Rac1 Signaling Is Critical to Cardiomyocyte Polarity and Embryonic Heart Development

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Background—Defects in cardiac septation are the most common form of congenital heart disease, but the mechanisms underlying these defects are still poorly understood. The small GTPase Rac1 is implicated in planar cell polarity of epithelial cells in Drosophila; however, its role in mammalian cardiomyocyte polarity is not clear. We tested the hypothesis that Rac1 signaling in the second heart field regulates cardiomyocyte polarity, chamber septation, and right ventricle development during embryonic heart development.

Methods and Results—Mice with second heart field–specific deficiency of Rac1 (Rac1SHF) exhibited ventricular and atrial septal defects, a thinner right ventricle myocardium, and a bifid cardiac apex. Fate-mapping analysis showed that second heart field contribution to the interventricular septum and right ventricle was deficient in Rac1SHF hearts. Notably, cardiomyocytes had a spherical shape with disrupted F-actin filaments in Rac1SHF compared with elongated and well-aligned cardiomyocytes in littermate controls. Expression of Scrib, a core protein in planar cell polarity, was lost in Rac1SHF hearts with decreased expression of WAVE and Arp2/3, leading to decreased migratory ability. In addition, Rac1-deficient neonatal cardiomyocytes displayed defects in cell projections, lamellipodia formation, and cell elongation. Furthermore, apoptosis was increased and the expression of Gata4, Tbx5, Nkx2.5, and Hand2 transcription factors was decreased in the Rac1SHF right ventricle myocardium.

Conclusions—Deficiency of Rac1 in the second heart field impairs elongation and cytoskeleton organization of cardiomyocytes and results in congenital septal defects, thin right ventricle myocardium, and a bifid cardiac apex. Our study suggests that Rac1 signaling is critical to cardiomyocyte polarity and embryonic heart development. (J Am Heart Assoc. 2014;3:e001271 doi: 10.1161/JAHA.114.001271)

Key Words: congenital heart defects • heart septal defects • Rac1 • second heart field

Adult cardiomyocytes are highly polarized, with a rod shape and the majority of their cell junction proteins localized to the intercalated discs joining the cells end to end to facilitate electrical conduction and contraction of the heart; however, during embryonic development, cardiomyocytes are initially formed in spherical shapes. They begin to elongate at E13.5 and gradually assume a rod shape during fetal and postnatal development. The spatial differences or asymmetries in shape, orientation and structure of cells define cell polarity, which is controlled by planar cell polarity (PCP) signaling, also known as the noncanonical Wnt pathway. Initially identified in Drosophila, the PCP pathway is well conserved in mammalian species. The ligands of this pathway include Wnt5a and Wnt11. The transmembrane receptors involved are Frizzled and Vangl2/ Scrib. Through recruitment of Dishevelled, the PCP signals are transduced to the small GTPases RhoA and Rac, which in turn regulate actin polymerization and cell polarity. The important role of PCP signaling in regulating cardiomyocyte polarity and embryonic heart development has recently emerged. Genetic knockout or mutation of PCP components such as Wnt11, Vangl2, Scrib, and Dishevelled2 in mice all show congenital heart defects (CHDs). In addition, Wnt11−/− and Vangl2 mutant mice show thin myocardium and defects in cardiomyocyte elongation, organization, and migration; however, the role of Rac in cardiomyocyte polarity and embryonic heart development is unknown.

Rac GTPases are small (20 to 30 kDa), monomeric, signaling GTP-binding proteins that are a subfamily of the...
Rho family of GTPases with 4 members: Rac1, Rac2, Rac3, and RhoG. Rac1 is a key molecule in the PCP pathway to promote cell polarity of the eyes and wings in Drosophila. Rac1 is also an important regulator of cell migration and survival. Whole-body Rac1−/− mice die before E9.5, with defects in germ layer formation due to reduced cell adhesion and motility and increased apoptosis within the mesoderm. Moreover, Rac1−/− embryos fail to specify an anterior–posterior axis because cells in the anterior visceral endoderm do not migrate, suggesting an important role of Rac1 in cell polarity during embryogenesis.

The heart develops from 3 distinct populations of cells: the first heart field, the second heart field (SHF), and the cardiac neural crest. Initially, the primary heart tube is formed mainly from the first heart field progenitors. SHF cells are then added to the heart tube to form the right ventricle (RV) and the outflow tract, with contributions from the cardiac neural crest cells. In addition, SHF progenitors are critical to the formation of the cardiac septum. Abnormalities in SHF development result in CHDs in mice including septal defects, which are some of the most common types of CHDs in humans. To specifically study the role of Rac1 in cardiomyocyte polarity and RV and cardiac septal development, we generated a novel mouse model with an SHF-specific (or anterior heart field-specific) deficiency of Rac1 using the Me2c-Cre mouse, which directs Cre activity in the SHF. Our results show that Rac1 signaling in the SHF is critical to cardiomyocyte polarity, cardiac septation, and RV development.

**Methods**

**Mice**

Rac1+/− mice (stock no. 5550) and membrane-targeted Tomato (mT)/membrane-targeted green fluorescent protein (mG) mice (stock no. 7676) were obtained from Jackson Laboratory (Bar Harbor, Maine). The mT/mG mouse is a global double-fluorescent Cre reporter mouse that expresses mT before Cre-excision and mG after excision of mT. The Me2c-Cre embryos were obtained from Mutant Mouse Regional Resource Centers (Chapel Hill, North Carolina) and rederived. A breeding program was carried out to generate Me2c-Cre:Rac1+/− (Rac1SHF), Me2c-Cre:mT/mG, and Rac1SHF:mT/mG transgenic mice. Genotyping was performed by polymerase chain reaction (PCR) using genomic DNA from tail biopsies. Primer sequences are shown in Table 1. All mouse experiments and procedures were carried out in accordance with the guidelines of the Canadian Council of Animal Care and approved by the animal use subcommittee at the University of Western Ontario.

**Histological Analysis**

Neonatal and embryonic samples were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated, and paraffin embedded. Embryos were serially sectioned at 5 µm from the top of the aortic arch to the apex with a Leica RM2255 microtome. Sections were mounted onto positively charged albumin/glycerin-coated microslides in a set of 5, with 25-µm intervals between each section. Slides were stained with hematoxylin and eosin for histological analysis. Images were captured using a light microscope (Observer D1, Zeiss).

**Immunohistochemistry**

Immunohistochemical staining was performed on heart sections (5 µm). Antigen retrieval was carried out in sodium citrate buffer (pH 6.0) at 92°C using a BP-111 laboratory microwave (Microwave Research and Applications). Immunostaining was performed with primary antibodies for Rac1 (Santa Cruz Biotechnology), phosphohistone-H3 (phospho S10) (Abcam), cleaved caspase-3 (Cell Signaling Technology), active (non-phosphorylated) β-catenin (Cell Signaling Technology), and green fluorescent protein (Abcam). All slides were imaged with a Zeiss Observer D1 microscope using AxiosVision Rel 4.7 software. For phalloidin and wheat germ agglutinin staining, P0 heart samples were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in FSC22 frozen section media (Leica), and sectioned with a Leica cryostat at 10-µm thick onto glass slides. Slides were incubated with Alexa Fluor 488 phalloidin (Life Technologies), Alexa Fluor 647 wheat germ agglutinin (Invitrogen), and Hoechst 33342 (Invitrogen). Confocal images were obtained with a Zeiss LSM 510 Duo microscope using ZEN 2012 software (Zeiss).

**Western Blot Analysis**

Rac1 protein expression from P0 RV was measured by Western blot analysis. Briefly, 40 µg of protein from isolated RV tissue was separated by 12% SDS-PAGE gel and transferred to nitrocellulose membranes. Blots were probed with antibodies against Rac1 (1:500; Santa Cruz Biotechnology) and GAPDH.
Blots were then washed and probed with horseradish peroxidase–conjugated secondary antibodies (1:2500; Bio-Rad) and detected using an enhanced chemiluminescence detection method. Signal quantification was performed by densitometry.

**RV Explant Culture**

Embryos were harvested at E12.5, and embryonic hearts were dissected to separate RV tissue from the whole heart. RV tissues were cultured on 1% collagen-coated tissue culture plastic multiwell plates, as described previously with the following adaptations. Explants were incubated for 6 days at 37°C with 5% CO₂ in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Media was refreshed every 2 days.

**Quantitative Reverse Transcription PCR**

Total RNA was isolated from E13.5 RV using the RNeasy Mini Kit (Qiagen). Reverse transcription reaction was performed using M-MLV reverse transcriptase (Invitrogen), and EvaGreen qPCR Mastermix (Applied Biological Materials) was used for real-time thermal cycling. Samples were amplified for 35 cycles using the Eppendorf Mastercycler Realplex Real-Time PCR machine. In addition, 28S ribosomal RNA was used as an internal control. Primer sequences are shown in Table 2. The mRNA levels in relation to 28S ribosomal RNA were determined using a comparative CT method.

**Scratch Assay**

Wild-type neonatal cardiomyocytes were prepared and cultured, as described previously. Cardiomyocytes were infected with adenoviruses expressing a dominant-negative form of Rac1 (Ad-Rac1N17; Vector Biolabs) or β-galactosidase (Ad-β-gal; Vector Biolabs) as a control at a multiplicity of infection of 10 plaque forming units per cell. The scratch was

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Table 2. The Real-Time Reverse Transcription Polymerase Chain Reaction Primer Sequences

| Gene  | Forward            | Reverse               |
|-------|--------------------|-----------------------|
| Arp2  | ggcttttctctctctcact| gcttctgctctgtcaata   |
| Arp3  | cggcttttctctctcaaga| cagctttgacctccacacc  |
| Abi1  | ttgacctccacagatcctctcac| ttcatctgcacagaggggtg |
| WAVE2 | gcaaggaagagagggaagagtg| cgagaaagaggggaagggag |
| Nkx2.5| tcaatgctctgtgccacag| gacgcaagactgccaagtgct |
| Gata4 | cactagggccacagcagctc| gctcagctgctgcgatgac  |
| Tbx5  | acacagagtggctggtatgc| gttaaggctggccgagaggt |
| Rac1  | aacctgcctgtctctgatttt| ttgctccagcggtccata   |
| Tbx20 | cactagggccagaggggtgctc| tctcaggtctgctgtactgt |
| Hand2 | gctactgctctctctctgtagt| tcttctgtttcgctctactt |
| 28S   | ttgaaactctccggggagag| accttgttcacacagtccag |

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**Figure 1.** A, Rac1 mRNA levels were significantly decreased in Rac₁SHF RV compared with Rac₁f/f controls at E13.5, as measured by quantitative reverse transcription polymerase chain reaction. **P<0.01 by unpaired Student t test. B,** Immunostaining shows that Rac1 protein levels were reduced in P0 Rac₁SHF RV compared with the left ventricle myocardium. **C,** Western blot analysis of P0 RV showed a significant decrease in Rac1 protein in Rac₁SHF compared with Rac₁f/f controls. *P<0.05 by Mann–Whitney test. RV indicates right ventricle; SHF, second heart field.
performed 6 hours after adenoviral infection. Cells were fixed and immunostained with primary antibodies for Rac1 (Santa Cruz Biotechnologies) and α-actinin (Sigma-Aldrich) 24 hours after scratch.

Statistical Analysis
Data are mean±SEM. Unpaired Student t test was used when data passed a normality test. Nonparametric Mann–Whitney test was used when samples size was 3 to 4 per group. Differences were considered significant at P≤0.05.

Results
Generation of a Transgenic Mouse With Rac1 Knockdown in the SHF
To elucidate the role of Rac1 in the SHF, the Mef2c-Cre mouse was crossed with the Rac1floxed/floxed mouse to generate Mef2c-Cre:Rac1floxed/floxed (Rac1SHF) offspring. Rac1SHF mice have the Rac1 gene specifically knocked down in SHF progenitors and their derivatives. Embryonic samples were collected, and loss of the Rac1 transcript was confirmed in Rac1SHF hearts by quantitative reverse-transcription PCR. Rac1 mRNA expression was reduced by 51% in the RV myocardium of E13.5 Rac1SHF compared with littermate controls (Figure 1A). Immunostaining for Rac1 protein was also performed to further confirm the loss of myocardial Rac1 expression in SHF derivatives. In P0 Rac1SHF hearts, robust staining for Rac1 was observed in the left ventricle (LV). In the same P0 Rac1SHF heart section, the Rac1 signal intensity was markedly decreased in the RV when compared with the LV (Figure 1B). Ratio of Rac1 to GAPDH protein was reduced by 40% in P0 Rac1SHF RV compared with littermate controls, as determined by Western blot analysis (Figure 1C). These data show that the Mef2c-Cre–mediated recombination downregulates Rac1 expression in SHF derivatives of the embryonic heart.

Deficiency of Rac1 in the SHF Results in CHDs
The most prominent defect on initial analysis of P0 Rac1SHF hearts was a gross morphological abnormality in the overall shape of the heart. Instead of a distinct apex, all P0 Rac1SHF hearts (28 of 28) had a deep interventricular groove and bifurcation of the 2 ventricles, resulting in a bifid cardiac apex, a rare CHD in humans (Table 3; Figure 2A and 2B). Atrial septal defects were observed in 82% (23 of 28) of Rac1SHF hearts but not in littermate control Rac1f/f hearts (0 of 19) (Table 3; Figure 2C and 2D). Ventricular septal defects were seen in 100% (28 of 28) of P0 Rac1SHF hearts (Table 3; Figure 2C and 2D). In addition, the RV myocardium had poor trabeculation and the compact myocardium was significantly thinner in Rac1SHF hearts compared with littermate controls (Figure 2E through 2G; Table 3). These findings suggest a critical role for SHF Rac1 signaling in cardiac septation and RV development.

Rac1SHF Hearts Exhibit Defective Cardiomyocyte Polarity
Cardiomyocyte organization and alignment were severely disrupted in P0 Rac1SHF hearts. Cell membrane staining with wheat germ agglutinin revealed rounded, spherically shaped cardiomyocytes in Rac1SHF RV (Figure 3B) compared with Rac1f/f RV, which had elongated cardiomyocytes (Figure 3A). Actin cytoskeleton organization was also severely disrupted in Rac1SHF RV, as shown by phalloidin staining to mark F-actin filaments (Figure 3D). Rac1SHF RV had an absence of long F-actin filaments, whereas Rac1f/f RV myocardium exhibited long, parallel running F-actin fibers (Figure 3C). In addition, active (nonphosphorylated) β-catenin staining between cell junctions was also reduced in E13.5 Rac1SHF RV compared

|                      | Rac1f/f (n=19) | Mef2c-Cre:Rac1f/f (n=10) | Rac1SHF (n=28)* |
|----------------------|---------------|--------------------------|------------------|
|                      | n  | %  | n  | %  | n  | %  |
| Normal               | 19  | 100| 10  | 100| 0  | 0  |
| ASD                  | 0  | 0  | 0  | 0  | 23 | 82 |
| VSD                  | 0  | 0  | 0  | 0  | 28 | 100|
| Bifid apex           | 0  | 0  | 0  | 0  | 28 | 100|
| Defective RV         | 0  | 0  | 0  | 0  | 28 | 100|

ASD indicates atrial septal defect; RV, right ventricle; VSD, ventricular septal defect.

*21 alive and 7 collected dead at P0. Because penetrance of congenital heart defects is 100% in Rac1SHF hearts, statistical analysis is not necessary.

Table 3. Incidence of Congenital Heart Defects in P0 Rac1SHF Hearts

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with Rac1f/f RV (Figure 3E and 3F). These data strongly suggest disruption of cardiomyocyte polarity in Rac1SHF hearts.

Expression of Scrib Protein Is Lost in Rac1SHF Hearts

Scrib, the mouse homolog of the Drosophila protein Scribble, is a component of the PCP pathway. Immunostaining shows Scrib protein was expressed mainly in the lower part of RV and the cardiac apex in Rac1f/f hearts (Figure 4A, 4B, 4E, and 4F), and the number of Scrib-positive cells was about 10 times higher in E15.5 than E12.5 Rac1f/f hearts (Figure 4I and 4J). Notably, Scrib expression was almost absent in both E12.5 and E15.5 Rac1SHF hearts (Figure 4C, and 4D, 4G, and 4H), and the number of Scrib-positive cells was significantly decreased in Rac1SHF hearts compared with littermate controls (Figure 4I and 4J). The loss of Scrib expression suggests disruption of the PCP pathway and further supports failure to establish cardiomyocyte polarity in Rac1SHF hearts.

Rac1 Is Required for Lamellipodia Formation and Cardiomyocyte Migration

Cell migration is a fundamental process of gastrulation, morphogenesis, and formation of organs and tissues during...
To determine whether Rac1 plays a migratory role in SHF derived-cells, RV from E12.5 Rac1\textsuperscript{SHF}:mT/mG and control Mef2c-Cre:Rac1\textsuperscript{f/+}:mT/mG hearts were isolated and explants cultured on collagen gels for 6 days. The mT/mG global double-fluorescent Cre reporter mouse expresses mT before Cre-mediated excision and mG after excision of mT.\textsuperscript{18} Thus all SHF-derived cells in Rac1\textsuperscript{SHF}:mT/mG and Mef2c-Cre:Rac1\textsuperscript{f/+}:mT/mG are positive for green fluorescent protein. It was observed that Mef2c-Cre:Rac1\textsuperscript{f/+}:mT/mG control explants had both non-SHF-derived (mT-labeled) and SHF-derived (mG-labeled) cells migrating from the RV explant by day 6 (Figure 5A through 5C). In contrast, Rac1\textsuperscript{SHF}:mT/mG RV explants had only non-SHF-derived (mT-labeled) cells migrating from the RV explant and very few to no SHF-derived (mG-labeled) cell migration (Figure 5D through 5F). The migration defects observed in the Rac1\textsuperscript{SHF}:mT/mG hearts indicate that Rac1 regulates migration of SHF-derived progenitor cells, likely through actin organization controlled by the Wiskott-Aldrich syndrome protein.
family verprolin-homologous protein (WAVE) and actin-related protein-2/3 (Arp2/3) pathway downstream of Rac1. A significant decrease was found in Abi1, WAVE2, Arp2, and Arp3 expression—components of the WAVE and Arp2/3 complex—in E13.5 Rac1SHF RV compared with littermate controls (Table 4).

To further assess cell migration, scratch assays were performed on neonatal cardiomyocytes infected with adenoviral constructs of β-galactosidase (Ad-β-gal) or dominant negative Rac1 (Ad-Rac1N17). It was observed that 24 hours after scratch, Ad-β-gal-treated cardiomyocytes were aligned perpendicular to the scratch edge and had projections along the scratch edge, indicative of actin organization and lamellipodia extension (Figure 5G). In contrast, no projections and lamellipodia formation were observed in Ad-Rac1N17 cardiomyocytes, and the cells were disorganized and arranged in a random orientation along the scratch edge (Figure 5H). These findings implicate Rac1 in the regulation
Cardiomyocyte Polarity and Heart Development

Leung et al.

was considered statistically significant. No significant differences were found in Tbx20 and β-actin mRNA expression.

Table 4. Real-Time Polymerase Chain Reaction Analysis of Gene mRNA Levels in E13.5 Rac1SHF Right Ventricle

| Gene     | Rac1f/f | Rac1SHF | P Values |
|----------|---------|---------|----------|
| Nkx2.5   | 0.0583±0.00423 (n=6) | 0.0462±0.00467 (n=6) | 0.0320 |
| Gata4    | 0.0410±0.00206 (n=6) | 0.0299±0.00405 (n=6) | 0.0338 |
| Tbx5     | 0.0124±0.00043 (n=8) | 0.0090±0.00135 (n=8) | 0.0320 |
| Hand2    | 0.0315±0.00281 (n=6) | 0.0211±0.00116 (n=6) | 0.0065 |
| Tbx20    | 0.0428±0.00338 (n=6) | 0.0331±0.00423 (n=8) | 0.1256 |
| Abi1     | 0.0268±0.00215 (n=8) | 0.0177±0.00341 (n=8) | 0.0404 |
| WAVE2    | 0.1174±0.00902 (n=8) | 0.0756±0.0140 (n=8) | 0.0247 |
| Arp2     | 0.0246±0.00239 (n=8) | 0.0167±0.00109 (n=8) | 0.0098 |
| Arp3     | 0.1306±0.00639 (n=8) | 0.0901±0.0109 (n=8) | 0.0063 |
| β-actin  | 0.2977±0.0395 (n=6) | 0.2220±0.0298 (n=6) | 0.1534 |

Data are ratios to 28S and are presented as mean±SEM. Significance in gene expression difference between Rac1SHF and Rac1f/f littermates analyzed by unpaired Student t test. P<0.05 was considered statistically significant.

of cell migration and lamellipodia extension in cardiomyocytes.

Rac1SHF Cardiomyocytes Are Shortened and Rounded

Neonatal cardiomyocyte cultures from Rac1SHF RV tissues showed that these cells had disrupted actin organization, as indicated by phalloidin staining and the absence of distinct cell projections compared with Rac1f/f cardiomyocytes. Interestingly, patterning of α-actinin also seemed to be disrupted in Rac1SHF cardiomyocytes (Figure 6A and 6B). Measurement of cardiomyocyte long-axis length revealed significantly shorter Rac1SHF cardiomyocytes compared with littermate control cardiomyocytes (Figure 6E). In addition, neonatal Rac1SHF cardiomyocytes were rounded compared with control Rac1f/f cardiomyocytes, which had an elongated morphology (Figure 6C and 6D). No rounded cardiomyocytes were found in Rac1f/f cultures, whereas >40% of Rac1SHF cardiomyocytes exhibited a rounded morphology (Figure 6F). These data strongly support the role of Rac1 in regulating elongation and polarization of cardiomyocytes.

Contribution of SHF Progenitors to Rac1SHF Hearts Is Decreased

Histological analysis showed that a deep fissure between the RV and LV was observed in E11.5 Rac1SHF hearts, whereas this was not evident and the early muscular interventricular septum was beginning to form in littermate control hearts (Figure 7A and 7B). At E12.5, bifurcation of the RV and LV in Rac1SHF hearts continued to be present (Figure 7C and 7D), and by E15.5, a distinct apex was formed in control hearts, whereas Rac1SHF hearts continued to have a deep cleft separating the ventricles (Figure 7E and 7F).

Fate mapping with the mT/mG global double-fluorescent Cre reporter mouse was performed to follow the developmental progression of the SHF progenitors. A deficiency in the SHF lineage contributing to the developing E11.5 Rac1SHF: mTmG hearts was evident, especially in the early forming muscular interventricular septum between the RV and LV (Figure 7G and 7H). At E12.5 and E15.5, the interventricular septum in Rac1SHF:mT/mG hearts had a major deficiency of SHF-derived cells, leading to formation of a bifid apex (Figure 7I and 7L). These findings suggest a critical role for SHF Rac1 signaling in muscular interventricular septum formation.

Rac1 Promotes Cell Survival in SHF-Derived Myocardium

Aberrant apoptosis or decreased proliferation in the SHF may contribute to CHDs. To determine whether an abnormality in proliferation was a factor in Rac1SHF hearts, phosphohistone-H3 protein staining was performed on E11.5 heart sections (Figure 8A through 8F). No differences in proliferation were observed between Rac1SHF and Rac1f/f control hearts (Figure 8G). Next, we stained for cleaved caspase-3 (the active form of caspase-3) on E11.5 heart sections to detect cell apoptosis (Figure 9A through 9D). Cleaved caspase-3 staining was quantified as a percentage of the total number of cells. E11.5 Rac1SHF hearts had significantly higher levels of apoptosis in the RV compared with littermate controls (P<0.05) (Figure 9E). Similarly, increased apoptosis was observed in the interventricular septum of E11.5 Rac1SHF hearts (P<0.05).
No significant difference in apoptosis was observed in the LV between E11.5 Rac1\textsuperscript{SHF} and control hearts ($P=0.27$) (Figure 9E). Together, these data define a requirement for Rac1 in cell survival but not proliferation in the development of SHF-derived cardiac structures.
Downregulation of Transcription Factors in Rac1\textsuperscript{SHF} RV Myocardium

Heart development is regulated by a complex network of transcription factors that interact synergistically and in a dose-dependent manner to activate target genes.\textsuperscript{24} To address the genetic pathways regulated by Rac1 signaling in the SHF, we analyzed known transcription factors important for heart development. Expression levels of Gata4, Nkx2.5, and Tbx5 mRNA were significantly decreased in the RV of E13.5 Rac1\textsuperscript{SHF} compared with Rac1\textsuperscript{f/f} hearts (Table 4). Hand2 and Tbx20 are 2 transcription factors shown to be expressed predominantly in the RV and to regulate RV development.\textsuperscript{25,26} Hand2 expression was significantly decreased in the RV of E13.5 Rac1\textsuperscript{SHF} compared with Rac1\textsuperscript{f/f} hearts (Table 4); however, no significant differences were found in Tbx20 expression between Rac1\textsuperscript{f/f} and Rac1\textsuperscript{SHF} RV (Table 4). Taken together, these findings indicate that transcriptional regulation of RV development was severely disrupted in the Rac1\textsuperscript{SHF} hearts.

Discussion

The present study was carried out to examine the role of Rac1 in SHF progenitors. Using the SHF-specific Mef2c-Cre mouse line to delete Rac1 in SHF progenitors and derivatives, we demonstrated that a Rac1 deficiency in the SHF leads to 100% penetrance of CHDs including septal defects, thin RV myocardium, and defective trabeculation and, most notably, a bifid cardiac apex. Our data showed, for the first time, that Rac1 signaling is critical to cardiomyocyte polarity and embryonic heart development, with contributions to cell migration and survival and cardiac gene expression (Figure 9F).

Rac1 has been shown to be a downstream effector of the PCP pathway by modulating the cytoskeleton and regulating actin dynamics in Drosophila and Xenopus;\textsuperscript{9,27} however, the role of Rac1 in mammalian cardiomyocyte polarity and heart development is not known. Studies have shown that disruption of the PCP pathway in mice by deletion of Scrib resulted in cellular disorganization, defective trabeculation, thin myocardium, and chamber septation defects,\textsuperscript{6} which are strikingly similar to the defects observed in this study and strongly support a disruption of PCP signaling in Rac1\textsuperscript{SHF} hearts. Interestingly, we observed that Scrib protein expression pattern is most abundant in the RV and interventricular septum junction during embryonic heart development. Notably, Scrib protein expression in this distinct cardiac tissue area is lost in Rac1\textsuperscript{SHF} hearts. We postulate that cardiomyocytes at the RV and interventricular junction must be highly polarized and oriented to align with the forming cardiac apex.
The rounded, nonpolarized, and disorganized Rac1SHF cardiomyocytes are likely unable to bridge the RV and interventricular junction to unify the 2 ventricles to form a cardiac apex, resulting in bifurcation of the muscular septum. In addition, a decrease in cell number due to increases in apoptosis in this area may also contribute to the development of bifid apex. It has been reported that genetic deletion of transcription factor Ets1 also results in ventricular septal defects and bifid cardiac apex; however, another group using the same Ets1 knock-out mouse did not observe a bifid cardiac apex. This makes the role of Ets1 still unclear in the formation of a bifid cardiac apex. Recorded cases of bifid cardiac apex in humans are very rare and have usually been reported concomitantly with other congenital heart anomalies including atrial septal defects, ventricular septal defects, and defective RV development, which are very similar to the type of defects that were observed in Rac1SHF hearts. Consequently, it is possible that the bifid cardiac apex and associated cardiac defects found in humans could be partially associated with abnormalities in Rac1 signaling and defects in cardiomyocyte polarity during embryonic heart development.

It is well established that actin is the most abundant cytoskeleton protein and plays a critical role in controlling cell orientation, cell shape, and migration. Rac1 is an important regulator of actin polymerization, ruffle formation, lamellipodia extension, and migration in numerous cell types. Rac1 is known to activate the WAVE complex, which is recruited to the plasma membrane and regulates the actin cytoskeleton through the ARP2/3 complex. ARP2/3 associates with actin filaments to promote formation of new filaments from the sides of existing filaments, creating a dendritic network of branched actin filaments to extend lamellipodia and propel cells forward during migration. The combined defects in cell polarity, morphology, lamellipodia formation, and actin organization observed in SHF-derived cells led to overall inhibition of cardiomyocyte migratory ability in Rac1SHF embryos. Furthermore, Rac1 has been shown to promote cell survival by activation of the PI3K/Akt pathway. Decreased migration ability and an increased rate of apoptosis in SHF-derived cells likely contribute concomitantly to an overall deficiency in SHF contribution to the developing Rac1SHF heart.

Numerous studies have found that Rac1 can regulate gene expression through different pathways. Interestingly, it has been shown that Rac1 can activate the canonical Wnt pathway by promoting nuclear accumulation of β-catenin, the effector of the Wnt pathway. Ai et al showed that deletion of β-catenin in the SHF led to abnormalities in development of the RV and interventricular septum, very similar to the defects observed in Rac1SHF mice in the present study. Whether the defects were also due to loss of β-catenin cell-cell adhesion was not addressed in their study. Our data showed a loss of β-catenin cell adhesions in the Rac1SHF RV myocardium, supporting a defect in polarity and placing noncanonical Wnt signaling as the main driver of the cellular and morphological defects observed. Our data are supported by Abhul-Ghani et al, who showed that noncanonical Wnt, which is highly active during cardiogenesis, antagonizes the canonical Wnt pathway. The contribution of canonical Wnt signaling cannot be ruled out in our study because it has been shown to play a role in specification and expansion of early

**Figure 8.** pH3 protein staining to assess cell proliferation in E11.5 Rac1SHF hearts. A through F, Representative pH3 staining in transverse heart sections. Arrows indicate positive signals. Scale bars: 20 μm. G, No significant differences in cell proliferation were found between E11.5 Rac1SHF and Rac1ff hearts. n=3 hearts per group. LV indicates left ventricle; pH3, phosphohistone-H3; RV, right ventricle; SHF, second heart field.
SHF progenitors. Rac1 can also promote gene expression through NF-κB and JNK pathways. Our data showed that Rac1 SHF hearts have decreased expression of Gata4, Tbx5, Nkx2.5, and Hand2 transcription factors in the developing RV. Consequently, it is plausible that a Rac1 deficiency in the SHF will lead to decreased NF-κB and/or JNK signaling and downregulation of key cardiac transcription factors during embryonic heart development. Tbx20 acts in a dose-sensitive manner as an essential regulator of outflow tract, cardiac valve, and RV development; however, no significant downregulation of Tbx20 expression was found in the Rac1 SHF hearts. A hierarchy of distinct cardiac transcription factor regulation exists in the developing heart. Our data suggest that Rac1 regulates a distinct network of cardiac transcription factors that do not include Tbx20 in SHF derivatives. Future studies will be necessary to determine the exact downstream pathways that Rac1 regulates in the SHF to promote cardiac gene expression.

Figure 9. Increased apoptosis in Rac1 SHF RV and interventricular septum. A through D, E11.5 heart sections were immunostained with cleaved caspase-3 antibody to mark apoptotic cells. Arrows indicate positive signals. B and D are magnifications of the boxes in A and C, respectively. E, Quantification of apoptosis in RV, LV, and the cardiac septum. *$P<0.05$ vs corresponding Rac1 f/f by Mann–Whitney test. Scale bars: 100 μm (A and C), 10 μm (B and D). F, The proposed pathway of Rac1 signaling in SHF derivatives during embryonic heart development. Rac1 regulates actin organization to promote cardiomyocyte polarity and cell migration. In addition, Rac1 promotes cell survival and the expression of cardiac developmental genes. These effects of Rac1 contribute to normal RV and septal development of the heart. LV indicates left ventricle; RV, right ventricle; SHF, second heart field.
Tbx5 is essential in the SHF for atrial septation, although it is predominantly expressed in the LV during early heart development. In addition, Späte et al recently challenged the view of Tbx5 as a classical first heart field marker; they showed an overlap of expression pattern between the posterior end of the Tbx5-positive first heart field domain and the Isl1-positive SHF domain, suggesting a role for Tbx5 in the SHF. Our data are compatible with the studies on Tbx5 from Nadeau et al and Xie et al, who showed that Gata4 and Tbx5 cooperatively regulate atrial septum development and that haplinsufficiency of Tbx5 in the SHF results in atrial septal defects.

Although further investigations are required to analyze potential RAC1 gene mutations in humans with bifid cardiac apex, the present study has shown that SHF RAC1 is critical to septal and RV development and that deficiencies in SHF RAC1 can give rise to bifid cardiac apex along with the associated CHDs. Our data are the first to show Rac1 as an essential regulator of cardiomyocyte polarity during heart development and to provide a possible mechanism for how bifid cardiac apex arises. Establishment of cellular polarity is essential for the processes regulating cell morphology, lamellipodia formation, and cell migration in the developing embryo, and this seems to be especially critical in formation of a distinct cardiac apex during embryonic heart development.

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Disclosures
None.

References
1. Henderson DJ, Chaudhry B. Getting to the heart of planar cell polarity signaling. Birth Defects Res A Clin Mol Teratol. 2011;91:460–467.
2. Niehrs C. The complex world of WNT receptor signalling. Nat Rev Mol Cell Biol. 2012;13:767–779.
3. Vivancos V, Chen P, Spassky N, Qian D, Dabdoub A, Kelley M, Studer M, Guthrie S. Wnt activity guides facial branchiomotor neuron migration, and involves the PCP pathway and JNK and ROCK kinases. PLoS One. 2013;8:e77611.
4. Song W, Lu X, Feng Q. Tumor necrosis factor-alpha induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. Cardiovasc Res. 2000;45:595–602.
5. Bruneau BG. The developmental genetics of congenital heart disease. Nature. 2008;451:943–948.
6. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. Genesis. 2007;45:593–605.
7. Kelly RG. The second heart field. Curr Top Dev Biol. 2012;100:33–65.
8. Munoz-Descalzo S, Gomez-Cabrero A, Mlodzik M, Paricio N. Analysis of the functions from in vivo studies. Nat Rev Mol Cell Biol. 2008;9:690–701.
9. Migeotte I, Omelchenko T, Hall A, Anderson KV. Rac1-dependent collective cell migration is required for specification of the anterior-posterior body axis of the mouse. PLoS Biol. 2010;8:e1000442.
10. Kelly SG, Marchal CC, Zhu F, Worku A, Clausen BE, Foerster I, Marks P, Downey GP, Dinauer M, Kwiatkowski DJ. Rac1 deletion in mouse neutrophils has selective effects on neutrophil functions. J Immunol. 2003;170:5652–5657.
11. Zaffran S, Kelly RG, Meilhac SM, Buckingham ME, Brown NA. Right ventricular myocardium derives from the anterior heart field. Circ Res. 2004;95:261–268.
12. Liu Y, Lu X, Xiang FL, Lu M, Feng Q. Nitric oxide synthase-3 promotes embryonic development of atrioventricular valves. PLoS One. 2013;8:e77611.
33. Stradal TE, Rottner K, Disanza A, Confalonieri S, Innocenti M, Scita G. Regulation of actin dynamics by WASP and WAVE family proteins. Trends Cell Biol. 2004;14:303–311.

34. Jiang K, Zhong B, Ritchey C, Gilvary DL, Hong-Geller E, Wei S, Djieu JY. Regulation of Akt-dependent cell survival by Syk and Rac. Blood. 2003;101:236–244.

35. Nishida K, Kaziro Y, Satoh T. Anti-apoptotic function of Rac in hematopoietic cells. Oncogene. 1999;18:407–415.

36. Gonzalez E, Kou R, Michel T. Rac1 modulates sphingosine 1-phosphate-mediated activation of phosphoinositide 3-kinase/Akt signaling pathways in vascular endothelial cells. J Biol Chem. 2006;281:3210–3216.

37. Esufali S, Bapat B. Cross-talk between Rac1 GTPase and dysregulated Wnt signaling pathway leads to cellular redistribution of beta-catenin and TCF/LEF-mediated transcriptional activation. Oncogene. 2004;23:8260–8271.

38. Wu X, Tu X, Joeng KS, Hilton MJ, Williams DA, Long F. Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell. 2008;133:3210–3216.

39. Ai D, Fu X, Wang J, Lu MF, Chen L, Baldini A, Klein WH, Martin JF. Canonical Wnt signaling functions in second heart field to promote right ventricular growth. Proc Natl Acad Sci USA. 2007;104:9319–9324.

40. Abdul-Ghani M, Dufort D, Stiles R, De Repentigny Y, Kothary R, Megeney LA. Wnt11 promotes cardiomyocyte development by caspase-mediated suppression of canonical Wnt signals. Mol Cell Biol. 2011;31:163–178.

41. Cohen ED, Wang Z, Lepore JJ, Lu MM, Taketo MM, Epstein DJ, Morrissey EE. Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. J Clin Invest. 2007;117:1794–1804.

42. Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ, Knaus UG. Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. Nat Immunol. 2000;1:533–540.

43. Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Guskind JS. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell. 1995;81:1137–1146.

44. Klaus A, Muller M, Schulz H, Saga Y, Martin JF, Birchmeier W. Wnt/beta-catenin and Bmp signals control distinct sets of transcription factors in cardiac progenitor cells. Proc Natl Acad Sci USA. 2012;109:10921–10926.

45. Mandel EM, Kaltenbrun E, Callis TE, Zeng XX, Marques SR, Yelon D, Wang DZ, Conlon FL. The BMP pathway acts to directly regulate Tbx20 in the developing heart. Development. 2010;137:1919–1929.

46. Xie L, Hoffmann AD, Burnicka-Turek O, Friedland-Little JM, Zhang K, Moskowitz IP. Tbx5-hedgehog molecular networks are essential in the second heart field for atrial septation. Dev Cell. 2012;23:280–291.

47. Bruneau BG, Logan M, Davis N, Levi T, Tabin CJ, Seidman JG, Seidman CE. Chamber-specific cardiac expression of Tbx5 and heart defects in Holt-Oram syndrome. Dev Biol. 1999;211:100–108.

48. Li QY, Newbury-Ecob RA, Terrett JA, Wilson DI, Curtis AR, Yi CH, Gebuhr T, Bullen PJ, Robson SC, Strachan T, Bonnet D, Lyonnet S, Young ID, Raeburn JA, Brook JD. Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachury (T) gene family. Nat Genet. 1997;15:21–29.

49. Spater D, Abramczuk MK, Buac K, Zangi L, Stachel MW, Clarke J, Sahara M, Ludwig A, Chien KR. A HCN4+ cardiomyogenic progenitor derived from the first heart field and human pluripotent stem cells. Nat Cell Biol. 2013;15:1098–1106.

50. Nadeau M, Georges RO, Laforest B, Yamak A, Lefebvre C, Beauregard J, Paradis P, Bruneau BG, Andelfinger G, Nemer M. An endocardial pathway involving Tbx5, Gata4, and Nos3 required for atrial septum formation. Proc Natl Acad Sci USA. 2010;107:19356–19361.