Sympathetic Reinnervation Is Required for Mammalian Cardiac Regeneration

Ian A. White, Julie Gordon, Wayne Balkan, Joshua M. Hare

Rationale: Although mammalian cardiac regeneration can occur in the neonatal period, the factors involved in this process remain to be established. Because tissue and limb regeneration require concurrent reinnervation by the peripheral nervous system, we hypothesized that cardiac regeneration also requires reinnervation.

Objective: To test the hypothesis that reinnervation is required for innate neonatal cardiac regeneration.

Methods and Results: We crossed a Wnt1-Cre transgenic mouse with a double-tandem Tomato reporter strain to identify neural crest-derived cell lineages including the peripheral autonomic nerves in the heart. This approach facilitated the precise visualization of subepicardial autonomic nerves in the ventricles using whole mount epifluorescence microscopy. After resection of the left ventricular apex in 2-day-old neonatal mice, sympathetic nerve structures, which envelop the heart under normal conditions, exhibited robust regrowth into the regenerating myocardium. Chemical sympathectomy inhibited sympathetic regrowth and subsequent cardiac regeneration after apical resection significantly (scar size as cross-sectional percentage of viable left ventricular myocardium, n=9; 0.87%±1.4% versus n=6; 14.05±4.4%; P<0.01).

Conclusions: These findings demonstrate that the profound regenerative capacity of the neonatal mammalian heart requires sympathetic innervation. As such, these data offer significant insights into an underlying basis for inadequate adult regeneration after myocardial infarction, a situation where nerve growth is hindered by age-related influences and scar tissue. (Circ Res. 2015;117:990-994. DOI: 10.1161/CIRCRESAHA.115.307465.)

Key Words: mice, transgenic ■ myocardium ■ regeneration ■ sympathectomy ■ sympathetic nervous system

It has recently been established that unlike the limited cardiac regeneration demonstrated by the injured adult mammalian heart, the neonatal heart remains permissive for near-complete cardiac regeneration during a finite developmental period.1,2 In a similar fashion to other regeneration-competent species including the salamander3 and zebrafish,4,5 the dominant mechanism underlying cardiac regeneration in the neonatal mouse seems to be dedifferentiation and proliferation of resident cardiac myocytes.6 However, the underlying basis for regeneration and revascularization of the neonatal tissue is not fully understood. Other vertebrate6-9 and invertebrate10 models of tissue regeneration exhibit a complete dependence on reinnervation of the regenerating tissue by nerves of the peripheral nervous system. Despite the clinical importance of cardiac autonomic innervation, the neuroanatomy of the sympathetic nerve plexus innervating the ventricular myocardium remains incompletely characterized.11

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To address these issues, we used a combination of genetic and pharmacological tools to map the cardiac peripheral nervous system and to test the role of peripheral nerve innervation in mammalian cardiac regeneration. Postganglionic, subepicardial sympathetic axons make up the bulk of nerve fibers in the ventricles,11 and we demonstrate, for the first time, that these nerve fibers undergo robust regrowth and reinnervation during the regeneration of resected ventricular tissue. Furthermore, sympathectomy abrogates cardiac regeneration and promotes collagenous scar formation, demonstrating that innate mammalian cardiac regeneration in neonates is dependent upon sympathetic innervation. Together these findings suggest that concurrent reinnervation of injured adult cardiac tissue is essential for functional and complete cardiac regeneration.
Methods

A detailed description of the experimental procedure and statistical analysis is provided in the online Data Supplement. Briefly, wingless-type MMTV integration site family, member 1 (Wnt1)-Cre recombinase (Cre) mice were crossed with double-tandem (td) Tomato reporter mice (The Jackson Laboratory). Cre+/tdTomato+ animals were used in experiments where direct observation of nerves was required. BALB/cJ animals (The Jackson Laboratory) were used in apical resection experiments. Studies were performed on 2-day-old neonates, and tissue was collected at either day 14 or day 21 post injury.

Results

The proto-oncogene Wnt1 is only expressed during the development of the central nervous system and demarcates lineages derived from the neural crest. The Wnt1-Cre transgenic mouse strain is a widely used and well-validated model for neural crest lineage tracing studies. Wnt1-Cre transgenic mice were mated with mice expressing the tdTomato reporter so that neural crest-derived cells, and their progeny, are permanently labeled with the red fluorescent tdTomato reporter. Using this system, together with whole-mount, broad focal plane, epifluorescent stereomicroscopy, we visualized the subepicardial neural network in unprecedented detail (Figure 1A and 1B).

The structures observed in whole-mount were confirmed to be nerves by staining for β-tubulin III (Figure 1C) and the presynaptic marker synapsin 1 (Figure 1D). To determine from which autonomic branch these nerves derive, we performed immunofluorescence histology against tyrosine hydroxylase, and choline acetyltransferase. Colocalization of tyrosine hydroxylase+ fibers only with Wnt1-Cre+ fibers was observed at the base of the heart where nerve density is greatest (Figure 1E–1G), which agrees with the published distribution of the sympathetic branch primarily to the subepicardium.

In contrast to the adult neonatal sympathetic neurons, sympathetic ganglia and dorsal root ganglia exhibit enhanced plasticity, both in vitro and in vivo. Therefore, we assessed the ability of neonatal subepicardial sympathetic nerves to reinnervate ventricular myocardium after injury. We resected the apex of the left ventricle of 2-day-old Wnt1-Cre;tdTomato mice, as described. Under normal conditions, the neonatal heart is capable of a robust regenerative

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**Nonstandard Abbreviations and Acronyms**

CRE Cre recombinase

td double-tandem

WNT1 wingless-type MMTV integration site family, member 1

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**Figure 1. Subepicardial distribution of postganglionic sympathetic nerve fibers in the mouse heart.** A, Crossing Wnt1-Cre recombinase transgenic mice with double-tandem (td) Tomato reporter mice identifies nerve fibers throughout the entire subepicardium from the base of the heart to the apex of the ventricles (bar, 1 mm). B, The fibers are heavily varicosed (arrows; bar, 200 μm). C, They stain positive for the neurofilament marker β-tubulin III (bar, 50 μm), the presynaptic marker synapsin 1 (D; bar, 20 μm) and the sympathetic nerve fiber marker tyrosine hydroxylase (TH; E–G; bar, 200 μm). LA indicates left atrium; LV, left ventricle; OT, outflow tract; RA, right atrium; RV, right ventricle.
response after apical resection within 21 days. At 14 days post resection, we observed an area of heavy dendrite hyperinnervation at the injury border (Figure 2A), which is consistent with that described in studies of acute myocardial infarction and varicose fibers emerging from the border into the site of active regeneration (Figure 2B). By day 21 post injury, the entire apex had regenerated and had become reinnervated by organized, arborized, and anastomosed fibers (Figure 2C–2F).

Denervation of tissue after injury in other animal models results in a block of the innate regenerative response with subsequent scar formation at the expense of functional tissue. Considering the vast abundance of sympathetic nerves associated with the ventricular myocardium and their ability to regrow in the model of neonatal cardiac regeneration, we sought to determine whether these nerves were a necessary component of the cardiac regenerative response. Beginning 48 hours after apical resection, chemical sympathectomy of adrenergic nerves was induced by treatment of neonatal mice with 3 doses of 6-hydroxydopamine hydrobromide (6-OHDA; 250 mg/kg IP injection) 48 hours apart. In response to 6-OHDA, the subepicardial sympathetic nerves demonstrated classical features of Wallerian degeneration throughout the surface of the heart (Figure 3A). Treatment resulted in robust denervation (n=9, 1.74×104±7447 pixels; n=9, 3.11×104±3863 pixels; P<0.01) of the subepicardial nerves in the heart, as quantified by densitometry (Figure 3B).

In the absence of 6-OHDA, neonatal mouse hearts underwent robust reinnervation and regeneration of the ventricular apex after resection with little or no signs of injury (Figure 3C; n=9, scar as a percentage of cross-sectional myocardial area, 0.87±1.4%). In contrast, the hearts of sympathectomized mice consistently exhibited extensive scarring, lack of regeneration, and failure to replace the ventricular myocardium after apical resection (Figure 3C) and denervation (n=6, 14.05±4.4%; P<0.01; Figure 3D).

In summary, we have demonstrated that sympathetic denervation completely inhibits the ability of the neonatal heart to regenerate after injury. These findings correlate with those from other animal models of tissue regeneration, which describe a critical dependence on peripheral nerves to regenerate injured tissue.

Discussion

Despite the clinical importance of the cardiac autonomic system, there is still much to learn regarding the neuroanatomy of the mammalian heart. Here, we combine a strong fluorescence lineage tracing reporter system with broad focal plane

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**Figure 2. Concurrent reinnervation and cardiac regeneration.** A and B, At day 14 post resection, the apex is in the process of regenerating (*) as nerves regrow into the injury (arrow marks site of hyperinnervation at injury border; bars, 500 μm and 100 μm, respectively). C–F, After 21 days, the apex has regenerated and fully reinnervated (bar, 500 μm). Boxes from C and E are expanded with greater magnification in D and F (bar, 100 μm). All images are of Wnt1-Cre;tdTomato hearts.
stereomicroscopy to visualize the vast cardiac sympathetic neural network in unprecedented detail. We demonstrate large nerve bundles entering the heart from the dorsal aspect, which then arborize and anastomose throughout all 4 chambers of the heart. The resulting dense network of nerve fibers makes extensive contact, via en-passant synapses, with subepicardial myocytes in the ventricles and with vessels of the cardiovasculature.

Direct visualization of these nerves facilitated investigation into the ability of the nerves to regenerate in vivo after injury and identified that cardiac regeneration is dependent on a nerve supply. Our data support the hypothesis that innervation is critical for innate cardiac regeneration exhibited by neonatal mice, as inhibition of sympathetic nerves completely blocks myocardial repair. Several other vertebrate and invertebrate models of tissue regeneration support this concept, including a recent report demonstrating that ablation of the parasympathetic branch of the autonomic system of neonatal mice by surgical vagotomy inhibits cardiac regeneration, despite an assumed presence of sympathetic nerves.9 Together, these results suggest that contributions from both branches of autonomic nerves are needed to support full cardiac regeneration. Additional studies are required to tease apart the relationship between the branches of the autonomic nervous system, the myocardium, and the cardiovasculature and to address the structural changes in cardiac neuroanatomy that result from injury.

Substantial evidence is accumulating that implicates peripheral autonomic nerves in several aspects of tissue regeneration and homeostasis, including stem cell niche maintenance, vasculogenesis and patterning, and tissue hyperplasia.6,7 Cardiac nerves clearly function as more than just simple impulse conduits. A recent report demonstrated that uninjured rats treated with 6-OHDA to ablate the sympathetic while sparing the parasympathetic nerves exhibit myocardial injury and fibrosis.21 This injury was prevented when a neuroprotectant was coadministered, demonstrating that myocardial injury was a secondary response to sympathetic nerve loss.

Taken together, these data support the idea that peripheral cardiac autonomic nerves play a role not only in repair but also in chronic disease. Peripheral neuropathies caused by diabetes mellitus or heart transplant are typically associated with idiopathic vasculopathy and heart failure.25,26 Further work is needed to investigate whether the loss of cardiac nerves in these patients results in a pathological loss of trophic support for the myocardium, vasculature, or cardiac stem cell niches. Considering the physiological differences between fetal/embryonic and adult postganglionic nerves, these findings have important implications for improving adult cardiac regeneration, which has, as of yet, remained elusive.

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Disclosures
Dr Hare reported having a patent for cardiac cell-based therapy. He holds equity in Vestion and maintains a professional relationship with Vestion as a consultant and member of the Board of Directors and Scientific Advisory Board. Vestion Inc did not play a role in the design and conduct of the study. The other authors report no conflicts.
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• Peripheral nerves are essential for tissue repair in newts and zebrfish.

What New Information Does This Article Contribute?
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Novelty and Significance
What Is Known?
• Whereas adult mammals, including humans, exhibit regenerative
capacity inadequate to repair injury caused by heart attack or other
damage, the neonatal mouse is capable of regenerating injured myo-
cardium during a finite developmental window.
• The mammalian heart is extensively innervated by peripheral nerves.
• Peripheral nerves are essential for tissue repair in newts and zebrfish.

What New Information Does This Article Contribute?
• We used a Wnt1 transgenic reporter mouse, which allowed for high-
resolution visualization of cardiac sympathetic nerves.
• Using this transgenic mouse, we demonstrated concurrent nerve and
myocardial tissue regrowth and repair at the site of cardiac regenera-
tion in the neonatal mouse.
• Cardiac regeneration in the neonatal mouse is critically dependent on
sympathetic nerves, as denervation blocks cardiac tissue regeneration.

Our understanding of cardiac regeneration has advanced sig-
ificantly in recent years. However, large-scale regeneration or
full recovery remains elusive, suggesting that an important com-
ponent of the regenerative process is being overlooked. Unlike
the adult, the neonatal mouse heart retains an innate ability to
regenerate after injury, providing a compelling model to study the
mechanisms of mammalian cardiac regeneration. Several animal
species exhibit a robust capacity to regenerate tissue and limbs,
and this process is critically dependent on intact innervation.
Here, we demonstrate that neonatal cardiac regeneration is criti-
cally dependent on sympathetic nerves and identify a potentially
novel strategic target for improving cardiac repair. This finding
has important implications for adult regeneration after myocar-
dial infarction where nerve growth is hindered by age-related
influences, disease processes, and scar tissue.
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Materials and Methods

Animals

Wnt1-Cre mice (The Jackson Laboratory, Stock No. 003829) express Cre recombinase in all neural crest-derived cells. tdTomato reporter mice (The Jackson Laboratory, Stock No. 007914) express the red fluorescent protein variant (tdTomato) in response to Cre-mediated recombination. Male Cre/+ mice were mated with homozygous female tdTomato mice to produce a litter including Cre/+;tdTomato/+ mice. The colony was maintained by mating these Cre/+;tdTomato/+ animals together. Progeny were screened directly using a portable dual fluorescent flashlight and filter unit, NIGHTSEA™ (Electron Microscopy Sciences, DFP1): Cre/+;tdTomato/+ animals could be identified directly by their red fluorescent heads, due to the fact that neural crest cells contribute to craniofacial structures. Cre/+;tdTomato/+ animals were used in experiments where direct observation of nerves was required. BALB/cJ animals (The Jackson Laboratory, Stock No. 000651) were used in apical resection experiments due to the relatively large litter size and enhanced maternal care following injury. Studies were performed on 2-day-old neonates and hearts were collected at either day 14 or day 21 post-injury.

Apical Resection

The surgical method was modified from that previously described. Briefly, 2-day-old pups were anesthetized by hypothermia by placing on ice, separated by a latex glove, until akinetic. The animals were secured in a supine position by applying surgical tape to the limbs. The thorax was sterilized with 10% iodine solution and a lateral incision was made into the skin at the level of the 4th intercostal. A perforation was introduced into the chest cavity with needle-nose tweezers and the ribs were spread with blunt-nose...
tweezers to avoid damage to the lungs. In a divergence from previous reports, we did not retract the heart by fixing it with tweezers. Instead, we applied gentle pressure to the abdomen, which presented the apex, then “hooked” the apex with a modified 30-gauge needle and retracted it through the surgical site (Supplemental Figure I). A minimal portion of the apex could then be resected in a reproducible manner by cutting proximal to the needle. This method significantly reduced trauma to the heart, compared to that previously caused by fixation\(^2,^3\). Following resection the heart was allowed to return to the chest cavity and a single suture (Prolene 6-0) was used to close the ribs. The skin was closed using GLUture topical tissue adhesive (Fisher Scientific, NC9855218) and the mice were allowed to recover on their bedding under a heat lamp. Once motile the pups were returned, en masse, to the mother as previously described\(^1\). The entire procedure was carried out in approximately 7 minutes per animal. Survival rate was approximately 80%.

**Denervation**

6-Hydroxydopamine hydrobromide (6-OHDA) (Santa Cruz Biotechnology, SC-256988), was dissolved in PBS containing 1% Sodium metabisulfite (Sigma, S-1516) antioxidant at a concentration of 25μg/μL and injected intraperitoneally into neonatal mice at a concentration of 250 μg/g. The 6-OHDA was administered three times at two-day intervals, beginning 48 hours after surgery.

**Epifluorescence and Immunofluorescence imaging**

Epifluorescent analysis of the neuroanatomical structure of the heart was performed on whole organs. Hearts were isolated from 14 day or 21-day-old mice, washed in cold PBS and fixed in 4% paraformaldehyde at room temperature for 15 minutes. The hearts were washed, submerged in PBS in a 60 mm plastic petri dish and immediately imaged at
592nm using a Zeiss Apo Stemi SV11 fluorescent stereomicroscope with a Spot Flex camera. Immunofluorescent analysis was performed on individually isolated ventricles. The tissue was prepared as described above. After fixation the hearts were incubated in blocking solution consisting of 20% Normal Donkey Serum (Millipore, S30-100KC), 5% Triton X-100 (Fluka analytical, #93443) in PBS for 30 minutes at room temperature. The hearts were transferred to primary antibodies diluted in 1% BSA (Sigma-Aldrich, #A2153)/PBS plus 0.1% Triton X-100 and incubated overnight at 4°C. Primary antibodies used were Rabbit anti-synapsin1 (Millipore, AB1543), Goat anti-ChAT (Millipore, AB144P), Rabbit anti-Tyrosine Hydroxylase (Millipore, AB152), Rabbit anti-Tuj-1 (β-tubulin III) (Covance, mrb-435p-100). Tissues were washed in PBS + 0.2% Tween (3 x 10 minutes) prior to incubation with secondary antibodies diluted in 1% BSA/PBS plus 0.1% Triton X-100 and incubated for 1 hour 15 minutes at room temperature. Secondary antibodies used were Donkey anti-Rabbit Alexa Fluor 488 (Life Technologies, #A21206), Donkey anti-Goat IgG Cy3 (Millipore, #AP180C). Immunofluorescent images were collected using an Olympus IX81 fluorescent microscope and Q Imaging Retiga EX camera. Relative nerve density was calculated using NIH Image J software.

**Immunohistochemistry**

Hearts were harvested from 21-day-old mice, with some requiring mechanical detachment from the wall of the chest cavity. The hearts were washed in cold PBS and fixed o/n at 4°C in 4% paraformaldehyde. After washing twice in cold PBS (5 minutes each) they were transferred to 30% sucrose/PBS solution at 4°C until equilibrium was reached. Hearts were then blotted and embedded in OCT (VWR, #95057-838) and frozen by floating on 100% EtOH cooled with dry ice. 10 μm thick tissue sections were cut using a cryostat (Leica, CM1850) onto Colorfrost Plus microscope slides (Fisher,
#12-550-17). After being allowed to dry, sections were stained with Masson’s Trichrome, and imaged using a Pathscan Enabler IV slide scanner. The scarred area was calculated using NIH Image J software and reported as a proportion of the area of the left ventricular myocardium.

**Statistical method**

Data are presented as mean ± SEM. Comparisons were conducted via unpaired, parametric $t$ test. Significant differences between groups are defined by $p<0.01$.

**Supplemental Figure I.** Modification of neonatal cardiac resection model. By hooking the apex of the heart with a modified 30-guage needle we reduced confounding trauma associated with fixation and retraction and permitted small, reproducible apical resections.