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Rac1 modulates cardiomyocyte adhesion during mouse embryonic development

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ABSTRACT

Rac1, a member of the Rho subfamily of small GTPases, is involved in morphogenesis and differentiation of many cell types. Here we define a role of Rac1 in cardiac development by specifically deleting *Rac1* in the pre-cardiac mesoderm using the *Nkx2.5-Cre* transgenic driver line. *Rac1*-conditional knockout embryos initiate heart development normally until embryonic day 11.5 (E11.5); their cardiac mesoderm is specified, and the heart tube is formed and looped. However, by E12.5-E13.5 the mutant hearts start failing and embryos develop edema and hemorrhage which is probably the cause for the lethality observed soon after. The hearts of *Rac1-cKO* embryos exhibit disorganized and thin myocardial walls and defects in outflow tract alignment. No significant differences of cardiomyocyte death or proliferation were found between developing control and mutant embryos. To uncover the role of Rac1 in the heart, E11.5 primary heart cells were cultured and analyzed in vitro. *Rac1*-deficient cardiomyocytes were less spread, round and loosely attached to the substrate and to each other implying that Rac1-mediated signaling is required for appropriate cell–cell and/or cellmatrix adhesion during cardiac development.

1. Introduction

Morphogenesis, a key process in embryogenesis, is driven by actin-myosin networks, where mechanical forces are produced and transmitted through adhesive complexes to other cells and tissues [1]. These networks are regulated via complex mechanisms, in which the small Rho-related GTPases Rac, Rho and Cdc42 are known to play crucial roles. In mammals, three *Rac* genes have been identified; however, only *Rac1* is expressed early in mice embryogenesis [2]. Systemic *Rac1* knockout embryos show severe defects in germ layer formation and die during early gastrulation (E6.5) [3]. Because the disruption of axis specification in *Rac1* null mutants causes early lethality, a conditional *Rac1* knockout (*Rac1*-cKO) allele has been widely used to circumvent this defect [4]. These studies have shown that in the visceral endoderm *Rac1* is required for formation of the anterior-posterior body axis by promoting migration of the anterior visceral endoderm [5]. However, epiblast-specific deletion of *Rac1* allows formation of the body axis, but results in defects in the paraxial mesoderm and in a failure of cardiac anlage to fuse [6]. In addition, increased apoptosis was observed, confirming earlier observations that loss of *Rac1* in embryoid bodies results in programmed cell death [7]. Apoptosis, however, is cell-type specific, since *Rac1* deletion in other cells types, e.g., keratinocytes in vivo does not cause apoptosis [8]. Neural crest cell-specific deletion of *Rac1* did not affect cell migration, but it unexpectedly resulted in defective proliferation and self-renewal at later stages leading to craniofacial and cardiac outflow tract defects [9,10]. Melanocyte-specific *Rac1* mutants showed less efficient melanoblast migration, and defective cell–cycle progression and cytokinesis [11], while in cerebellar granule neurons *Rac1* was shown to be required for cell migration and axon formation via the WAVE complex [12]. In the cardiovascular system [13], *Rac1* was shown to be required for endothelial adhesiveness and permeability [14], while *Rac1* over-expression or constitutive activation resulted in cardiac hypertrophy [15], which could be partially rescued by *Rac1* haploinsufficiency [13,16–18]. Despite these advances, the role of Rac1 in early heart development remains unknown. Here, we deleted *Rac1* in the precardiac mesoderm using the well-characterized *Nkx2*-5Cre knockin mouse line [19]. The resulting *Rac1:Nkx2-5Cre* mutants die after embryonic day 12.5 (E12.5) with disorganized ventricular walls and defective outflow tract alignment, additionally, mutant cardiomyocytes in culture showed defects in adhesion to substrate and to each other. Our results imply that during cardiac development *Rac1* is required cell-autonomously in cardiomyocytes for appropriate cell–cell and cell–matrix adhesion.

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2. Materials and methods

2.1. Mouse breeding, genotyping and generation of embryos for analysis

Nkx2.5-Cre mice were obtained from R. Schwartz [19] and generation of *Rac1*^{FXFX} and *R26R* mice has been described earlier [20,21]. *Rac1*$^{Fx/Fx}$ or *Rac1*$^{Fx/Fx}$*:R26R*$^{+/+}$ female mice were crossed with *Rac1*$^{Fx/+}$*:Nkx2.5-Cre* male mice to obtain timed pregnancies. As the dark period was 2 am–2 pm, the presence of vaginal plug was designated as embryonic day 0 (E0). DNA for genotyping was prepared from yolk sac or tail lysate using DirectPCR Lysis Reagents (Viagen Biotech). *Rac1*$^{Fx/Fx}$ and *Nkx2-5Cre* mice were genotyped by PCR as described previously [19,20]. Pregnant females were euthanized with CO_2 according to National and Institutional guidelines.

2.2. Histology, immunochemistry, cell death and proliferation assays

Embryos were collected at stages of interest, rinsed in PBS, fixed overnight in 4% buffered paraformaldehyde at 4 °C, washed, dehydrated and embedded in Leica Histowax, .7 μm sections were stained with hematoxylin and eosin using a standard protocol. Anti-striated muscle myosin antibody (MF20, 1:500 after heat retrieval, DSHB) binding was detected using Alexafluor goat anti-mouse (Invitrogen) and mounted as below. Apoptotic cells were detected using Dead End Fluorometric TUNEL system (Promega) following manufacturer's instructions. Cell proliferation was assessed using cell proliferation labeling reagent (RPN201, 200 μl ip injection), and then anti *BrdU* antibody (RPN202, GE Healthcare/Amersham) on tissue sections following antigen retrieval, detected using Alexafluor-488 goat anti-mouse antibodies (Invitrogen) and mounted in Vectashield with propidium iodide or with DAPI nuclear stain (Vector Labs Inc.). Fluorescent images were viewed on an Olympus BX51 with fluorescence attachments and photographed using an Olympus DP71 camera and DP controller and manager software.

2.3. R26R fate determination assay

Embryos at stages of interest, were rinsed several times in DPBS, fixed in 4% buffered paraformaldehyde or 0.25% buffered glutaraldehyde for 5–15 min at 4 °C, washed and stained using a standard β-galactosidase staining protocol including X-gal [21], washed in detergent rinse or PBS and fixed. Whole-mounts were examined using a Leica MZ95 dissecting microscope and photographed as above. Some embryos were processed for wax embedding and sectioned at 7 μm and mounted in Immu-mount (Thermoscientific), then examined on Olympus BX51 microscope and photographed as described previously.

2.4. Isolation of primary cardiomyocytes from the embryonic hearts

Hearts were dissected from E11.5 embryos and dissociated using trypsin (0.25%). Single cell suspensions were plated on fibronectin, laminin or collagen-coated dishes (all from BD Biosciences), cultured at 37 °C, 8% CO_2 for 2–4 days and processed for immunostaining. Cultured cells were fixed for 5 min with 4% buffered paraformaldehyde. Antibodies specific for FAK (05-537, Upstate) and FITC-phalloidin (77415, Sigma), were detected, visualized and recorded as described previously [10].

3. Results

3.1. Rac1-cKOs develop heart defects

To delete *Rac1* in the mouse precardiac mesoderm, female mice homozygous for the floxed *Rac1* allele (*Rac1*$^{Fx/Fx}$) were crossed with male mice heterozygous for both the floxed *Rac1* allele and the *Nkx2.5Cre* transgene (*Rac1*$^{Fx/WT}$*:Nkx2-5Cre*$^{-/+}$) [19,20]. When the resulting homozygote *Rac1*$^{Fx/Fx}$*:Nkx2-5Cre*$^{-/+}$ (*Rac1-cKO*) embryos were analyzed at different stages, it was uncovered that the mutant embryos start dying at E12 and no live embryo were recovered after E14. At E11.5, mutant embryos appeared indistinguishable from controls (Fig. 1A and B; the *Rosa26* reporter assay was used to show that the expected cardiac-specific recombination pattern was obtained and that the extent of recombination was comparable between mutants and controls). Similarly, mutant embryos harvested at E12.5-13.5 were recovered close to the expected Mendelian ratio (Table 1). However, several of them showed severe cardiac and vascular defects with edema and hemorrhaging, and some were necrotic. The myocardial wall in mutant embryos appeared thin and disorganized and particularly in more severely affected embryos, cardiomyocytes appeared round and detached (Fig. 1C-F).

3.2. Normal cardiomyocyte proliferation and survival in Rac1:Nkx2.5Cre mutants

To identify cellular processes responsible for a cardiac phenotype seen in *Rac1-cKOs* we first compared levels of programmed cell death and proliferation between controls and mutants. As the mutant mice were variably abnormal at E12.5-E13.5, we focused our analyses on embryos harvested at E11.5 prior to the manifestation of mutant phenotype to uncover possibly primary and not secondary defects. Sections from E11.5 embryos were first subjected to TUNEL staining to reveal dying (apoptotic and necrotic) cells. The number of TUNEL-positive cardiac cells was very low in both controls and mutants, both in the heart and the rest of the embryonic tissues (Fig. 2A and B). Next, we injected pregnant female mice with a nucleotide analog *BrdU*, harvested embryos 2 h later and analyzed them for label-retaining MF20-positive cardiomyocytes using immunohistochemistry. The mutant embryos showed about 15% reduction in a number of positively-staining cardiomyocytes when compared to the Cre-negative control samples (Fig. 2C-E); however the observed differences were not statistically significant (Fig. 2E) suggesting that cardiac cell proliferation was not grossly affected in *Rac1-cKO* mouse embryos.

3.3. Cardiomyocytes deficient in Rac1 show cell adhesion defects in vitro

After showing that the mutant embryos presented no significant changes in cell proliferation and cell death relative to their wild type littermates, we examined whether cardiomyocyte adhesion is affected in *Rac1-cKOs*. To this end, we isolated and dissociated cardiac cells from E11.5 embryos and plated them on fibronectin-coated dishes [4]. After 2 days in culture, the cells were stained for a cell adhesion molecule β-catenin and for MF20 to visualize adherence junctions and to identify cardiomyocytes, respectively (Fig. 3A–D). The control cells showed MF20-negative cells, presumably fibroblasts, and flattened MF20-positive cardiomyocytes (Fig. 3A and C), which were organized to small aggregates (Fig. 3A). Higher magnification showed in more detail how the cardiomyocytes formed cell clusters composed of 2–10 cells that express β-catenin in cell–cell junctions (Fig. 3C). In contrast, *Rac1*-deficient cells were round and only a few cell clusters were

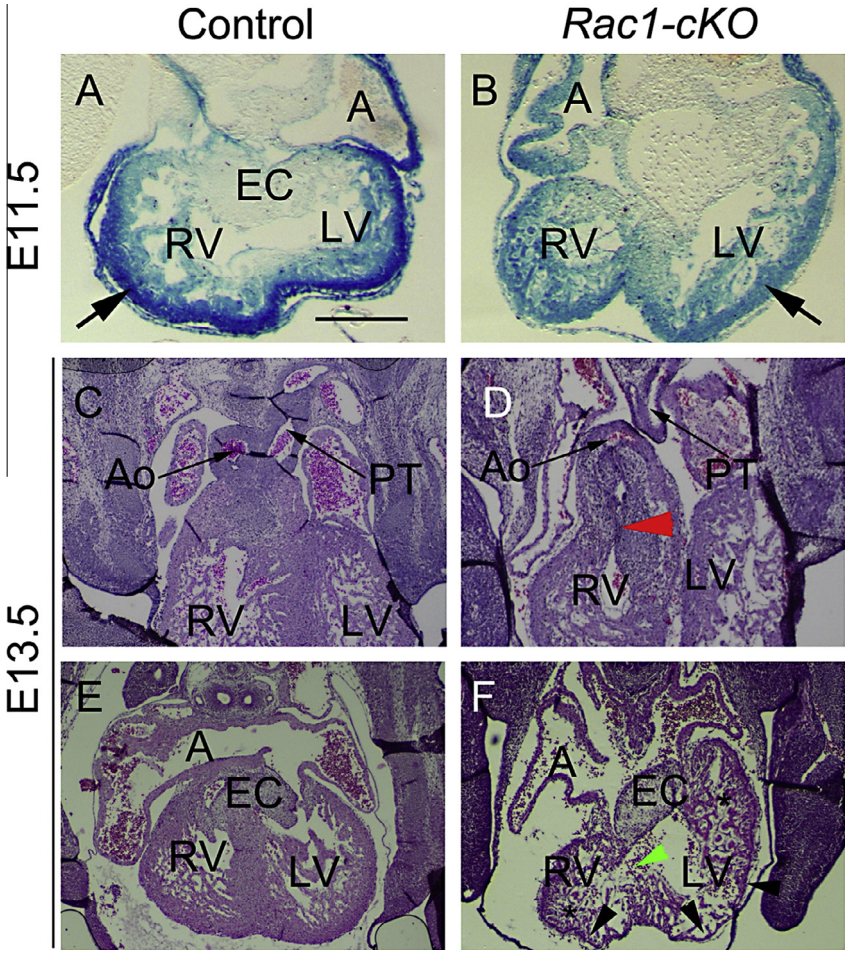

Fig. 1. *Rac1-cKO* embryos show severe cardiac defects at E12.5-E13.5. Embryonic histological sections of control (A, C, E) and *Rac1-cKO* (B, D, F) samples at E11.5 (A, B) and E13.5 (C, F); 4-chamber view. (A and B) X-Gal staining of *Nkx2-5Cre*-positive control and mutant samples (black arrows in A and B) carrying the *Rosa26:lacZ* reporter at E11.5 shows no differences between mutants (B) and controls (A). (C and D) H&E-stained sections at OFT level of control (C) and mutant (D) samples. In addition to the myocardial phenotype (which is milder than that seen in F), the mutant (D) shows misalignment of the OFT (dual outlet right ventricle) (red arrowhead points to an abnormal passage from the right ventricle to the aortic trunk). (E and F) H&E-stained sections at AVJ level of control (E) and mutant (F) samples. The mutant (F) shows a severe myocardial phenotype with disorganized ventricular wall (black arrowheads), trabeculae (asterisk) and ventricular septum (green arrow). A, atrium; V, ventricle; EC, endocardial cushion; RV, right ventricle; LV, left ventricle; Ao, aortic trunk; PT, pulmonary trunk; OFT, outflow tract; AVJ, atrio-ventricular junction. Scale bar shown in A: (A and B) 200 μm; (C and D) 500 μm; (E and F) 1 mm.

Table 1
Recovery of various genotypes at E11.0–E13.0.

Genotype/embryonic day	Total	$Rac1^{Fx/+}$:Nkx2-5Cre$^{-/-}$ (%)	$Rac1^{Fx/+}$:Nkx2-5Cre$^{-/+}$ (%)	$Rac1^{Fx/Fx}$:Nkx2-5Cre$^{-/-}$ (%)	$Rac1^{Fx/Fx}$:Nkx2-5Cre$^{-/+}$ (%)
E11.0	41	13 (32)	14 (34)	7 (17)	7 (17)
E12.0	87	22 (26)	26 (30)	20 (23)	19 (22)
E13.0	40	12 (30)	12 (30)	7 (16)	9 (23)
Total	168	47 (27)	52 (29)	34 (19)	35 (20)

seen (Fig. 3B and D), while the MF20-negative fibroblastoid cells were flattened comparable to those seen in control cultures (Fig. 3B). Higher magnification (Fig. 3D) showed that cardiomyocytes were rarely organized into aggregates and in a few cases where cell clusters composed of 2–5 cells were seen, the cells were less spread than corresponding cells in control cultures. In addition, cardiac cells harvested from control and *Rac1-cKO* embryos were stained for focal adhesion kinase (FAK), which constitutes a critical component of focal adhesion complexes, and for filamentous F-actin [5]. Again, we detected well-spread cells in controls (Fig. 3E). Cardiomyocytes showed prominent staining for F-actin, weak staining for FAK and barely detectable focal adhesion complexes, and they were organized in small clusters (Fig. 3E) as described above (Fig. 3A). In contrast, the fibroblastoid cells showed prominent FAK-staining in focal adhesion complexes, and characteristic stress fibers (Fig. 3E). In mutants, the cardiomyocytes appeared as single, rounded cells, while the fibroblast-like cells seen in mutants were indistinguishable from the corresponding control cells (Fig. 3F). These findings suggest that in cardiomyocytes Rac1 is required for appropriate cell–cell and cell-matrix interactions during heart development.

4. Discussion

In this study, we examined the role of *Rac1* in embryonic heart development using a tissue-specific gene knockout approach; the

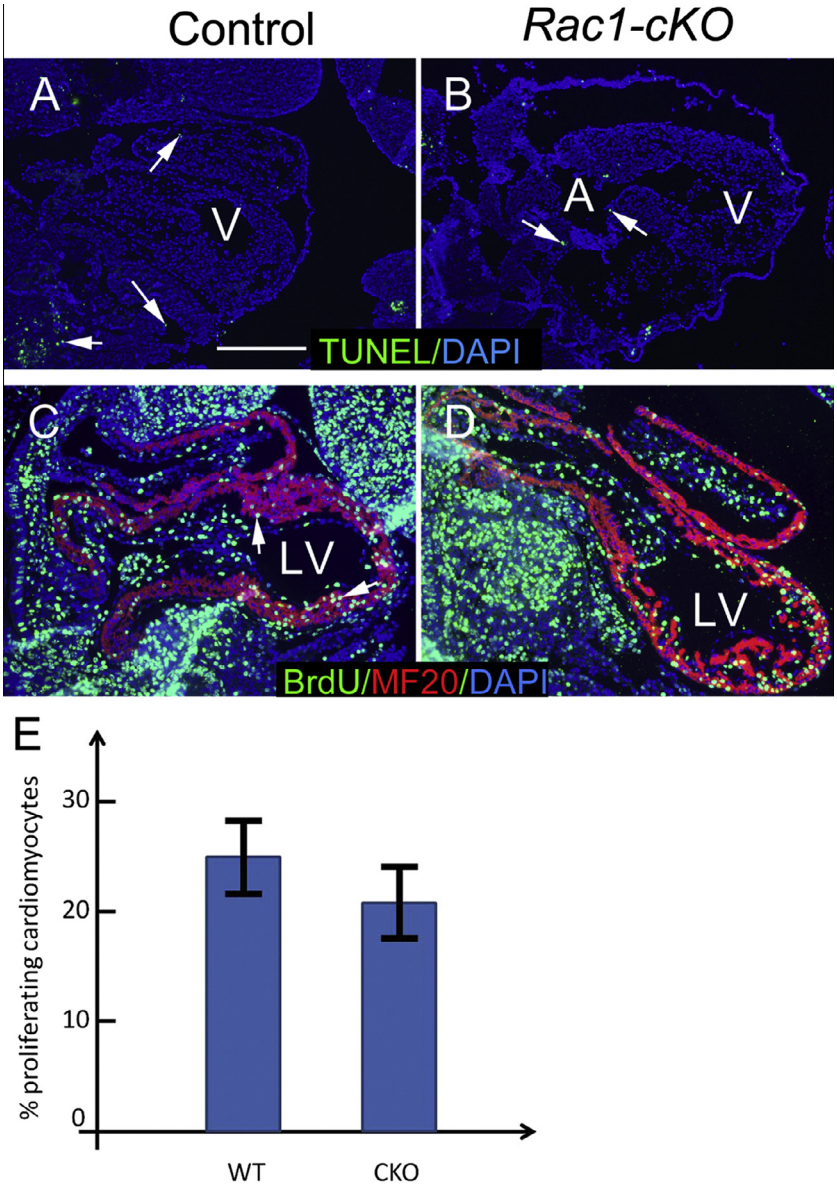

Fig. 2. Programmed cell death and proliferation is not significantly affected in cardiac tissues of *Rac1-cKO* embryos at E11.5. (A and B) TUNEL staining to visualize both apoptotic and necrotic cells (white arrows) in control (A) and *Rac1-cKO* (B) samples. TUNEL-positive nuclei appear green; counterstaining with DAPI (blue). (C and D) BrdU-staining (green nuclei) to detect proliferating cells (white arrows) in control (C) and mutant (D) samples. Cardiomyocytes were identified by MF20 immunostaining (red); counterstaining with DAPI (blue). The bar graph in E illustrates differences in percentage of BrdU-labeled cardiomyocyte nuclei of the total; C, control; cKO, *Rac1-cKO* mutant. Scale bar in A, 100 μm (for A–D).

Rac1 gene was deleted using the *Nkx2-5Cre* mouse line, which induced recombination in cardiac precursors just after their migration and organization into cardiac crescent around E7.5 [19,22]. This line induces deletion of *Rac1* from cardiac precursor cells soon after they have been determined to the cardiac fate, but it does not interfere with Rac1 function prior to *Nkx2-5* expression in the specification and migration of heart precursor cells from the primitive streak into the cardiac crescent as part of the migrating mesoderm [23]. Embryos that lack *Rac1* in the *Nkx2-5* recombination domain died around E12.5-E13.5 with edema and hemorrhages, which are characteristic landmarks of heart failure. Since the mutant embryos developed normally up to E11.5, we conclude that *Rac1* is dispensable for cardiac fate determination, formation of heart tube, rightward heart looping and initial cardiac function. *Rac1* becomes indispensable after E12.5 stage, as *Rac1-cKO* embryos display disorganized heart wall with the likely consequences of misalignment, edema and lethality. Using Nkx2.5 heterozygote (*Nkx2-5Cre*$^{-/+}$) to disrupt Rac1 may contribute to the observed phenotype as Nkx2.5 is involved in several aspects of heart development [28]. The reason of disorganized myocardial wall is due to neither abnormal cell proliferation nor apoptosis. Instead, cardiomyocytes showed defective spreading on a 2D substrate and impaired cell–cell adhesion resulting in a failure of cultured cardiomyocytes to form characteristic cell aggregates. On the other hand, MF20 negative heart fibroblast cell behaved like wild types that are usually segregated, flat and spread on the substrate demonstrating that the defect is specific to cardiomyocytes [24].

What can we learn about Rac1 function, when Rac1 is first needed and what is the specific role of Rac1 in cardiomyocyte differentiation and morphogenesis? To answer these key questions requires us to reconcile our in vitro and in vivo findings. The observed adhesion defects are manifested only after E12.5 even

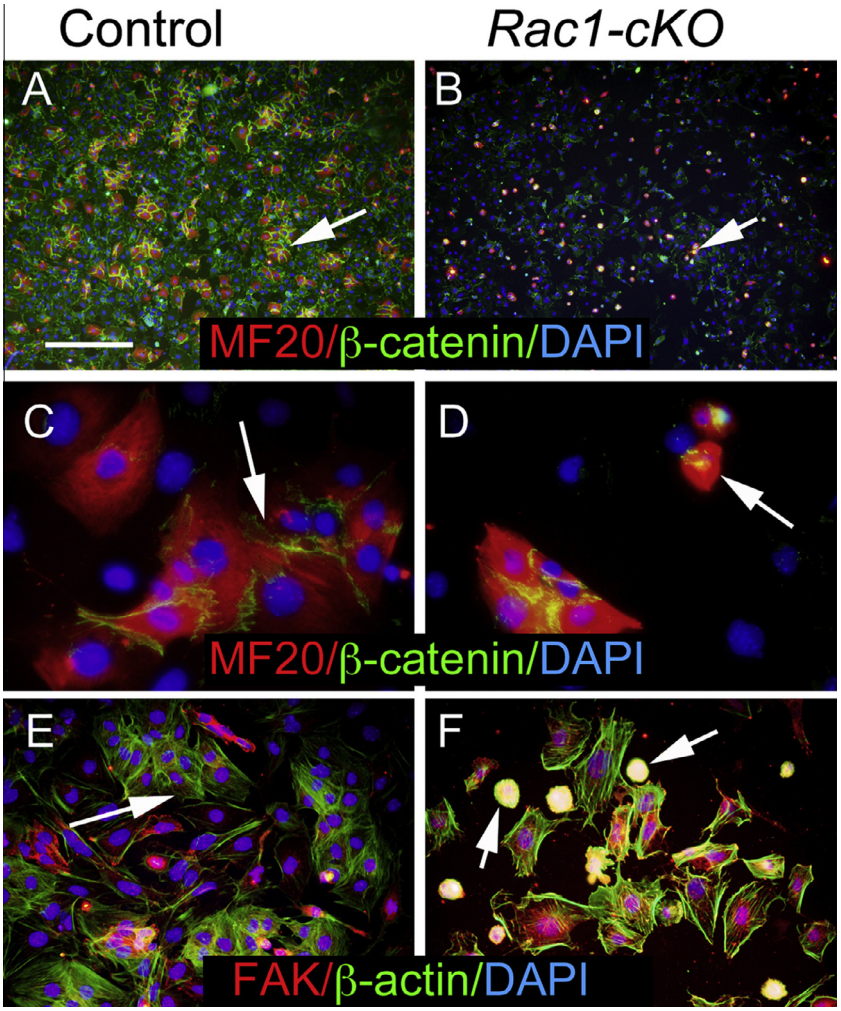

Fig. 3. Cardiomyocytes shows adhesion defect. Cultured heart cells harvested at E11.5 from control (A, C, E) and mutant (B, D, F) embryos. (A–D) Immunostaining for β-catenin (green), MF20 (red), counterstaining, and DAPI (blue). Control cultures show frequent cell clusters composed of 2–10 cardiomyocytes (A, C, white arrows) and dispersed fibroblast-like cells, while mutant cultures show a few, if any, cardiomyocyte clusters (B, D, white arrows). (E and F) Cultured heart cells of control (E) and mutant (F) samples stained for FAK (red), F-actin (green); counter staining with DAPI (blue) (E) and (F). Control culture shows cell aggregates of 2–10 cells of cardiomyocytes (E, white arrow) and dispersed fibroblast cells (E, arrowhead), while mutant culture shows many poorly spread (round) cells (F, white arrows) and many dispersed fibroblast-like cells (F). Scale bar in A, 200 μm (A and B); 25 μm (C and D); 50 μm (E and F).

though cardiomyocytes differentiate and start contracting few days earlier at E8.5 and Cre is expressed even a day earlier at E7.5. The 'in vitro' results demonstrate that cardiomyocytes present adhesion defects at E11.5, however, cardiomyocyte adhesion is not completely abolished as these cells still do weakly adhere to the substrate and one another. This suggests that *Rac1* is not required for initial adhesion, but is needed for enhancement and maturation of adhesive properties of cardiomyocytes when the heart beating begins to carry higher loads at E12.5 and beyond. The mechanism may involve, sensing mechanical forces generated by the working myocardium and by supporting maturation of cardiomyocyte adhesion complexes through the Arp2/3 as seen in other contexts [25–27]. At later stages and in adults, Rac1 may be inhibited or down regulated which would explain the dispensability of *Rac1* during later stages and the effect of over-expression on the heart size (reviewed in [13,18]). Results of this paper improve our mechanistic understanding of the Rac1 action in cardiomyocyte differentiation and maturation, and imply that abnormalities in Rac1-mediated signaling may be causally related to human congenital cardiac defects.

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