

Genetic lineage tracing reveals poor angiogenic potential of cardiac endothelial cells

Short title: Poor angiogenic potential of the adult heart

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Abstract

Aims. Cardiac ischemia does not elicit an efficient angiogenic response. Indeed, lack of surgical revascularization upon myocardial infarction results in cardiomyocyte death, scarring and loss of contractile function. Clinical trials aimed at inducing therapeutic revascularization through the delivery of pro-angiogenic molecules after cardiac ischemia have invariably failed, suggesting that endothelial cells in the heart cannot mount an efficient angiogenic response. To understand why the heart is a poorly angiogenic environment, here we compare the angiogenic response of the cardiac and skeletal muscle using a lineage tracing approach to genetically label sprouting endothelial cells.

Methods and results. We observed that overexpression of the Vascular Endothelial Growth Factor in the skeletal muscle potently stimulated angiogenesis, resulting in the formation of a massive number of new capillaries and arterioles. In contrast, response to the same dose of the same factor in the heart was blunted and consisted in a modest increase in the number of new arterioles. By using Apelin-CreER mice to genetically label sprouting endothelial cells we observed that different pro-angiogenic stimuli activated Apelin expression in both muscle types to a similar extent, however only in the skeletal muscle these cells were able to sprout, form elongated vascular tubes activating Notch signaling, and became incorporated into arteries. In the heart, Apelin-positive cells transiently persisted and failed to give rise to new vessels. When we implanted cancer cells in different organs, the abortive angiogenic response in the heart resulted in a reduced expansion of the tumor mass.

Conclusion. Our genetic lineage tracing indicates that cardiac endothelial cells activate Apelin expression in response to pro-angiogenic stimuli but, different from those of the skeletal muscle, fail to proliferate and form mature and structured vessels. The poor angiogenic potential of the heart is associated with reduced tumor angiogenesis and growth of cancer cells.

Keywords: Apelin, Lineage tracing, Angiogenesis, VEGF, cancer

Translational perspective

Cardiac ischemia is a leading cause of morbidity and mortality worldwide. Various clinical trials have attempted to revascularize ischemic hearts through the delivery of pro-angiogenic molecules, but they have invariably failed.

Our results indicate that cardiac endothelial cells sense pro-angiogenic stimuli but are not able to drive an efficient angiogenic response. This poor angiogenic potential is also associated with reduced growth of cancer cells in the heart.

This work provides some mechanisms behind the poor angiogenic capacity of the heart and paves the way to future strategies for the therapy of both cancer and cardiac ischemia.

Introduction

It is common experience that heart ischemia does not elicit an efficient angiogenic response, able to restore an appropriate supply of oxygen and nutrients to the hypoxic region. If not promptly re-vascularized, the infarcted myocardium inevitably heals by scarring, with detrimental consequences on heart function¹. Various angiogenic factors are upregulated early after ischemia, indicating a spontaneous attempt of the heart to induce the formation of new blood vessels and re-vascularize the ischemic region^{2,3}. Yet, this process does not result in an efficient angiogenic response, consistent with the failure of clinical trials aimed at inducing therapeutic angiogenesis in the heart^{4,5}. In addition, recent experimental evidence showed that collateral vessels after myocardial infarction are formed by arteriogenesis (i.e. the appearance of visible arteries from pre-existing small arterioles by rapid lumen opening and vascular wall thickening) and not by angiogenesis⁶.

Different from the heart, tumors have a remarkable capacity to form new blood vessels in response to hypoxia. During tumor growth, the central core becomes progressively hypoxic, inducing the expression of multiple angiogenic factors, among which the most potent is the Vascular Endothelial Growth Factor (VEGF). The VEGF gene encodes for a transcript that is alternatively spliced to produce multiple isoforms, each one characterized by different angiogenic potential⁴. The most potent isoform is the one composed by 165 amino acids (VEGF165)⁷, herein referred to as VEGF. Tumor-derived VEGF potently promotes the formation of new capillaries, thus supporting the further growth of cancer cells⁸. The reasons why a hypoxic tumor efficiently induces an angiogenic response, leading to new blood vessel formation, whereas a hypoxic heart fails to do the same are currently not understood.

Our laboratory has a long-lasting interest in the use of vectors derived from the Adeno-Associated Virus (AAV) for the induction of therapeutic angiogenesis through the delivery of VEGF. This strategy was very effective in promoting the massive formation of new blood vessels after either hind limb ischemia, or other ischemic diseases, in which the vector was delivered to the skeletal muscle⁹⁻¹². However, when we applied the same approach to cardiac ischemia, we have never observed any remarkable angiogenic response and eventually discovered a direct, protective effect of VEGF on cardiomyocytes, independent from angiogenesis¹³. In particular, the delivery of AAV-VEGF after myocardial infarction did not result in a significant endothelial cell proliferation and generation of new capillaries, however we observed an increased number of arteries^{13,14}, again consistent with ongoing arteriogenesis⁶.

To experimentally assess whether the skeletal and cardiac muscle differentially respond to angiogenic stimuli, we took advantage of a genetic lineage tracing approach, in which the Tamoxifen-inducible form of the Cre recombinase (CreER) is expressed under the

control of the Apelin (Apln) promoter¹⁵. Apelin is a highly conserved secreted peptide, acting as the endogenous ligand for the human G protein-coupled receptor APJ¹⁶. Endothelial cells express abundant levels of Apelin during embryonic development, when angiogenesis is active in almost all organs, and markedly down-regulates its expression in adulthood, when they become quiescent^{15, 17}. However, in response to hypoxia, under tissue ischemia or in the context of a tumor, Apelin expression is typically reactivated, particularly by tip cells, which induce stalk cell proliferation through Dll4/Notch1 signaling^{18, 19}, during sprouting angiogenesis^{17, 20}. Thus, the Apln-CreER mouse is a powerful tool to genetically label sprouting endothelial cells and their progeny^{6, 15}. Here, we take advantage of this mouse strain to analyse the response of cardiac and skeletal muscle endothelial cells to pro-angiogenic stimuli and follow their fate in the two organs *in vivo*.

Methods

Extended materials and methods are provided as Supplementary information.

AAV vector production and administration

All rAAV vectors were produced by the AAV Vector Unit at ICGEB Trieste (<https://www.icgeb.org/facilities/avu-core-facility/>)⁷. AAV9 (herein referred to as AAV) vectors were injected into either the tibialis anterior muscle or the left ventricular wall of the adult heart using a 30G syringe^{7,21}.

DNA and RNA extraction and quantification.

Total DNA was isolated using the DNeasy Blood and Tissue Kit to detect and quantify vector DNA by real time PCR²¹. Total RNA was isolated using TRIzol Reagent. Reverse transcription was performed using First Strand cDNA Synthesis Kit with random primers. The expression of *VEGF* and *Gapdh* was detected using specific primers and TaqMan® probes, while the expression of *Notch1*, *Hes1* and *Hey* was detected using SYBR Green.

Protein extraction and VEGF quantification.

Proteins were extracted from the skeletal and cardiac muscle using RIPA buffer supplemented with protease inhibitors. The amount of mouse and human VEGF were quantified using Mouse VEGF Quantikine ELISA (R&D systems) and VEGF human ELISA (Abcam) kits. Data were normalized on protein content.

Mice

All animal experiments were conducted in accordance with guidelines from the Directive 2010/63/EU of the European Parliament on animal experimentation in compliance with European guidelines and International Laws and Policies (EC Council Directive 86/609, OJL 34, 12 December 1987). CD1, Balb/c and C57BL/6 mice were purchased from Harlan Laboratories. Adult *ApInCreER*⁶ mice were crossed with *Rosa26-mT/mG* mice²² to obtain *ApInCreER;Rosa26-mT/mG* animals. To activate Cre-mediated recombination, 4-hydroxytamoxifen was dissolved in corn oil and injected intraperitoneally. To label proliferating cells, 5-ethynyl-2'-deoxyuridine (EdU) was administered intraperitoneally.

B16-F10 melanoma and LG1233 lung adenocarcinoma cells were injected into C57BL/6 mice either into the tibialis anterior skeletal muscle or the left ventricular wall of the heart. Breast cancer 4T1 cells were injected into Balb/c mice. B16-F10 and 4T1 cells were also injected subcutaneously into the right flank. Tumor volume was measured using the formula: $V = \pi/6 * (d_{max}^2 * d_{min}/2)$ ²³.

Mice were anesthetized with isoflurane 1% for intramuscular injections and with ketamine-xylazine (100 mg/kg-40 mg/kg) for intracardiac injections. At the end of the experiment, mice were anesthetized with isoflurane 5% and euthanized by cervical dislocation.

Primary endothelial cell cultures and spheroids

Endothelial cells were selected by magnetic separation and plated in primary 96-well plates coated with fibronectin/gelatin in EGM-2 medium. For Apelin expression assay, cells were cultured in the presence of both 4-hydroxytamoxifen and recombinant human VEGF. For endothelial spheroid formation, cells were seeded into agarose-coated round bottom plates. Spheroids were transferred on top of a collagen layer and cultured in EGM-2 medium supplemented with 4-hydroxytamoxifen to allow endothelial cell sprouting and EGFP expression.

Western Blot

Protein lysates were run in 10% polyacrylamide gels and nitrocellulose membranes probed with the following antibodies: VEGFR2 (Santa Cruz Biotechnology, sc-504), Akt1 (Cell Signaling Technology, 2938), phospho-Akt (Cell Signaling Technology, 4056), ERK1/2 (Cell Signaling Technology, 9102), phospho-Erk1/2 (Santa Cruz Biotechnology, sc-136521), tubulin (Merck, T5168), β -actin (Merck, A3854).

Immunofluorescence, image acquisition and analysis

Hearts and skeletal muscles were fixed in paraformaldehyde (PFA) and equilibrated in sucrose before embedding in OCT and cryosectioning. Sections were transferred on microslides, permeabilized in Triton X-100, blocked in BSA and incubated with primary antibodies. Cells were fixed in PFA, permeabilized, blocked and incubated with primary antibodies. Alexa Fluor-conjugated secondary antibodies were used to detect primary antibodies, while nuclei were counterstained with Hoechst. EdU incorporation was visualized using the EdU Click-iT assay. Images were acquired using a Nikon fluorescent microscope, a Zeiss LSM 880 confocal microscope and analysed using ImageJ2 software²⁴. Three-dimensional rendering of 100 μ m thick sections were obtained using Surface module of Imaris (Bitplane).

Flow cytometry and cell sorting

Samples were stained using antibodies specific for CD31, Dll4, VEGFR1 and CD45. Cells were acquired using a FACSCelesta and sorted using a FACS Aria II (BD).

Statistical analysis

Statistical analysis was performed using Excel and GraphPad Prism 7.0 on data expressed as mean \pm standard error. Unpaired student's T-test and One-Way ANOVA

with Student-Newman-Keuls correction were used to determine statistical significance for the normally distributed datasets, whereas non-normally distributed datasets were analysed by ANOVA on ranks and Mann-Whitney U test. p-value <0.05 was considered significant.

Results

The cardiac muscle is less responsive to VEGF than the skeletal muscle

To analyze the angiogenic response elicited by VEGF in either the skeletal or cardiac muscle, we injected the same dose of AAV-VEGF into the tibialis anterior and the left ventricular wall of 8 weeks old CD1 mice, using an empty AAV vector as a control (AAV-control). Animals were injected with the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) every other day starting from day 14 after vector administration, to specifically assess the proliferative response of endothelial cells to VEGF. All animals were eventually sacrificed at day 30 for molecular and histological evaluation, as schematically represented in **Fig. 1a**. As expected, the number of AAV viral genomes was comparable in the two tissues (**Fig. 1b**) and this resulted also in comparable levels of both human *VEGF* mRNA and protein in both tissues (**Fig. 1c, d**).

Consistent with previous data^{7,9}, we found that VEGF induced massive formation of new α -SMA⁺ arterioles (with a diameter in the 20-200 μ m range) in the skeletal muscle. While the AAV-MCS-injected muscle contained 4 ± 0.25 arterial vessels per field, this number raised up to 13.7 ± 1.1 upon the injection of AAV-VEGF. In contrast, the increase in the number of arterial vessels was modest in the heart (4.8 ± 0.2 and 6.3 ± 0.8 arteries per field in response to AAV-control and AAV-VEGF, respectively). Similar results were obtained by quantifying the number of pixels corresponding to α -SMA⁺ signal, as shown in **Fig. 1e, g**. The difference in the number of capillaries formed in response to VEGF in the two organs was even more evident. While the number of either CD31⁺ (**Fig. 1e, h**) or ERG⁺ endothelial cells (**Fig. 1f, i**) markedly increased upon AAV-VEGF injection in the skeletal muscle, no induction of angiogenesis was observed in the adult heart (**Fig. 1e, f, h, i**). In contrast, VEGF-induced angiogenesis was robust in neonatal hearts (**Supplementary Fig.1 a-g**).

By assessing EdU incorporation in endothelial cells, we observed a massive proliferative response of CD31⁺ endothelial cells in the skeletal muscle and in the neonatal heart, but only a minimal effect in the adult heart (**Fig. 1j, k** and **Supplementary Fig. 1h, i**).

Taken together, these results indicate that the response of endothelial cells to VEGF is blunted after birth in the heart, while it remains robust in the adult skeletal muscle.

VEGF induces sprouting angiogenesis in the skeletal but not in the cardiac muscle

To further characterize the angiogenic capacity of adult skeletal muscle and heart, we took advantage of a transgenic mouse enabling the genetic tracing of Apelin-expressing cells and their progeny (Apln-CreER;R26mT/mG). In this mouse, membrane-localized tdTomato is produced by all cells, while it switches to membrane-EGFP upon tamoxifen-

inducible Cre recombination, occurring only in Apelin⁺ cells. This mouse has been previously used to genetically label sprouting endothelial cells ¹⁵.

We injected either AAV-VEGF or control AAV-control in the skeletal muscle and heart of these mice, followed by tamoxifen administration at days 1 and 7 (**Fig. 2a**). Also in this mouse strain, VEGF overexpression resulted in an increased number of endothelial cells in the skeletal muscle but not in the heart, as detected by the two endothelial markers CD31 (labelling the cell membrane) and ERG (present in the nucleus). In both muscle types the fraction of Apelin⁺ endothelial cells was significantly increased in response to VEGF, indicative of proper response to the growth factor at the individual cell level (**Figs. 2b, c and e**). These Apelin⁺ cells were incorporated in the VEGF-induced arterioles in the skeletal muscle, but not in the heart (**Figs. 2d, f**). Apelin⁺ cells were negative for the pan-leukocytic marker CD45 (**Supplementary Fig. 2**), consistent with their endothelial identity.

To assess whether cardiac endothelial cells were able to respond to a higher dose of VEGF, we injected a 100-fold concentrated viral preparation (**Supplementary Fig. 3a**) in either the heart or the skeletal muscle of ApIn-CreER;R26mT/mG mice. We did not detect any increase in the number of arteries containing EGFP⁺ endothelial cells in any of the two organs compared to the initial concentration of the virus (compare **Fig. 2d** with **Supplementary Fig. 3b, d**). On the other hand, a 100-fold diluted preparation did not elicit any angiogenic response in any muscle type (**Supplementary Fig. 3c, d**).

Thus, VEGF is sensed by endothelial cells in both skeletal and cardiac muscle. In the latter tissue, however, it fails to induce further steps of angiogenesis and Apelin⁺ cells do not contribute to the formation of arterioles.

Tumor cell-induced angiogenesis is impaired in the heart

We wondered whether the response to other angiogenic stimuli might also be impaired in the adult heart. In particular we investigated cancer-associated angiogenesis, knowing that the heart is one of the exceptional organs in the body in which both primary and secondary tumors are extremely rare ²⁵. By taking advantage of the documented capacity of ApIn-CreER;R26mT/mG mice to label sprouting endothelial cells within tumors ¹⁵, we implanted lung cancer LG cells in either the tibialis anterior or the left ventricle of these mice, followed by tamoxifen administration at days 1 and 4. Animals were sacrificed at day 3, 6 and 9 after tumor cell injection for histological evaluation of angiogenesis (**Fig. 3a**). Within the tumors implanted in the skeletal muscle, the number of EGFP⁺CD31⁺ endothelial cells progressively increased over time, resulting markedly higher at day 9 compared to the surrounding, healthy tissue. In contrast, in the heart, endothelial cells switched on Apelin expression early after cancer cell implantation, but the number of these cells within the tumor area progressively decreased, becoming similar to that found

in the surrounding, non-infiltrated myocardium at day 9 (quantification in **Fig. 3b** and representative images at day 9 in **Fig. 3d**; images of the entire time course are in **Supplementary Fig. 4a**).

Next, we wondered whether the progressive decrease in the number of Apelin⁺ endothelial cells in tumor-implanted hearts could be due to their death. By staining tissue sections for the apoptosis marker cleaved Caspase-3, we observed the presence of numerous apoptotic EGFP⁺ cells, particularly evident at day 6 after cancer cell implantation in the heart (representative images and quantification in **Supplementary Fig. 5**). No apoptotic cells were detected in the skeletal muscle.

In the same samples, the number of EGFP⁺ arterioles in the skeletal muscle significantly increased over time in the tumor region compared to the surrounding, non-infiltrated tissue (quantification in **Fig. 3c** and representative images at day 9 in **Fig. 3e**; images of the entire time course are in **Supplementary Fig. 4b**). In contrast, very few EGFP⁺ arteries were detected in the heart, either within tumors or in the surrounding area (**Fig. 3c, e**).

These results reiterate that, similar to what we observed in response to AAV-VEGF, cancer cells induce Apelin expression in endothelial cells of both skeletal muscle and heart, but in the latter organ Apelin⁺ cells die and do not become incorporated into the cancer-associated vessels.

To investigate whether the different angiogenic capacity of skeletal muscle and heart might impact on tumor cell survival and proliferation, we monitored LG tumor volume over time. We found that cancer cells grew at a slower rate in the cardiac than in the skeletal muscle at all analyzed time points (**Fig. 4a**). This marked difference was attributable to both reduced proliferation and increased apoptotic death of cancer cells in the heart, as shown by staining for the proliferation marker Ki67 and the apoptosis marker cleaved Caspase-3 (quantification in **Fig. 4b, c** and representative images in **Fig. 4d, e**).

We also wanted to confirm these findings using two additional cancer cell lines. We injected the same number of 4T1 breast cancer cells and B16-F10 melanoma cells into either the skeletal or cardiac muscle of syngeneic Balb/c and C57BL/6 mice, respectively. At 15 days tumors were clearly detectable by gross examination of the injected organs. Representative pictures of control skeletal and cardiac muscles and the same organs injected with LG, B16-F10 and 4T1 cells are shown in **Fig. 4f-i**. For all the tested cell lines, tumors formed in the skeletal muscle were invariably larger than those formed in the heart (**Fig. 4j-l**) and similar to those implanted subcutaneously (**Supplementary Fig. 6**).

Collectively, these results are consistent with the conclusion that sprouting endothelial cells efficiently contribute to cancer-associated angiogenesis in the skeletal muscle, but fail to do so in the heart.

Cardiac endothelial cells sense the VEGF stimulus but fail to sprout *ex vivo*

Since we detected a similar expression of Apelin in both organs early after the angiogenic stimulus, we aimed at assessing the angiogenic potential of primary endothelial cells isolated from both tissues. First, we isolated endothelial cells from *Apln-CreER;R26mT/mG* mice and exposed them to both tamoxifen and VEGF for 48 hours. Consistent with the *in vivo* results, we observed that both cell types activated Apelin expression in response to VEGF (**Fig. 5a, b**). Next, we assessed their proliferation and found that the number of Ki67⁺ cells isolated from the skeletal muscle significantly increased upon VEGF administration, while that of cardiac cells did not (**Fig. 5c, d**). In both cases, proliferating endothelial cells were largely negative for Apelin (the percentage of Ki67⁺ Apelin⁺ cells was invariably lower than 0.05, as shown by representative images in **Supplementary Fig. 7a**). While we did not detect any major difference in the expression of the major pro-angiogenic receptor VEGFR2, downstream signaling pathways resulted more active in basal conditions in endothelial cells derived from the skeletal muscle compared to those derived from the heart. Phosphorylation of both Akt and ERK1/2 tended to further increase in skeletal muscle, but not in cardiac endothelial cells, stimulated with VEGF in culture, consistent with their different proliferative potential (**Supplementary Fig. 7b-f**).

We also cultured the same cells in non-adhesive round bottom plates favoring their organization into spheroids, which were eventually transferred onto a collagen substrate to allow endothelial cell sprouting. Primary endothelial cells from skeletal muscle efficiently sprouted and formed tubular structures extending out of the spheroids over 2 days, while cardiac endothelial cells essentially remained inside the spheroid body and minimally migrated out of it (**Fig. 5e, f**). Consistent with their higher sprouting capacity, muscle-derived spheroids contained a remarkable number of Apelin⁺ cells in their sprouts (**Fig. 5e, g**), often exhibiting the classic tip cell morphology (inset in **Fig. 5e**). In contrast, cardiac endothelial spheroids contained numerous Apelin⁺ cells in their bodies, similar to what was observed in muscle spheroids (**Fig. 5e, h**), but these cells were not able to sprout (**Fig. 5e, g**).

Thus, endothelial cells from both muscle types respond to VEGF inducing Apelin expression, but those derived from the heart do not proliferate and are incapable of forming angiogenic sprouts in culture.

Apelin-positive endothelial cells fail to form elongated vascular structures in the heart in response to VEGF

Our data on cultured cells indicate that a comparable number of Apelin⁺ cells is induced by VEGF in both skeletal and cardiac muscle, but they differentially proliferate and form

vascular sprouts. We wondered whether similar differences were also occurring *in vivo* in the context of VEGF- and tumor-induced angiogenesis.

First, we focused on the capacity of Apelin⁺ endothelial cells to form elongated vascular structures 30 days after AAV-VEGF injection in either skeletal or cardiac muscle of ApIn-CreER;R26mT/mG mice (as schematically represented in **Fig. 6a**). In control heart and skeletal muscle, the few EGFP⁺ERG⁺ endothelial cells were all individually spaced. Delivery of AAV-VEGF to the skeletal muscle resulted in a massive increase in the number of EGFP⁺ERG⁺ endothelial cells, which were often incorporated in elongated vascular structures, reaching up to 15 EGFP⁺ERG⁺ endothelial cells positioned in the same tube. In contrast, most of the EGFP⁺ERG⁺ endothelial cells in the heart remained as single cells or formed very short vascular structures, even after VEGF stimulation. The distribution of the number of EGFP⁺ERG⁺ endothelial cells in the same vascular tube is shown for both muscle types in **Fig. 6b, c**, while representative images are shown in **Fig. 6d**. Only in the skeletal muscle, this distribution was skewed towards the right in response to VEGF, indicating the generation of long capillaries by EGFP⁺ERG⁺ endothelial cells, while no significant changes could be observed in the heart. Comparable results were obtained in 100 μ m thick muscle sections, that better show the three-dimensional organization of the vascular network (**Fig. 6e, Supplementary Fig. 8a** and **Movies 1-4**). In parallel, we also assessed the effect of a 100-fold higher or lower dose of vector, and again we did not detect any improvement in vessel length by increasing the dose of VEGF in any of the two muscle types (**Supplementary Fig. 3e**). The lowest dose of the vector did not result in any angiogenic response (**Supplementary Fig. 3f**).

To shed light on the molecular mechanisms underlying the poor elongation of vascular tubes in response to VEGF in the heart, we used flow cytometry to characterize the expression of VEGFR1 and Dll4, which are well known to regulate endothelial cell proliferation and the cross-talk between tip and stalk cells during sprouting angiogenesis ^{19, 26}. We followed the gating strategy shown in **Supplementary Fig. 9 a-c** to separately analyze EGFP⁺ cells and tdTomato⁺EGFP⁻ cells (possibly representing tip and stalk cells, respectively). We found that the percentage of VEGFR1⁺ endothelial cells was significantly decreased at day 7 after AAV-VEGF injection in the skeletal muscle, but not in the heart (**Fig. 6f**). In parallel, Dll4 was significantly more expressed in response to VEGF in endothelial cells purified from the muscle compared to those purified from the heart (**Fig. 6g**). This up-regulation in the muscle was almost totally sustained by Apelin⁺ cells, consistent with their possible tip cell function (**Fig. 6h**). As a result of increased Notch signaling from tip cells, the Notch-target genes *Hes1*, *Hey1* and *Notch1* itself were more expressed by tdTomato⁺EGFP⁻ cells purified from the skeletal muscle compared to those purified from the heart (**Supplementary Fig. 9d-f**).

Thus, VEGF stimulates effective angiogenesis and the formation of elongated vascular structures, relying on Notch signaling, in the skeletal muscle, but fails to do the same in the heart.

Apelin-positive endothelial cells fail to form elongated vascular structures in the heart in response to cancer cell implantation

Next, we moved to the cancer model and assessed tumor vessels at 10 days after LG cell implantation (**Fig. 7a**). In the skeletal muscle, vessels included up to 9 EGFP⁺ cells lining in a row in the same vascular tube inside the tumor area. In contrast, most of the cardiac vessels contained only 1 or 2 EGFP⁺ cells. The results of this quantification are shown in **Fig. 7b** and representative images in **Fig. 7c**. A comparable pattern was observed in 100 μ m thick sections (**Fig. 7d**, **Supplementary Fig. 8b** and **Movies 5-6**). Similar to what was observed upon VEGF overexpression, Apelin⁺ cells were able to persist and generate elongated vascular structures only in the context of cancers growing in the skeletal muscle, skewing the distribution to the right compared to the one observed in the heart (**Fig. 7b**).

We also analyzed the spatial localization of these cells. While EGFP⁺ vessels were abundantly present within the central part of the tumor in the skeletal muscle, most of the EGFP⁺ cells were concentrated in the healthy tissue surrounding the tumor mass in the heart. By quantifying the EGFP⁺ area in the border region (as defined in methods), we found that in the heart most of the Apelin⁺ cells were still present in the healthy portion of the border 10 days after tumor implantation. In the skeletal muscle, the same cells had already penetrated inside the neoplastic mass (quantification is shown in **Fig. 7e** and representative images in **Fig. 7f**). Thus, in the heart sprouting Apelin⁺ endothelial cells have a low capability to form elongated vascular structures and penetrate the tumor mass, consistent with their apoptotic phenotype particularly evident at day 6 (**Supplementary Fig. 5**).

Overall, these data support a model in which endothelial cells in both skeletal and cardiac muscle similarly start expressing Apelin in response to pro-angiogenic stimuli (i.e. VEGF or molecules secreted by cancer cells). In the skeletal muscle, Apelin⁺ cells activate Notch signaling and drive the formation of new vascular sprouts, which are eventually incorporated into arterial vessels. In contrast, Apelin⁺ cells in the heart persist for a short period of time, undergo apoptosis and fail to drive an efficient angiogenic response (**graphical abstract**).

Discussion

The results presented in this work provide a definitive evidence that the adult mammalian heart has a low angiogenic potential. While in the skeletal muscle angiogenic stimuli induce Apelin expression in a few endothelial cells, which sprout and lead to the formation of mature new vessels, in the heart Apelin⁺ cells are unable to support an efficient angiogenesis.

The poorly angiogenic nature of the adult heart was suggested by our serendipitous observation that the presence of the same amount of VEGF into either the skeletal or the cardiac muscle resulted in a very different angiogenic response. While VEGF induced a massive increase in the number of both capillaries and small arteries in the skeletal muscle, in the heart it resulted in the formation of a modest number of new arteries, with almost null endothelial cell proliferation.

Thus, we used *Apln-CreER;R26mT/mG* mice to genetically trace sprouting endothelial cells and follow their fate upon administration of pro-angiogenic stimuli. Besides VEGF, which is the most potent and best characterized angiogenic molecule, we also exploited the capacity of cancer cells to secrete pro-angiogenic factors. In both cases, i.e. the injection of either AAV-VEGF or cancer cells, a comparable proportion of endothelial cells started expressing Apelin in both muscle types. These cells efficiently formed new capillaries and were incorporated into small arteries in the skeletal muscle, but not in the heart, in which VEGF-induced arterioles were essentially Apelin-negative. These data are consistent with those obtained by He and co-workers, who used the same genetic model to assess the mechanism responsible for the formation of new vessels during embryonic development and after myocardial infarction⁶. These investigators found that arteries appearing in the adult heart after ischemia did not bear any Apelin⁺ cell and concluded that new vessels can be formed in the ischemic heart only through arteriogenesis, namely the expansion of pre-existing arterioles, and not by angiogenesis⁶. Thus, the modest increase in the number of arterioles that we observed in the heart after AAV-VEGF administration can be reasonably ascribed to the recruitment and expansion of pre-existing vessels, in the absence of a true angiogenic response.

When we assessed the basal expression of Apelin in the two muscle types, we observed that the number of arteries containing Apelin⁺ endothelial cells was relatively higher in the untreated skeletal than in the cardiac muscle. Whether this indicates a spontaneous turnover of endothelial cells within arterial vessels in the skeletal muscle, which, different from the heart, is an organ endowed with a high regenerative capacity²⁷, is an interesting hypothesis that requires further validation. Indeed, the different angiogenic capacity of the heart and the skeletal muscle well correlates with the different regenerative potential of the two muscle types. The regenerative response activated upon any kind of muscle injury entails massive activation of endothelial cells and profound remodeling of the vascular

bed²⁸. Angiogenesis and myogenesis are tightly coupled and controlled by a set of key molecules, including Periostin, Oncostatin M and Apelin itself²⁸. In contrast, the adult mammalian heart has a minimal, if not null, regenerative potential^{29, 30} and any damage is repaired by scarring. Lower vertebrates, such as fish and amphibians, are able to regenerate the myocardium after damage³¹. In these animals, endothelial cells invade the damaged area well before cardiomyocytes start proliferating, indicating that angiogenesis precedes and is possibly essential for heart regeneration³². The neonatal mammalian heart also retains some regenerative potential for a few days after birth³³, yet the vascular network observed in the regenerating neonatal heart following apical resection is rather incomplete as compared to the normal myocardium³⁴, again consistent with the poor capacity of the post-natal heart to form new arteries.

Previous studies claimed the effective formation of new blood vessels in response to VEGF and other angiogenic molecules in the heart of multiple species³⁵. At a deeper analysis, most of these studies either used transgenic models, in which the angiogenic factor was active already during embryonic development, or they did not discriminate between spouting angiogenesis and other mechanisms of vessel formation. For instance, the cardiac-specific overexpression of another member of the VEGF family, VEGF-B, resulted in a massive angiogenic response in transgenic animals, but did not increase capillary density when delivered to adult hearts using AAV vectors³⁶. In addition, many studies delivered the angiogenic stimulus either as adenoviral vectors or recombinant proteins, which both have a short half-life *in vivo* and results in a very transient effect^{37, 38}. This requires a very early analysis of the angiogenic response, which likely starts in the heart as in other organs, but fails to progress to form structured vessels. Indeed, we report that the pro-angiogenic stimulus is sensed by cardiac endothelial cells, as they do activate Apelin expression, and that the problem occurs at a later stage, during the vascular elongation phase.

Another difference between the two muscle types, is endothelial cell density, which appears higher in the heart compared to skeletal muscle, when normalized per field. This difference is importantly reduced when the number of capillaries is normalized on the number of nuclei, as nuclei in the cardiac tissue are definitely denser than in the skeletal muscle. Thus, we would tend to exclude that the increased vessel density in the heart results in higher oxygenation and justifies the unresponsiveness of the cardiac muscle to VEGF, as also indicated by the comparable activation of Apelin expression in the two organs. Indeed, both muscle types up-regulated Apelin early after the injection of either VEGF or cancer cells, indicating that they are equally able to sense pro-angiogenic stimuli at the individual cell level. Increasing the dose of VEGF, by injecting a 100-fold more concentrated AAV vector, did not rescue the responsiveness of cardiac endothelial cells to angiogenic stimulation, and not even increased the angiogenic response in the skeletal

muscle, consistent with recent evidence that excessive VEGF stimulation does not result in functional angiogenesis³⁹.

Apelin up-regulation was also observed in response to VEGF in primary endothelial cells, purified from both skeletal and cardiac muscle, and grown *ex vivo* as either a monolayer or spheroids. While skeletal muscle-derived endothelial cells increased their proliferation and sprouting upon VEGF stimulation, cardiac endothelial cells did not increase their growth rate and failed to sprout from the spheroid body. Despite comparable levels of VEGFR2, downstream signaling was more active in cells purified from the skeletal muscle compared to those purified from the heart, both in basal conditions and upon VEGF stimulation. These data suggest that endothelial cells in both muscle types can specialize into tip cells, characterized by high levels of Apelin expression, and that a failure in the subsequent induction of stalk cell proliferation specifically occurs in cardiac endothelial cells. Consistently, in the skeletal muscle, the angiogenic process effectively proceeded and Apelin⁺ endothelial cells, along with their progeny, were incorporated into elongated vascular structures. In contrast, in the heart most of the Apelin⁺ endothelial cells stayed as single cells and died by apoptosis. When we followed their fate over time in the tumor model, we observed that these cells were mostly retained within the tumor border and failed to penetrate the neoplastic mass.

Can this poor angiogenic response be associated to the low incidence of cancer and metastasis in the heart? Primary and secondary cardiac tumors are indeed extremely uncommon⁴⁰, despite the fact that the heart is among the most vascularized organs. Our results show that the experiential injection of cancer cells in the heart invariably resulted in poor cancer growth and inefficient angiogenesis.

Understanding the mechanisms blocking angiogenesis in the heart could thus offer novel opportunities for both interfering with cancer growth and promoting cardiac revascularization. We provide evidence that VEGFR1 and Dll4/Notch1 signaling are differentially regulated in endothelial cells purified from either the skeletal or the cardiac muscle, previously stimulated with VEGF. This could reflect a defective tip-to-stalk cell communication specifically occurring in the heart. Alternatively, the mechanism behind vessel elongation could be organ-specific and the traditional model of Notch1-dependent tip-to-stalk cell signaling, described in the retina, may not be not necessarily applicable to other tissues. Our findings provide the base and experimental models for future investigation, aimed at clarifying the organotypic features of cardiac angiogenesis and their possible therapeutic exploitation.

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Author's contribution

TK, MR, ACo, NV, GZ, ACa performed most of the experiments, EG and GMP performed flow cytometry and cell sorting, ML contributed to image analysis of tumor models, SV performed in vivo experiments, ZB, FB, MH, MG and RA provided genetic models, reagents and critically discussed the experiments, LZ produced AAV vectors, TK, ACo and SZ conceived the work and wrote the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. VEGF induces a potent angiogenic response in the skeletal but not in the cardiac muscle. **a.** Schematic of the AAV and EdU injection. **b.** Real-time PCR quantification of the number of AAV viral genomes (vg) in the skeletal muscle (SM) and cardiac muscle (CM) normalized on tissue weight. **c.** Real-time PCR quantification of human VEGF165 mRNA normalized on mouse GAPDH. **d.** Quantification of mouse and human VEGF by ELISA assay normalized on tissue weight. **e.** Representative immunofluorescence staining of skeletal and cardiac muscle, injected with either AAV-control (left) or AAV-VEGF (right), using anti- α -SMA and anti-CD31 antibodies. **f.** Representative immunofluorescence staining of skeletal and cardiac muscle, injected with either AAV-control (left) or AAV-VEGF (right), using anti-ERG antibodies. **g.** Quantification of the area covered by α -SMA⁺ cells. **h.** Quantification of the area covered by CD31⁺ cells. **i.** Quantification of the percentage of ERG⁺ nuclei. **j.** Quantification of the percentage of EdU⁺ CD31⁺ cells. **k.** Representative immunofluorescence staining of skeletal and cardiac muscle, injected with either AAV-control (left) or AAV-VEGF (right) showing EdU⁺ nuclei and CD31⁺ endothelial cells. The inset shows a higher magnification to visualize EdU⁺CD31⁺ endothelial cells (indicated by arrows). In **b-d**, **g-j** data are shown as mean \pm s.e.m. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test, * $P < 0.05$, *** $P < 0.001$, n=3-5 per group. Scale bar in **e**, **f**, and **k** 100 μ m.

Figure 2. Sprouting Apelin⁺ endothelial cells differentially contribute to the formation of arterial vessels in response to VEGF in the skeletal and cardiac muscle. **a.** Schematic of AAV and tamoxifen (Tam) injection in Apln-CreER;R26mT/mG mice. **b.** Quantification of the number of EGFP⁺ CD31⁺ endothelial cells (% of CD31⁺ cells). **c.** Quantification of the number of EGFP⁺ ERG⁺ endothelial cells (% of ERG⁺ cells). **d.** Quantification of the number of arteries containing EGFP⁺ endothelial cells (% of total arteries). **e.** Representative immunofluorescence staining of skeletal and cardiac muscle of Apln-CreER;R26mT/mG mice injected with either AAV-control (left) or AAV-VEGF (right), labelled with anti-CD31 antibodies. Boxed regions are shown as split channels in the panels on the right. **f.** Representative immunofluorescence staining of skeletal and cardiac muscle of Apln-CreER;R26mT/mG mice injected with either AAV-control (left) or AAV-VEGF (right), labelled with anti- α -SMA antibodies. In **b-d** data are shown as mean \pm s.e.m. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test, ** $P < 0.01$, *** $P < 0.001$, n \geq 7 per group. Scale bar in **e**, **f**, 100 μ m.

Figure 3. Sprouting Apelin⁺ endothelial cells differentially contribute to tumor angiogenesis in the skeletal and cardiac muscle. **a.** Schematic of LG cell and tamoxifen (Tam) injection in Apln-CreER;R26mT/mG mice. **b.** Quantification of the number of EGFP⁺ endothelial cells (% of CD31⁺ cells) at the indicated time points in either

the tumor region or the surrounding, healthy tissue. **c.** Quantification of the number of arteries containing EGFP⁺ endothelial cells (% of total arteries) at the indicated time points in either the tumor region or the surrounding, healthy tissue. **d.** Representative immunofluorescence staining of skeletal and cardiac muscle of A^{pln}-CreER;R26mT/mG mice injected with LG cells, labelled with anti-CD31 antibodies 9 days after cancer cell injection. Panels on the left show regions highly infiltrated by cancer cells, whereas panels on the right show the surrounding, healthy tissue. Boxed regions are magnified in the lower panels to show split channels. **e.** Representative immunofluorescence staining of skeletal and cardiac muscle of A^{pln}-CreER;R26mT/mG mice injected with LG cells, labelled with anti- α -SMA antibodies 9 days after cancer cell injection. Panels on the left show regions highly infiltrated by cancer cells, whereas panels on the right show the surrounding, healthy tissue. In **b,c** data are shown as mean \pm s.e.m. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n=3$ per group. Scale bar in **d, e** 200 μ m.

Figure 4. Tumors grow less in the heart than in the skeletal muscle. **a.** Quantification of tumor size at different time points after LG cell implantation into either the skeletal or the cardiac muscle. **b.** Quantification of Ki67⁺ nuclei (% of total nuclei) in sections of either skeletal or cardiac muscle at 10 days after LG cell implantation. **c.** Quantification of Cleaved Caspase-3⁺ cells in sections of either skeletal or cardiac muscle at 10 days after LG cell implantation. **d.** Representative immunofluorescence staining of skeletal and cardiac muscle injected with LG cells using anti-Ki67 antibodies. Scale bar, 100 μ m. **e.** Representative immunofluorescence staining of skeletal and cardiac muscle injected with LG cells using anti-Cleaved Caspase-3 antibodies. Scale bar, 50 μ m. **f-i.** Representative whole organ pictures of hearts and skeletal muscle not treated (**f**) or injected with LG lung adenocarcinoma (**g**), B16/F10 melanoma (**h**) and 4T1 breast cancer cells (**i**). Tumor area is indicated by a dashed yellow line. Scale bar in **f-i**, 2 mm. **j-l.** Quantification of LG (**j**), B16 (**k**) and 4T1 (**l**) tumor size in skeletal and cardiac muscle at 10 days after cell implantation. In **a-c, j-l** data are shown as mean \pm s.e.m.. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test for data in **a**, and by Student's t-test in **b, c, j, k, l**, * $P < 0.05$, ** $P < 0.01$, $n \geq 4$ per group.

Figure 5. Cardiac endothelial cells upregulate Apelin but fail to form vascular structures upon VEGF stimulation *in vitro*. **a.** Representative images of cultured skeletal muscle and cardiac endothelial cells (SMECs and CECs, respectively) isolated from A^{pln}-CreER;R26mT/mG mice, treated with tamoxifen, either in the presence or in the absence of VEGF (100 ng/ml) for 48 hours. Endothelial cells are labelled with anti-CD31 antibodies. **b.** Quantification of EGFP⁺ endothelial cells (percentage of CD31⁺ cells). **c.** Representative images of SMECs and CECs isolated from C57Bl/6 mice either in the presence or in the absence of VEGF (100 ng/ml) for 48 hours. Endothelial cells are labelled with anti-CD31 antibodies and proliferating cells with anti-Ki67 antibodies. **d.**

Quantification of Ki67⁺ endothelial cells (percentage of CD31⁺ cells). **e.** Representative images of SMEC and CEC spheroids isolated from *Apln-CreER;R26mT/mG* mice and let to sprout for 48 hours. The boundary between the spheroid body and the sprouts is marked with a dashed white line. The inset shows an EGFP⁺ cell, presenting numerous filopodia classically associated to tip cells, at the forefront of a growing sprout. **f-g.** Quantification of tdTomato⁺ (**f**) and EGFP⁺ (**g**) area in the sprouts (normalized to the length of the spheroid boundary visible in each field). **h.** Quantification of EGFP⁺ area within SMEC and CEC spheroid body (EGFP⁺/Hoechst⁺ area). In **b,d,f-h** data are shown as mean±s.e.m.. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test for data in **b** and **d**, and by Student's t-test in **f, g, h**, ** $P < 0.01$, *** $P < 0.001$, $n \geq 4$ per group. Scale bar in **a, c, e**. 100 μm .

Figure 6. Cardiac endothelial cells upregulate Apelin but fail to form elongated vascular structures upon VEGF stimulation *in vivo*. **a.** Schematic of AAV and tamoxifen (Tam) injection in *Apln-CreER;R26mT/mG* mice for characterisation of sorted tdTomato⁺ and EGFP⁺ cells. **b-c.** Histograms showing the number of EGFP⁺ cells lining in a vascular tube in either skeletal (**b**) or cardiac (**c**) muscle injected with AAV-control (left) and AAV-VEGF (right). Superimposed to each histogram is the density plot (in blue). **d.** Representative immunofluorescence staining of skeletal and cardiac muscle of *Apln-CreER;R26mT/mG* mice injected with either AAV-control or AAV-VEGF, labelled with anti-ERG antibodies. **e.** Representative 3D renderings of 100 μm thick sections from skeletal and cardiac muscle of *Apln-CreER;R26mT/mG* mice injected with AAV-VEGF. **f-h.** Flow cytometry analysis of CD31⁺ VEGFR1(VR1)⁺ cells (**f**) and DLL4 intensity in either total CD31⁺ cells (**g**) or sorted tdTomato⁺ and EGFP⁺ cells (**h**), purified from cardiac and skeletal muscle of *Apln-CreER;R26mT/mG* mice, 7 days after AAV-VEGF injection. Data in **f-h** are shown as mean±s.e.m. Statistical significance was determined using Student's t-test and one-way ANOVA followed by Tukey's multiple comparison test for data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $n=3-6$ per group. Scale bar in **d, e** 100 μm .

Figure 7. Cardiac endothelial cells upregulate Apelin but fail to form elongated vascular structures upon cancer cell injection *in vivo*. **a.** Schematic of LG cells and tamoxifen (Tam) injection in *Apln-CreER;R26mT/mG* mice. **b.** Histograms showing the number of EGFP⁺ cells lining in a vascular tube in tumors within either skeletal or cardiac muscle. Superimposed to each histogram is the density plot (in blue). **c.** Representative immunofluorescence staining of skeletal and cardiac muscle of *Apln-CreER;R26mT/mG* mice injected with LG cells, labelled with anti-CD31 antibodies. **d.** Representative 3D renderings of 100 μm thick sections of skeletal and cardiac muscle from *Apln-CreER;R26mT/mG* mice injected with LG cells. **e.** Quantification of the area covered by EGFP⁺ cells within each side of the tumor border in both the skeletal and cardiac muscle ($P < 0.001$). **f.** Representative immunofluorescence of tumor border, defined as the region extending for 200 μm in both directions starting from the interface between tumor and

healthy tissue, in both the skeletal and cardiac muscle. Boxed regions are shown as split channels in the right panels. Scale bar in **c** and **f**, 100 μm . n=6 per group.

Abstract figure. Model showing the differential response of endothelial cells from the cardiac and skeletal muscle to pro-angiogenic stimuli. In response to a pro-angiogenic stimulus, such as VEGF or additional molecules secreted by cancer cells, a few endothelial cells in the skeletal muscle start expressing Apelin. These Apelin⁺ cells efficiently sprout, activate Dll4/Notch1 signaling and drive the formation of new vessels, to be eventually incorporated into arteries. In the heart, the same stimulus leads to a similar activation of Apelin in endothelial cells, which, however, only transiently persist and fail to form elongated vascular structures.















