduced investiture with mural cells. We conclude that progranulin promotes exaggerated vessel growth in vivo, with subsequent effects in the formation of the mural cell layer and weakening of vessel integrity. These results demonstrate that overexpression of progranulin in endothelial cells influences normal angiogenesis in vivo.

Introduction

Angiogenesis, the formation of new blood vessels from preexisting vessels, is critical whenever tissues undergo extensive remodeling processes, including during normal growth and development, wound healing [1] [2], in the female reproductive cycle [3], mammary gland development during pregnancy and lactation [4] and the expansion of fat mass during obesity [5] [6]. Angiogenesis contributes to many pathologies, including the formation of tumor blood supply [7], and contributes to diabetic complications such as retinopathy and kidney failure [8] [9]. Angiogenesis is regulated by vascular-specific growth factors, notably those of the vascular endothelial growth factor (VEGF) family and the angiopoietins, and by less cell-restricted growth factors including members of the fibroblast growth factor family, the Notch family proteins and proteins in the Eph–signaling family [10]. We have proposed previously that the secreted glycoprotein progranulin may also have a role in vascular development [11].

Progranulin (which is also called granulin-epithelin precursor, PC cell derived growth factor and acrogranin) is a pleiotropic regulatory protein that promotes cellular proliferation, cell migration and survival (reviewed in [12]). It is often overexpressed in cancers and contributes to tumor progression (reviewed in [13,14]) and stroma formation [15]. Mutation of the gene for progranulin (Grn) results in the neurodegenerative disorder, frontotemporal dementia [16] [17]. Progranulin regulates inflammation [18] [19] [20]. It is an inhibitor of the tumor necrosis factor receptors [21], and is a cofactor in the presentation of CpG oligonucleotides to the Toll-9 receptor [22]. Quiescent endothelial cells typically express little progranulin [23], however progranulin expression is induced in activated endothelial cells after tissue wounding [11], and in the developing placenta [24]. Progranulin staining has been reported in endothelial cells in ovarian tumors [25] [26], breast cancer [27], esophageal carcinomas, [28], and in gliomas [29]. Indeed, cumulative survival in patients with glioblastoma was significantly decreased for those with overexpression of progranulin in the tumor vasculature [29]. Progranulin...
stimulates the expression of the angiogenic growth factor VEGF in breast cancer cell lines [30], and breast cancers with elevated progranulin levels also have higher VEGF levels and microvascular density [27]. Monoclonal antibodies that deplete progranulin levels reduced cancer growth of hepatocellular carcinomas in mice, in part by reducing tumor microvascular density [31] by decreasing progranulin mediated VEGF expression. Progranulin increased capillary size and number when it was added to rodent dermal wounds [11]. In tissue culture, progranulin stimulated proliferation and migration of endothelial cells in a mitogen-activated protein kinase (MAPK) and phosphatidyl-inositol-3-kinase (PI3K)-dependent fashion [11]. It also accelerated tubule formation of primary microvascular endothelial cells growing on Matrigel [11]. Tube-formation is an assay of organized self-association in vitro that recapitulates the formation of luminal endothelial structures during angiogenesis in vivo [32].

Despite the production of progranulin by endothelial cells in tissues that are actively proliferating and remodeling, including tumors, and the ability of progranulin to stimulate endothelial proliferation and migration in tissue culture, it remains uncertain whether progranulin influences angiogenic processes in the more complex in vivo environment. To address this issue, we developed mice that overexpress Grn in endothelial cells under the control of the Tie2 promoter. These transgenic mice exhibited expanded vessel size, and a progressive disruption of vascular integrity that leads to high rates of perinatal mortality. This supports the hypothesis that progranulin influences angiogenic processes in vivo.

Results

Characterization of the Tie2-Grn Founder Transgenic Mice

The Tie2-Grn gene (Fig. 1A) was detected in 3 of 47 mice tested. We estimated that Tie2-Grn transgenic line 1 (female founder MIL-535) had 2–3 copies (2.45 ± 0.23), Tie2-Grn transgenic line 2 (male founder MIL-772) had 13–16 copies (14.3 ± 1.72), Tie2-Grn transgenic line 3 (female founder MIL-794) had 6–8 copies (7.12 ± 1.23) respectively (Fig. 1B). Line 1 was subsequently referred to as GrnLo, line 3 as GrnMid, and line 2 as GrnHi to reflect their respective copy numbers. The three Tie2-Grn founders appeared healthy and showed no obvious gross external abnormalities.

Expression of the Tie2-Grn Transgene in Transgenic Mice

The founder lines were backcrossed on a C57BL/6 background. The expression of Tie2-Grn mRNA was established in various mouse tissues by real time qPCR (Fig. 1C). The transgene was expressed in all the organs tested, with the highest levels detected in the lungs. Agarose gel analysis of the amplified PCR products confirmed that the Tie2-Grn was detected only in the transgenic mice (Fig. 1D). Progranulin protein levels were higher in the GrnHi positive animals compared to the GrnLo and GrnMid positive animals (Fig. 1E).

Serial sections stained for progranulin and the endothelial marker protein PECAM (CD31) show overlay of progranulin with endothelial cells in Tie2-Grn positive mice (Fig. 1F). Over-production of progranulin by endothelial cells in the Tie2-Grn positive mice was demonstrated by immunohistological staining (Fig. 1G and H). This confirms that the Tie2-Grn transgene has a widespread tissue distribution, and results in a stable increase in progranulin production in endothelial cells.

Higher Mortality in Transgenic Litters

Mortality was higher in crosses between Tg × WT litters than in WT × WT litters both at birth and at three weeks. All three transgenic lines had lower litter sizes than corresponding WT × WT crosses. Figure 2A shows that the average litter size drops from 8.7 at birth for wildtype crosses to 4.7 for the GrnHi crosses. The average litter sizes, both at birth and at three weeks, showed a statistically significant inverse linear correlation with respect to the copy number of Tie2-Grn in the three transgenic lines (Fig. 2A). A comparison of litters that were genotyped at three weeks of age (Fig. 2B) indicates that the diminishing litter sizes were due specifically to lower numbers of mice carrying the Tie2-Grn gene rather than a non-specific effect on survival regardless of genotype. The germline transmission ratios of Tie2-Grn positive mice at three weeks were calculated as 26.01%, 19.61% and 4.72% for GrnLo, GrnMid and GrnHi respectively (Fig. 2C), and show an inverse linear correlation with Tie2-Grn copy number. Assuming a predicted germline transmission of 50% based on a Tg × WT breeding scheme, this indicates losses of approximately half, three fifths and nine tenths of the Tie2-Grn positive mice in the GrnLo, GrnMid, and GrnHi lines respectively. This clearly shows a detrimental effect of Tie2-Grn on survival before or close to birth that is gene dosage-dependent. The survival of Tie2-Grn negative littermates was similar between GrnLo and GrnMid litters, while in the GrnHi litter, there was increased loss of Tie2-Grn negative mice. This suggested a possible maternal effect on the litter survival in the GrnHi line. The maternal placenta is highly vascular, and defects in its development might influence the survival of litters. To investigate whether maternal effects influence survival of pups, we compared the survival ratios obtained from breeding either Tie2-Grn positive mothers or Tie2-Grn positive fathers with WT mice of the corresponding gender. Tests of correlation between the number of Tie2-Grn positive litter sizes (GrnLo, GrnMid and GrnHi lines) and two variables, gender of the transgenic parent and Tie2-Grn copy number, showed a statistically significant maternal effect for Tie2-Grn only in GrnHi litters. The maternal effect was not sufficient in magnitude to account for frequent loss of Tie2-Grn positive animals (Fig. 2D).

When we performed Tg × Tg crosses to obtain homozygous mice, we noticed several aborted litters from GrnHi × GrnHi crosses and GrnMid × GrnMid crosses. Among those litters that were not aborted, we observed multiple deformed newborns and at E17.5, the uterus showed identifiable resorption sites. Therefore, the homozygous crosses appeared to be present with earlier lethality. Given the difficulty in obtaining reliable live births in Tg × Tg crosses, all experiments were performed using Tg × WT crosses.

Mortality occurs Predominantly in the Perinatal Period

Given that the GrnHi transgenic line shows the greatest premature mortality amongst the Tie2-Grn transgenic lines, we focused on this line to further evaluate the effect of the Tie2-Grn transgene on litter size throughout development. We collected and genotyped embryos pooled at between embryonic days E9.5 to E14.5, later fetuses pooled at between E15.5 and E17.5, neonates (postnatal days 0–3), and young mice at three weeks. As before, an under representation of Tie2-Grn positive mice at birth and at weaning was observed (Fig. 2E). In contrast, in the E9.5–14.5 embryos and E15.5–17.5 fetuses, the ratio of Tie2-Grn positive to negative animals is close to 1:1. Death continues postnatally until day three (Fig. 2F). Tie2-Grn positive pups that survive beyond the third day after birth are likely to be alive at three weeks. Clearly, therefore, the lethality of the Tie2-Grn transgene occurs close to birth.
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**Figure A**

- HindIII and NotI enzymes
- Mouse GRN cDNA
- p[A] promoter
- Tie2 promoter
- Tie2 enhancer

**Figure B**

- Copy Number of Tie2-Grn
- *** significance

**Figure C**

- Log10(RER) of Tie2-Grn
- Brain, Lung, Liver, Kidney, Skin

**Figure D**

- Male and Female Tie2-Grn and beta-Actin expression

**Figure E**

- Mean Progranulin [ug/mL]
- GmLo, GmMid, GmHi

**Figure F**

- Tie2-Grn (+) adult cerebellum
- Anti-PECAM ab, Anti-Pgrn ab

**Figure G**

- Tie2-Grn (-) adult cerebellum

**Figure H**

- Tie2-Grn (+) adult cerebellum
Abnormal Coloration and Subcutaneous Hemorrhage in Tie2-Grn Positive Mice

There are a number of abnormalities in the external appearance of the Tie2-Grn positive fetuses and neonates in the GrnHi and GrnMid lines. Figure 3 shows a representation of these abnormal mice from the GrnHi line. These were first observed from E15.5 when a subset of Tie2-Grn positive mice showed subcutaneous focal hemorrhage or ruptured vasculature (Fig. 3A and B). At E16.5, some Tie2-Grn positive fetuses observed in utero had a pale appearance (Fig. 3C and D). Pale fetuses and pale neonates were collected from both the GrnMid (not shown) and the GrnHi line but were more frequently noted in the GrnHi line (Fig. 3D and E).

Finally, a subset of Tie2-Grn positive embryos appeared indistinguishable from Tie2-Grn negative littersmates with respect to external appearance (Fig. 3D and E).

Some pallid Tie2-Grn positive fetuses were carried to term and delivered alive (Video S1), thus pale coloration preceded death. Pallor was more immediately obvious at birth than in utero. Externally visible subcutaneous hemorrhages were not observed in mice at birth suggesting that these fetuses may have succumbed to hemorrhaging in utero. Visual observation revealed that certain live-born pallid mice had apparent respiratory difficulty and died shortly after birth. The frequencies of externally evident abnormalities for embryos and neonates are summarized in figures 3F and 3G. The pale coloration might be due either to anemia or vascular malformation. We therefore evaluated red blood cell count, and although the red blood cell count was slightly lower in Tie2-Grn positive embryos, this did not correlate with pallor (Fig. S1). The overexpression of endothelial progranulin significantly increased secreted progranulin levels in the amniotic fluid of Tie2-Grn positive fetuses compared to the negative littermates at E15.5 (Fig. 3H).

Although Tie2-Grn mRNA is expressed in adult organs (Fig. 1C and D) some transgenic mice survived to maturity. No gross morphological differences were seen among survivor Tie2-Grn positive mice when compared with WT mice, at birth or at weaning. Overall, Tie2-Grn positive survivor mice had equivalent body weights at 3 weeks as their Tie2-Grn negative counterparts except in GrnHi litters, where the WT littermates were heavier (Fig. S2). The difference is relatively small but statistically significant. Subsequent analysis, showed, however, that the major determinant of weight at 3 weeks is litter size rather than genotype, with WT littermates from GrnHi litters gaining weight because of small litter size (Table S1). We investigated the structure of blood vessels and progranulin expression in the adult survivor mice (Fig. S3). Blood vessels in positive and wildtype littersmates appeared indistinguishable with respect to diameter and coverage with a well-developed vascular smooth muscle cell layer (Fig. S3A-D).

In the Tie2-Grn positive adult survivor mice, endothelial cells stained for progranulin (Fig. S3C) but less robustly than observed in the Tie2-Grn positive fetuses, and fewer progranulin positive vessels (Fig. S3E) were observed in the positive adult mice than in the fetuses and neonates. This may contribute to the lack of vascular abnormalities in the transgenic adults.

Vascular Abnormalities in the Neonatal Tie2-Grn Positive Mice

We investigated the histological basis of perinatal mortality in Tie2-Grn positive mice and observed a number of vascular abnormalities of varying severity. Massive internal bleeding was detected in a number of the Tie2-Grn positive mice (Fig. 4A, B and D) but not in Tie2-Grn negative mice (Fig. 4C). Figures 4A and 4D show Tie2-Grn positive neonates that were collected alive but died shortly after birth. Both succumbed to cardiac tamponade, where blood collected around the heart in the pericardial cavity. An abnormal epicardial vessel with the appearance of rupture that may have contributed to the blood hemorrhage was observed in the ventricle wall from a Tie2-Grn positive neonate with cardiac tamponade (Fig. 4D). Epicardial vessels were enlarged in some mice (Fig. 4E) compared to the Tie2-Grn negative counterparts (Fig. 4C) even when cardiac tamponade did not precede post-natal death. Blood hemorrhage was also observed in the pleural cavities of some GrnHi positive neonates (Fig. 4F). Intriguingly, the interstitial wall of the Tie2-Grn positive neonatal lung appears to be hypercellular (Fig. 4G) compared to the Tie2-Grn negative counterpart (Fig. 4F).

Bleeding into body cavities was not detected in all Tie2-Grn positive neonates; however, widely disseminated local internal hemorrhage affected several organs, including the brain, the kidney, the liver and the uterus (Fig. 4H-K). The affected organs were not always the same in every Tie2-Grn positive neonate. Some of the Tie2-Grn positive neonates did not have any observable abnormalities; however, since it is not possible to sample all regions exhaustively, it is likely that small, localized hemorrhages may have been missed in some apparently normal Tie2-Grn positive neonatal mice. Other than vascular abnormalities, the tissues examined appeared to be normal. Regardless of the organs affected, the hemorrhages and the enlarged vessels demonstrate that the blood vessels in Tie2-Grn positive fetuses and neonates were abnormal and prone to rupture.

Structurally Intact and Functional Vasculature in Early Tie2-Grn Positive Embryos

Given that Tie2-Grn positive mice show elevated mortality around birth, we sought to establish the time of onset of Tie2-Grn abnormalities during development. To trace back the initial time-point of the disruption in blood vessel formation of the Tie2-Grn positive mice, we investigated mice from the GrnHi line in mid gestation from E10.5–14.5 (Fig. 5) and late gestation from E15.5–17.5 (Fig. 6). Tie2-Grn positive E10.5 embryos exhibited normal endothelial sprouting (Fig. 5A). Cardiac development appeared normal. Endocardial cushions were properly formed (Fig. 5B and C). Since endocardial cushion are formed when endothelial cells undergo an endothelial to mesenchymal transition, the transdiffer-
Figure 2. The Tie2-Grn transgene results in elevated mortality during perinatal period. (A) Mean litter size at birth and weaning decreases as a function of higher Tie2-Grn copy number. N = 1220 pups. Correlation between litter size at birth and copy number is significant at the 0.01 level (2 tailed), $r = -0.405$. Correlation between litter size at weaning and copy number is significant at the 0.01 level (2-tailed), $r = -0.371$. (B) Comparison of Progranulin levels in angiogenesis.
entiation of the endothelial cells during development must occur normally in the Tie2-Grn positive embryos. Endothelial lining of the trabeculae of the heart in the Tie2-Grn positive embryos appeared similar to that of the Tie2-Grn negative counterpart (Fig. 5D and E). Figures 5F and 5G confirmed the over-production of progranulin at E10.5. Immunohistochemical analysis of the embryonic aortic vessel revealed strong expression of progranulin in the endothelial lining, strong expression of laminin in the basement membrane underlying the endothelial cells and a thick band of vascular smooth muscle cells enveloping the aorta as revealed by robust smooth muscle α-actin staining (not shown). We conclude therefore that the Tie2-Grn transgene has no overt effect on early vasculogenesis and angiogenesis, nor does it interfere with embryonic endothelial functions in endocardial cushion formation and consequently valve formation.

Enlarged Blood Vessels with Abnormally Sparse Mural Cell Coverage in Late Tie2-Grn Positive Fetuses

Compromised vasculature was first observed in Tie2-Grn positive fetuses at E15.5, both in the GrnMid and GrnHi lines (GrnLo mice were not investigated). As reported in figure 3, visual inspection of Tie2-Grn positive fetuses revealed some that were pale looking and some that harbored subcutaneous hemorrhages (GrnLo mice were not investigated). As reported in figure 3, visual inspection of Tie2-Grn positive fetuses revealed some that were pale looking and some that harbored subcutaneous hemorrhages. Independent samples T-test was performed for individual transgenic lines. N = 1069. Correlation is significant between germline transmission ratio and copy number at the 0.01 level (2-tailed), R = -0.469. (C) Comparison of germline transmission at weaning of the Tie2-Grn gene expressed as a function of copy number number. N = 1069. Correlation is significant between germline transmission ratio and copy number at the 0.01 level (2-tailed), R = -0.446. (D) Litter sizes from matings with Tie2-Grn positive father and mother. Independent samples T-test for variable (%tg) was conducted between groups E9.5–14.5 vs. E15.5–17.5 (p = 0.8); E9.5–14.5 vs. P0–3 (p < 0.001); and E0–14.5 vs. 3 weeks (p < 0.001). (F) Death continued after birth (newborn), and the first, second and third days of life. Thereafter, most mice survived to weaning. Error bars denote ±95% C.I. N = 17, GrnHi line. Asterisks indicate statistical significance in 2A–D; significance of <0.001 is ***, <0.01 is ** and <0.05 is *.

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Discussion

There are many indications that progranulin might contribute to angiogenic processes [11] [24] [25] [26] [27] [28] [29]. It was however uncertain whether elevated progranulin levels in endothelial cells would influence vessel development and structure in vivo.

We addressed the question of the role that progranulin has in endothelial cells in vivo by developing mice with targeted endothelial expression of murine progranulin. The Tie2 gene is expressed throughout vascular development, including in angioblasts, and in sprouting blood vessels [33]. A Tie2-promoter-enhancer construct supports the expression of heterologous genes in endothelial cells during embryonic development and in adults

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Figure 3. Tie2-Grn dependent abnormalities. (A–D) Appearance of embryos from E15.5 to E16.5 showed subcutaneous hemorrhage, often behind the head, (indicated by arrowheads; P is placenta) (A and B) or pale appearance (C and D) (indicated by arrowhead in C). Litters were genotyped either ex utero (D) or post-natally (E). Experimentally visible abnormalities were found only in Tie2-Grn positive mice. The phenotype is variable, as some Tie2-Grn positive mice were indistinguishable from the negative littermates. The genotype is indicated in the figure by (+) or (−). The distribution of externally visible abnormalities in E15.5–17.5 fetuses, N = 29 from the GrnHi line and, (G) from P0–P1 live neonates, N = 59 from the GrnHi line. The neonates found dead were not included. (H) Levels of progranulin protein were higher in the amniotic fluid of the Tie2-Grn positive fetuses than negative fetuses (E16.5). Independent samples T-test showed significant difference between the transgenic and negative population, p = 0.007 (two-tailed). Errors bars denote ± S.E. N = 6 from the GrnHi line only. Asterisks indicate statistical significance in 3H; significance of <0.01 is indicated by **.

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[34] [35]. Three Tie2-Grn mouse lines were developed, with different Tie2-Grn copy numbers. The Tie2-Grn transgene was reproducibly expressed in multiple tissues of transgenic adults with highest levels in the lung, which is among the most vascular of the tissues tested.

The expression of the Tie2-Grn gene was associated with an elevated risk of perinatal death in all three Tie2-Grn mouse lines. Mortality and transmission of the Tie2-Grn gene correlated inversely with Tie2-Grn copy number, with greatest mortality and lowest germ line transmission observed in the GrnHi litters. Thus, Tie2-Grn mortality is reproducible between different transgenic lines and is gene dosage-dependent. The death of the Tie2-Grn positive animals occurred at or around birth, then continued for three days after birth, after which time those Tie2-Grn positive neonates that remained alive would generally survive to maturity and appeared fully viable. Deaths were caused by vascular abnormalities of varying severity that included massive internal bleeding into body cavities, localized hemorrhage within organs, enlarged vessels with thin walls, and apparent vessel rupture in tissues, including the epicardium (Fig. 4D). Taken together, elevated endothelial expression of progranulin has a profound effect on vascular formation and integrity.

The Tie2-Grn positive mice that survived continued to transmit the phenotype of vascular defects to their offspring but displayed no obvious vascular abnormalities. Adult Tie2-Grn expression appears less active than in the fetuses and neonates (Fig. S3). Phenotypic variability and incomplete penetration with elevated transgene expression, as occurred with Tie2-Grn, is observed in mice even with a pure genetic background [36], and the underlying causes have been discussed [37].

Vasculogenesis, that is, the de novo formation of vessels and their subsequent maturation to form a capillary nexus with mural cell recruitment occurs between E6.5 and E9.5 in mice [38]. The earliest embryos examined, E10.5, showed normal vasculature and evidence for normal capillary sprouting and endothelial lining of cardiac trabeculae. Cardiac development appeared normal. Progranulin was overexpressed in endothelial cells of Tie2-Grn positive embryos at this time. During development some endothelial cells undergo a transition to mesenchymal cells, for example in the formation of endocardial cushions, structures which will later develop into heart valves [39] [40]. The endocardial cushions of TIE2-Grn positive pups are fully formed, indicating normal endothelial-mesenchymal transition. Clearly, therefore, the absence of any vascular phenotype at E10.5 in Tie2-Grn positive embryos precludes a major role for progranulin during vasculogenesis. At present, it is unclear why progranulin has little effect on early vascular development, given the dramatic actions of progranulin later in gestation. One possibility is that receptors or cofactors that modulate progranulin action are missing at the earlier stages. Definitive receptors for progranulin have not been identified (review in [14]) although progranulin binds other receptors such as the TNFα receptor [21] and the Toll-like receptor 9 [22]. Progranulin is, however, known to interact with domain V of perlecan, (basement membrane-specific heparan sulfate proteoglycan) [41], a protein whose expression in endothelial cells commences around E10.5 [42]. Domain V of perlecan promotes endothelial cell angiogenesis but inhibits vascular smooth muscle cell growth [43] and its interaction with progranulin has been suggested to regulate tumor angiogenesis [26].

The earliest vascular abnormalities occurred at E15.5. Focal hemorrhages colocalized with reduced basement membrane deposition and limited mural cell recruitment and, as in the neonatal vessels, the fetal vessels were enlarged and disorganized. Some vessels, however, had high levels of progranulin, but were, nevertheless invested with a well-formed basement membrane and mural cell envelope. These tended to be smaller than the vessels that lacked well defined mural cell coverage. Their presence demonstrates that the production of progranulin by endothelial cells is not, by itself sufficient to prevent mural cell recruitment. Progranulin activates endothelial proliferation and migration in vitro [11] suggesting that the vessel dilation seen in Tie2-Grn mice is, at least in part, due to a direct action of progranulin on the endothelia. Importantly, however, mural cells exert a suppressive effect on endothelial angiogenesis, and their absence results in endothelial hyperplasia and microaneurysms [44] [45]. It is likely, therefore, that the lack of mural cell restraint in thin walled Tie2-Grn positive vessels would amplify progranulin-mediated endothelial vessel enlargement, and contribute significantly to the disruption of vessel integrity seen in Tie2-Grn positive mice from E15.5 onwards. The production of progranulin in endothelial cells is tightly regulated in wildtype mice [11] [23] [24], which presumably limits progranulin angiogenic action under physiological conditions only to situations where it is required.

Other than vascular defects, the tissues appear mostly normal with a few exceptions. Some Tie2-Grn positive neonates had hypercellular alveolar walls indicative of underdevelopment of the alveolus process. This may be due to the increased circulating progranulin. Pulmonary angiogenesis is critical in alveolation and its disruption in late development results in a marked inhibition of alveolus [46]. The placenta, in which the maternal vessels have high levels of progranulin even in wild-type mice showed no structural alterations in the Tie2-Grn positive mice, but did exhibit increased numbers and size of syncytial knots (Fig. S4). The syncytial knots are indicators of placental maturity, and their number increases following placenta hypoxia [47] [48] [49]. The non-vascular features of the Tie2-Grn positive phenotype are, therefore, likely to be secondary indications of abnormal blood vessel development or function.

Figure 10 summarizes the timing of Tie2-Grn action on the blood vasculature, comparing it to the landmarks in vascular development and the action of critical regulatory proteins in these processes. Genetic disruption of VEGFR2 [50] or VEGF [51] [52] results in a failure of vasculogenesis. Deletion of angioptoin-1, the ligand for Tie2, does not impede vasculogenesis but results in cardiac abnormalities with failure to form trabeculae by E9.5, and poor stabilization of blood vessels [53]. Loss of PDGF-beta or its receptor, PDGF-receptor β, cause a failure in mural cell
Figure 4. **Vascular abnormalities and altered lung structure in Tie2-Grn positive neonatal mice.** Extravasated blood (*) in the pericardial cavity (A) and in the pleural cavity adjacent to the lung (B) of Tie2-Grn positive neonates. (C) The heart from a Tie2-Grn negative neonate; note the absence of blood in the pericardial space. The insert shows a higher power view of the normal epicardial wall (arrow). (D) Blood pooling in the pericardial cavity of a Tie2-Grn positive neonate; note the ruptured vessel shown at higher power magnification in the insert (arrow). The neonates shown in (A–D) were collected alive, the transgenic neonates in A, B and D died soon after collection. (E) The heart from a post-mortem Tie2-Grn positive neonate; note the absence of bleeding into pericardial space but the presence of enlarged epicardial vessels (arrow) in the ventricle wall, shown at higher power magnification in the insert. Appearance of the lungs in a (F) Tie2-Grn negative neonate and (G) a matched Tie2-Grn positive neonate. Note that the alveolation is incomplete in the Tie2-Grn positive neonate with small alveoli and thickened mesenchymal alveolar wall (G). Localized hemorrhages (arrows) were observed in multiple organs of Tie2-Grn positive mice at birth including (H) brain, (I) renal cortex, (J) liver, and (K) uterus. All sections stained with H&E. Scale bars denote 20 μm. Animals were obtained from the GrnHi line. Abbreviations used: A, atrium; D, diaphragm; L, lung; Lv, liver; PC, pericardial cavity; RC, rib cage; T, thymus; V, ventricle; VC, vertebral column.

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recruitment in mid-gestation [45]. Progranulin acted only late in fetal life, suggesting that it regulates unique aspects of angiogenesis distinct from the well-established angiogenic factors.

Progranulin is expressed in tumor vasculature [25] [26] [27] [28] [29]. Strong vascular progranulin expression correlated with tumor recurrence in astrocytoma and with decreased patient survival in glioblastomas [29]. Cancer cells also overproduce progranulin, and this has been associated with increased VEGF production [27] [28] [30] [31]. In the Tie2-Grn positive mice, however, there were no changes in VEGF levels or changes in VEGFR2 expression. Progranulin stimulates endothelial cell proliferation and migration in culture [11], suggesting that it exerts angiogenic actions independently of VEGF. In a tumor, both mechanisms may occur, with progranulin produced by the tumor vasculature acting as an autocrine angiogenic factor, while progranulin produced by cancer cells may increase their VEGF secretion. The structure of tumor capillaries is often atypical, with large disorganized vessels that are poorly covered by mural cells.

Figure 5. The development of the vasculature and heart appears normal at mid-gestational Tie2-Grn positive embryos (E10.5). (A) Sprouting capillaries (white arrow heads) in the brain and meninges of a Tie2-Grn positive embryo (PM, pia mater). (B) and (C) illustrate the appearance of the heart in a Tie2-Grn negative (B) and Tie2-Grn positive embryo (C), which are structurally similar. EC, endothelial cushion. (D–E) Trabeculation and the formation of the endothelial lining of the trabeculae are indistinguishable in a Tie2-Grn negative (D) and Tie2-Grn positive embryo (E) embryos. (Black arrow heads point to the endothelium covering the cardiac muscle of the trabeculae). A Tie2-Grn negative (F) and Tie2-Grn positive embryo (G) stained for progranulin demonstrate the increased production of progranulin in the endothelia (black arrow heads) of the Tie2-Grn positive embryo at E10.5. M, mandible; EC, endocardial cushion; T, trabeculae. Scale bars denote 20 μm. Images were obtained from the GmHi line.

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Figure 6. Vascular abnormalities were first observed at E15.5 in the Tie2-Grn positive embryos. A comparison of mesenteric vessels (black arrow heads) in (A) a Tie2-Grn negative and (B) a Tie2-Grn positive fetus stained with H&E. Note the presence of highly distended vessels in (B). Blood vessels stained strongly for laminin, a basement membrane protein in Tie2-Grn negative fetuses (insert, black arrow head) (C), but in Tie2-Grn positive fetuses (D) laminin stained weakly, especially in the most distended vessels. Mesenteric vessels from Tie2-Grn negative fetuses (E) showed extensive coverage with smooth muscle α-actin positive mural cells, while in Tie2-Grn positive fetuses (F) distended vessels were in large part negative for smooth muscle α-actin positive mural cells (black arrow heads). P, pancreas; I, intestines; M, mesentery. Scale bars denote 20 μm. Images were obtained from the GmMid line.

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This is strikingly similar to the abnormal vessels of the Tie2-Grn positive mice. Taken together, there is strong support for a role for progranulin in angiogenesis and vessel formation. This may prove relevant in tumor angiogenesis, tissue repair and normal physiological tissue remodeling.

Figure 7. Compromised subcutaneous vessel structure at E15.5 in the Tie2-Grn positive embryos. Some Tie2-Grn positive fetuses exhibited subcutaneous hemorrhage at E15.5, particularly in the head region. (A) The scalp from a Tie2-Grn negative littermate and (B) from a Tie2-Grn positive littermate showing highly enlarged vessels not present in the negative counterpart. Sections were stained with H&E, the arrow heads point to blood vessels; the insert shows the external appearance of the Tie2-Grn positive fetus with the arrow head showing the extent of subcutaneous hemorrhage. The small vessels of the Tie2-Grn negative fetus (C) are enclosed in smooth muscle α-actin positive mural cells (insert). (D) The smaller vessels in the Tie2-Grn positive fetus is enclosed smooth muscle α-actin positive mural cells but the more enlarged vessels show incomplete association with smooth muscle α-actin positive mural cells (arrow heads indicate vessels). B, bone; M, muscle. Scale bars denote 20 μm. Images were obtained from the GrnHi line.

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Figure 8. Cerbrocortical hemorrhaging in Tie2-Grn positive fetus at E15.5 associated with reduced basement membrane and pericyte enclosure of capillaries. H&E staining of the cerebral cortex of (A) a Tie2-Grn negative fetus with no hemorrhage sites and (B) a Tie2-Grn positive fetus. Small localized hemorrhages were observed in the cerebral cortex of the Tie2-Grn positive fetus (insert) but not the Tie2-Grn negative fetus. Capillaries (black arrow heads) were poorly stained for progranulin in the Tie2-Grn negative fetus (C), but displayed high levels of progranulin in the Tie2-Grn positive fetus (D). The basement membrane protein laminin clearly demarcated capillaries in the Tie2-Grn negative fetus (E, and insert). Some capillaries in the Tie2-Grn positive fetus showed strong laminin staining (tailed arrowhead) while other showed reduced laminin (flat arrowhead). Desmin, a marker for pericytes, was associated with capillaries in the Tie2-Grn negative fetus (F, and insert). Few capillaries in the Tie2-Grn positive were positive for desmin (arrowhead).

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Methods and Materials

Ethics Statement

For experiments requiring animals, the mice were housed in facilities managed by the McGill University Animal Resources Centre. Animal experiments were conducted under a McGill University approved Animal Use Protocol (#5124) in accordance with guidelines established by the Canadian Council on Animal Care. Every effort was made to minimize suffering of the animals.

Production of Tie2-Grn Targeting Vector

Mouse progranulin full-length cDNA plasmid, a RIKEN clone (clone # 2900053G23) pFLC1 (modified Bluescript II) plasmid, was a gift from Max-Planck Institute for Biophysical Chemistry, Germany. A vascular endothelial promoter/enhancer package vector pSPTg.T2FpAXK (#52) was employed to make the Tie2-Grn transgene construct (gift from Montreal Clinical Research Institute [IRCM], originated from Dr. Sato, Texas Southwestern University at Dallas) [37]. This vector contains a Tie2 promoter, a SV40 polyA signal and a Tie2 minimum enhancer fragment. The mouse progranulin cDNA was inserted in the multiple cloning site (MCS) located between the Tie2 promoter and the polyA signal (Fig. 1A).

Since the Tie2 vector contains the unique HindIII and NotI cloning sites, which are not present in the full-length Grn cDNA, HindIII and NotI were created at the ends of the Grn cDNA by PCR. Briefly, the mouse Grn cDNA was amplified by PCR (RIKEN clone as a template) using a forward primer containing a HindIII site and the Grn start codon, and a reverse primer containing a NotI site and the Grn stop codon. The HindIII/NotI Grn fragment was inserted into the pSPTg.T2FpAXK vector downstream of the Tie2 promoter (Fig. 1A). The fidelity and accuracy of the PCR amplification and cloning was confirmed by DNA sequencing.

Generation of Tie2-Grn Transgenic Mice

The new 5757 bp Tie2-Grn vector construct fragment was excised by SalI digestion and purified using a gel extraction kit (ZymoClean), followed by an Elutip column (Schleicher & Schuell), and the purified Tie2-Grn DNA fragment was used for

Figure 9. Relationship between Tie2-Grn expression and VEGF-A/VEGFR2 expression. (A) VEGF-A protein levels in the amniotic fluid. Independent samples T-test revealed no significant difference in VEGF-A levels between the Tie2-Grn positive fetuses and the Tie2-Grn negative fetuses (p = 0.423). Age of fetuses are E15.5–16.5. N = 8, GrnHi line. Error bars denote +/- S.E. VEGFR2 endothelial expression in (B) Tie2-Grn negative fetus and (C) Tie2-Grn positive fetus. Arrows show endothelial cells within the mesenteric vessels. (D) Serial sections of the Tie2-Grn vessel in (B) expressed progranulin. Scale bars denote 20 μm. Images were obtained from the GrnHi line. doi:10.1371/journal.pone.0064989.g009
microinjection into fertilized eggs from B6C3F1 mice (Jackson Laboratories) at the Transgenic Mouse Facility, IRCM. Three *Tie2-Grn* transgenic founder mice were picked out from screening 47 mice produced including two females (MIL-535, MIL-794) and one male (MIL-772).

The transgenic founder mice were identified by PCR of tail genomic DNA using transgene-specific primers and confirmed by DNA sequencing analysis.

**Forward primer:** AGCGGGAAGTCGCAAAGTTGTTGAGTTGT;  
**Reverse primer 1:** CTTGCCAAGAAGGTTGAGAAGCTAC;  
**Reverse primer 2:** AGAAATGCCCTTCTCCACTCCA.

Both the reverse primers are paired up with the same forward primer. The forward primer is located on the *Tie2* promoter region of the transgene construct, whereas the reverse primers are located within the full-length mouse *Grn* cDNA region.

The housekeeping gene employed is mouse β-actin gene.

**Forward primer:** TGTGATGGTGGGAATGGGTCAGAA;  
**Reverse primer:** TGTGGTGCCAGATCTTCTCCATGT.

Congenic strains were created by backcrossing the *Tie2-Grn* positive donors with the pure background inbred strain recipient mice (C57BL/6). The descendents of 5–10 generations after backcross can be used for phenotypic analysis [55]. Transgenic lines were established and maintained using this approach.

**Tie2-Grn** Copy Number Estimation by Real Time qPCR

A quantitative real time PCR strategy was used to estimate the number of copies of *Tie2-Grn* transgene in each of the three transgenic lines. To selectively detect the *Tie2-Grn* transgene, we used primers that span exon junctions, since the *Tie2-Grn* transgene is intronless whereas the endogenous *GRN* has introns.

**Forward primers:** TCTTCTGGACACATGGCCTA (spanning the *GRN* exon 2–3 junction);  
**Reverse primers:** ATCTGACATCTGGAAGCAGGAT (spanning the *GRN* exon 4–5 junction).

A copy number standard curve was prepared by spiking a serial dilution of the *Tie2-Grn* transgene construct plasmid DNA into

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**Figure 10. Summary of the effect of expression of progranulin during vascular development.** The upper bar summarizes major landmarks in vascular development. VEGF and its receptors (yellow bar) are required for early vasculogenesis and for angiogenesis throughout development. *Tie2* and its ligand angiopoietin (red bar) are required for cardiac development and for vessel stabilization, but not for early vasculogenesis (see text for details). When progranulin was expressed developmentally (purple bar) it had no effect on vasculogenesis, the formation of the primary capillary plexus, sprouting or endothelial-mesenchymal transition. Its major actions were on vessel size and structural stability in late embryonic and neonatal phases. The timing of its actions is therefore distinct from those of the well-established angiogenic growth factors, suggesting that its effect on vessel growth and stability constitutes a novel angiogenic pathway.

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10 ng of WT mouse genomic DNA (gDNA). First, we calculated how much plasmid DNA should be spiked into 10 ng of gDNA in order to reflect one copy of the transgene. In this instance, a single copy is equivalent to 0.013594 pg of Tie2-Grn plasmid DNA spiked in 10 ng of gDNA. The amplification efficiency was 1.86, r = 1, error = 0.11, indicating the qPCR condition was optimized and this was a good standard curve. An external standard curve was created and re-used for copy number estimation. Measurements were performed in triplicate.

Determination of Tie2-Grn Transgene mRNA Expression in Mouse Tissue by Real Time RT-PCR

Total RNAs were extracted from mouse tissues (brain, lung, liver, kidney, and skin) using Qiagen RNeasy Micro Kit according to the manufacturer’s instructions. Real-time quantitative RT-PCR analysis for Tie2-Grn mRNA and endogenous GRN mRNA expression was performed using a LightCycler 2.0 System (Roche).

To selectively detect Tie2-Grn mRNA expression and exclude endogenous GRN expression, we used the following primers:

**Forward primer:** TTGGGAAAGAAGATTCCCTGGCTGG

**Reverse primer:** ACCTCTCATAGTGTATGCTGCTG (polyA region on the transgene construct).

To specifically detect endogenous GRN mRNA expression, we designed a primer set close to 3′-UTR, but after the GRN stop codon (in the end of exon 13).

**Forward primer:** TCTTCCGGTTTCTGTGGACCTTGT;

**Reverse primer:** TTATGGAGCAACACGCACACG.

Mouse β-actin was used as housekeeping control using the same primers as above.

All the PCR primers were obtained from AlphaDNA (Montreal, Quebec).

Amplification reactions were performed using the QuantiTect SYBR Green PCR kit. (Roche) The thermal cycling conditions comprised an initial denaturation step at 95°C for 15 min followed by 40 cycles amplification at 95°C for 10 s, 66°C for 5 s, and 72°C for 10 s to amplify Tie2-Grn transgene, endogenous Grn and β-Actin. The PCR products were subsequently melted at 60°C for 30 s. The melting curve analysis shows a single peak with no primer-dimers at the described PCR working conditions for all the genes amplified. PCR without reverse-transcribed cDNA were used as negative controls.

Genotyping

Embryo yolk sacs, fetal, neonatal or adult tail snips were incubated in lysis buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) with protease K (0.4 mg/mL) at 55°C overnight with gentle shaking at 80 rpm. Genomic DNA was extracted and purified using phenol chloroform. Proteinase K and phenol chloroform were purchased from Bioshop, Canada.

ELISA

Amniotic fluid was collected from amniotic cavities within the uterus (E14.5–E16.5) and snap-frozen in liquid nitrogen. Blood was collected from the heart of the adult mice (2–3 months old) immediately after euthanasia, and allowed to clot at room temperature for 2 hours, and spun at 2000 rpm for 20 minutes. The serum from the blood samples were also snap-frozen in liquid nitrogen. The frozen amniotic fluid and serum samples were thawed on ice and subjected to respective ELISA. The progranulin ELISA kit was purchased from Cayman Chemical Company and the VEGF ELISA kit was purchased from R&D Systems.

Immunohistochemistry

Mouse embryos or tissues were collected for histological assessment. Samples were fixed in 10% neutral buffered formalin (Fisher) and embedded in paraffin. Sections were deparaffinized in Citrosolv (Fisher) and rehydrated in a graded series of ethanol. Antigen retrieval was performed by boiling the slides in a 10 mM sodium citrate solution for 20 min in a microwave oven. The sections were incubated with Dako Dual Endogenous Enzyme Block (DakoCytomation) for an hour at room temperature using the LSAB+ System-HRP (DakoCytomation) as per the manufacturer’s recommended protocol. Positive reactions were detected using Dako Liquid DAB+ Substrate Chromogen System (Dako-Cytomation). The primary antibodies employed were diluted in Dako Antibody Diluent and the anti-progranulin antibody (dilution 1:70) was purchased from R&D systems, the anti-laminin antibody (dilution 1:50) was purchased from Abcam, the anti-smooth muscle α-actin antibody (dilution 1:100) and the anti-desmin (dilution 1:100) were purchased from Dako; the anti-PECAM1 antibody (dilution 1:25) was purchased from Santa Cruz and the VEGFR2 antibody (dilution 1:600) was purchased from Cell Signaling.

Supporting Information

**Figure S1** Total red blood cell counts from Tie2-Grn positive and negative newborn mice. Independent samples T-test revealed insignificant differences (p = 0.054) in red blood cell number between the Tie2-Grn positive and the negative mice. Error bars denote 95% CI. Within the Tie2-Grn positive population, the total red blood cell count was not correlated with pallor, p = 0.821 (not shown).

**Figure S2** Mean weight of Tie2-Grn positive and negative mice. Data taken from 3-week-old mice (weaning age). Independent samples T-test was performed on individual lines, p = 0.943 (GrnLo), p = 0.940 (GrnMid) and p<0.001 (GrnHi). Tie2-Grn positive mice that survived birth had similar average weight as Tie2-Grn negative littersmates, except in the GrnHi line where Tie2-Grn negative mice were heavier than their Tie2-Grn positive counterparts. Tie2-Grn positive mice from GrnHi were of similar average weight as Tie2-Grn positive and negative mice from the GrnLo and GrnMid lines. Error bars indicate +/- S.E. Asterisks indicate statistical significance in S2; significance of <0.001 is ***, <0.01 is ** and <0.05 is *.

**Figure S3** Lung section of a Tie2-Grn negative adult showing (A) absence of endothelial progranulin protein expression and (B) positive aSMA protein expression in the same blood vessel (serial sections), as indicated by the arrowheads. Lung section of a Tie2-Grn positive adult showing (C) positive endothelial progranulin protein expression and (D) positive aSMA protein expression in the same blood vessel (serial sections), as indicated by the arrowheads. (E) Weak progranulin expression in a neighboring vessel of the same lung section as C and D. Scale bars denote 20 μm. Images were obtained from the GrnHi line. GrnHi adults photographed were about 3 months old.

**Figure S4** Fetal placenta at E15.5 from Tie2-Grn positive and negative mice. The endothelial walls of blood vessels in the fetal placenta stain for progranulin in the Tie2-Grn negative mice (A and Tie2-Grn positive (B) mice (arrowheads). H&E stains of the placenta indicate that placental structure is apparently unaffected by the expression of Tie2-Grn transgene comparing the
**Table S1** Statistical analysis of the mean weights (g) of mice at 3 weeks. Independent variables, gender, genotype and litter size were tested using a Univariate General Linear Model. No statistical significance was attained between mean weight at birth and genotype ($p = 0.144$) or gender ($p = 0.627$), but it was significant for litter size ($p < 0.001$).

**Video S1** Pale colored pups were born alive. The video was taken within hours of birth.

**Video S2** Some pale colored neonates displayed apparent respiratory distress. The video was taken shortly after birth.

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**Author Contributions**

Conceived and designed the experiments: HT MC. Analyzed the data: HT AB MC ED. Wrote the paper: HT AB.

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**Progranulin in Angiogenesis**

**Tie2-Grn negative (C) and positive (D) mice. However, the Tie2-Grn positive plaques showed larger, and more frequent syncytial knots than their Tie2-Grn negative counterparts (see insert boxes in C and D). Scale bars denote 20 μm. (THF)**

**Author Contributions**

Conceived and designed the experiments: HT MC. Analyzed the data: HT AB MC ED. Wrote the paper: HT AB.
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