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Adventitial MSC-like cells are progenitors of vascular smooth muscle cells and drive vascular calcification in chronic kidney disease

Rafael Kramann[1,2,*], **Claudia Goettsch**[3], **Janewit Wongboonsin**[4], **Hiroshi Iwata**[3], **Rebekka K Schneider**[5,6], **Christoph Kuppe**[1], **Nadine Kaesler**[1], **Monica Chang-Panesso**[4], **Flavia G. Machado**[4], **Susannah Gratwohl**[7], **Kaushal Madhurima**[4], **Joshua D. Hutcheson**[3], **Sanjay Jain**[4], **Elena Aikawa**[3,8], and **Benjamin D. Humphreys**[4]

[1]Division of Nephrology and Clinical Immunology RWTH Aachen University Medical Faculty RWTH Aachen University, Germany

[2]Renal Division, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA

[3]Center for Interdisciplinary Cardiovascular Sciences, Cardiovascular Division, Brigham and Women's Hospital, Boston, Massachusetts, USA

[4]Division of Nephrology, Department of Medicine, Washington University School of Medicine, USA

[5]Division of Hematology, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA

[6]Division of Hematology, RWTH Aachen University, Aachen, Germany

[7] Deparment of Surgery, Washington University School of Medicine, USA

[8]Center for Excellence in Vascular Biology, Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

[*]Corresponding author: Rafael Kramann, MD, Division of Nephrology and Clinical Immunology, Medical Faculty RWTH Aachen University, Pauwelsstrasse 30, 52074 Aachen, Germany, Phone.: 0049-241-80 37750, Fax.: 617-525-5965, rkramann@gmx.net.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, five figures and two excel spreadsheets with single cell qPCR data can be found with this article online.

Human arteries

We examined femoral arteries obtained from consented clinical autopsies of 12 dialysis patients (8 men, age 68 ± 15 years, dialysis vintage 6 ±2 years) and 10 age-matched non-CKD patients (6men, age 66 ± 10 years) between April 2009 and April 2011 at the Department of Pathology of the RWTH Aachen University. The study was approved by the ethical committee of the RWTH Aachen University (ethical votes EK 180/14 and EK239/11) and carried out according to the declaration of Helsinki.

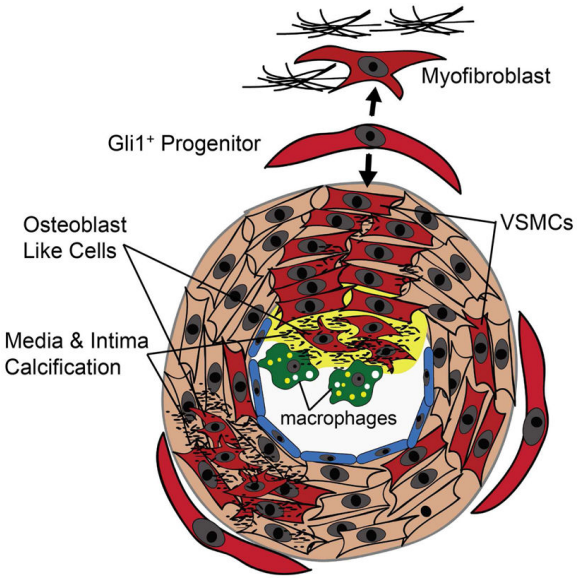
Author contributions

R.K. designed and carried out experiments, analyzed results and wrote the manuscript, C.G., J.W. H.I., R.K.S., C.K., N.K., M.C.P., S.G., F.M., and J.D.H. carried out experiments, analyzed the data and reviewed the manuscript, K.M. and S.J. performed bioinformatics of the single cell qPCR data and reviewed the manuscript, E.A. and B.D.H. designed experiments, analyzed results and contributed to data interpretation and writing of the manuscript

Abstract

Mesenchymal stem cell-like (MSC-like) cells reside in the vascular wall but their role in vascular regeneration and disease is poorly understood. Here, we show that Gli1$^+$ cells located in the arterial adventitia are progenitors of vascular smooth muscle cells, and contribute to neointima formation and repair after acute injury to the femoral artery. Genetic fate tracing indicates that adventitial Gli1$^+$ MSC-like cells migrate into the media and neointima during athero- and arteriosclerosis in ApoE−/− mice with chronic kidney disease. Our data indicate that Gli1$^+$ cells are a major source of osteoblast-like cells during calcification in the media and intima. Genetic ablation of Gli1$^+$ cells before induction of kidney injury dramatically reduced the severity of vascular calcification. These findings implicate Gli1$^+$ cells as critical adventitial progenitors in vascular remodeling after acute and during chronic injury and suggest that they may be relevant therapeutic targets for mitigation of vascular calcification.

Graphical abstract



eTOC:

Kramann et al show that Gli1$^+$ MSC-like cells that reside in the vascular wall differentiate into osteoblast like cells after injury and make a major contribution to calcification. Ablation of these cells before injury eliminates calcification, and therefore suggests that they could be a target for therapeutic intervention.

Introduction

It has become evident in recent years that the perivasculature (e.g., adventitia and pericyte) represents the *in vivo* niche for mesenchymal stem cells (MSC). However, the *in vivo* role of these perivascular MSC has remained unclear due to the absence of a specific marker to enable genetic fate tracing experiments. We and others recently reported that Gli1 represents a specific MSC marker in adult tissues (Kramann et al., 2015; Zhao et al., 2015; Zhao et al.,

2014). Gli1$^+$ cells with tri-lineage differentiation capability are located in the perivasculature across major organs from the pericyte niche of microcapillaries to the adventitia of large arteries (Kramann et al., 2015). We demonstrated that Gli1$^+$ cells are major contributors to the myofibroblast pool after solid organ injury in kidney, heart, liver and lung (Kramann et al., 2015). Progenitors of the adventitia have been suggested to play roles in vascular regeneration and disease (Psaltis and Simari, 2015), however, definitive proof is lacking due the absence of lineage analysis results to clarify the role of adventitial progenitors in vascular repair and disease.

Vascular calcification is a tightly regulated process resembling bone morphogenesis (Sage et al., 2010). Indeed, vascular calcification was described as a form of extraskeletal ossification over a century ago (Bunting, 1906; Virchow, 1863). Arterial calcification is of major clinical importance as it predicts cardiovascular events (Criqui et al., 2014; Martin et al., 2014), it might affect plaque stability (Hutcheson et al., 2014) and also stiffens the aorta increasing afterload and contributing to chronic heart failure (Demer and Tintut, 2008). The current dogma is that mature vascular smooth muscle cells (vSMC) dedifferentiate upon injury, become synthetically active and differentiate into osteoblast-like cells driving the calcification process in both media and intima (Paloian and Giachelli, 2014; Sage et al., 2010; Speer et al., 2009). While strong recent genetic fate tracing evidence implicates mature vSMC in contributing significantly to atherosclerotic plaque remodeling (Shankman et al., 2015), a role for adventitial progenitors such as MSC in this process remains undefined. The occurrence of ectopic bone formation, including hydroxyapatite mineral and even fully formed marrow cavities with hematopoiesis inside the artery wall has led to speculation that indeed progenitor cells such as MSC might be involved (Sage et al., 2010).

Multiple groups have described vascular wall progenitor cells (Psaltis and Simari, 2015). Understanding the role of these resident cells in the vascular wall during homeostasis, injury repair and disease might have major therapeutic implications including identification of potential approaches to manipulate these progenitors therapeutically towards tissue repair and plaque stabilization.

Peault's group was the first to demonstrate that MSC are located in the perivasculature (Corselli et al., 2012; Crisan et al., 2008). Sca1 and/or CD34 are two among many nonspecific markers that several groups have used to isolate vascular smooth muscle progenitor cells from arteries (Hu et al., 2004; Passman et al., 2008; Sainz et al., 2006). Passman et al. reported that Sca1$^+$, CD34$^+$, PDGFRβ$^+$ cells residing in an adventitial niche characterized by sonic hedgehog (Shh) signaling could be differentiated into smooth muscle-like cells *in vitro*. They demonstrated that these precursors co-localize with the Hedgehog receptor Ptc1 using Ptc1-LacZ mice and express mRNA of most Hh pathway members including Gli1. When incubated with bone morphogenic protein 2 (BMP-2) the Ptc1$^+$ progenitor cells differentiated into osteoblasts *in vitro*.

These observations prompted us to evaluate the role of Gli1$^+$ adventitial cells in acute vascular injury and repair and chronic injury during arterio- and atherosclerosis in chronic kidney disease (CKD). Using inducible genetic fate tracing, we demonstrate that Gli1$^+$ progenitor cells are an important adventitial cell source for vSMC, and contribute to repair

after acute arterial injury. However, during chronic injury in ApoE$^{-/-}$ mice subjected to CKD, Gli1^{+} cells differentiate into osteoblast-like cells and contribute significantly to vascular calcification. Importantly, genetic ablation of Gli1^{+} cells prior to injury substantially reduced vascular calcification CKD.

Results

Gli1^{+} cells with tri-lineage differentiation capacity are located in the arterial adventitia and express CD34, Sca1 and PDGFRβ

We have recently demonstrated that Gli1^{+} cells are located in the pericyte niche with direct contact to microcapillary endothelial cells but also in the arterial adventitia (Kramann et al., 2015). These Gli1^{+} cells sorted from whole organs fulfill all criteria for a cell that has been termed MSC before (Kramann et al., 2015). However, we isolated Gli1^{+} cells from both the pericyte and adventitial niche because we enzymatically dissociated the whole organ followed by fluorescence activated cell sorting (FACS) (Kramann et al., 2015). Because the adventitia has been described as the stem cell niche of the vascular wall we sought to examine whether the adventitial Gli1^{+} cell population represent progenitor cells of the arterial wall contributing to injury repair and disease.

Gli1-CreER^{t2}; R26tdTomato mice were pulsed with tamoxifen at 8 weeks (Figure 1 A) and 48 hours later the aorta with carotid and subclavian arteries was isolated by microdissection. Immunostaining revealed that Gli1^{+} cells are located in the arterial adventitia distinct from CD31^{+} endothelial cells and calponin expressing vSMC (Figure 1B). Gli1^{+} cells co-expressed PDGFRβ, Sca1 and CD34 (Figure 1B). Importantly, CD34 expression has been reported as a surface marker of adventitial progenitor cells by various groups (Psaltis and Simari, 2015). We have also previously detected a small population of Gli1^{+}, CD34^{+} cells in cardiac, muscular and hepatic tissue (Kramann et al., 2015), which could be Gli1^{+} cells from arterial adventitia.

We then used FACS to isolate Gli1^{+} cells from micro-dissected adventitia of aortic arches. Importantly, in these experiments we carefully microdissected the aortic arch, removed periadventitial adipose tissue and stripped the adventitia from the media to isolate only adventitial Gli1^{+} cells and avoid isolation of Gli1^{+} pericytes. Gli1^{+} cells represent a small population of the aortic arch (0.02% of all cells) (Fig 1C) and possess *in vitro* differentiation capacity towards osteoblasts, adipocytes and chondrocytes (Fig 1C). Since it has been recently reported that tamoxifen might induce browning of adipose tissue (Hesselbarth et al., 2015) and Gli1^{+} cells show adipogenic potential we tested whether tamoxifen might effect adipogenic differentiation of Gli1^{+} cells. Tamoxifen treatment of Gli1^{+} cells, however, did not show any effect on expression of the adipocyte marker FABP4 and brown fat marker UCP1 in Gli1^{+} cells (Figure S1).

Adventitial Gli1^{+} cells are vSMC progenitors and contribute to repair of the arterial wall after acute injury

Since it has been previously reported that CD34^{+}, Sca1^{+}, PDGFRβ^{+} cells co-express the Hh receptor Ptc1 and can be differentiated into vSMC *in vitro* (Passman et al., 2008)., we

sought to determine whether isolated Gli1$^+$ cells of the adventitia, which also express CD34$^+$, Sca1$^+$ and PDGFRβ$^+$ (Fig 1B) can differentiate into vSMC. Five days after tamoxifen injection, we isolated aortic arches from 8 week old, bigenic Gli1CreER^{t2}; tdTomato mice by micro-dissection. Gli1$^+$ cells were then isolated by FACS after enzymatic digestion of the whole aortic arch (Fig 2A). Under differentiation conditions with addition of PDGF-BB and TGF-β, the Gli1$^+$ cells acquired expression of SMC markers including α-SMA (ACTA2), smoothelin and calponin (Figure 2A-C). By contrast, none of these markers was detectable early after isolation (d0) or under control conditions without addition both growth factors (Figure 2A-C). It has been previously reported that sphingosylphosphorylcholine (SPC) induces differentiation of MSC into smooth muscle cells (Jeon et al., 2006; Jeon et al., 2008). We therefore asked whether SPC might also induce vSMC differentiation of Gli1$^+$ cells. Indeed, following 7 days of SPC treatment >80% of Gli1$^+$ cells showed expression of SM-calponin (Figure S2A-B).

We next sought to determine whether Gli1$^+$ cells might also be able to differentiate into vSMC *in vivo* and contribute to arterial repair after injury. The wire injury model of the femoral artery is a well-established model of acute arterial injury with apoptosis of vSMC followed by neointimal hyperplasia (Sata et al., 2000). To perform genetic fate tracing of Gli1$^+$ cells in acute arterial injury repair, bigenic Gli1CreER^{t2}; tdTomato mice received tamoxifen and were subjected to wire injury of the right femoral artery (Figure 2D). To eliminate the possibility of recombination after injury the wire injury surgery was performed 10 days after the last tamoxifen dose (Goritz et al., 2011). Mice were sacrificed 4 weeks after injury. The contralateral non-injured femoral artery served as control. We detected a significant number of Gli1$^+$ cells in the media and hyperplastic neointima after wire injury (Figure 2E-F). The vast majority of Gli1$^+$ cells in media and neointima expressed α-SMA (Figure 2E, G). As α-SMA has been suggested to be insufficient for definitive identification of SMC, we also examined the coexpression of Gil1 and the mature SMC maker SM-calponin (Iwata et al., 2010). A significant number of Gli1$^+$ cells in media and neointima co-expressed SM-calponin (Figures 2E, S2C). To elucidate the relative contribution of Gli1$^+$ cells to newly formed vSMCs we repeated the wire injury experiment with daily injections of 5-ethynyl-2-deoxyuridine (EdU) (Figures S2D). Quantification of EdU$^+$ neointimal cells indicated that >50% of newly formed VSMCs were derived from Gli1$^+$ progenitors (Figures 2H, S2 E).

These data suggest that adventitial Gli1$^+$ cells are actively involved in the injury repair process of the arterial wall and migrate into media and neointima after injury. Moreover our data also suggests that Gli1$^+$ cells might differentiate into highly differentiated vSMC *in vivo*, replacing lost vSMC after arterial wall injury.

Gli1$^+$ cells migrate into media and intima plaques during arterio- and atherosclerosis

Our data so far suggested that Gli1$^+$ cells are vSMC progenitors of the adventitia and contribute to acute arterial injury repair. We next asked whether these cells might also be involved in chronic injury during athero- and arteriosclerosis in ApoE$^{-/-}$ mice with superimposed CKD. Triple transgenic Gli1CreER$^{t2+/-}$; tdTomato$^{+/-}$; ApoE$^{-/-}$ mice received tamoxifen and were subjected to a surgical two-step model of subtotal nephrectomy

removing 5/6 of the kidney tissue at 10 days after the last tamoxifen dose (Figure 3A). Mice received a western diet and were sacrificed 10 or 16 weeks after surgery. Littermates with the same genotype subjected to sham surgery and standard chow served as a control group.

We detected increased levels of blood urea nitrogen (BUN) in the serum of 5/6 nephrectomized mice indicating development of CKD due to loss of kidney tissue (Figure 3B). Representative low-magnification images of the aortic arch indicate significant remodeling in the 5/6 nephrectomized mice with severe atherosclerosis largely occluding the aortic lumen at 16 weeks after CKD induction (Figure 3C). Genetic fate tracing revealed a high number of $Gli1^+$ cells in the media and neointima during CKD (Figure 3 C-E). Quantification of $Gli1^+$ cells in media indicated a slight increase of $Gli1^+$ cells during aging in the sham group while significantly higher numbers of $Gli1^+$ cells were present in the media of CKD mice at both early and late time points (Figure 3 D, E). While we never detected $Gli1^+$ cells in the intima of the sham groups, we noticed a dramatic increase of $Gli1^+$ $tdTomato^+$ cells in the neointima at 16 weeks after CKD induction (Figure 3 C, E). A plasticity of cells from the vascular wall has been described and it has been recently reported that vSMC in atherosclerotic plaque acquire expression of macrophage markers such as CD68 (Feil et al., 2014; Shankman et al., 2015). We therefore performed CD68 co-staining; however, while we detected massive numbers of macrophages in the neointima during CKD, we did not observe Gli^+ cells expressing CD68 (Figure 3F).

Adventitial $Gli1^+$ cells differentiate into osteoblast-like cells during medial and intimal calcification in CKD

Our results indicated that adventitial $Gli1^+$ cells are MSC-like with osteogenic differentiation potential *in vitro*. It has been hypothesized that MSC of the vascular wall are drivers of vascular calcification by differentiating into osteoblast-like cells (Sage et al., 2010). We therefore asked whether $Gli1^+$ cells differentiate into osteoblast-like cells during vascular calcification in CKD.

To assess the severity of vascular calcification and to co-localize calcification with tdTomato signal, a subgroup of mice (n=4 each) were injected with near-infrared (NIR) calcium tracer, a fluorochrome-conjugated bisphosphonate for detection of hydroxyapatite formation (Aikawa et al., 2007). We isolated the heart with the whole aortic arch and subclavian and carotid arteries for *ex vivo* Fluorescence Reflectance Imaging (FRI) of tdTomato and NIR calcium tracer (Figure 4A-C). While tdTomato signal intensity was relatively low soon after induction of recombination (10d), it increased over time in the sham group, and was substantially elevated in the CKD group, reflecting the increased number of Gli1-$tdTomato^+$ cells in chronic injury (Figure 4A-C). We detected NIR calcium tracer limited to the aortic arch at 10 weeks after CKD induction and considerable hydroxyapatite mineralization in the whole thoracic aorta and the carotid arteries at 16 weeks after subtotal nephrectomy (Figure 4C). Of note, the areas with the highest intensity of hydroxyapatite (green) co-localized with the areas of the highest tdTomato signal (Figure 4C). High magnification images of the aortic arch confirmed the co-localization and showed that NIR calcium tracer positive areas appeared in close proximity to $tdTomato^+$ cells (Figure 4E).

While (10d) Gli1$^+$ cells were restricted to the adventitia, distinct from calponin expressing vSMC directly after recombination (Figure 4D), we detected increased numbers of tdTomato$^+$ cells in the media in sham and CKD mice and in the neointima of the CKD group over time (Figure 4E). We quantified the percentage of Gli1-derived cells that co-localized with the mature SMC marker calponin (Fig 4F-H). While Gli1 cells localized in the adventitia never expressed calponin, a majority of Gli1-derived cells in the media co-expressed calponin in the sham groups (Figure 4F-G). However, in the media of the CKD mice (16 wk) a significantly lower percentage of Gli1$^+$ cells co-stained for calponin (Figure 4G). Similarly, a high number of Gli1-derived cells in the neointima co-expressed calponin (70%) 10 weeks after CKD induction, whereas at 16 weeks after nephrectomy, where severe calcification occurred in the vascular wall, a significantly lower percentage of intimal Gli1-derived cells co-localized with calponin (20%) (Figure 4E, G-H). These results suggest that Gli1$^+$ cells might first differentiate towards the vSMC lineage, but during chronic injury in CKD they dedifferentiate and lose expression of SMC markers.

We next asked whether these dedifferentiated Gli1-derived cells indeed become osteoblast-like cells with nuclear expression of the master osteogenic differentiation transcription factor Runx2 (Cbfa1) during calcification of media and intima in CKD. Co-staining for Runx2 revealed that Gli1$^+$ cells within NIR calcium tracer positive areas in media and intima and expressed nuclear Runx2 (Figure 5A) whereas we did not detect Runx2 expression in the control group. Quantification indicated that a high fraction of Gli1$^+$ cells in both media (Figure 5B) and intima (Figure 5C) of CKD mice showed nuclear expression of Runx2. Alkaline phosphatase (ALP) activity is another important characteristic of osteoblast differentiation. To detect activity of ALP in Gli1$^+$ cells, we performed co-staining with an ALP substrate and co-stained for tdTomato on frozen sections. We identifed many Gli1-derived cells with high ALP activity in the aortic wall of CKD mice (Figure S3A), thus providing further evidence that Gli1$^+$ cells differentiate into osteoblast-like cells during vascular calcification in CKD. Osteochondrogenic differentiation has also been reported to be involved in vascular calcification (Speer et al., 2009) and Gli1$^+$ cells possess chondrogenic differentiation capacity *in vitro*. We therefore asked whether Gli1$^+$ cells differentiate into chondrocytes *in vivo* and performed co-staining for Sox9 and Runx2 (Figure S3B). Interestingly, we detected very few tdTomato$^+$ cells expressing Sox9 or both Runx2 and Sox9 (Figure S3B-D), suggesting that chondrogenic differentiation of Gli1$^+$ cells is a rare event in our mouse model.

Our data so far suggested that Gli1-derived cells first differentiate towards vSMC lineage, but then lose expression of vSMC markers such as calponin during chronic injury in CKD. However, an alternate interpretation might be that adventitial Gli1$^+$ cells directly differentiate towards osteoblast like cells without prior differentiation towards vSMC. To distinguish between these possibilities, we asked whether the Gli1-derived cells that differentiate into osteoblast-like cells (Runx2$^+$) express markers of contractile or synthetic vSMC. Co-staining for α-SMA and calponin revealed that the Runx2 expressing Gli1-derived cells in both media and intima did not express markers of contractile vSMC (Figure 5 D-E). However, costaining for tropomyosin 4 (TPM4), nonmuscle myosin heavy chain 2b (nmMHCIIb) and vimentin indicated that most Gli1$^+$ derived osteoblast-like cells did express markers of synthetic vSMCs (Figures 6A-F, S4A-C). Of note, while adventitial

Gli1$^+$ cells already expressed vimentin, they were negative for both TPM4 and nmMHCIIb, whereas Gli1$^+$ derived cells in media and neointima showed expression of vimentin, TPM4 and nmMHCIIb (Figures 6A-F, S4A-C).

Together these results indicate that Gli1$^+$ cells differentiate into osteoblast-like cells during arterio- and athero-sclerosis in CKD. The co-staining data for contractile and synthetic vSMC markers suggest that Gli1-derived cells first differentiate towards a vSMC lineage and maintain their progenitor potential to de-differentiate into osteoblast-like cells in CKD. However, it might also be possible that adventitial Gli1$^+$ cells directly differentiate towards osteoblast like cells co-expressing synthetic vSMC markers without prior differentiation towards the vSMC lineage.

We next asked whether Gli1$^+$ cells and Gli1 derived vSMC differ in their *in vitro* osteogenic differentiation capacity. Gli1$^+$ cells sorted from mouse adventitia were cultivated in vSMC differentiation conditions or standard MSC medium for 35 days followed by 14 days of osteogenic differentiation medium (Figure S4D-E). Interestingly, quantification of calcium content showed significantly lower levels in the Gli1 derived vSMCs (Figure S4F). This suggests that undifferentiated Gli1$^+$ cells might have a higher calcification potential compared to Gli1-derived vSMCs.

Gli1$^+$ cells show a heterogenous expression profile in single cell qPCR analysis

It remained unclear whether the Gli1$^+$-labeled cell population is a homogenous progenitor population or instead a heterogenous population. To address this question we sorted a total of 96 Gli1$^+$ single cells from adventitia of bigenic Gli1CreER;tdTomato mice 10 days after tamoxifen pulse and performed Single cell PCR. We chose MSC markers, Hh pathway members, vSMC markers and markers that have been reported in the literature for adventitial progenitor cells. The analysis clearly indicated heterogeneity of the progenitor population, as the cells clustered into three subgroups (Figure 6G). Interestingly, while all cells expressed the mesenchymal marker vimentin and most cells expressed reported MSC/adventitial progenitor markers such as CD29, Sca1, CD105, CD34 we also observed expression of smooth muscle cell markers such as SM22α and smoothelin in various cells. In addition, only a small fraction of Gli1$^+$-labeled cells exhibited Gli1 expression in the qPCR analysis, while significantly more cells showed expression of other Hh pathway members such as ptch1, GILZ and cyclin D2 (Figure 6G).

Since this result suggested that the Gli1-labeled cells are indeed a heterogeneous population, we next asked whether Gli1$^+$ cells differ in their *in vitro* capacity to differentiate towards the osteoblast or vSMC lineage. We sorted single Gli1$^+$ cells from the adventitia of bigenic Gli1CreER^{t2};tdTomato mice 10 days after tamoxifen into 96 well plates and assessed the osteogenic and myogenic differentiation capacity of the single cell clones. Interestingly, following sorting and osteogenic differentiation, all detectable living colonies were von Kossa positive (Figure S4G). Similarly following sorting and vSMC differentiation all detectable living colonies were calponin positive (Figure S4H). This data suggested that despite heterogeneity in the single cell qPCR analysis all Gli1$^+$-labeled cells possess both myogenic and osteogenic potential *in vitro*. However, we cannot exclude that some unipotent Gli1$^+$ cells that have been sorted did not survive in vitro.

Activation of smoothened drives Gli1 proliferation and enhances calcification *in vitro*

Our data so far suggested that Gli1^{+} cells are progenitors of vSMC and osteoblast like cells of the vascular wall. Because Gli1 is a readout of the canonical Hh pathway we asked whether activation of smoothened might affect osteogenic or vSMC differentiation of Gli1^{+} cells. While treatment with smoothened agonist (SAG) significantly increased Gli1 mRNA expression of adventitial Gli1^{+} cells, it did not affect expression of SM-calponin (Figure S5A). However, interestingly, while SAG treatment did not induce osteogenic differentiation of Gli1^{+} cells by itself it enhanced the calcification of Gli1^{+} cells during osteogenic differentiation (Figure S5D). There are many published examples of Hh signaling driving cell proliferation, we therefore asked whether smoothened activation affects proliferation of Gli1^{+} adventitial cells. Indeed, SAG treatment significantly increased proliferation of Gli1^{+} cells as assessed by cell-cycle flow cytometry (Figure S5D). These data suggest a potential role of canonical Hh signaling in Gli1^{+} cell expansion and calcification.

Genetic ablation of Gli1^{+} cells before injury ameliorates vascular calcification in CKD

Since Gli1^{+} cells massively migrate into media and intima during arterial remodeling in CKD and acquire expression of osteogenic markers within areas of matrix mineralization, we next asked whether the adventitial progenitors are required for arterial calcification in the vascular wall. We generated triple transgenic ApoE$^{-/-}$ mice in which Gli1^{+} cells specifically express the simian diphtheria toxin receptor (iDTR) (Gli1CreER$^{t2+/-}$;iDTR$^{+/-}$;ApoE$^{-/-}$). To induce expression of the iDTR in Gli1^{+} cells mice received tamoxifen at 8 weeks of age (Figure 6 A). To genetically ablate Gli1^{+} cells, we injected a high dose of diphtheria toxin (DTX, 50ng/g) or vehicle (VEH) 10 days after the last tamoxifen dose (Figure 6A). We previously verified that this dosage of DTX sufficiently ablates Gli1^{+} cells (Kramann et al., 2015). Mice were subjected to subtotal nephrectomy followed by western diet and sacrificed 16 weeks after CKD induction (Figure 6A). BUN measurements showed no significant difference between ablated (DTX) and non-ablated (VEH) group (Figure 6B). Aortic mRNA expression of iDTR showed a significant decrease in the DTX group confirming successful Gli1^{+} cell (Figure 6C). Atherosclerotic plaque size was quantified in scanned, hematoxylin-eosin stained whole aortic arches (Figure 6D-E). While we detected a tendency towards smaller plaque size in the DTX group, this was not statistically significant (Figure 6E). Immunostaining for CD68 showed high numbers of macrophages within intima plaques of both DTX and VEH group, as expected in this model of severe atherosclerosis (Figure 6 F). *Ex vivo* FRI for NIR calcium tracer confirmed significantly reduced vascular calcification in the DTX group when compared to the VEH group (Figure 6 G-H). This was accompanied by abolished Runx2 mRNA expression in the aorta of the mice injected with DTX (Figure 6I). Von Kossa staining and ALP activity confirmed this finding of significantly reduced mineralization in the vascular wall of mice where Gli1^{+} cells have been ablated (Figure S5F-G). This data provides strong direct evidence that Gli1^{+} cells are an important cause of vascular calcification in CKD.

Evidence for Shh and Gli1^{+} expressing cells in human calcified arteries from CKD patients

Our data indicated that Gli1^{+} adventitial cells are progenitors of osteoblast-like cells in mouse models of CKD induced vascular calcification and suggest a potential role of

canonical Hh signaling in Gli1$^+$ cell calcification and expansion. This prompted us to ask whether Gli1$^+$ cells also exist in human arteries and whether we find any evidence for Shh secreting cells. We therefore stained human arteries obtained from consented clinical autopsies from dialysis patients and non-CKD patients for Gli1 and Shh (Figure 6J). In arteries from non-dialysis patients Gli1 expression was predominantly in the adventitia while in arteries of dialysis patients we observed strong Gli1 expression in calcified media and atherosclerotic plaques (Figure 6J). An adventitial domain of Shh signaling has been reported by Passman et al. in mice (Passman et al., 2008). In line with this finding we detected Shh expressing cells in the arterial adventitia of arteries from non-CKD patients including endothelial cells of the adventitial vasa vasorum (Figure 6J). However, in the calcified arteries from dialysis patients we detected strong Shh staining in media and neointima (Figure 6J). Based on these results we speculate that adventitial Gli1$^+$ progenitors also exist in human arteries and that Shh signaling might be involved in their expansion. However, further studies are needed to prove this hypothesis.

Discussion

Vascular diseases are the leading cause of death in the developed world (Tabas et al., 2015). Therefore, understanding the critical cell-types involved in vascular regeneration and disease is of major importance and will guide the development of novel targeted therapeutics. It has been hypothesized that adventitial progenitor cells might contribute to acute injury repair of the vascular wall and also to vascular disease such as vascular calcification (Majesky et al., 2012; Sage et al., 2010). However, no inducible genetic fate tracing experiments convincingly demonstrated a role of adventitial progenitors in vascular regeneration and disease (Nguyen et al., 2013). Our inducible genetic fate tracing data indicate that Gli1$^+$ MSC-like cells of the adventitia are vSMC progenitors that contribute to repair after acute injury to the femoral artery. In addition, during chronic injury in CKD adventitial Gli1$^+$ cells de-differentiate into osteoblast-like cells contributing to vascular calcification. The fact that genetic ablation of Gli1$^+$ cells before injury dramatically reduces vascular calcification in the arterial wall during CKD clearly indicates that adventitial Gli1$^+$ MSC-like cells are a critical cause of vascular calcification in CKD.

A classical hypothesis holds that in response to injury, terminally differentiated vSMC undergo a phenotypic switch, downregulating contractile proteins, and proliferating and migrating into the neointima (Tabas et al., 2015). Indeed, Nemenoff *et al.* showed in genetic fate tracing of vSMCs using a tamoxifen-inducible genetic mouse model (MHC-CreER^{t2}; R26-floxStop/βGal), that terminally differentiated vSMC down-regulate α-SMA and contribute to neointima formation at 7 days after femoral artery wire-injury (Nemenoff et al., 2011). While a significant role for mature vSMC in acute vascular injury and atherosclerosis is becoming evident (Shankman et al., 2015), a role of resident vascular progenitor cells is controversial and heavily debated (Cho et al., 2013; Nguyen et al., 2013; Psaltis and Simari, 2015; Tang et al., 2012).

The arterial adventitia has been reported as a progenitor niche for the vascular wall (Majesky et al., 2012). Several groups have reported SMC progenitors in the adventitia (Hu et al., 2004; Majesky et al., 2011; Passman et al., 2008). Adventitial cells that express stem cell

markers such as Sca1 and/or CD34 can be differentiated into vSMC *in vitro* and mural cells *in vivo* (Hu et al., 2004; Majesky et al., 2011). Gli1$^+$ cells express several of the previously reported vascular progenitor markers, including CD34, Sca1 and PDGFRβ. Therefore, it is likely that Gli1$^+$ cells are similar to some of the previously reported adventitial progenitor cells. In fact the Ptc1$^+$, Sca1$^+$, PDGFRβ$^+$ and CD34$^+$ adventitial cells with *in vitro* vSMC differentiation capacity described by Passman *et al.* may be identical to the Gli1$^+$ cell population we describe here (Passman et al., 2008). We provide the first inducible genetic fate tracing data suggesting that adventitial MSC-like progenitor cells can differentiate into vSMC and contribute to neointima formation after wire-injury of the femoral artery.

Vascular calcification is an important contributor to cardiovascular morbidity and mortality (Paloian and Giachelli, 2014). CKD represents one of the most common causes of vascular calcification (Aikawa et al., 2009; Paloian and Giachelli, 2014). Vascular calcification can occur in the intima during late stage atherosclerosis and in the media mostly in patients with CKD and / or diabetes. Calcification in the media, also described as Monckeberg's arteriosclerosis, is more specific for CKD and even children with CKD develop this form of calcification (Shroff et al., 2008). Once considered a passive precipitative process, it is now appreciated that vascular calcification is an active cell-mediated process resembling osteogenesis (Shroff et al., 2013). Because all cells involved in endochondral ossification share a common mesenchymal progenitor cell, it has been hypothesized that MSC might drive the calcification process of the vessel wall (Sage et al., 2010). However, the current dogma favors a model similar to neointima formation after acute vascular injury where mature vSMCs undergo a phenotypic switch and (de)differentiate into osteoblasts and chondrocytes driving the calcification process in media and intima (Shroff et al., 2013).

Brighton et al. first decribed the osteogenic potential of pericytes (Brighton et al., 1992) and subsequently Bostrom and Demer described 3G5$^+$ pericyte-like cells cloned from nodules of SMC from human aorta with calcification capacity *in vitro* (Bostrom et al., 1993). They subsequently termed these cells calcifying vascular cells (CVC) (Watson et al., 1994) (Tintut et al., 2003). Surgical stripping of the adventitia has been reported to reduce calcification of the media (Bujan et al., 1996). Towler and colleagues described that genes of the Msx family might regulate calcification of SM22α expressing adventitial myofibroblasts (Cheng et al., 2003; Shao et al., 2005; Towler et al., 1998).

Hu et al. transferred genetically β-Gal-tagged Sca1$^+$ adventitial cells to the adventitial side of vein grafts in ApoE$^{-/-}$ mice and reported that 20% of cells in atherosclerotic lesions expressed β-Gal (Hu et al., 2004). Similarly, Chen *et al.* performed vein graft experiments and reported that adventitial Sca1$^+$ cells contribute to 30% of neointimal vSMC (Chen et al., 2013). Grudzinska *et al.* transplanted aortae with labeled adventitial tissue and reported that a large proportion (79%) of intimal cells originated from the adventitia (Grudzinska et al., 2013). The authors further hypothesized, based on surface marker expression, that these adventitial cells might be MSC (Grudzinska et al., 2013). However, the Owens group recently demonstrated in elegant genetic fate tracing experiments using Myh11CreERt2; R26-floxStop-eYFP; ApoE$^{-/-}$ mice that mature vSMC undergo a phenotypic switch and lose expression of classical vSMC markers contributing to as much as 30% of all cells in atherosclerotic lesions (Shankman et al., 2015). This dedifferentiation even lead to

expression of macrophage and MSC markers by a sub-population of vSMC (Shankman et al., 2015).

Thus, there is evidence for both adventitial progenitor cells and medial vSMCs contributing to atherosclerotic plaque formation. However, it remains unclear which cells contribute to the population of osteoblast-like cells driving vascular calcification. Two groups have performed genetic fate tracing experiments using a non-inducible SM22αCre driver to trace the fate of vSMC in vascular calcification in different mouse models (Naik et al., 2012; Speer et al., 2009). This work clearly indicates that SM22α expressing cells become osteoblast-like cells in calcifying arteries. However, a contribution of adventitial progenitor cells that either already express SM22α or acquire expression of SM22α after injury, and thus become genetically marked by Cre recombinase during the experiment, cannot be excluded.

Indeed, our single cell qPCR analysis demonstrates that many $Gli1^+$ adventitial progenitors express SM22α, and as mentioned above, adventitial $SM22\alpha^+$ myofibroblasts have been reported to contribute to vascular calcification (Cheng et al., 2003; Shao et al., 2005; Towler et al., 1998). Our data suggests that $Gli1^+$ cells acquire vSMC marker expression during both aging and injury. During vascular calcification in CKD, $Gli1^+$ cells acquire Runx2 and ALP expression suggesting that they differentiate towards the osteoblast lineage. Because most Runx2 expressing $Gli1^+$ cells did not show co-expression of contractile vSMC markers but expressed secretory vSMC markers, we speculate that adventitial $Gli1^+$ cells first differentiate towards a vSMC lineage, but eventually retain their stem cell-like potential and de-differentiate to osteoblast-like cells during vascular calcification. Alternatively, adventitial $Gli1^+$ cells might directly differentiate towards osteoblast like cells and acquire expression of secretory vSMC markers. The fact that genetic ablation of $Gli1^+$ cells before CKD induction abolished vascular calcification suggests that adventitial $Gli1^+$ cells are a major source of osteoblast-like cells in the vascular wall and an important source of vascular calcification in CKD. Future studies will reveal whether this finding can be translated into novel therapeutic approaches and whether pharmacologic targeting of $Gli1^+$ cells might reduce vascular calcification severity.

Of note, while there is some evidence that bone marrow-derived cells also contribute to the pool of calcifying vascular cells (Cho et al., 2013; Wang et al., 2014), other groups have reported that there is no significant contribution of bone marrow-derived cells to atherosclerotic plaque formation (Bentzon et al., 2007; Daniel et al., 2010). Importantly, we have previously shown in bone marrow transplantation and parabiosis experiments that $Gli1^+$ cells do not circulate (Kramann et al., 2015), suggesting that resident vascular cells are driving the vascular calcification process. Furthermore, mouse arteries do not have significant numbers of vasa vasorum in homeostasis (Ritman and Lerman, 2007); however, although unlikely, we can not completely exclude that $Gli1^+$ cells that are pericytes of vasa vasorum or the periadventitial adipose tissue might also contribute to arterial remodeling in our mouse models.

Our staining data of human arteries suggests that a population of adventitial $Gli1^+$ cells might exist in human arteries, and we observed strong Gli1 expression in media calcification

and atherosclerosis. Further studies are needed to determine whether Gli1^{+} cells are MSC in human adventitia and novel therapeutic targets in vascular calcification.

Experimental procedures

Mice

All mouse experiments were performed according to the animal experimental guidelines issued by the Animal Care and Use Committee at Harvard University and Washington University. Gli1CreER^{t2} (i.e. Gli1$^{tm3(re/ERT2)Alj}$/J, JAX Stock #007913), Rosa26tdTomato (i.e. B6-Cg-Gt(ROSA)26Sort$^{tm(CAG-tdTomato)Hze}$/J JAX Stock # 007909) iDTR mice (i.e. C57BL/6-Gt(ROSA)26Sor$^{tm1(HBEGF)Awai}$/J, JAX Stock # 007900) and ApoEKO mice (ApoEtm1Unc, JAX Stock # 002052) were purchased from Jackson Laboratories (Bar Harbor, ME). Offspring were genotyped by PCR according to the protocol from the Jackson laboratory. For lineage tracing studies 6-7 week old mice received 3× 10 mg tamoxifen in corn oil / 3% ethanol (Sigma) via oral gavage 10days before surgery. Subtotal nephrectomy surgery (5/6Nx) was performed in a two step method as described previously (Aikawa et al., 2009) with subtotal nephrectomy of the left kidney followed by right uninephrectomy one week later.

Mice received a high fat diet (21% fat and 0.21% cholesterol) that was obtained from Research Diets (D12079B, New Brunswick, NJ). In the sham group kidneys were exposed by flank incision. Sham mice received standard mouse chow. Transluminal arterial injury was induced surgically as previously described, (Sata et al., 2000). Briefly, a straight spring wire (0.38mm, C-SF-15-15, Cook) was inserted into the femoral artery under microscopic observation. The wire was moved 10 times in and out of the femoral artery. Mice were sacrificed at day 28 after surgery.

Gene / Protein expression

Primers, antibodies and methods are described in the Supplementary Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Gli1^{+} adventitial MSC-like cells are vascular smooth muscle cell progenitors
- After arterial injury Gli1^{+} cells contribute to neointima formation and arterial repair
- Gli1+ adventitial cells form osteoblast like cells during vascular calcifcation
- Genetic ablation of Gli1^{+} cells before injury abolishes vascular calcification

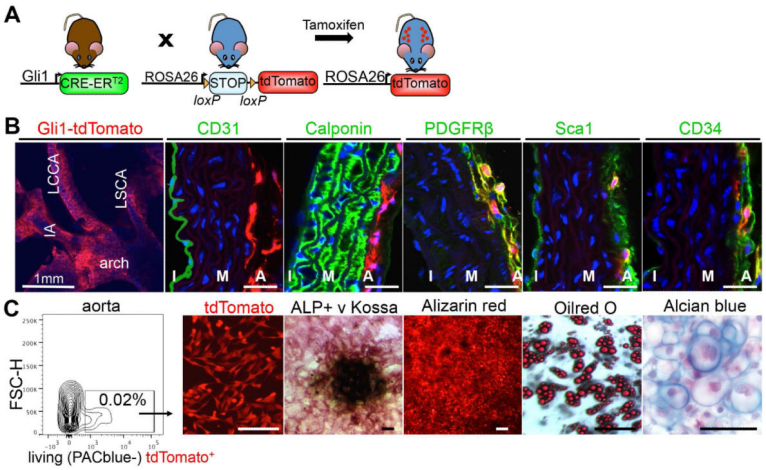

Figure 1. Gli1 defines an adventitial population of Mesenchymal Stem Cells

(A) Adult Gli1Cre-ER^{t2}; tdTomato mice were injected with tamoxifen at 8 weeks of age to genetically tag Gli1 expressing cells with the bright red fluorochrome tdTomato.

(**B**) Gli1-tdTomato$^+$ form an extensive network in the adventitia of arteries distinct from endothelial and vascular smooth muscle cells. (IA, innominate artery; LCCA, left common carotid artery and LSCA, left subclavian artery). Scale bars 25μm

(C) Sorted Gli1-tdTomato$^+$ cells possess trilineage differentiation capacity towards osteoblasts (ALP-alkaline phosphatase & von Kossa and Alizarin red staining), adipocytes (Oilred O staining) and chondrocytes (Alcian Blue staining) Scale bars 50μm

See also figure S1

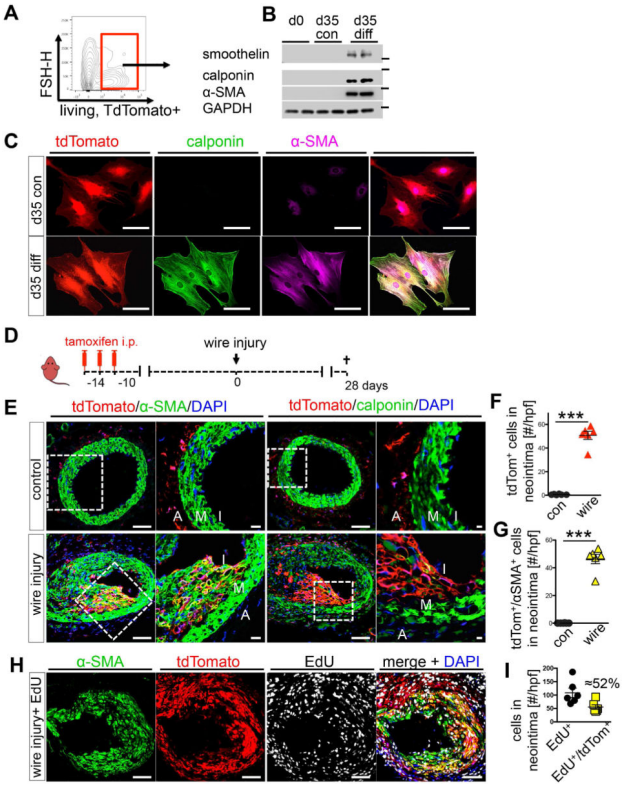

Figure 2. Adventitial Gli1^{+} cells are progenitors of vascular smooth muscle cells and contribute to neointima formation after acute vascular injury

(A) Gli1-tdTomato^{+} cells were isolated from micro dissected and digested aortic arches by fluorescence activated cell-sorting for tdTomato.

(B) Representative Western blots of adventitial Gli1^{+} cells directly after isolation (d=0), after cultivation in Mesenchymal Stem Cell Medium for 35 days (d35 con) and after cultivation in smooth muscle cell differentiation medium with transforming growth factor beta (TGF-β) and platelet derived growth factor BB (PDGF-BB) for 35 days (d35 diff)

(C) Representative images of adventitial Gli1+ cells stained for calponin and alpha smooth muscle actin after cultivation in control (d35 con) or smooth muscle differentiation medium (d35 diff) for 35 days. Scale bars 25μm.

(D) Bigenic Gli1CreER^{t2};tdTomato mice (n=6, 3 females, 8 week old) received tamoxifen (3×10mg p.o.), were subjected to wire injury of the right arteria femoralis 10 days after the last tamoxifen dose and sacrificed 4 weeks after the wire injury surgery. The contralateral arteria femoralis of each mouse served as control.

(E) Genetic fate tracing of Gli1^{+} cells after wire injury. Representative images of the injured (wire injury) and contralateral (control) femoral artery 28 days after injury costained for alpha-smooth muscle actin (α-SMA) and calponin. (A, adventitia; M, media; I, intima). Scale bars 50μm

(F-G) Quantification and co-expression of smooth muscle cells markers of Gli1^{+} derived cells (tdTom^{+}) in neointima after wire injury (wire) of the femoral artery compared to contralateral non injured femoral artery (con). ***p<0.001 by t-test, mean ± SEM.

(H-I) Represenative images and quantification of a fate tracing experiment with wire injury in bigenic Gli1CreER^{t2};tdTomato mice (n=6 males, 8 week old) with daily injections of 5-ethynyl-2-deoxyuridine (EdU) to label proliferating cells.
See also Figure S2

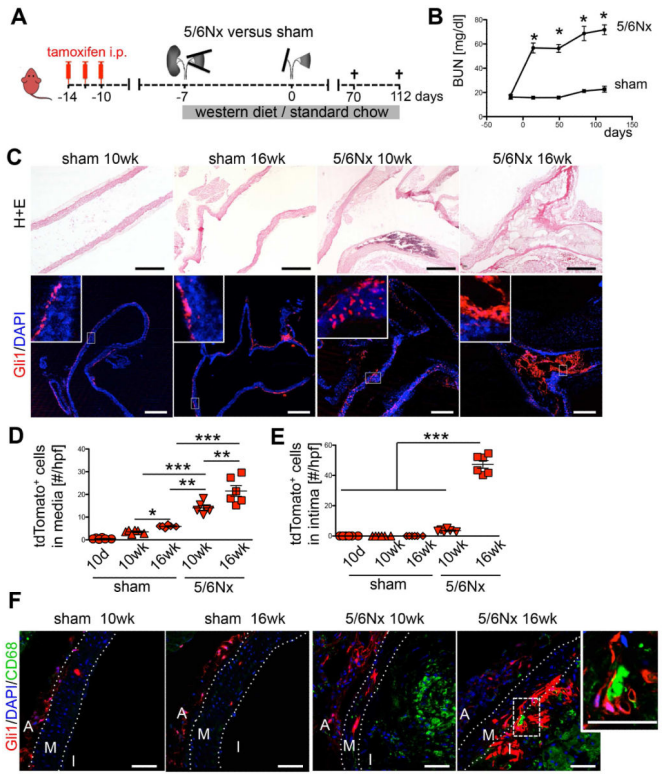

Figure 3. Gli1^{+} cells migrate in media and neointima during vascular remodeling in chronic kidney disease

(A) Experimental schedule of genetic fate tracing analysis of Gli1^{+} cells during athero- and arteriosclerosis in CKD using triple transgenic Gli1CreER^{t2};tdTomato;ApoEKO mice. Subtotal nephrectomy (5/6Nx; n=18, 6 females) or sham (n=12, 4 females) surgery was performed at 8 weeks of age mean ± SEM. (n=6 mice, 2 females of the 5/6Nx group died during the follow up).

(B) Blood urea nitrogen (BUN) stratified for the sham and subtotal nephrectomy (5/6Nx) group. *p<0.001 by t-test

(C) Representative low magnification images of the aortic arch (scale bar 500μm).

(D-E) Quantification of Gli1 derived cells in media and intima/neointima by counting in high power field (hpf, 400x, n=6 each). **p<0.01; ***p<0.001 by one was ANOVA with posthoc Tukey, mean ± SEM.

(F) Representative images of costaining for the macrophage marker CD68 Scale bars 50μm, inserts 25μm (A, adventitia; M, media; I, intima)

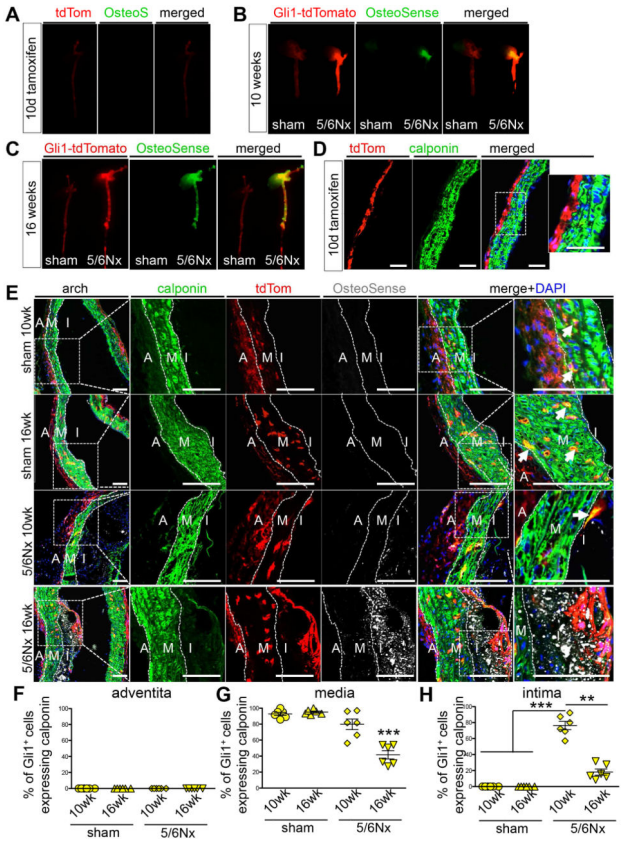

Figure 4. Gli1^{+} cells acquire smooth muscle cell markers during aging but de-differentiate during chronic injury with vascular calcification

(**A-C**) Representative images of hydroxyapatite imaging (OsteoSense; Cy5 conjugated bisphosphonate) indicating severe vascular calcification at 16 weeks after subtotal nephrectomy (5/6Nx) with increased tdTomato signal in areas of hydroxyapatite deposition. (**D**) Representative image of the aortic wall from a Gli1CreER;tdTomato;ApoEKO mice at 10days after tamoxifen injection. Scale bars 50μm

(**E**) Representative images of the aortic arch from Gli1CreER;tdTomato;ApoEKO mice at 10 and 16 weeks after sham surgery or subtotal nephrectomy (5/6Nx). Mice received OsteoSense intravenously 24 hours before sacrification to stain for hydroxyapatite deposition. Scale bars 50μm, arrows indicating co-localization of calponin and tdTomato **(F-H)** Quantification of the fraction of Gli1^{+} cells in adventitia, media and intima that coexpress the smooth muscle marker calponin (n=6 each). **p<0.01; ***p<0.001 by one way ANOVA with posthoc Tukey, mean ± SEM.

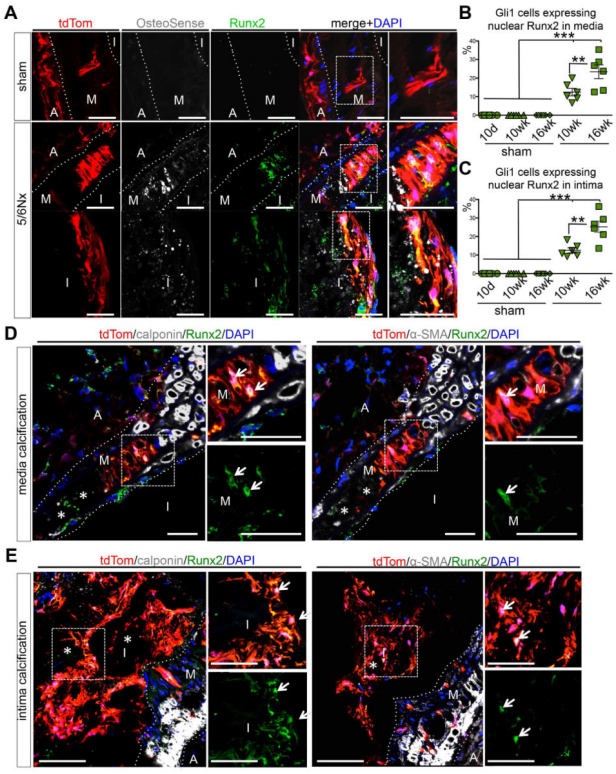

Figure 5. Gli1^{+} cells differentiate into osteoblast-like cells during media and intima calcification in CKD

(A) Representative images of OsteoSense^{+} calcified areas (hydroxyapatite deposition) in media and neointima costained for Runx2 (Cbfa1). Of note, we always observed close spatial relation of calcified areas with Gli1 derived cells (tdTomato+) that showed nuclear expression of Runx2. Scale bars 25μm (A, adventitia; M, media; I, intima)

(B-C) Quantification of the fraction of Gli1 derived cells (tdTOmato+) that showed nuclear expression of Runx2. **p<0.01; ***p<0.001 by one way ANOVA with posthoc Tukey, mean ± SEM

(**D-E**) Representative images of calcified areas (asterisks) in media (M) and neointima (I) at 16 weeks after subtotal nephrectomy. Costaining was performed for the smooth muscle cell markers alpha smooth muscle actin (α-SMA, ACTA2) and calponin with the osteoblast transcription factor Runx2 (Cbfa1, arrows). Of note, Gli1 derived cells (tdTomato+) within calcified areas of media and intima express Runx2 indicating the osteoblastic differentiation but do not express smooth muscle cells markers. Scale bars 50μm, inserts 25μm, A, adventitia, M, media, I, intima.

See also Figure S3.

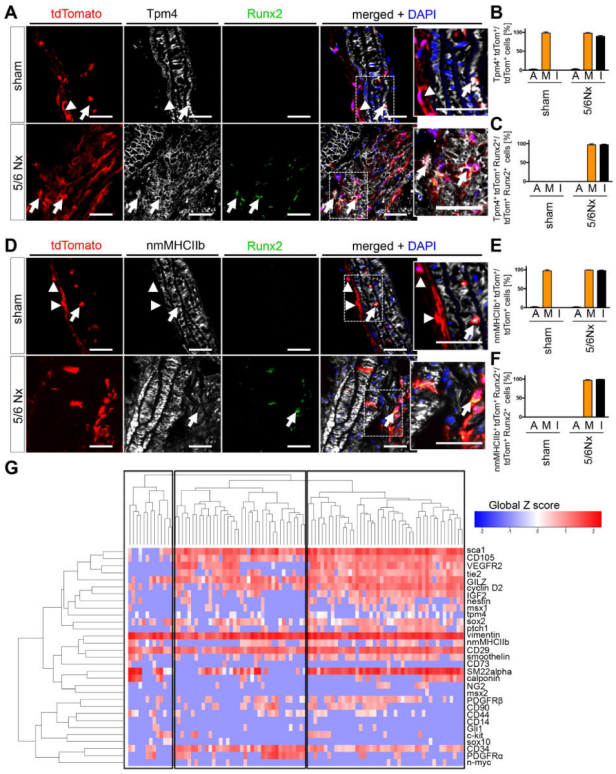

Figure 6. Single cell qPCR indicates heterogeneity of the Gli1^{+} cells population that acquires expression of secretory vSMC markers upon migration into media and intima

(A-C) Representative images and quantification of tdTomato^{+} cells co-stained for Runx2 and tropomyosin 4 (Tpm4) at 16 weeks. arrowheads indicating adventitial tdTomato^{+} cells that do not express TPM4, arrows upper panel indicating tdTomato+ cells in media coexpressing Tpm4 , arrows lower panel indicating tdTomato^{+} cells coexpressing Runx2 and Tpm4. Scale bars 25μm. A, adventitia, M, media, I, intima.

(D-F) Representative images and quantification of tdTomato^{+} cells co-stained for Runx2 and nonmuscle myosin heavy chain IIb (nmMHCIIb) at 16 weeks. arrowheads indicating adventitial tdTomato^{+} cells that do not express nmMHCIIb, arrows upper panel indicating tdTomato+ cells in media coexpressing nmMHCIIb , arrows lower panel indicating tdTomato^{+} cells coexpressing Runx2 and nmMHCIIB. Scale bars 25μm. A, adventitia, M, media, I, intima.

(G) Hierarchical clustering of quantitative realtime PCR (qPCR) data of 31 genes from 98 sorted single Gli1^{+} cells producing a matrix of 2976 values. The data shows three cell clusters and three gene clusters. Gli1+ cells were sorted from from bigenic Gli1CreER;tdTomato mice at the age of 8 weeks 10 days after tamoxifen pulse (3×10mg). See also Figure S4, for single cell qPCR raw data please see the supplemental data i.e. single cell qPCR standard output and single cell qPCR heatmap output

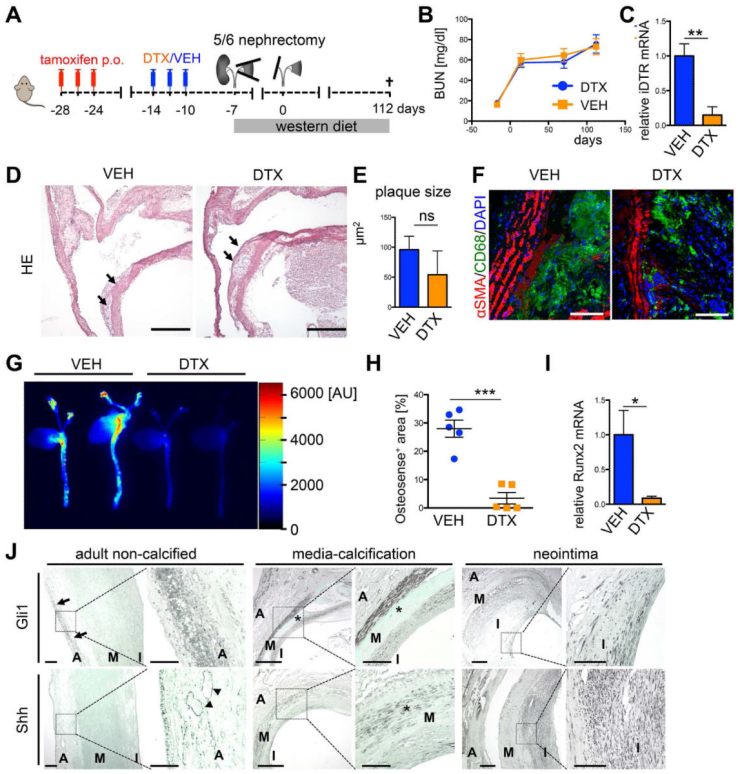

Figure 7. Genetic ablation of Gli1^{+} cells before injury abolishes vascular calcification in CKD

(A) Triple transgenic Gli1CreER^{t2};iDTR;ApoEKO mice (n=18, 12 males) received tamoxifen (3×10mg p.o.) at 8 weeks of age and were injected with either diphtheriatoxin (DTX, 50ng/g BW 3x i.p.) or vehicle (VEH, PBS) 10 days after the last tamoxifen dose. Mice were subjected to subtotal nephrectomy (5/6Nx) 7 days after DTX injection and uninephrectomy of the contralateral kidney one week later. Mice were sacrificed 16 weeks (112 days) after uninephrectomy. All mice received a western diet and OsteoSense i.v. 24hours before euthanasia. During the follow up n=8 mice, 2 females died.

(B) Blood urea nitrogen (BUN) measurements showed no difference between mice that received diphtheriatoxin (DTX) or vehicle (VEH). mean ± SEM

(C) Confirmation of ablation by reduction in mRNA expression for DTR receptor (iDTR) in whole aortic arch. **p<0.01 by t-test, mean ± SEM

(D) Representative hematoxylin-eosin (HE) stained images of the aortic arch from mice that received either vehicle (VEH) or diphtheria toxin (DTX) to ablate Gli1^{+} progenitors. Arrows indicating atherosclerotic plaques. Scale bars 500μm

(E) Quantification of plaque size of whole aortic arches did show a non-significant tendency towards smaller plaque size in the group where Gli1+ cells were ablated (DTX).

(F) Representative images of atherosclerotic intima plaques stained for alpha smooth muscle actin and the macrophage marker CD68. Scale bars 50μm

(G-H) Representative images and quantification of OsteoSense^{+} vascular calcification (hydroxyapatite) indicating dramatic reduction of calcification in mice were Gli1+ progenitors had been ablated before induction of chronic kidney disease. ***p<0.001 by t-test, mean ± SEM

(I) Reduction of Runx2 mRNA expression in the aortic arch of mice where the Gli1+ progenitor pool had been ablated before chronic kidney disease induction. *$p<0.05$ by t-test, mean±SEM

(J) Representative images of human arteries immunostained for Gli1 and sonic hedgehog (Shh), counterstained with methyl green. (A, adventitia; M, media; I, intima; aarrows, $Gli1^+$ cells in the adventitia; arrowheads, endothelial cells of adventitial vasa vasorum, asterisks, calcified media. Scale bars 300μm, inserts 100μm)

See also Figure S5