Neural crest-derived resident cardiac cells contribute to the restoration of adrenergic function of transplanted heart in rodent

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Aims

We investigated whether neural crest-derived cardiac resident cells contribute to the restoration of intrinsic adrenergic function following transplantation in mice. Transplanted heart shows partial restoration of cardiac adrenergic activity with time. Both the intrinsic cardiac adrenergic system and extrinsic sympathetic re-innervation contribute to neuronal remodelling in the transplanted heart. Little is known about the origin and function of the intrinsic system in the transplanted heart.

Methods and results

Heart from the protein O-Cre/Floxed-Enhanced Green Fluorescent Protein double-transgenic mouse was transplanted onto the abdominal aorta of the non-obese diabetic/severe combined immunodeficient mouse to trace the fate of cardiac resident neural crest-derived cells. Sympathetic nerve fibres, which are predominantly localized to the epicardial surface of the heart, disappeared in the transplanted heart. Intramyocardial neural crest cells increased immediately, while neural crest-derived nucleated tyrosine hydroxylase (TH)-immunoreactive cells increased over 2 weeks following transplantation. The mRNA expression levels of TH, dopamine-β-hydroxylase and phenylethanolamine N-methyltransferase, and the tissue content of catecholamines in the transplanted hearts increased with time in association with an increase in the number of neural crest-derived nucleated TH-immunoreactive cells and tissue nerve growth factor levels. Iodine-123-metaiodobenzylguanidine scintigraphy showed that the uptake ability of transplanted heart for catecholamines also recovered with time. Finally, the chronotropic response to tyramine both in vivo and ex vivo reappeared 2 weeks after transplantation.

Conclusion

Neural crest-derived adrenergic cells increased following heart transplantation. The restoration of cardiac sympathetic activities in transplanted heart is tightly coupled with an increase in the number of neural crest-derived adrenergic cells.

Keywords

Transplantation • Sympathetic activity • Regeneration

1. Introduction

An intrinsic adrenergic system, which is independent of the intracardiac sympathetic nerve ending and adrenal medulla, appears to be present in both fetal and adult mammalian myocardium. Intrinsic catecholamine synthesis in the primitive heart tube is critical for a wave of contraction propagating from the inflow to the outflow end of the tube to pump the blood that supports fetal development.1 In mice, targeted disruption of tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, is lethal in utero because of heart failure at an embryonic stage before sympathetic innervation.2 After sympathetic innervation, the cardiac sympathetic activities are predominantly controlled by efferent sympathetic axons originating from the stellate ganglion. However, in patients with advanced heart failure or heart transplantation, functional or anatomical denervation of cardiac sympathetic nerves awakens the latent catecholamine synthetic capability of an intrinsic adrenergic system in the myocardium.3
**2. Methods**

**2.1 Transgenic animals**
To trace the fate of neural crest-derived cells and catecholaminergic cells, we used transgenic mice harbouring protein-0 (P0) promoter-Cre and DBH-Cre. Protein 0-Cre and DBH-Cre recombinase transgenic mice were provided by Yamamura (Kumamoto University, Japan) and Kobayashi (Fukushima Medical University, Japan), respectively. CAG-CAT-EGFP transgenic mice were gifts from Miyazaki (Osaka University, Japan). Protein 0-Cre and DBH-Cre recombinase transgenic mice were crossed with CAG-CAT-EGFP transgenic mice were used as a donor. No immunosuppressive agents served within 72 h were excluded from evaluation. The viability of the donor's heart was assessed based on the beating of the transplant heart. In this study, NODE-SCID mice were used as a recipient and Protein 0-Cre/EGFP transgenic mice were used as a donor. No immunosuppressive agents were administered.

**2.2 Heterotopic cardiac transplantation**
Isogenic heterotopic cardiac transplantation was performed according to the method of Corry et al. Briefly, the donor hearts were transplanted to the recipients' abdomen as primary vascularized grafts by the microvascular technique. An end-to-side anastomosis was then made by running suture of the donor's aorta to the recipient's descending aorta, and suture of the donor's pulmonary artery to the recipient's inferior vena cava, respectively (Figure 2A). Both donor and recipient mice were anaesthetized with 1–2% inhaled isoflurane in oxygen (1 L/min). Technical failures observed within 72 h were excluded from evaluation. The viability of the donor heart was assessed based on the beating of the transplant heart. In this study, NODE-SCID mice were used as a recipient and Protein 0-Cre/EGFP transgenic mice were used as a donor. No immunosuppressive agents were administered.

**2.3 RNA extraction and quantitative RT–PCR**
Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Predesigned gene-specific primer and probe sets (TaqMan Gene Expression Assays) were used. The 18s rRNA was amplified as an internal control. All samples (n = 5 per group) were run in triplicate.

**2.4 [125I]-metaiodobenzylguanidine accumulation**
The metaiodobenzylguanidine (MIBG) accumulation experiment was performed as described previously (n = 3 per group). Briefly, [125I]-MIBG (25 μCi/kg) was injected via the tail vein while the mice were under anaesthesia with pentobarbital (30 mg/kg intraperitoneally). Mice were killed at 3 h after injection under anaesthesia with pentobarbital (30 mg/kg intraperitoneally). Biodistribution of MIBG in graft heart, original heart, spleen, and blood was measured with an automatic well-type gamma counter (ARC 2000, Aroka, Japan).

**2.5 Monitoring ECG of transplanted heart and tyramine administration**
Mice undergoing heterotopic heart transplantation were anaesthetized with 4% isoflurane and maintained with 1.5% isoflurane. In addition to 12-lead ECG monitoring, perigraft leads were placed bilaterally on the abdominal wall (Figure 1B). The ECG was evaluated using a commercially available machine (ECG-1400; Nikon Kohden). Body temperature was kept at 37 ± 0.5°C. The animal’s head was covered with a cloth to prevent light stimulation to sympathetic reflexes. An intraarterial bolus injection of tyramine hydrochloride (200 μg/kg in 20 μL saline) was administered to stimulate the release of endogenous norepinephrine, with a saline bolus injection (20 μL) used as a control (n = 5 per group).

**2.6 Langendorff perfusion of the transplanted hearts**
The donor hearts were quickly excised under anaesthesia with ketamine/xylazine combination (100 and 10 mg/kg intraperitoneally) and perfused with modified Krebs–Henseleit buffer according to the Langendorff procedure (n = 6 per group). Coronary perfusion pressure was maintained at 70 mmHg, and a plastic catheter with a latex balloon was inserted into the left ventricle through the left atrium. The left ventricular end-diastolic pressure was adjusted to 10 mmHg by filling the balloon with water, as described previously. After a period of stabilization, the perfusion buffer was switched to Krebs–Henseleit buffer containing tyramine (10 μM). ECG was continuously measured every minute before and after tyramine administration.

**2.7 Statistical analysis**
Values are presented as means ± standard error. Differences between groups were examined for statistical significance using Student’s t-test or analysis of variance with Tukey test. P-values < 0.05 were regarded as significant.

Detailed methods of histochemistry, echocardiography, flow cytometry and tissue norepinephrine, epinephrine, and dopamine measurement are provided in Supplementary Information.

**3. Results**

**3.1 Neural crest-derived TH-immunoreactive cells existed in the fetal heart before the initiation of sympathetic innervations**
To trace the fate of neural crest-derived cells in the heart, we used double-transgenic mice harbouring Protein 0-Cre and Floxed-EGFP, in which the neural crest-derived cells express EGFP (see Supplementary material online, Figure S1). Our group previously showed that...
EGFP-positive cells in these mice were consistent with known neural crest populations, including those found in the peripheral nervous system, adrenal glands, epidermis, and sympathetic nerve ganglion. At embryonic day 10.5 (E10.5), immunohistological analysis revealed neural crest-derived cells densely distributed in the outflow tract of Protein 0-Cre/Floxed-Enhanced Green Fluorescent Protein (EGFP) double-transgenic mice in E10.5 for EGFP (green), TH (red), and Topro-3 (blue, nuclei). (C) High-power photomicrographs of neural crest-derived adrenergic cells. (D) Double immunostaining for EGFP (green) and α-actinin (red) in the adult Protein 0-EGFP heart. (E) Triple immunostaining for EGFP (green), α-actinin (red), and Topro-3 (blue) in the adult Protein 0-EGFP mice. (I) Three-dimensional image analysis of neural crest-derived adrenergic cells. Note that the neural crest-derived adrenergic cells have neurites and nucleus. (G) Triple immunostaining for EGFP (green), TH (red), and Topro-3 (blue) of ICA cells in Protein 0-EGFP transgenic ventricular myocardium. (H) Triple immunostaining for EGFP (green), TH (red), and Topro-3 (blue) in DBH-EGFP transgenic ventricular myocardium. White box corresponds to the high-power photomicrographs in the insets and in I. (J) Triple immunostaining for EGFP (green), TH (red), and Topro-3 (blue) of cardiac resident adrenergic cells in DBH-EGFP transgenic ventricular myocardium. DBH, dopamine β hydroxylase; EGFP, enhanced green fluorescent protein; TH, tyrosine hydroxylase.

**Figure 1** Distribution and characterization of neural crest-derived adrenergic cells in the heart. (A) HE staining of E10.5 neonatal heart section. Black box indicates the area used for immunofluorescent microscope imaging in (B) and (C). (B) Triple immunofluorescent staining of the outflow tract of Protein 0-Cre/Floxed-Enhanced Green Fluorescent Protein (EGFP) double-transgenic mice heart in E10.5 for EGFP (green), TH (red), and Topro-3 (blue, nuclei). (C) High-power photomicrographs of neural crest-derived adrenergic cells. (D) Double immunostaining for EGFP (green) and α-actinin (red) in the adult Protein 0-EGFP heart. (E) Triple immunostaining for EGFP (green), α-actinin (red), and Topro-3 (blue) in the adult Protein 0-EGFP mice. (I) Three-dimensional image analysis of neural crest-derived adrenergic cells. Note that the neural crest-derived adrenergic cells have neurites and nucleus. (G) Triple immunostaining for EGFP (green), TH (red), and Topro-3 (blue) of ICA cells in Protein 0-EGFP transgenic ventricular myocardium. (H) Triple immunostaining for EGFP (green), TH (red), and Topro-3 (blue) in DBH-EGFP transgenic ventricular myocardium. White box corresponds to the high-power photomicrographs in the insets and in I. (J) Triple immunostaining for EGFP (green), TH (red), and Topro-3 (blue) of cardiac resident adrenergic cells in DBH-EGFP transgenic ventricular myocardium. DBH, dopamine β hydroxylase; EGFP, enhanced green fluorescent protein; TH, tyrosine hydroxylase.

EGFP-positive cells in these mice were consistent with known neural crest populations, including those found in the peripheral nervous system, adrenal glands, epidermis, and sympathetic nerve ganglion. At embryonic day 10.5 (E10.5), immunohistological analysis revealed neural crest-derived cells densely distributed in the outflow tract of the developing heart (Figure 1A and B), and some of these cells were positive for TH. High-power magnification revealed that most TH-positive cells were 10 μm in diameter, round, and nucleated (Figure 1C). Sympathetic innervation of the heart does not occur before E15.5; thus, these neural crest-derived TH-positive cells existed in the fetal heart before the initiation of sympathetic innervations.

### 3.2 Neural crest-derived TH-immunoreactive cells still exist in the adult heart

The number of neural crest-derived TH-immunoreactive cells decreased after cardiac sympathetic nerve innervation, but some remained within the adult murine ventricular myocardium. Immunohistological analyses of hearts from 8-week-old Protein 0-Cre/Floxed-EGFP double-transgenic mice (Figure 1D) revealed EGFP-positive neural crest-derived cells scattered throughout the left ventricular myocardium. There were two distinct types of TH-positive neural
crested-derived cells identified. One type comprised cardiac sympathetic nerve ending originating from the stellate ganglion as non-nucleated cells, markedly localized in the myocardium (Figure 1E). The other types of cells were localized in the myocardium. Some of these cells appeared to have neurites (Figure 1F) but were different from intracardiac sympathetic nerve ending, since they were nucleated and frequently seen in clusters. Three-dimensional image analysis of these cells confirmed the presence of nuclei (Figure 1G).

We also analysed double-transgenic mice harbouring DBH-Cre and Floxed-EGFP to trace the lineage of catecholaminergic cells (see Supplementary material online, Figure S2). Immunohistological analysis demonstrated nucleated TH/DBH double-positive cells within the adult ventricular myocardium (Figure 1H and I).

### 3.3 Neural crest-derived adrenergic cells increased in transplanted heart with time

We examined the role of intrinsic cardiac neural crest-derived cells in cardiac neuronal remodelling after heterotopic heart transplantation. The time course of change in neural crest-derived cells was charted in heart transplanted from the Protein 0-Cre/Floxed-EGFP double-transgenic mice onto the abdominal aorta of NOD-SCID mice (Figure 2A). We successfully recorded electrical activities of the heterotopic transplanted heart that were independent of those recorded from the orthotopic recipient heart. We pinned needle electrodes on the original sites for 12-lead ECG recording and on the recipient mice’s abdominal walls by removing abdominal hairs and opening the abdomen skin (Figure 2B). In this way, we simultaneously recorded the electrical activity of the recipients’ original hearts and the transplanted hearts. In the precordial chest leads, the ECG voltages from recipient hearts were higher than those from transplanted heart, whereas voltages recorded through the perigraft abdominal leads from transplanted hearts were higher than those from transplanted heart, whereas voltages recorded through the perigraft abdominal leads from transplanted hearts were higher than those from transplanted heart (Figure 2C). ECG recordings from the recipient hearts were independent of those from the transplanted heart. The cardiac echocardiogram also revealed that the heterotopic transplanted hearts continued to beat throughout the study (data not shown).

As a consequence of surgical denervation, sympathetic nerve fibres from the stellate ganglion, which is predominantly localized at the epicardial surface of the heart, disappeared in the transplanted heart (Figure 3A). The number of EGFP-positive neural crest-derived cells in the ventricles of the transplanted hearts increased immediately following transplantation (Figure 3B). The number of neural crest-derived cells in the donor hearts before, at 1, 2, and 4 weeks after transplantation was 24 ± 6, 211 ± 32, 292 ± 36, and 632 ± 92 cells per 10⁵ cells, respectively (Figure 3C). The neural crest-derived TH-immunoreactive cells increased over the 2 weeks following transplantation. These cells were nucleated. Some of the cells were round and did not generate neurites, while others appeared to have long axons (Figure 3D, E, G). The number of neural crest-derived nucleated TH-immunoreactive cells in the donor hearts before, at 1, 2, and 4 weeks after transplantation was 4.2 ± 0.8, 5.4 ± 2.3, 42.2 ± 19.8, and 212.2 ± 26.2 cells per 10⁵ cells, respectively (Figure 3F). These cells in the transplanted heart were also immunopositive for phenylethanolamine N-methyltransferase (Figure 3G), and the long axons obtaining nucleated TH-immunoreactive cells were positive for synaptophysin (Figure 3H).

To determine whether the increase in number of neural crest-derived nucleated TH-immunoreactive cells in the donor hearts is associated with the presence of nerve growth factor (NGF), we found that cardiac NGF expression levels increased in response to sudden cardiac sympathetic denervation after transplantation (see Supplementary material online, Figure S3). Consistent with this, sorted residual neural crest-derived cells in P0-EGFP neonate heart significantly proliferated and differentiated into neural cells expressing TH in the presence of NGF in vitro (see Supplementary material online, Figure S4).

### 3.4 Catecholamine-synthesizing activities recovered over time in the transplanted heart

We used quantitative real-time PCR to determine the mRNA expression levels of catecholamine-synthesizing enzymes in the heart to

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**Figure 2** Heterotopic cardiac transplantation with physiological and histological analysis of the donor heart. (A) Scheme of the heterotopic cardiac transplantation. IVC, inferior vena cava; PA, pulmonary artery. (B) Procedure for electrocardiography recording of the transplanted heart. (C) ECG of precordial chest lead V2 and V5, and perigraft abdominal lead. ‘R’ indicates the QRS waves from the recipient’s original heart, and ‘D’ indicates the QRS waves from the donor heart.
examine the time course change in the ability of the transplanted heart to produce catecholamine. The mRNA expression levels of TH, DBH, and phenylethanolamine N-methyltransferase dropped markedly 1 week after transplantation compared with pre-transplanted recipient heart, and then gradually recovered with time after transplantation (Figure 4A).

We next examined the tissue content of catecholamines in the transplanted hearts using HPLC. The tissue content of dopamine, epinephrine, and norepinephrine in the transplanted hearts dropped initially at 1 week after transplantation and increased thereafter (Figure 4B), in parallel with the change in mRNA expression levels of catecholamine-synthesizing enzymes. These results suggest that intrinsic catecholamine-synthesizing activities in the transplanted heart were salvaged in parallel to the change in neural crest-derived TH-immunoreactive cells.

To confirm the contribution of NGF in the recovery of catecholamine-synthesizing activity in vivo, we administrated anti-NGF neutralizing antibodies on the recipient mice after the cardiac transplantation procedures. Interestingly, the recoveries of tissue content catecholamines in the transplanted hearts were suppressed by the administrations of anti-NGF neutralizing antibodies compared with isotype control administrations at 4 weeks after transplantation (see Supplementary material online, Figure S5).

Figure 3 Neural crest-derived adrenergic cells increased after cardiac transplantation. (A and B) Triple immunostaining for EGFP (green), α-actinin (red), and Topro-3 (blue) in Protein 0-EGFP hearts in pre-transplantation heart, and at 1, 2, and 4 weeks after cardiac transplantation. While EGFP-positive sympathetic nerves in the epicardium (first panel) were de-innervated, EGFP-positive cells in the myocardium increased with time after the cardiac transplantation (panels 2–4). (C) Time course change in the number of EGFP-positive neural crest-derived cells in the heart after cardiac transplantations (n = 5), (*P < 0.01; **P < 0.001 compared with the number observed before cardiac transplantation). (D and E) Triple immunostaining of neural crest-derived adrenergic cells for EGFP (green), TH (red), and Topro-3 (blue) in Protein 0-EGFP hearts 1, 2, and 4 weeks after cardiac transplantation. (F) Time course change in the number of neural crest-derived adrenergic cells in the heart after cardiac transplantations (n = 5) (*P < 0.01; **P < 0.001). The number of neural crest-derived nucleated TH-immunoreactive cells per 10^5 nucleated cells was determined by FACS. (G) Triple immunostaining for EGFP (green), phenylethanolamine N-methyltransferase (red), and Topro-3 (blue), (H) triple immunostaining for synaptophysin (green), TH (red), and Topro-3 (blue), of neural crest-derived adrenergic cells in Protein 0-EGFP hearts 4 weeks after the cardiac transplantation. EGFP, enhanced green fluorescent protein; TH, tyrosine hydroxylase.
3.5 Functional significance of neural crest-derived adrenergic cells in the transplanted hearts

Myocardial catecholamine uptake and storage are associated with the ventricular inotropic response to exercise in human cardiac transplant recipients. We examined the ability of transplanted heart to uptake catecholamine using iodine-123-MIBG scintigraphy. The uptake of MIBG initially dropped 1 week after transplantation, but began to recover at 2 weeks after transplantation (Figure 5A and B).

We injected tyramine into the peritoneal cavity of recipient mice 2 weeks after transplantation to determine the restoration of intrinsic adrenergic activity in transplanted hearts. The administration of tyramine induces intra-cellar catecholamine secretion that is independent from direct stimulation of adrenergic transporters. (Figure 5C). Although the tyramine injection did not affect mean heart rate in the heterotopic transplanted heart at 1 week after transplantation (data not shown), heart rate was significantly increased from 188 ± 3.4 to 217 ± 1.8 bpm in the heterotopic transplanted heart at 2 weeks after transplantation (Figure 5D and see Supplementary material online, Figure S6). The serum concentrations of catecholamines were not affected by tyramine injection at this time point, and the serum dopamine levels in control, transplanted rats with/without tyramine treatments were 5.5 ± 1.8, 4.1 ± 0.3, and 5.0 ± 1.5 ng/mL, the serum epinephrine levels were 0.16 ± 0.01, 0.15 ± 0.04, and 0.21 ± 0.3 ng/mL, and the serum norepinephrine levels were 1.35 ± 0.30, 0.95 ± 0.24, and 1.30 ± 0.20 ng/mL, respectively (see Supplementary material online, Figure S7).

To exclude the contribution of sympathetic reinnervation and adrenal release of catecholamines, transplanted heart was rapidly

Figure 4 Recovery of catecholamine biosynthetic activity after cardiac transplantation. (A) The mRNA expression levels of TH, dopamine-β-hydroxylase (DBH), and phenylethanolamine N-methyltransferase in control hearts, and at 1, 2, and 4 weeks after cardiac transplantations were determined by quantitative real-time PCR (n = 5) (**P < 0.001 compared with the expression levels at 1 week after cardiac transplantation). (B) Tissue content of catecholamine (dopamine, epinephrine, and norepinephrine) in control heart, and at 1, 2, and 4 weeks after cardiac transplantations were measured by HPLC (n = 5) (**P < 0.001 compared with the contents 1 week after the cardiac transplantation; #P < 0.01 compared with the contents 2 weeks after the cardiac transplantation).
excised from the mouse abdominal cavity and mounted on a Langen-
dorff perfusion system. Langendorff-perfused hearts were then treated
with tyramine and their heart rates monitored. Tyramine treatment did
not affect heart rate of the transplanted hearts excised from mice 1 week
after transplantation, but heart rate was significantly increased by 23% in
the transplanted hearts excised from mice 2 weeks after transplantation
(at 1 week after the transplantations, heart rates changed from 101 ± 5 to
98 ± 7 bpm after tyramine treatment, while at 2 weeks they changed
from 103 ± 4 to 127 ± 8 bpm) (Figure 5E).

4. Discussion

Sympathetic denervation of transplanted heart occurs due to surgical
interruption of the postganglionic sympathetic fibres at the time of
transplantation.14 There is some evidence of partial restoration of car-
diac adrenergic activity in the allograft with time, demonstrated by an
improvement in the chronotropic and inotropic response to exercise,
and the restitution of catecholamine uptake and storage by the myocar-
dium.13 Re-establishment of the cardiac adrenergic activity can be as-
cribed to both the sympathetic re-innervation and the activation of
an ICA system.15 However, little is known about the origin and function
of the ICA system after heart transplantation.

Using genetically tagged cells arising from the neural crest, we de-
monstrated that surgical denervation following heart transplantation
ablates the sympathetic nerve fibres from the stellate ganglion, while
stimulating an increase in the number of neural crest-derived
TH-immunoreactive-nucleated cells in the heterotopically transplanted
mouse heart. The restoration of cardiac sympathetic activities, includ-
ing mRNA expression of catecholamine-synthesizing enzymes; myocar-
dial catecholamine uptake and storage, and chronotropic response to
tyramine, is tightly coupled to the increased number of neural crest-
derived TH-immunoreactive-nucleated cells.

Although neural crest-derived cells in the transplanted heart immedi-
ately increased at 1 week after transplantation, neural crest-derived
TH-immunoreactive cells only started to increase at 2 weeks following
transplantation and rose thereafter. Consistent with this, tyramine ad-
ministration caused a positive chronotropic response in the transplanted
heart both in vivo and ex vivo from 2 weeks after transplantation. Cardiac
NGF expression also increased in response to the sudden cardiac sym-
pathetic denervation after transplantation, while sorted residual neural
crest-derived cells from P0-EGFP neonate heart proliferated and dif-
ferentiated into neural cells expressing TH in the presence of NGF.
Together, these findings support our conclusion that the subsequent dif-
ferrntiation of neural crest stem cells into catecholamine-biosynthetic

Figure 5 Functional recovery of cardiac adrenergic activity after transplantations. (A) Representative autoradiograms of [125I]-MIBG in short-axis view
of the hearts in controls, and at 1 and 2 weeks after cardiac transplantations. (B) Quantitative analysis of MIBG uptake, determined from the radioactivity
standardized against mouse body weights. ([125I]-MIBG accumulation in hearts of the controls, and at 1, 2, and 4 weeks after cardiac transplantations
(n = 3) (*P < 0.01 compared with the accumulation of 1 week after the cardiac transplantation). (C) Schema of the catecholamine synthesis, release,
and reuptake of norepinephrine into the ICA cells, and the mechanisms of tyramine-induced norepinephrine release. (D) Increase in the heart rates of the
transplanted heart at 2 weeks after cardiac transplantations by tyramine administration (n = 5) (*P < 0.01 compared with heart rate prior to tyramine
treatments). (E) The chronotropic response to tyramine in Langendorff-perfused transplanted heart. Transplanted heart was isolated from recipient
mice at 1 and 2 weeks after heart transplantation (n = 6) (*P < 0.01).
cells contributed to activation of the ICA system with the support of NGF.

High-power magnification revealed that the some TH-immunoreactive cells were 10 μm in diameter, round, and nucleated. The morphology of these cells is consistent with ICA cells originally reported by Huang et al. Some of these cells appear to have neurites but are distinguishable from intracardiac sympathetic nerve ending, since they are nucleated and frequently appear in clusters. It seems possible that neural crest-derived ICA cells display neurite outgrowth in response to certain stimuli, as seen in adrenal medullary cells. And the sprouting TH-immunoreactive-nucleated cells have also expressed synaptophysin. It strongly indicates that these cells functionally contribute neuro-hormonal endocytosis.

We did not confirm whether an increase in the number of neural crest-derived TH-immunoreactive-nucleated cells improves the capacity for exercise. However, there is evidence that myocardial catecholamine uptake and storage are surrogate markers of the exercise-induced increase in ejection fraction in heart transplant recipients. Notably, iodine-123-MIBG scintigraphy showed that the ability of transplanted heart to uptake catecholamine recovered with time, and that this functional recovery correlated with the increase in neural crest-derived TH-immunoreactive-nucleated cells. Together, these findings supported the functional importance of neural crest-derived TH-immunoreactive-nucleated cells in the transplanted heart.

In conclusion, neural crest-derived TH-immunoreactive-nucleated cells and their precursor, neural crest stem cells, remain resident in the heart even after sympathetic innervation has stopped, serving as an emergency ‘backup’ system for cardiac adrenergic activity that can be reactivated by sympathetic denervation following heart transplantation.

Cardiac NGF expression increased in response to sudden cardiac sympathetic denervation after transplantation, and it may be implicated in the differentiation and/or proliferation of neural crest-derived adrenergic cells in the transplanted heart.

Our use of the murine heterotopic cardiac transplantation model may limit the findings of this study. As the transplanted heart is located in the abdomen, it would be difficult for the cardiac sympathetic nerves originating in the stellate ganglia to reconnect to these hearts. In addition, the retrogradely perfused and beating, but non-working, heterotopic transplantation model in the mouse is different from human orthotopic transplantation, particularly in the time course of restoration of cardiac adrenergic activity. It takes several years to restore cardiac adrenergic uptakes, while this occurs within a few weeks in the murine model of heterotopic cardiac transplantation. In human, it could be evaluated with the catecholamine analogue [11C] hydroxyephedrine in heart transplant patients, and it affects the improvements of quality of life after cardiac transplantations. Moreover, such phenomenon sometimes results in frequent angina or syncopal episodes. However, orthotopic transplantation is technically challenging in the mouse. Different coronary flow patterns and mechanical unloading in the murine heterotopic cardiac transplantation model might also affect the activation of an intrinsic adrenergic system.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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