GFAP (Glial Fibrillary Acidic Protein)-Positive Progenitor Cells Contribute to the Development of Vascular Smooth Muscle Cells and Endothelial Cells—Brief Report

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OBJECTIVE: While GFAP (glial fibrillary acidic protein) is commonly used as a classical marker for astrocytes in the central nervous system, GFAP-expressing progenitor cells give rise to other cell types during development. The goal of this study was to investigate whether GFAP-expressing progenitor cells contribute to the development of vascular cells in major arteries.

APPROACH AND RESULTS: To label GFAP-expressing progenitor cells and their progeny, we crossed GFAP promoter-driven Cre recombinase mice (GFAP-Cre) with transgenic mice expressing the Cre-dependent mTmG dual fluorescent reporter gene. Using this genetic fate-mapping approach, here we demonstrate that GFAP-positive progenitor cells contribute to the development of vascular smooth muscle cells in both neural crest- and non-neural crest-derived vascular beds. In addition, GFAP-positive progenitor cells contribute to a subset of endothelial cells in some vasculature. Furthermore, fate-mapping analyses at multiple time points of mouse development demonstrate a time-dependent increase in the contribution of GFAP-positive progenitor cells to vascular smooth muscle cells, which mostly occurs in the postnatal period.

CONCLUSIONS: Our study demonstrates that vascular smooth muscle cells and endothelial cells within the same vascular segment are developmentally heterogeneous, where varying proportions of vascular smooth muscle cells and endothelial cells are contributed by GFAP-positive progenitor cells.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: astrocytes, endothelial cells, genes, reporter, muscle, smooth, neural crest

Vascular smooth muscle cells (VSMCs) are a major component of the vascular wall. Lineage tracing studies have demonstrated that VSMCs are derived from multiple distinct developmental origins. For instance, VSMCs in the aortic arch and the carotid arteries are derived from the neural crest; VSMCs in the ascending aorta are derived from both the neural crest and the secondary heart field; VSMCs localized in the aortic root are solely derived from the secondary heart field; VSMCs within coronary arteries are predominantly derived from the proepicardium; and VSMCs in the thoracic and abdominal aortae are of mesodermal origin. Similarly, endothelial cells (ECs) originate mainly from the mesoderm. Importantly, previous studies have demonstrated that lineage-specific VSMCs may respond differently to the same environmental cues when tested under identical conditions, suggesting that the distinct developmental origins of VSMCs may influence their relative contribution to vascular diseases. A better understanding of the developmental origins of VSMCs is key to unravel the molecular mechanisms that underlie their differentiation and to provide novel insights into the mechanisms of vascular disease.
GFAP (glial fibrillary acidic protein) is an intermediate filament protein that is commonly used as a classical marker for astrocytes in the central nervous system. However, during embryonic development, GFAP is also expressed in multipotent neural stem cells that give rise to neurons and glial cells in the brain and the spinal cord. Notably, previous studies have shown that GFAP-positive progenitors, acting as endogenous stem cells, are also involved in brain regeneration and gliogenesis in the mouse cortex in vivo. Furthermore, in peripheral organs such as the liver, GFAP-expressing quiescent hepatic stellate cells contribute to regeneration in the injured liver. These reports lead us to hypothesize that the progeny of GFAP-expressing precursors may also contribute to vascular cell development and the response to vascular injuries.

Using a genetic fate-mapping approach, here we demonstrate that GFAP-positive progenitor cells contribute to the development of VSMCs and ECs in a wide range of vascular beds that have distinct developmental origins. In particular, our findings demonstrate that VSMCs and ECs within the same vascular bed are heterogeneous, where varying proportions of VSMCs and ECs are contributed by GFAP-positive progenitor cells.

### MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and its Data Supplement.

**Mouse Breeding**

GFAP-Cre (Stock #: 004600), Wnt1-Cre (Stock #: 009107), and mTmG dual fluorescent reporter mice (Stock #: 007676) were purchased from the Jackson Laboratory. GFAP-Cre or Wnt1-Cre male mice were bred to female mTmG dual fluorescent reporter mice to trace GFAP-expressing or neural crest-derived cells, respectively. Cre negative mice served as negative control. All mice used in this study were maintained on a C57BL/6J background. This study used both male and female mice. Mice were sacrificed at multiple time points, including embryonic day (E) 14.5, postnatal day (P) 0, P14, and at 3 to 6 months of age. The use of experimental animals has been approved by the Institutional Animal Care and Use Committee and Biosafety committee at Augusta University in accordance with the National Institutes of Health guidelines.

**Tissue Sectioning, Histological Examination, and Immunofluorescence Staining**

Mice were euthanized by an overdose of 4% isoflurane via inhalation, then systemically perfused with PBS followed by fixation with 4% paraformaldehyde in PBS via the left ventricle. Isolated tissues were further fixed overnight with 4% paraformaldehyde in PBS at 4°C, washed 3× with PBS, then kept in 30% sucrose in PBS overnight at 4°C. Fixed tissues were embedded in optimal cutting temperature compound and kept at −80°C before cryo-sectioning at 8 to 10 µm thickness.

For direct visualization of mEGFP (membrane-targeted enhanced green fluorescent protein) and mTomato signals, sections were air-dried for 15 minutes, washed 3× with PBS, then mounted with ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific) and imaged using confocal microscopy (780 upright, Zeiss).

For immunofluorescence, sections were air-dried for 15 minutes, washed with PBS, and antigen retrieval was performed by heating at 98°C for 10 minutes in citrate buffer (10 mmol/L, pH 6.0). Sections were blocked and permeabilized with goat serum (10%, Thermo Fisher Scientific) plus 0.1% Tween for 30 minutes, then incubated with anti-GFP, anti-ACTA2 (SM α-actin), anti-PECAM1 (CD31), or anti-GFAP primary antibodies overnight at 4°C. After washing with PBS, sections were incubated with secondary antibodies (488 nm-conjugated anti-rabbit, 647 nm-conjugated antimouse, or 633 nm-conjugated anti-rat secondary antibodies; 1:250 dilution; Thermo Fisher Scientific) diluted in blocking buffer for 1 hour at room temperature. Sections incubated without primary antibodies but with secondary antibodies only served as negative control. After washing with PBS, sections were mounted with ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific) and imaged using confocal microscopy (780 upright, Zeiss).

In select experiments, a modified immunofluorescence protocol was used that does not include the antigen retrieval step but treats sections with 0.1% Triton-x 100 in PBS for 5 minutes as an alternative permeabilization approach. This protocol maintains the strong elastic lamina auto-fluorescence and the endogenous mTmG dual fluorescence without the need to co-stain fluorescent reporter proteins using antibodies.

**Quantification of mEGFP-Positive Cells**

The total number of VSMCs in a confocal image was defined as the total number of DAPI-positive nuclei enclosed between...
GFAP-Positive Progenitor Cells Contribute to Vascular Cell Development

To assess the contribution of GFAP-positive progenitor cells in vascular cell development, we utilized GFAP-Cre; mTmG double-transgenic mice, where Cre recombinase mediates the excision of floxed membrane-targeted tandem dimer Tomato (mTomato, mT) and leads to the simultaneous expression of membrane-targeted EGFP (mEGFP, mG). Thus, GFAP-positive progenitor cells and their progeny are permanently labeled with mEGFP even if these cells no longer express GFAP (Figure 1A).

Using this genetic fate-mapping approach, we examined several vascular tissues of 3- to 6-month-old male mice as shown in Figure 1B and directly visualized mEGFP and mTomato signals using confocal microscopy. Consistent with previous studies that demonstrated the role of GFAP-positive progenitor cells in the development of neurons,14,15 we found that in Cre-positive mice, the vagus nerve adjacent to the carotid artery was almost exclusively labeled with mEGFP, which thus can serve as a positive control for GFAP-positive-derived cells (Figure 1C, indicated by a white arrow). Next, we tested if these cells no longer express GFAP (Figure 1A).

The total number of ECs within a confocal image was defined as the total number of DAPI-positive nuclei that reside inside the internal elastic lamina toward the luminal side. VSMCs or ECs were scored as being either mEGFP- or mTomato-positive and were quantified as a percentage of their respective total cell populations within a given field of interest.

**RESULTS**

GFAP-Positive Progenitor Cells Contribute to Vascular Cell Development

To confirm the identity of mEGFP-labeled vascular cells, we co-labeled mEGFP-positive cells in carotid arteries, thoracic aortae, or femoral arteries using antibodies against ACTA2 (SM α-actin) or PECAM1 (CD31), to label VSMCs and ECs, respectively. As expected, mEGFP-labeled vascular cells within the elastic laminae of the carotid artery or the thoracic aorta were ACTA2-positive, indicating they are indeed authentic VSMCs (Figure 2A, 2C, and 2G, indicated by green arrows). Notably, we also confirmed the presence of a subset of mEGFP-positive cells in the internal and the external elastic laminae of the blood vessel, which were visualized owing to the strong elastin autofluorescence. The total number of ECs within a confocal image was defined as the total number of DAPI-positive nuclei that reside inside the internal elastic lamina toward the luminal side. VSMCs or ECs were scored as being either mEGFP- or mTomato-positive and were quantified as a percentage of their respective total cell populations within a given field of interest.

**Identification of Vascular Cells Derived From GFAP-Positive Progenitors in the Vessel Wall**

To confirm the identity of mEGFP-labeled vascular cells, we co-labeled mEGFP-positive cells in carotid arteries, thoracic aortae, or femoral arteries using antibodies against ACTA2 (SM α-actin) or PECAM1 (CD31), to label VSMCs and ECs, respectively. As expected, mEGFP-labeled vascular cells within the elastic laminae of the carotid artery or the thoracic aorta were ACTA2-positive, indicating they are indeed authentic VSMCs (Figure 2A, 2C, and 2G, indicated by green arrows). Notably, we also confirmed the presence of a subset of mEGFP-positive cells in the internal and the external elastic laminae of the blood vessel, which were visualized owing to the strong elastin autofluorescence.
Figure 1. GFAP (glial fibrillary acidic protein)-positive progenitor cells contribute to vascular development.

A, Diagram to depict the strategy used to fate-map GFAP-expressing cells and their progeny. B, Diagram to show the vascular tissues harvested from 3- to 6-month-old adult male GFAP-Cre\(^{+}\); mTmG\(^{+/-}\) (Cre \(+ve\)) or GFAP-Cre\(^{-}\); mTmG\(^{+/-}\) (Cre \(-ve\)) mice for direct visualization of membrane-targeted enhanced green fluorescent protein (mEGFP) and mTomato signals by confocal microscopy. C, Right carotid artery and the adjacent vagus nerve of Cre \(+ve\) mice, (D) right carotid artery and the adjacent vagus nerve of Cre \(-ve\) mice, (E) aortic arch of Cre \(+ve\) mice, (F) thoracic aorta of Cre \(+ve\) mice, (G) abdominal aorta of Cre \(+ve\) mice, and (H) femoral artery and the adjacent femoral nerve of Cre \(+ve\) mice were sectioned for direct visualization of mEGFP and mTomato signals. Nuclei were counter-stained with DAPI (blue). Magnified areas are depicted by dashed boxes and shown below. White arrows point to the peripheral nerves. Green arrows and white arrowheads point to mEGFP \(+ve\) presumptive vascular smooth muscle cells and mEGFP \(+ve\) presumptive endothelial cells, respectively. *Designates mEGFP \(+ve\) adventitial cells. N=6 per group.
ECs in the carotid arteries but not in the thoracic aorta as indicated by PECAM1 co-labeling (Figure 2B, 2D, and 2G, indicated by a white arrowhead). Co-labeling of mEGFP-labeled vascular cells that reside inside the internal elastic lamina of the femoral artery demonstrated that these cells were negative for ACTA2 but positive for PECAM1, confirming that they are ECs and not VSMCs (Figure 2E and 2F, indicated by white arrowheads). In addition, a sparse population of mEGFP-positive adventitial cells in the femoral artery (Figure 1H) were negative for both ACTA2 and PECAM1 labeling (Figure 2E and 2F, indicated by asterisks).

**Carotid Artery VSMCs Are Almost Exclusively Derived From the Neural Crest**

Next, we compared the pattern of mEGFP expression in GFAP-Cre; mTmG double-transgenic mice to Wnt1-Cre; mTmG double-transgenic mice, which label neural crest-derived cells with mEGFP (Figure IVA in the Data Supplement). Similar to our observations with GFAP-Cre; mTmG mice, peripheral nerves accompanying the carotid and femoral arteries from Wnt1-Cre; mTmG mice, but not Wnt1-Cre-negative mice, were also mEGFP-positive, suggesting that these neural cells are derived from GFAP- and Wnt1-double-positive progenitor cells (Figure IVB through IVD in the Data Supplement, indicated by white arrows). However, unlike the heterogeneous pattern of mEGFP expression in carotid artery VSMCs of GFAP-Cre; mTmG mice (Figure 1C), carotid artery VSMCs were almost uniformly mEGFP-positive in the Wnt1-Cre; mTmG double-transgenic mice, suggesting that these cells are almost exclusively derived from Wnt1-positive progenitor cells (Figure IVB in the Data Supplement). Furthermore, none of the ECs of the carotid or femoral arteries of Wnt1-Cre; mTmG mice double-transgenic mice were mEGFP-positive, suggesting no contribution of Wnt1-positive progenitors to EC development in these vascular tissues (Figure IVB through IVD in the Data Supplement).

**Time-Dependent Contribution of GFAP-Positive Progenitor Cells to VSMC Development**

Next, we determined whether GFAP-positive progenitor cells contribute to vascular cells during embryonic development. Surprisingly, however, we did not detect any mEGFP-positive cells in the carotid arteries or thoracic aorta of E14.5 embryos (Figure VC and VD). As a positive control, we detected a high percentage of mEGFP-positive cells in the brain sections of the same embryos (Figure VA and VB in the Data Supplement). Notably, microvessels in the brain were mEGFP-negative (Figure VB in the Data Supplement, indicated by a white arrowhead). Next, we tested the contribution of GFAP-positive progenitors to vascular cells in neonates (P0) and in postnatal day 14 (P14) mice from both sexes. Interestingly, we detected a few scattered (<1%) mEGFP-positive presumptive VSMCs in the carotid arteries and thoracic aorta of P0 neonates (Figure VE and VF in the Data Supplement, indicated by green arrows). Furthermore, we detected an increased number of mEGFP-positive presumptive VSMCs in the carotid arteries (≈12%) and thoracic aorta (≈8%) of P14 mice. Notably, most of the mEGFP-positive signals in the vascular tissues of P14 mice were detected in duplets of neighboring cells (Figure VG and VH in the Data Supplement, indicated by green arrows). Together, these data demonstrate a time-dependent increase in the percentage of mEGFP-positive VSMCs that mostly occurs after birth.

**mEGFP-Positive Cells in the Thoracic Aortae Do Not Express Detectable GFAP**

Given that there were no mEGFP-positive cells detected in the vasculature in E14.5 embryos, we tested whether the postnatal time-dependent increase in the percentage of mEGFP-positive VSMCs (Figure V in the Data Supplement) corresponds to endogenous expression of GFAP in the vascular wall after birth. As a positive control, adult brain tissue of wild-type mice demonstrated a high expression of GFAP by immunofluorescence (Figure VIA in the Data Supplement). In contrast, data from immunofluorescence assays did not reveal any detectable expression of GFAP in mEGFP-positive VSMCs in the thoracic aorta of P0 or P14 mice (Figure VIIB and VIC in the Data Supplement) or in the carotid arteries of adult wild-type mice (Figure VID in the Data Supplement).

**DISCUSSION**

Using genetic fate-tracing, herein, we demonstrate, for the first time, that GFAP-positive progenitor cells are an important cell source for both VSMCs and ECs in the mouse vasculature. Notably, we found that GFAP-positive progenitor cells contribute to the vascular development of both neural crest- and non-neural crest-derived VSMCs. In contrast to previous studies, which demonstrated that there is little or no intermixing between VSMCs of distinct developmental origins within the same vascular segment, we demonstrate here a highly mosaic distribution of these VSMCs derived from GFAP-positive progenitors in vascular segments that have been previously thought to arise from a single developmental origin.

In the adult mouse, GFAP is considered a rather specific marker for differentiated astrocytes. However, during development, GFAP is widely expressed in the brain and the spinal cord in multipotent neural progenitor cells. Accordingly, GFAP-Cre-mediated activation of reporter gene expression takes place in many neural progenitor-derived cells, including neurons and astrocytes. Consistently, we found here that brain tissues...
Figure 2. Cellular identity of vascular cells derived from GFAP (glial fibrillary acidic protein)-positive progenitor cells. Sections prepared from 3- to 6-month-old male GFAP-Cre\(^{+}\); mTmG\(^{-/−}\) mice were used for immunofluorescence (IF) staining of mEGFP (membrane-targeted enhanced green fluorescent protein)-positive cells with anti-GFP (α-GFP; green) and anti-ACTA2 (actin alpha 2, smooth muscle; red) primary antibodies using (A) right carotid artery or (C) thoracic aorta. To maintain the mTmG dual fluorescence without the need to co-stain fluorescent reporter proteins using antibodies and to visualize the elastin autofluorescence, a modified IF staining protocol was used for co-staining mEGFP-positive cells with α-ACTA2 (red) or α-PECAM1 (platelet and endothelial cell adhesion molecule 1; red) primary antibodies in (B) right carotid artery, (D) thoracic aorta, and (E and F) right femoral artery. G, Right carotid artery sections incubated with fluorophore-labeled secondary antibodies (2nd Abs) and DAPI served as negative control. Nuclei were counter-stained with DAPI (blue). Green arrows and white arrowheads point to mEGFP +ve vascular smooth muscle cells and mEGFP +ve endothelial cells, respectively. *Designates mEGFP-positive adventitial cells. N=4 per group.
of E14.5 embryos (Figure VA and VB in the Data Supplement) and nerves peripheral to the carotid and femoral artery of adult mice (Figure 1C through 1H) were highly labeled with mEGFP in GFAP-Cre; mTmG+/− double transgenic mice. These observations demonstrate the high efficiency of the used GFAP-Cre system. Importantly, our study extends these earlier observations and suggests that GFAP-expressing multipotent neural progenitors not only contribute to the development of cells in the nervous system but they also contribute to the development of both VSMCs and vascular ECs.

In this study, we found that the distribution of neural crest-derived cells in the carotid and femoral arteries is consistent with previous reports. However, we report here that although carotid artery VSMCs are almost exclusively derived from Wnt1-positive neural crest progenitors, at the adult stage, only about one-third of these cells were derived from GFAP-positive progenitors. Furthermore, the ascending aorta and aortic valve interstitial cells, which are partially derived from the neural crest, exhibited varying proportions of GFAP-positive progenitor-derived cells. Conversely, aortic root VSMCs, which are derived from the secondary heart field, were devoid of any GFAP-positive progenitor-derived cells. Furthermore, coronary artery VSMCs around the aortic root, which are derived from neural crest cells during development, were negative for GFAP-positive progenitor-derived cells as well. It remains to be determined whether GFAP-positive progenitor-derived cells contribute to VSMCs in more distal positions of coronary arteries that are derived from proepicardium. Together, these observations suggest that a varying proportion of neural crest-derived cells arise from GFAP-positive progenitors.

Equally important is that, at the adult stage, we detected varying proportions of GFAP-positive-derived vascular cells in the thoracic aorta, abdominal aorta, and the femoral artery, which lie outside the neural crest domain and are generally considered to be mesodermal-derived. These observations suggest that neural progenitors may have a broader role in vascular development than previously recognized.

Previous studies have demonstrated that the descending aorta develops in a rostral-to-caudal fashion. Interestingly, we observed here that, at the adult stage, the proportion of VSMCs derived from GFAP-positive progenitors markedly decreases caudally from the thoracic aorta to the abdominal aorta, until there is no detectable contribution to VSMCs in the femoral artery. Surprisingly, however, we did not detect any mEGFP-positive cells in the carotid arteries or thoracic aortae of E14.5 embryos. Analysis of comparable vessels at later time points demonstrated a few scattered (<1%) mEGFP-positive VSMCs in P0 neonates, an increased number of mEGFP-positive VSMC duplets at P14, and the presence of multiple patches of mEGFP-positive VSMCs in adult mice that ultimately constitute up to ≈30% of total VSMCs. Notably, the mEGFP-positive vascular cells in P0 or P14 mice do not reflect ongoing GFAP expression as these cells did not stain positive with GFAP antibody (Figure VI in the Data Supplement). The time-dependent increase in mEGFP-positive VSMCs after birth is likely dependent on the expansion of the existing mEGFP-positive small population of VSMCs at P0, rather than ectopic expression of GFAP in these mEGFP-positive VSMCs. Notably, the lack of significant contribution of GFAP-positive progenitors to vascular wall development during embryonic development is consistent with previous studies that have not revealed any arterial morphology or patterning defects using GFAP-null mice.

In contrast to the observed poor investment of GFAP-positive progenitors to vascular beds during embryonic development (<1% of mEGFP-positive VSMCs in P0 neonates), previous studies demonstrated a rich investment of neural crest-derived cells in the cardiac outflow tract and aortic arch arteries during embryonic development. These observations suggest that GFAP-positive progenitors’ contribution (<1%) to neural crest-derived vascular segments likely occurs during late embryonic and fetal stages (between E14.5 and P0), and that these cells seem to expand over time to reach ≈30% at the adult stage. Our results suggest that a similar expansion of GFAP-positive progeny likely occurs in the thoracic aorta, which is mesodermal-derived and lies outside the neural crest domain. Interestingly, although VSMCs exhibit a very slow turnover in the adult stage, previous studies demonstrated that postnatal VSMCs still continue to divide physiologically in vivo during the early postnatal period. For instance, Olivetti et al demonstrated that the number of rat aortic wall VSMCs doubles during the first 5-day interval after birth. Consistently, Cook et al demonstrated that rat aortic VSMCs exhibit a replication rate of 20% per day at P0 that slows down to 10% at P16 before they reach 0.6% in adult animals. Future studies using a confetti reporter mouse model, for instance, are warranted to test whether the ≈30% mEGFP-positive VSMCs in the carotid arteries and thoracic aortae at the adult stage indeed arise from clonal expansion of few mEGFP-positive VSMCs in the postnatal period.

Previous studies have demonstrated that lineage-specific VSMCs may exhibit different molecular signatures and may respond differently to the same environmental factors when tested under identical conditions. Accordingly, origin-specific VSMCs are speculated to contribute to the well recognized site-specific presentation of some acquired vascular diseases. Since we report here that different vascular segments exhibit varying proportions of VSMCs derived from GFAP-positive progenitors, future studies are warranted to determine if the VSMCs derived from GFAP-positive and GFAP-negative progenitors are functionally distinct and whether they have specific roles in vascular diseases.
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