cKit+ cardiac progenitors of neural crest origin

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The degree to which cKit-expressing progenitors generate cardiomyocytes in the heart is controversial. Genetic fate-mapping studies suggest minimal contribution; however, whether or not minimal contribution reflects minimal cardiomyogenic capacity is unclear because the embryonic origin and role in cardiogenesis of these progenitors remain elusive. Using high-resolution genetic fate-mapping approaches with cKitCreERT2/− and Wnt1:Flp mouse lines, we show that cKit delineates cardiac neural crest progenitors (CNCs). CNCs possess full cardiomyogenic capacity and contribute to all CNC derivatives, including cardiac conduction system cells. Furthermore, by modeling cardiogenesis in cKitCreERT2-induced pluripotent stem cells, we show that, paradoxically, the cardiogenic fate of CNCs is regulated by bone morphogenetic protein antagonism, a signaling pathway activated transiently during establishment of the cardiac crescent, and extinguished from the heart before CNC invasion. Together, these findings elucidate the origin of cKit cardiac progenitors and suggest that a nonpermissive cardiac milieu, rather than minimal cardiomyogenic capacity, controls the degree of CNC contribution to myocardium.

Heart development is a highly regulated process during which cell lineage diversification and growth programs are dynamically coordinated in temporal and spatial manners (1). These programs are activated sequentially, in parallel, or intersect to give rise to distinct heart domains. For example, the myocardial lineage originally develops from cardiac progenitors (CPs) of mesodermal origin (2–5), which form the first and second heart fields. However, later during morphogenesis, the cardiomyogenic program diverges and activates cardiomyocyte proliferation signals, along with CPs from the hemogenic endothelium, epicardial, pulmonary, and cardiac neural crest (CNC) lineages, to produce new cardiomyocytes (1, 6–11). Gauging the relative contribution of each lineage for scaling their cardiomyogenic—and consequently therapeutic—capacity is a challenge. For example, many of the CP lineages are heterogeneous and incompletely characterized, and therefore cannot always be traced under a straightforward genetic fate-mapping experiment. Furthermore, it is unknown whether and how changes in the cardiac milieu (i.e., morphogens, tissue composition, and size) regulate the final proportions of heart muscle derived from each lineage.

cKit is a receptor tyrosine kinase that marks several cell lineages, including neural crest (NC), hematopoietic, and germ-line stem cells (12–15). Following the seminal description by Beltrami et al. (16) of clusters of cKit cells in the postnatal mammalian heart, several laboratories, including ours, suggested that cKit marks CPs (16–19), a finding that led to the clinical testing of these cells for heart repair (20). Recently, a straightforward genetic fate-mapping study showed that a relatively small proportion of murine myocardium is derived from cKit+ CPs, leading to the conclusion that the cardiomyogenic capacity of cKit+ CPs is functionally insignificant (21). However, the identity of cKit+ CPs and the mechanisms controlling their differentiation into cardiomyocytes remain controversial (22). Here, by using a high-resolution genetic lineage-tracing strategy, as well as induced pluripotent stem cell (iPSC)-based models of cardiogenesis, we demonstrate that cKit marks CNCs. Furthermore, we show that their relatively small contribution to myocardium during embryogenesis is not related to poor cardiomyogenic capacity, but rather to changes in the cardiac activity of the bone morphogenetic protein (BMP) pathway that prevent their differentiation into cardiomyocytes.

Results

Genetic Lineage-Tracing of cKit+ CPs. We used a well-characterized cKitCreERT2/+ mouse line to lineage-trace cKit+ CPs (23–25). cKitCreERT2/+ are healthy, fertile, and express the white spotting phenotype (12, 23, 24, 26) (Fig. 1A).

We first investigated whether cKit marks mesodermal CPs (e.g., first- or second-heart field CPs; or primitive hematogenic lineage) (1), by administering pregnant mice carrying cKitCreERT2/Irg embryos with tamoxifen (TAM) from embryonic days (E)7.5 to E8.5 (Fig. 1B and Table S1). At E18.5, EGFP expression was detected in mesodermal cells (13, 14, 21, 26), including gonads, blood, and lungs (Fig. 1 C and D). At this stage of labeling (21), EGFP was rarely detected in the heart, and EGFP+ heart cells were non-cardiomyocytes with rare colocalization with the cardiac transcription factor Gata4 (Fig. 1 E and F).

Next, to test whether cKit marks other cardiomyogenic lineages (e.g., proliferating cardiomyocytes; or CPs of the epicardial, CNC, and definitive hematogenic lineages) (1), we administered TAM to pregnant mice at selected time points during E9.5–E12.5.


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genitors (6, 7], and derived from an extracardiac CP lineage. Transdifferentiating cardiac fibroblasts (29) and hemogenic pro-cells during the period of TAM-induced recombination do not arise.

Results were similar using this reporter compared with EGFP (Fig. 1). Escape is detected within the cardiac outflow tract (OFT), enteric cells, gonads, and pulmonary cells (Fig. 1).

Because our findings are consistent with a CNC origin of cKit progenitors are of different origins. These studies illustrate a lineal relationship between the cKit+/ progenitors of different origins. These studies illustrate a lineal relationship between the cKit+ CPs and Wnt1+ CNCs (CNCs).

CNC+ Derivatives in the Heart. Expression of Cre-reporters was detected in all expected cardiac NC derivatives (37), including the OFT (Figs. 1J and 3A and B, and Figs. S2B and S5), the
Consistent with their CNC origin (38), CNC progenitors, and sensory cells (Fig. S5), the tunica media of the aortic arch (Fig. 3C), cardiac and aortic valves (Fig. 3D and E), atria (Fig. 1K), inflow tract, satellite glial progenitors, and sensory cells (Fig. S5 A and B and Movie S1). Consistent with their CNC origin (38), CNCkit contributed to endothelium and smooth muscle layers of the OFT (Fig. 3A and B), although coronary vascular cell differentiation was not observed (Fig. 3F).

In agreement with previous reports in zebras (8, 11) and mice (10, 21, 31–33), our analysis with the Wnt1-Cre (Fig. S6 and Movie S2) and cKitCreERT2/+ alleles suggests that CNCs contribute to the myocardial lineage. Particularly, we documented contribution of CNCkit to atrial and ventricular cardiomyocytes (29.9% ± 3.1% of total EGFP+ derivatives) (Fig. 3 K and L), and pericardial, endocardial, and epicardial cells (Figs. 1 I, J, and N, and 3 G–K, and Movie S3). The majority of CNCkit-derived cardiomyocytes was localized in the interventricular septum (Figs. 1 J and 3 K and L), which, unlike the left and right ventricular myocardium, is partly derived from mesoderm posterior 1 homolog nonexpressing (Mexp1−) CPs of undefined origin (9).

CNCkit Identity. To better characterize the identity of CNCkit, we studied the expression of microphthalmia-associated transcription factor (Mitf), a direct target and transactivator of cKit signaling, expressed not only in cranial NC derivatives and mast cells but also in cardiomyocytes (39). If analysis demonstrated that Mitf is also expressed in CNCkit and their cardiomyocytic derivatives (Fig. S7 A–C). However, EGFP+ cells in the heart did not express the melanocyte-specific markers tyrosinase or trp1, suggesting that Mitf+ CNCkit derivatives in the heart are not melanocytes (Fig. S7C).

Next, we investigated the expression of Isl1, a homebox transcription factor that specifies the majority of the mammalian CP lineages, including CNCs (36). Accordingly, cKitCreERT2;IRG mice were crossed with mice carrying an Isl1 nuclear lacZ (Isl1nLacZ) allele (40). When pregnant mice were administered TAM from E9.5–E11.5, (Table S1), colocalization of EGFP and nLacZ was documented in cells of the NT, DRGs, and the OFT (Fig. S7 D–J) in E12.5 embryos.

**Fig. 2.** Intersectional genetic fate-mapping of cKit and Wnt1. (A) Schematic of the two different approaches of the study. (B–D) Live epifluorescence imaging (B), followed by salmon-gal histochemical detection of nLacZ (C and D), in an E10.5 cKitCreERT2, RC:Felo embryo exposed to TAM during E8.5–E9.5. The flip- and intersectional indicators are not expressed in the absence of Flpe and Flpe/Cre-mediated recombination, respectively. (E–H) A Wnt1::FpexCreERT2;RC:Felo littermate exhibits widespread GFP epifluorescence (E) and a few salmon-gal cells in the NT and heart. (I–L) Live embryo imaging of mCherry and GFP expression in a E17.5 Wnt1::FpexCreERT2;RC:Felo embryo. EGFP+ CNCkit in the craniofacial region (l, skin (J), OFT (K), and the epicardial wall of the heart (L). (M and T) X-gal+ CNCkit derivatives in the OFT (M and G) and epicardium (P and T) of E17.5 Wnt1::FpexCreERT2;RC:Felo embryos. Arrows in M–P are depicted in higher magnification in panels Q–T, respectively. Panels B, E are photomerged image tiles. OFT, outflow tract; Ht, heart; Lu, lung. (Scale bars, 50 μm in M–P and 300μp in L–M). (Magnification, 100× in B–H and Q–T).
Fig. S8), were significantly down-regulated compared with controls. Final-ly, expression of cKit increased significantly over time (Fig. S8D), although the level of expression was similar between the different treatment groups (Fig. 4G). As previously shown (15, 41), decreased expression of myocardin specification of cKit+ CPs commenced via codelivery of NKX2.5 (Fig. 5 and Fig. S9 A-D). Remarkably, BMP antagonism enhanced the development of EGFP+/NKX2.5 progenitors by ~sevenfold (Fig. 5, Fig. S9 A-D, and Movie S5) (P = 0.0057). Moreover, iPSCs+/derived CNCs gave rise to all CNC derivatives, including EGFP+/smooth muscle cells (Fig. S9 E and F), Isl1+ (Fig. S9 G and H), and Pax3+ CPs (Fig. S9 I and J), while inverting the beating EBs with neurofilament-M+ and TuJ1+ neurons (Fig. S9 K-N).

Discussion

The major findings are that cKit marks CPs of CNC origin, which enter the embryonic mouse heart at ~E9.5 and contribute to the relatively small proportion of myocardium and other derivatives of the CNC, but not coronary vascular cells. In addition, we show that CNC samples with cardiomyocyte differentiation capacity can be derived in vitro from mouse iPSCs following transient antagonism of the BMP pathway, which drives the stage-specific differentiation of iPSCs toward the cardiac mesoderm and CNC lineages. Our findings confirm previous developmental studies in mice showing that, during gastrulation, cKit is expressed in extraembryonic mesoderm and embryonic ectoderm, but not mesodermal CPs (4, 13, 14, 42). Furthermore, the findings are in agreement with previous reports supporting the existence of Kit+ CPs, which do not contribute to coronary endothelium (15), as well as with recent endothelial lineage fate-mapping analyses suggesting that the coronary endothelium is unlikely to originate from Kit+ cells (6, 29).

Notably, although much controversy exists over the contribution of CNCs to the myocardium (8, 10, 11, 30–33), our studies with the cKitCreERT2/+; Wnt1-Cre, and Wnt1::Flpe alleles strongly support the hypothesis that the mammalian CNC holds full cardiomyogenic capacity, which our findings now suggest is undermined by developmental changes in the activity of BMP and Wnt pathways preceding their invasion in the heart (5, 31, 45). It is also noteworthy that a pool of multipotent postmigratory NC progenitors (47, 48), some of which express cKit (49), has been reported in other tissues; hence, it would be interesting to examine their relationship to CNCs.

Our study differs from a recent cardiac genetic fate-map of cKit, using different cKit alleles (21). First, in contrast to findings presented here and elsewhere (6, 15, 29), van Berlo et al. (21) reported that cKit CPs contribute extensively to coronary endothelium. However, it is noteworthy that mutations in the mouse Wnt locus have not been associated with tangible cardiovascular defects (12), as would be likely if cKit+ CPs comprised a major source of coronary vascular cells. Second, van Berlo et al. (21) concluded that the minimal cardiomyocyte contribution of cKit+ CPs reflects minimal differentiation capacity. However, although our study agrees that the in vivo cardiomyocyte contribution of cKit+ CPs is lower than expected from previous reports (50, 51), we show that this is not a result of minimal differentiation capacity, but rather, because of their developmental origin in the CNC, which comprises a minor contributor of cardiomyocytes to the mammalian heart. Importantly, using iPSC modeling we demonstrate that differentiation of CNCs to cardiomyocytes requires the BMP signaling pathway, which also directs differentiation of mesodermal CPs to the myocardium. This finding suggests that, although CNCs hold full cardiomyocyte differentiation capacity, their in vivo contribution is repressed by spatiotemporal changes in BMP activity, which render the cardiac milieu nonconducive for cardiomyocyte differentiation during CNC invasion to the heart (45).

Our findings have several important implications. First, they resolve the current controversy over the existence and cardiomyogenic capacity of cKit+ CPs (22). We show that cKit+ CPs invested within the developing heart are fully capable of producing new cardiomyocytes, both in vivo and in vitro. Therefore,
coupled with the findings from many laboratories that cKit+ CPs are present in the postnatal heart, they represent an important therapeutic target for heart regeneration (17–19, 52, 53). For example, the activity of BMP in the damaged myocardium could be modulated pharmacologically, or via transplantation of cells capable of regulating BMP activity, to support production of myocardium from endogenous or exogenously supplied cKit+ CPs (18, 52).

Second, the findings advance our understanding of the cellular and molecular mechanisms underlying mammalian cardiomyogenesis, by illustrating a previously unknown relationship between the spatiotemporal modulation of the BMP pathway and the generation of myocardium from mesodermal and CNC CPs. Third, the generation of CNCs from iPSCs provides a unique opportunity to study and understand the biology and function of CNCs, as well as to test their regenerative capacity in novel cell-based therapeutic strategies. Finally, considering the technical limitations often associated with conditional gene-targeting approaches, our findings do not exclude the possibility that, in addition to CNC+ kit, the adult heart contains other cKit+ cells with full cardiovascular differentiation capacity, as those reported by others (21, 28, 50, 51), which may have remained undetectable with our reagents.

In conclusion, our findings support the hypothesis that the mammalian heart is invested with a cKit+ CP lineage, with full capacity to generate cardiomyocytes in vivo and in vitro, and therefore provide an important therapeutic target for the prevention and treatment of heart disease. Modulation of the activity...
of the BMP pathway in the heart may enhance the therapeutic regeneration of damaged myocardium from CNCs.

Materials and Methods

All animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility at the University of Miami, Miller School of Medicine, and procedures were performed using Institutional Animal Care and Use Committee-approved protocols according to NIH standards. cKit\textsuperscript{CreERT2} mice were developed as previously described (24). The Wnt\textsuperscript{1-Cre, RC:\texttt{tdTomato}, IRG, and R26\textsuperscript{Rosa26}} mouse lines were purchased from Jackson Laboratories. The iPS\textsuperscript{LacZ} mice have been described elsewhere (40). Wnt\textsuperscript{1-Flip}, RC::Fela and RC::Prepe mice were developed as previously described (35, 36). iPS\textsuperscript{C} were generated from adult c-kit\textsuperscript{pos} midfield tail-tip fibroblasts. Geno- type verification was performed by PCR. Immunohistochemistry, gene-expression analysis, lineage-tracing, and histological analysis of mouse embryos was performed as described (24). See SI Materials and Methods for more detailed discussion.

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SI Materials and Methods

Mice. cKitCreERT2/+ mice were developed at the Medizinische Klinik und Poliklinik der Technischen Universität München, in Germany (24). The endogenous cKit locus was targeted by homologous recombination in embryonic stem (ES) cells as previously described for the Rosa26 locus (24). In brief, the targeting vector with the CreERT2 cassette, an internal ribosome entry site and an ft-flanked neomycin resistance cassette was electroporated into 129S6 ES cells. Homologous recombination and single-copy insertion was verified by Southern blot analysis and correctly targeted ES cells were injected into C57Bl/6J blastocysts.

Wnt1::Flpe4351 were generated as previously described (36, 54). Briefly, sequences encoding a synthetic intron fused to Flpe-encoding sequence were inserted into the Erov site of WEXP2 harboring 11 kb of the Wnt1 gene. The 5′-flank fragment was purified and injected into B6Sil2 fertilized eggs using standard methods to generate transgenic mice, which were subsequently backcrossed to achieve a largely C57Bl6 genetic background. All experiments involving these mice were approved by the University of Miami Institutional Animal Care and Use Committee.

For genetic fate-mapping, CreER T2 was activated by intraperitoneal injections of 100 μL of TAM (Sigma), dissolved in peanut oil (Sigma) at a concentration of 20 mg/mL at desired time points, as previously described (40).

For assessing the role of cKit in first- or second-heart field progenitors, mice carrying cKitCreERT2;IRG or cKitCreERT2;R26RlacZ embryos received a daily injection of TAM for 2 consecutive days, during E7.5–E8.5. Embryos were harvested at E18.5. For accessing the expression of cKit in NC cells, mice carrying cKitCreERT2;IRG or cKitCreERT2;R26RlacZ embryos received a daily injection of TAM for 3 consecutive days, either during E9.5–E11.5 or during E10.5–E12.5. Embryos were harvested at E12.5 or E18.5. To trace the coexpression of Isl1-driven nLacZ and cKitCreERT2;IRG-driven EGFP in cKitCreERT2;IRG; Isl1lacZ embryos, microscopic analysis was performed within 24 h after the last injection of TAM. This strategy allowed us to detect cells in which Cre-mediated recombination was induced for a period that was sufficient to report expression of EGFP before expression of nLacZ disappear. The same approach was used for intersectional genetic fate-mapping in mice carrying cKitCreERT2; Wnt1::Flpe;RC::Fela or cKitCreERT2; Wnt1::Flpe;RC::Fela embryos.

Mouse Embryo Dissections. Females with a vaginal plug were considered at E0.5, as previously described (40). Embryos at different time points were harvested in ice-cold HBSS (Gibco). For E12.5 and E18.5 embryos carrying the cKitCreERT2;IRG Wnt1-Cre;RC::tdTomato, cKitCreERT2; Wnt1::Flpe;RC::Fela or cKitCreERT2; Wnt1::Flpe;RC::Fela alleles, live-tissue imaging was performed immediately after dissection under a fluorescence microscope (Olympus) and expression of EGFP, DsRed, tdTomato, or mCherry epifluorescence were photodocumented. Samples were stored then fixed for 1–1.5 h in 4% (vol/vol) PFA (EM) at room temperature followed by overnight incubation in 30% (wt/vol) sucrose (Calbiochem) at 4 °C. For embryos carrying the RC::Fela allele, tissues were fixed for 20 min in 0.2% glutaraldehyde/0.4% PFA. The next day, samples were embedded in OCT (EM) and flash-frozen in liquid nitrogen. Cryosectioning was performed as previously described (40).

For cKit immunohistochemical analyses, E12.5–E14.5 wild-type mouse embryos were harvested and fixed overnight in 10% (vol/vol) buffered formalin. For RC::Fela and RC::Fela embryos, the nLacZ reporter gene followed by humanized nLacZ reporter gene before expression of nLacZ driven by the R26RlacZ reporter gene was followed by humanized nLacZ reporter gene.

All animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility at the University of Miami, Miller School of Medicine, and procedures were performed using Institutional Animal Care and Use Committee-approved protocols according to NIH standards.

Generation of iPSCs. Mouse iPSCs were generated from adult tail-tip fibroblasts obtained from a 3.5-mo-old female cKitCreERT2;IRG mouse, using a commercially available kit (STEMCCA, Millipore). Briefly, tail-tip fibroblasts at passage one were plated in gelatin-coated 12-well plates at a density of 2 × 10^5 cells per well (day 0) with DMEM (Gibco), 2 mM l-glutamine, 10% (vol/vol) FBS (Gibco), and 1% penicillin-streptomycin (Gibco). The next day (day 1), fibroblasts were transduced with STEMCAA at a multiplicity of infection of 100 on day 1, followed by a repeated l-glutamine, 10% (vol/vol) FBS (Gibco), and 1% penicillin-streptomycin (Gibco). The next day (day 1), fibroblasts were transduced with STEMCAA at a multiplicity of infection of 100 on day 1, followed by a repeated
infection at an multiplicity of infection of 75 on day 2. Approximately 48 h later, cells were collected by trypsinization and replated on fresh plates coated with irradiated mouse embryonic fibroblasts (MEFs; Millipore) and fed with mouse ES medium [DMEM, 2 mM t-glutamine, 15% (vol/vol) FBS (Gibco), 0.1 mM nonessential amino acids (Gibco), 0.1 mM 2-mercaptoethanol, and (Gibco, and 1,000 units/mL LIF (Millipore)] supplemented with a cocktail of small molecules to enhance reprogramming (Mouse iPS reprogramming Boost Supplement, Millipore). IPS colonies began to emerge on day 7. Each colony was manually picked and individually transferred to fresh plates coated with MEFs. A total of 32 IPS<sup>diff</sup> clones were generated, 25 of which stably expressed the D<sup>a</sup>k<sup>o</sup> cassette as indicated by DsRed fluorescence. Clones 8, 21, and 31 were selected to be further expanded for the purpose of the study. All IPS<sup>diff</sup> lines where grown on MEFs and adapted gradually to a modified ES+2i medium [DMEM, t-glutamine, 15% (vol/vol) knockout serum replacement (Gibco), 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1,000 units/mL LIF, 1 µM PD0325901 (Tocris) and 3 µM CHIR99021 (Tocris)].

The expression of pluripotency markers from IPS<sup>diff</sup> was evaluated using a commercially available iPS immunohistochemical characterization kit (Abcam) and an alkaline phosphatase live stain (Invitrogen).

**Differentiation of IPS<sup>diff</sup>**. Two different methods were used to drive differentiation of IPS<sup>diff</sup> into cardiomyocytes. In the first method, IPS<sup>diff</sup> were trypsinized into single cells and plated for 30–45 min with ES+2i medium on 100-mm<sup>2</sup> gelatin-coated dishes to deplete MEFs. Feeder-depleted MEFs were then resuspended at a concentration of ∼4–8 × 10<sup>5</sup> cells/mL and allowed to grow for 3 d in MEF-conditioned differentiation medium [DM; IMDM (GIBCO), t-glutamine, 20% (vol/vol) FBS (Gibco), 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol], supplemented with 2,000 units/mL LIF (day −3 to day 0). On day 0, IPS<sup>diff</sup> were trypsinized and resuspended at a concentration of 2.5 × 10<sup>6</sup> cells/mL to generate EBs via the hanging-drop method, as previously described (15). EBs were then grown in hanging drops for 2 d in DM supplemented with 50 µg/mL AA or PBS, without LIF. On day 2, EBs were transferred into Petri dishes with DM+ AA or PBS and were grown in suspension for 2 more days. On day 4, EBs were transferred on gelatin-coated dishes with DM+ AA or PBS. Cells were fed every other day with DM+ AA or PBS. The first spontaneously beating cells emerged between late-afternoon of day 10 to day 12.

For the second method, feeder-depleted IPS<sup>diff</sup> were grown in MEF-conditioned DM for 3 d (day −3 to day 0), supplemented with 2,000 units/mL LIF, and 150 ng/mL recombinant mouse NOG (Noggin-FC; R&D) or 2 µM Dorso (Dorsomorphin; Tocris) or PBS, as previously described (43, 45). On day 0, EBs were generated as previously described in DM+ NOG or Dorso or PBS, without LIF. On day 1, treatment with NOG, Dorso, or PBS was discontinued and EBs were transferred into new Petri dishes or 12-well low-cluster plates (Corning) with fresh DM medium. EBs were grown in suspension for 2 more days before they were transferred into gelatin-coated plates.

To induce Cre-mediated recombination, (Z)-4-hydroxytamoxifen (Abcam) was supplemented to the cells at selected time points at a final concentration of 1 µM.

**X-Gal and Salmon-Gal Histochemistry**. X-gal histochemistry was performed using a β-galactosidase staining kit in fixed intact mouse embryonic hearts or embryos carrying the c<sup>Ki</sup>CreERT2; R26<sup>LoxP</sup> or Isl<sup>1<sup>LoxP</sup></sup> alleles, as well as their respective littermates, according to the manufacturers’ instructions (Molecular Probes). Briefly, samples were incubated overnight in X-gal solution at 37 °C. The next day, X-gal was washed away with PBS, samples were photodocumented under a light microscope (Nikon), and incubated overnight in 30% (wt/vol) sucrose at 4 °C. Fixed tissue samples were then embedded in OCT and processed for cryosectioning. In some instances of c<sup>Ki</sup>CreERT2; IRG; Isl<sup>1</sup>LoxP and c<sup>Ki</sup>CreERT2; Wnt1::Flpe; RC: Fela embryos, X-gal was performed after cryosectioning, following EGFP immunostaining. Staining with salmon-gal was performed as previously described (55).

Brieﬂy, samples were ﬁxed for ~20 min at room temperature in 0.2% glutaraldehyde/0.4% PFA, 5 mM EGTA and 2 mM MgCl<sub>2</sub> in 0.1 M phosphate buffer (pH 7.3). Embryos were then incubated for 3–4 h in staining solution consisting of wash buffer supplemented with 1 mg/mL salmon-gal (Indoﬁne) and 0.4 mM of Tetranitro blue tetrazolium salts dissolved in absolute ethanol (TCI).

**Immunofluorescence Confocal Microscopy**. For cryosections, 10-µm-thick sections were postﬁxed for 10 min with 4% (vol/vol) PFA. For parafﬁn-embedded tissues, 4- to 5-µm-thick tissue sections were deparafﬁnized and rehydrated, as previously described (18). Antigen unmasking was performed by microwaving the slides for 2 x 10 min in citrate buffer Solution, pH = 6 (Dako). Sections were then blocked for 1 h at room temperature with 10% (vol/vol) normal donkey serum (Chemicon International), followed by overnight incubation at 4 °C with the primary antibody.

The following antibodies were used: c<sup>Ki</sup> (DAKO, eBiosciences, and R&D), EGFP (Abcam, Aves, Molecular Probes), β-galactosidase (Molecular Probes), MITF, PECAM1, α-smooth muscle actin (Sigma), antismooth muscle myosin heavy chain (SM1; Kamiya Biomedical), SM22α, cardiac troponin-I, cardiac troponin-T, Calponin, Tyrosinase, Oct4, Sox2, Nanog, SSEA1 (Abcam), cardiac myosin light chain-2v (cmLC2v), neurofilament-M, FABP, Wnt1 (Novus Biologicals), Nkx2.5 (R&D and Santa Cruz Biotechnologies), GATA-4 (Santa Cruz Biotechnologies), Pax3, Isl-1 (40.2D6, Developmental Studies Hybridoma Bank), Factor VIII-related antigen (Biocare Medical), Tuj1 anti-Cre (Covance). Subsequently, the antibodies were visualized by incubating the sections for 1 h at 37 °C with FITC, Cy3 and Cy5-conjugated F(ab)2 fragments of afﬁnity-puriﬁed secondary antibodies (Jackson Immunoresearch) or Alexa 488 and Alexa 546 dyes (Molecular Probes). For MITF and SM1, tyramide signal amplification was used according to the manufacturer’s instructions (Perkin-Elmer). Slides were counterstained with DAPI, mounted with ProLong Antifade Gold reagent (Molecular Probes), and stored at 4 °C until further examination. Microscopic images and image acquisitions were performed with a Zeiss LSM-710 Confocal Microscope (Carl Zeiss Micro Imaging). The Zeiss ZEN software (v2009, Carl Zeiss Imaging Solutions) was used.

**Gene Expression Analysis**. Total RNA was extracted from IPS<sup>diff</sup> at selected time points before and during the course of their differentiation into cardiomyocytes, using the RNasey mini plus kit, according to the manufacturers’ instructions (Qiagen). cDNA synthesis was performed using the high-capacity cDNA reverse-transcription kit, according to the manufacturer’s instructions (Applied Biosystems). Quantitative PCR was performed using Taqman Universal Master mix in a iQ5 real-time PCR detection system (Bio Rad). All samples were run in triplicates and normalized to a GAPDH endogenous control. Relative fold-change was calculated using the 2<sup>ΔΔCt</sup> method. The IDs for the Tagman Gene-expression assays are the following: GAPDH, Mm00840746_m1; BRACHYURY, Mm01318252_m1; MESP1, Mm00801883_g1; NKX2.5, Mm01309813_s1; ISL1, Mm00517585_m1; HNK1, Mm00661498_m1; SNAI2, Mm00441531_m1; MITF, Mm00445212_m1; WNT1, Mm01300555_g1; PAX3, Mm00435491_m1; MITF-H (Variant 3), Mm01182481_m1; MITF (Variants, 1,2,3), Mm00434954_m1, SOX10, Mm0130162_m1; TBX18, Mm00470177_m1;
WT1, Mm01337048_m1; TCF21, Mm00448961_m1; KDR, Mm01222421_m1.

**Statistical Analysis.** For genetic lineage-tracing experiments, we estimate that to detect a minimum difference of 30 cardiomyocytic derivatives per cryosection between groups, with an expected SD of ±6 cardiomyocytes with a power of 90% and a 0.05 α-level, at least two embryos per group are necessary. Here, we used 10 embryos to perform genetic lineage-tracing of cKit during E7.5–E8.5; 12 embryos during E9.5–E12.5; 6 embryos of the ckit<sup>CreERT2</sup>, IRG:Jsr<sup>plLacZ</sup> genotype; and 5 embryos for intersectional genetic fate-mapping. Randomization and blinding were not applicable for this animal study. Statistical analyses were performed using GraphPad Prism v5.00 for Windows. A one-way ANOVA followed by Tukey’s post hoc tests were used for comparing changes in gene expression and beating EBs. Differences in the generation of NKX2.5<sup>+/EGFP</sup> progenitors between groups were compared using a Kruskall–Wallis test, followed by a Dunn’s post hoc analysis. All data met the assumptions of the tests. P < 0.05 was considered statistically significant. All values are reported as mean ± SEM.
Fig. S1. ckit is not expressed in the embryonic myocardium at the time of TAM administration. (A) Colocalization of X-gal with anti-ckt immunohistochemistry (arrow) in an E13.5 NT of a ckit<sup>CreERT2</sup>:R26R<sup>LacZ</sup> embryo. (Magnification, 200×.) Inset depicts a higher-magnification image of the indicated cell. (B) Live embryo imaging of EGFP and DsRed epifluorescence in an E12.5 ckit<sup>CreERT2</sup>;IRG embryo. Two EGFP<sup>+</sup> cells are detected in proximity to the OFT and two more in the epicardium (arrows). EGFP expression is absent in the myocardium. In contrast, strong expression of EGFP is seen in the NT and the skin. (C–F) A transverse section from an E12.5 ckit<sup>CreERT2</sup>;IRG mouse embryo in which immunohistochemistry against EGFP has been performed. EGFP cells are detected in the skin (Inset 1 in higher magnification), neural tube (Inset 2 in higher magnification), and the conotruncus (Inset 3 and F in higher magnification). No EGFP signal is detected in the myocardium. (G and H) A transverse section of an E12.5 ckit<sup>CreERT2</sup>;IRG heart illustrating expression of EGFP in the epicardium and left atrium. No signal is detected in the myocardium. Panel B is a photomerged image tile. (Magnification, 100×/tile.) Panel C is a confocal tile-scan. Ht, Heart. (Scale bars, 10 μm in D–F, and 100 μm in G–H.)
Fig. S2. Expression of cKit in the cKit<sup>+</sup> developing mouse heart. Representative confocal immunofluorescence of cKit antibody localization in E14.5 wild-type mouse embryos. (A) Consistent with cKit<sup>c<sub>Cre</sub>ERT2<sup>+</sup></sup> genetic fate map, CNC<sup>cre</sup> (red fluorescence) are detected in the NT, DRG, as well as in the dermis. CNC<sup>cre</sup> are detected in proximity of both the dorsolateral (arrows) and ventral (arrowheads) sites of the NT. (B) cKit expression in the embryonic lungs, RA, OFT, and epicardium (boxes). (C) Magnification of the solid box in B, highlights CNC<sup>cre</sup> (arrowheads) in the RA appendage, pericardial and epicardial walls. (D) Magnification of the dashed box in B, highlights CNC<sup>cre</sup> (arrowheads) accessing the RA, epicardium, and OFT. (E) CNC<sup>cre</sup> are dispersed in dermis, parietal pericardium, epicardium, and cardiac ventricular walls. (F) Magnification of the boxed area in E, illustrates CNC<sup>cre</sup> accessing the LV through the pericardial (arrows) and epicardial (arrowheads) walls. (Scale bars, 100 μm in A and 10 μm in B–F.) RA, right atrium; RV, right ventricle.
Fig. S3. cKit immunohistochemistry labels weakly expressing tdTomato+ cells in Wnt1-Cre;RC::tdTomato mouse embryos. Confocal immunofluorescence analysis following anti-cKit immunohistochemistry in E12.5 Wnt1-Cre;RC::tdTomato embryos illustrates colocalization of cKit in a population of cells with weak tdTomato epifluorescence, located dorsally (A, arrows) and ventrally from the NT (B, arrow), as well as within the outflow tract (C, arrows). A total of n = 3 Wnt1-Cre;RC::tdTomato embryos were analyzed. (Scale bars, 20 μm.)
Fig. S4. Intersectional genetic fate map of cKit and Wnt1. A–C, Wnt1::Flpe4351 reliably marks the cardiac neural crest as indicated by the expression of EGFP in the OFT (A), epicardium (B), and myocardium (C) of an E18.5 Wnt1::Flpe;Rc::Fela heart. (D–I) When both Wnt1::Flpe and cKitcreERT2/ become activated in cKitcreERT2;Wnt1::Flpe;Rc::Fela embryos, expression of EGFP is still widely expressed in the NC derivatives, including melanoblasts (D, arrows), DRGs (E), and OFT (G). In addition, a population of nLacZ+ cells are also present within the skin (F, arrows), DRGs (F, arrowheads), OFT (H) and, rarely, within the compact myocardium (I). [Scale bars, 100 μm (A, D, and E), 10 μm (B and C), 20 μm (G) and 80 μm (I).] (Magnification, 100× in H.)
**Fig. S5.** CNC<sup>-kit<sup> derivatives in the heart and their identity. (A) Tilescan image illustrating extensive contribution of EGFP<sup>+</sup> CNC<sup>kit<sup> derivatives in the lung, IFT, atria, ventricles and OFT. Notably, EGFP<sup>+</sup> cells are consistently detected to be closely associated with BFABP<sup>+</sup> satellite glial progenitors. Occasionally, EGFP and BFABP colocalize, illustrating that CNC<sup>kit<sup> contribute to glia and glial progenitor lineages (B, boxed area). (C and D) Higher magnification of the IFT from A, illustrating extensive contribution from CNC<sup>kit<sup>. BFABP, brain fatty acid-binding protein; IFT, inflow tract; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.
Fig. S6. Wnt1-expressing CNCs contribute cardiomyocytes in the murine heart. Similar to the CNCkit derivatives, confocal microscopy of tdTomato (epifluorescence; pseudocolored green) in postnatal day 1 (PN1) Wnt1-Cre;RC::tdTomato mouse hearts illustrates that the Wnt1-expressing CNCs contribute extensively in the OFT (A, arrowheads), tricuspid valve (A, arrow), and mitral valve (B, arrows). In addition, tdTomato+ cells are detected within the ventricular myocardium (C, arrows, and D; yellow Inset) and epicardium (D, arrowheads). Immunostaining against the cardiomyocyte-specific marker cmlc2v illustrates that, similar to the CNCkit, the Wnt1-expressing CNCs contribute both noncardiomyocytes (C, arrows; tdTomato+/cmlc2v−) and fully differentiated cardiomyocytes (D, Inset; tdTomato+/cmlc2v+) in the mouse ventricle. (E–G) Higher magnification of the tdTomato+/cmlc2v+ clone of ventricular cardiomyocytes in the yellow Inset in D. (Scale bars, 20 μm.)
Fig. S7. Mitf and Isl1 expression in CNC studies. (A) EGFP+ melanoblasts in the skin (arrows) coexpress Mitf. (B) EGFP+ derivatives in the heart (arrowhead, inset) coexpress Mitf. (C) Tyrosinase+ melanocytes in the tricuspid valve (arrows) do not colocalize with EGFP (arrowheads). (D) Colocalization of X-gal and EGFP epifluorescence in cKitCreERT2;IRG;Isl1IRES-CreERT2 embryos. (Magnification, 100×.) (E) X-gal and EGFP colocalization in the NT (inset, 1) and DRGs (arrows, 2). (F) X-gal and EGFP colocalization (1, 2, arrows) in the OFT. (G) Colocalization of EGFP and β-gal in the NT, DRGs, and OFT (arrow). (H) EGFP and β-gal colocalization in the OFT. (I) EGFP+β-gal+ neurons (arrows) in the NT. (J) Higher magnification of the OFT in (G, arrow). [Scale bars, 10 μm (B, C, E, F, I, J), 100 μm (G), and 20 μm (A and H).]
Fig. S8. Transient BMP antagonism induces CNC and suppresses epicardium. (A–J) NOG promotes the generation of cardiac mesoderm from iPSC as indicated by the transient induction of Mesp1 (A) and up-regulation in the expression of Nkx2.5 (B) and Isl1 (C) at ∼EB day 7. Subsequently, establishment of cardiac mesoderm is followed by the induction of CNC at ∼EB day 10, as indicated by the dramatic up-regulation in the expression of cKit (D), Pax3 (E), Wnt1 (F), SNAI2 (G), and Mitf-H (H). Notably, the noncardiac variants of Mitf (variants 1 and 2) remain unchanged. (I–K) Expression of the proepicardial lineage markers WT1 (I) and TBX18 (J) is significantly repressed before reaching baseline values by EB day 10. (L) Summary of the iPSC-based lineage tracing experiments. Values represent means ± SEM. ***P < 0.0001, **P < 0.005. n = 3 per group.
Fig. 59. Derivation of CNC kit from mouse iPSCs following NOG-mediated BMP antagonism. (A and B) A cluster of iPSC-derived EGFP+/Nkx2.5+ un-differentiated (cTnnT−) CNC kit (arrows) next to a cluster of non-CNC kit-derived (EGFP−) cTnnT+ differentiated cardiomyocytes. (C and D) An iPSC-derived CNC kit fully differentiated into cTnnT+/Nkx2.5+ cardiomyocyte (arrowhead). (E and F) Differentiation of iPSC-derived CNC kit into SM22a+ smooth muscle cells. (G and H) Colocalization of iPSC-derived CNC kit with Isl1. (I and J) iPSC-derived CNC kit generate Pax3 progenitors. (K and L) EGFP/neurofilament M+ motorneurons within a spontaneously beating EB. (M and N) An EGFP+/Tuj1+ neuron within the beating EBs. n = 3 per group. (Scale bars, 10 μm in A–F and I–N; 20 μm in G and H.)
Table S1. Exact sample sizes of genetic lineage-tracing experiments

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Summary of sample sizes of genetic lineage-tracing experiments. For the purpose of cKit genetic fate-mapping studies, a total of 150 mouse embryos from 20 different litters were analyzed. Thirty-three embryos carried the desired genotypes.

Movie S1. Confocal z-stack imaging of an E18.5 \textit{cKit}\textsuperscript{CreERT2};IRG heart, following TAM-induced recombination at E9.5–E11.5. Colocalization of EGFP immunofluorescence with neurofilament-M illustrates contribution of CNC\textsuperscript{kit} into conduction system cells of the heart.
Movie S2. Confocal z-stack imaging of a PN1 Wnt1-cre;RC:tdTomato heart illustrating the contribution of Wnt1-expressing cardiac neural crest cells in cmlc2v+ ventricular cardiomyocytes. epi, epifluorescence; IF, immunofluorescence. (Scale bar 10 μm.)

Movie S2

Movie S3. Confocal z-stack imaging of an E18.5 cKitCreERT2;IRG heart, following TAM-induced recombination at E9.5–E11.5. A cluster of cmlc2v+ ventricular cardiomyocytes with strong EGFP colocalization are shown, illustrating the capacity of CNC[IRG] to contribute cardiomyocytes in the developing heart.

Movie S3
Movie S4. Clusters of CNCkit-derived EGFP+ (white) spontaneously beating cardiomyocytes (EB-D14), generated from Dorso-treated EBs. Recombination was induced on EB-D10 with 4-OH TAM. The movie switches from EGFP epifluorescence to brightfield. (Magnification, 100×.)

Movie S5. Confocal z-stack imaging of a Dorso-induced day 14 EB, following 4-OH TAM-induced recombination on EB-D10. Colocalization of cTnnT with EGFP illustrates that transient BMP antagonism enhances the contribution of CNCkit to myocardium. The panels on right depict a still image of the inset in higher magnification, illustrating coexpression of cTnnT, EGFP, and NKX2.5.