Control of cytokinesis by β-adrenergic receptors indicates an approach for regulating cardiomyocyte endowment

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INTRODUCTION
Congenital heart disease (CHD) is the most common birth defect. Improvements in diagnosis and treatment have increased survival rates (1), enabling 1 million patients to live with CHD in the United States. Patients with CHD have a high lifetime risk for developing heart failure, and current thinking about this risk is directed by studies of heart failure in adult patients (1). Unlike adults in whom there is little proliferation of cardiomyocytes, heart muscle in infants and children without heart disease grows by proliferation and differentiation of cardiomyocytes (2, 3). Here, we hypothesized that CHD may entail altered cardiomyocyte proliferation and differentiation.

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We studied these mechanisms in tetralogy of Fallot with pulmonary stenosis (ToF/PS), a common form of CHD that has relatively uniform structural defects (anterior deviation of the infundibulum, pulmonary stenosis, ventricular septal defect, and right ventricular hypertrophy). Despite extensive research to understand the genetic causes of ToF/PS, little is known at the molecular level. Although infants and children with ToF/PS rarely have heart failure, the disease leads to morbidity and mortality in adults (4–11). This is currently explained with the sequelae of cardiac surgery. However, prior studies showed severe cardiomyocyte changes in patients with ToF/PS before surgery (12, 13), leading us to consider the basis for those changes. We characterized changes in cardiomyocyte proliferation and differentiation in patients with ToF/PS by examining the prevalence and molecular mechanisms of binucleation in cardiomyocytes. Binucleation has previously been linked to the decrease in cardiomyocyte proliferative capacity; when cardiomyocytes stop proliferating in mice and rats in the first week after birth, they undergo incomplete cell cycles, leading to binucleated cardiomyocytes (14). Multiple studies have suggested that cardiomyocytes become binucleated by incomplete cytokinesis (15–17). The molecular alterations of the cytokinesis machinery in cardiomyocyte binucleation are largely unknown, as are the regulatory mechanisms.

By examining mouse cardiomyocytes in culture and in vivo, we sought to determine the cause of cytokinesis failure and its relationship to cardiomyocyte proliferation. Multiple signaling pathways regulate cardiomyocyte proliferation (18). How these pathways cooperate has not been fully characterized; however, the Hippo tumor suppressor pathway is thought to be a central node. The Hippo pathway was reported to be activated in the heart by β-adrenergic receptors (β-ARs) (19). β-ARs are known to regulate cardiomyocyte contractile function by adjusting the intracellular second messenger cyclic adenosine monophosphate (cAMP) (20).

In CHD, and specifically in ToF/PS, adrenergic signaling is over-activated (21–25). Adrenergic signaling has been assessed in the context of heart regeneration in neonatal mice (26, 27) and cell cycle activity in cultured rat cardiomyocytes (28–30); however, these studies were performed without genetic disruption of signaling pathways.

Our present study builds on these findings by demonstrating a function of β-AR signaling in regulating cardiomyocyte cytokinesis in vivo. Using formation of binucleated cardiomyocytes as readout for the definitive endpoint of cell division, we found an extensive decrease in cardiomyocyte proliferation in ToF/PS. We identified the mechanisms of formation of binucleated cardiomyocytes, establishing a new connection between β-AR signaling and regulation of cardiomyocyte cytokinesis.

RESULTS

Infants with ToF/PS have a higher proportion of multinucleated cardiomyocytes

We examined samples from the right ventricle of patients with ToF/PS and made the unexpected observation that the percentage of binucleated cardiomyocytes was increased to 50 to 60% (Fig. 1, A and B), suggesting increased cytokinesis failure. Temporal analysis revealed that newborns with ToF/PS showed the expected percentage of 20% binucleated cardiomyocytes (2, 3) but that the increase happened in the first 6 months after birth (Fig. 1B). All patients with ToF/PS >2 months of age had cardiomyocytes with >2 nuclei (Fig. 1C); this is a very rare phenotype in humans without heart disease (2, 3), suggesting that multiple serial cytokinesis failures occurred. Bi- and multinucleated cardiomyocytes were present in a 6- and a 13-year-old patient with ToF/PS, suggesting that bi- and multinucleated cardiomyocytes persist. The proportion of polyploid mononucleated cardiomyocytes increases in humans after birth, prompting us to investigate the ploidy of nuclei in mononucleated cardiomyocytes in infants with ToF/PS. We found that this was not different in comparison with published, age-matched controls (Fig. 1, D and E) (2, 3).

To directly assess the generation of mono- and binucleated cardiomyocytes in patients with ToF/PS, we labeled a 1-month-old infant with ToF/PS with 15N-thymidine and examined uptake and retention using multi-isotope imaging mass spectrometry (MIMS) (31, 32) at 7 months of age (Fig. 1, F and G). Twelve percent of cardiomyocytes were 15N-thymidine positive, indicating that these were generated between label administration at 1 month and removal of myocardium at 7 months after birth. Of mononucleated cardiomyocytes, 8.9% were 15N-thymidine positive (Fig. 1H). Of these, 80% had diploid nuclei (Fig. 1, I and J, and fig. S1), in agreement with the results shown in Fig. 1E. Because mononucleated cardiomyocytes make up about 45% of all cardiomyocytes in ToF/PS hearts at this age (Fig. 1B), this shows that 0.089 × 0.8 × 0.45 = 3.2% of all cardiomyocytes were generated by division between 1 and 7 months after birth. Of binucleated cardiomyocytes, 19.2% were 15N-thymidine positive (Fig. 1H). The 15N-thymidine label intensity in the two nuclei of binucleated cardiomyocytes was the same (fig. S2), suggesting that they shared the same cell cycle history. Because binucleated cardiomyocytes make up the other 55% of all cardiomyocytes, this indicates that 0.192 × 0.55 = 10.6% of all cardiomyocytes were generated by cytokinesis failure in the period between 1 and 7 months after birth. Thus, cytokinesis failure was 3.3 times more common than division as a cell cycle outcome during this period, which explains the higher percentage of binucleated cardiomyocytes generated in hearts with ToF/PS. Prior research showed that an equal proportion of mono- and binucleated cardiomyocytes should be generated because their relative prevalence does not change in humans without heart disease (2, 3). These findings motivate us to determine the mechanisms controlling cytokinesis in cardiomyocytes.

Cardiomyocyte cytokinesis failure is associated with low expression of Ect2

To determine the cellular mechanisms of cytokinesis failure in cardiomyocytes, we performed live cell imaging with neonatal rat ventricular cardiomyocytes (NRVMs) that undergo binucleation (Fig. 2A and movie S1). Cleavage furrow ingression was observed in 80% of the cardiomyocytes studied, followed by cleavage furrow regression. We used a transgenic mouse model expressing the fluorescent ubiquitination-based cell cycle indicator (Fucci) to highlight cell cycle progression (33); we noted normal cell cycle progression until cleavage furrow regression (movies S2 and S3). This finding demonstrates that failure of abscission generates binucleates from mononucleated cardiomyocytes.

To identify the molecular mechanisms of cleavage furrow regression, we separated cycling from noncycling cardiomyocytes and took a single-cell transcriptional profiling approach to compare gene expression (fig. S3). We isolated embryonic [embryonic day 14.5 (E14.5)] and neonatal [postnatal day 5 (P5)] cardiomyocytes, identified cycling cardiomyocytes with the mAG-hGem (monomeric Azami Green-human Geminin) reporter of the Fucci indicator (33), and separated them by...
fluorescence-activated cell sorting (fig. S4). We performed deep, genome-wide, single-cell transcriptional analysis using the Eberwine method (34), followed by validation of the results (fig. S5). During cytokinesis, a contractile ring forms at the future division plane (35). Constriction of this ring is triggered by the cytokinesis protein ECT2, a RhoA guanine-nucleotide exchange factor. RhoA–guanosine 5′-triphosphate (GTP) activation of non-muscle myosin II via Rho-associated protein kinase (ROCK) constricts the cleavage furrow. Because of the critical function

**Fig. 1. Cardiomyocytes in infants with ToF/PS fail to divide.** (A) Immunostaining and (B and C) quantification of cardiomyocytes in heart tissue from patients with tetralogy of Fallot and pulmonary stenosis (ToF/PS). Multinucleated (≥2) cardiomyocytes are indicated by filled symbols and solid lines in (B). Each symbol in (B) and bar in (C) represents one human heart (ToF/PS; n = 12; no heart disease; n = 5). (D) Immunostaining and (E) quantification of ploidy of nuclei in mononucleated cardiomyocytes determined with microscopy (ToF/PS; n = 5). Quantification in (E) includes age-matched published results for no heart disease (n = 5) (2). (F to J) Myocardium analyzed from a 4-week-old human infant with ToF/PS labeled with 15N-thymidine (given orally). Myocardium was analyzed by multiple-isotope imaging mass spectrometry (MIMS) at 7 months. 31P staining in nuclei and 32S morphologic detail, including striated sarcomeres, in (F) mononucleated and (G) binucleated myocytes. Nuclei are dark in the 32S image. The 15N/14N ratio image reveals 15N-thymidine incorporation. The blue end of the scale is set to natural abundance (no label uptake), and the upper bound of the rainbow scale is set to 100% (F) or 50% (G) above natural abundance. The white arrows in (F) indicate the boundaries of a labeled mononucleated cardiomyocyte, and in (G) the nuclei of a binucleated cardiomyocyte. (H) Quantification of labeled binucleated and mononucleated cardiomyocytes [mononucleated cardiomyocytes analyzed: n = 282; 15N+ mononucleated cardiomyocytes: n = 25; binucleated cardiomyocytes analyzed: n = 104 (208 total nuclei); 15N+ binucleated cardiomyocytes: n = 20 (40 total nuclei)]. (I) Ploidy of 15N-thymidine–positive mononucleated cardiomyocytes analyzed by microscopy of Hoechst staining of adjacent sections. (J) Quantification of diploid and polyploid cardiomyocytes. Statistical significance was tested with Student’s t test (E). Scale bars, 20 μm (A and D) and 10 μm (F, G, and I).
Fig. 2. Ect2 regulates cardiomyocyte cytokinesis and binucleation. (A) Live cell imaging of neonatal rat cardiomyocytes (NRVM, P2-P3, n = 52 cardiomyocytes), corresponding to movie S1. Cleavage furrow (white arrows) ingression is between 300 and 335 min, regression at 355 min, and formation of a binucleated cardiomyocyte at 510 min. (B) Transcriptional profiling of single cycling (+) and not cycling (−) cardiomyocytes at embryonic day 14.5 (E14.5) and 5 days after birth (P5) for 61 DbI homology family Rho-GEFs. Ect2 is significantly repressed in cycling P5 cardiomyocytes (P < 0.05). The color code is given below, and the frequency of genes with corresponding expression is indicated with a black line. (C) Immunostaining and quantification of RhoA-GTP at the cleavage furrow (E14.5: n = 3 cell isolations; P2: n = 4 cell isolations) in binucleating cardiomyocytes. (D to H) NRVM transduced with Adv-GFP-Ect2 or Adv-GFP. (D) Live cell imaging of GFP-ECT2 in cycling NRVM, corresponding to movie S4. (E) Images and quantification of binucleated cardiomyocytes (n = 3 cell isolations). (F) Quantification of cardiomyocytes in the S phase (n = 3 cell isolations). (G) Images and quantification of cardiomyocytes in the M phase (n = 3 cell isolations). (H) Analysis of ploidy of nuclei (GFP: n = 3 cell isolations; GFP-Ect2: n = 2 cell isolations). Statistical significance was tested with Student’s t test. Scale bars, 20 μm (C) and 50 μm (E and G).
of RhoA activation for cleavage furrow constriction, we examined the expression of Dbl homology Rho guanine nucleotide exchange factors (GEFs) in the single-cell transcriptional dataset (Fig. 2B and table S1). Ect2 mRNA was present in cycling E14.5 cardiomyocytes but not in binucleating P5 cardiomyocytes (Fig. 2B). Other genes controlling cytokinesis, such as Racgap1 (inactivating RhoA), RhoA, Anillin, Aurkb, and Mklp1, were present in P5 cycling cardiomyocytes (fig. S6), indicating that Ect2 may be uniquely regulated. In accordance with the decreased Ect2 expression, RhoA activation (RhoA-GTP) was decreased in binucleating cardiomyocytes (Fig. 2C). Together, these results show insufficient Ect2 expression in cardiomyocytes leads to less RhoA activation, weakening their cleavage furrow constriction (35).

We tested whether increasing Ect2 expression enabled cardiomyocyte abscission by expressing a fusion construct of green fluorescent protein with a wild-type (WT) Ect2 (GFP-Ect2) (36). Live cell imaging showed the functionality of GFP-ECT2 in cardiomyocytes (Fig. 2D, fig. S7, and movie S4). Transduction with GFP-Ect2 decreased the formation of binucleated cardiomyocytes twofold (Fig. 2E) without altering the proportion of cardiomyocytes in the S phase [measured by quantifying 5-bromo-2′-deoxyuridine (BrdU)–positive cardiomyocytes] (Fig. 2F) or M phase [measured by quantifying phosphohistone H3–positive (H3P) cardiomyocytes] (Fig. 2G), or inducing apoptosis (fig. S8). In addition, expressing GFP-Ect2 did not alter the ploidy of cardiomyocyte nuclei (Fig. 2H). In conclusion, increasing Ect2 expression in cardiomyocytes has a specific positive effect on abscission without changing cell cycle entry or progression.

**Lowering Ect2 expression reduces cardiomyocyte endowment and heart function**

To determine the effect of lowering the expression of Ect2 in vivo, we inactivated the Ect2<sup>fl</sup> gene in mice with αMHC-Cre (fig. S9A) (37). αMHC-Cre<sup>−/−</sup>;Ect2<sup>fl-fl</sup> mice showed a 3.2-fold increase in binucleated cardiomyocytes (23.3%; Fig. 3A) compared with control (EF = 85.9%; Fig. 3G and movie S4). Transduction with GFP-Ect2 decreased the formation of binucleated cardiomyocytes twofold (Fig. 2E) without altering the proportion of cardiomyocytes in the S phase [measured by quantifying 5-bromo-2′-deoxyuridine (BrdU)–positive cardiomyocytes] (Fig. 2F) or M phase [measured by quantifying phosphohistone H3–positive (H3P) cardiomyocytes] (Fig. 2G), or inducing apoptosis (fig. S8). In addition, expressing GFP-Ect2 did not alter the ploidy of cardiomyocyte nuclei (Fig. 2H). In conclusion, increasing Ect2 expression in cardiomyocytes has a specific positive effect on abscission without changing cell cycle entry or progression.

The Hippo tumor suppressor pathway regulates Ect2 gene transcription and cardiomyocyte division

We next sought to identify the mechanisms responsible for decreasing transcription of the Ect2 gene. Previous publications suggested that the Hippo tumor suppressor pathway regulates cardiomyocyte proliferation (41–43). YAP1 (yes-associated protein 1), the central transcriptional coregulator controlled by the Hippo pathway, forms a protein complex with TEAD (TEA domain family member) transcription factors (42). We identified five binding sites for TEAD transcription factors in the Ect2 promoter (fig. S11). Removing these TEAD-binding sites individually or en bloc decreased Ect2 promoter activity in luciferase assays (Fig. 4A). Small interfering RNA (siRNA) knockdown of TEAD1/2 reduced Ect2 mRNA expression (Fig. 4B) and increased the proportion of binucleated cardiomyocytes (Fig. 4C).

Adenovirus-directed increase in TEAD1 expression decreased the formation of binucleated cardiomyocytes (Fig. 4D). Adenoviral-mediated expression of YAP1-WT and a nongeodegradable version (YAP1-S127A) in NRVMs increased Ect2 mRNA expression (Fig. 4E) and reduced the proportion of binucleated cardiomyocytes (Fig. 4F). These results show that YAP1 and TEAD transcription factors regulate the expression of Ect2 and cardiomyocyte abscission.

β-AR gene inactivation increases cardiomyocyte Ect2 expression, cytokinesis, and endowment

Because the Hippo pathway was shown to be activated by β-AR in the heart (19), we examined β<sub>1</sub>-AR<sup>−/−</sup>; β<sub>2</sub>-AR<sup>−/−</sup> [double-knockout (DKO)] (44, 45) pups. DKO pups showed increased transcription of the Hippo target genes Cyr61 and CTGF (Fig. 5A), as well as Ect2 (Fig. 5B), in the heart. These pups showed a lower proportion of binucleated cardiomyocytes (Fig. 5C) and a higher endowment at P4 and P10 (Fig. 5D). Their cardiomyocyte M phase activity did not change (Fig. 5E). To determine the functional relationship between ß-AR signaling and Ect2 in generation of binucleated cardiomyocytes, we used siRNA (fig. S12) to knock down Ect2 in DKO cardiomyocytes. This experiment showed a significant increase in the percentage of binucleated cardiomyocytes generated (P = 0.0032; Fig. 5F).

β-AR signaling regulates cardiomyocyte Ect2 expression, cytokinesis, and endowment

We explored pharmacological ways to control formation of binucleated cardiomyocytes and endowment growth. β-AR directly activates large heterotrimeric GTP-binding proteins (G proteins) of the stimulatory family (Gs). The natural compound forskolin (Fsk) mimics the active conformation of Gs (46). Accordingly, we added Fsk to cultured NRVMs, which decreased Ect2 mRNA expression (Fig. 6A). We administered Fsk to newborn mice and found a 37% increase in the proportion of binucleated cardiomyocytes after 4 days and a 21% increase after 8 days (Fig. 6B). We then administered propranolol, a blocker of β<sub>1</sub>- and β<sub>2</sub>-AR, to newborn mice. Propranolol decreased the proportion of binucleated cardiomyocytes by 21% after treatment from P1 to P4, and by 17% after treatment from...
P1 to P8 (Fig. 6C). This was associated with a 22 and 30% increase in cardiomyocyte endowment at P4 and P8, respectively (Fig. 6D), without a change in cardiomyocyte cell cycle entry (quantified by BrdU uptake; Fig. 6E and fig. S13) or progression to the M phase (quantified by H3P staining; Fig. 6F) at P8. The heart weight was not changed (fig. S14A). We examined the effect of another blocker of β1- and β2-AR, alprenolol. Administration of alprenolol from P1 to P8 decreased the formation of binucleated cardiomyocytes by 13% (Fig. 6G), corresponding to a 24% increase in cardiomyocyte endowment (Fig. 6H), but did not result in a change of the heart weight (fig. S14A).
weight (fig. S14B). These results show that reducing β-AR signaling by β-blocker administration during the neonatal phase enables cardiomyocyte abscission, thus increasing the endowment.

**Neonatal propranolol-mediated increased endowment improves heart function and remodeling in adult mice after MI**

The increased cardiomyocyte endowment resulting from neonatal propranolol administration persisted until adulthood (Fig. 7A) but did not alter cardiac function (Fig. 7B). A larger endowment should confer a benefit after large-scale cardiomyocyte loss, for example, after myocardial infarction (MI). We tested this by administering propranolol during the neonatal period and then inducing MI in adult mice (Fig. 7C). We determined cardiac structure and function with magnetic resonance imaging (MRI) (Fig. 7C). Two days after MI, control and propranolol-treated mice had the same infarct size as determined by late gadolinium enhancement (LGE; Fig. 7D) and EF (measured by MRI; Fig. 7E). However, 12 days after MI, mice with propranolol-induced endowment growth had an MRI-measured EF of 42%, compared with 18% in control mice (Fig. 7E and movie S8). The thinned region of the left ventricular (LV) myocardium after MI was significantly smaller ($P = 0.0032$, Fig. 7F), and the relative systolic thickening was higher (Fig. 7, G to I), indicating less adverse remodeling. The region of myocardium affected by ischemia, visualized by LGE (Fig. 7D), and the scar size, determined by histology (Fig. 7J), were not different. Propranolol-treated hearts had a 30% higher cardiomyocyte endowment after MI (determined by stereology; Fig. 7K and fig. S15), in keeping with the increased endowment before MI (Fig. 7A). The heart weight was not changed (Fig. 7L), indicating that the higher endowment
reduced the maladaptive hypertrophy, which drives adverse remodeling after MI. Together, these results demonstrate that rescuing cardiomyocyte cytokinesis failure with propranolol during development reduces adverse ventricular remodeling in adult mice.

**β-Blockers rescue cytokinesis failure in cardiomyocytes from infants with ToF/PS**

We determined to what extent the molecular mechanisms of cardiomyocyte cytokinesis failure we found are responsible for the increased proportion of binucleated cardiomyocytes in ToF/PS. To this end, we transcriptionally profiled single cardiomyocytes from infants with ToF/PS. Human control samples, corresponding in age and quality to the available freshly resectedToF/PS myocardium, are not available because infant hearts without disease are used for transplantation. As such, we turned to available human fetal hearts for comparative single-cell transcriptional analysis. We normalized cardiomyocytes from human fetuses without ToF/PS and infants with ToF/PS together and imposed a rigorous quality control to ensure equal quality. Although expression of structural and functional genes may differ between fetuses and infants, we reasoned that expression of the cell cycle program, which is evolutionarily conserved, should be similar. In other words, we investigated whether single non-ToF/PS fetal and ToF/PS infant cardiomyocytes express cell cycle genes at the same amount when they enter the cell cycle. The normalized mRNA expression of 16 cell cycle genes was similar between non-ToF/PS fetal and ToF/PS infant cardiomyocytes (Fig. 8A and table S2). We then compared the expression of the mRNAs encoding for Ect2’s direct protein interaction partners in cytokinesis, RhoA and RacGAP1, which also showed similar mRNA expression. However, while fetal cardiomyocytes expressed Ect2, ToF/PS did not. We then identified cycling cardiomyocytes by the expression of cell cycle genes. The portion of cycling cardiomyocytes in infants with ToF/PS that did not express Ect2 was >90% (Fig. 8B), corresponding to the proportion of cycling cardiomyocytes that fail cytokinesis. This suggests that the majority of cycling cardiomyocytes in infants with ToF/PS experience cytokinesis failure, which agrees with the results shown in Fig. 1. In contrast, 75.6% of cycling human non-ToF/PS fetal cardiomyocytes expressed Ect2, indicating that the majority divided (Fig. 8B). This is consistent with the prior finding that human newborns have approximately 20 to 30% binucleated cardiomyocytes, which must be generated during fetal life (2, 3). In conclusion, a large proportion of cycling cardiomyocytes in infants with ToF/PS exhibits decreased Ect2 expression, similar to cardiomyocytes in neonatal mice (Fig. 2B).

This finding prompted us to examine whether regulating β-AR signaling would alter cytokinesis failure in human cardiomyocytes. We used cultured human fetal cardiomyocytes and added Fsk, which maximally increased cardiomyocyte cytokinesis failure (Fig. 8C). We then treated human fetal cardiomyocytes with dobutamine to mimic the in vivo microenvironment of increased β-AR stimulation. Dobutamine increased binucleated cardiomyocytes to 95.2% of the Fsk-induced increase (Fig. 8C). Addition of propranolol blocked the dobutamine-stimulated increase in cardiomyocyte cytokinesis failure completely (Fig. 8C). We examined cardiomyocytes in cytokinesis by immunofluorescence microscopy, which showed that Ect2-positive midbodies were increased with propranolol (Fig. 8D). We then generated organotypic cultures of heart pieces from infants with ToF/PS and added BrdU to label cycling cardiomyocytes (Fig. 8E and figs. S16 and S17). Fsk and dobutamine induced a maximal increase in the

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**Fig. 5. β-AR signaling regulates cardiomyocyte abscission and binucleation.** (A) Cardiac expression of YAP target genes Cyr61 and CTGF and of (B) Ect2 (n = 3 hearts per group) after inactivation of β1- and β2-AR genes (DKO) in mice (P4). (C) Immunostaining and quantification of multinucleated (P4; n = 4 hearts per group; P10: n = 6 hearts for WT, n = 3 hearts for DKO) and (D) total cardiomyocytes in DKO mice (quantified by counting fixation-digested hearts, P4: n = 7 hearts for WT, n = 5 hearts for DKO; P10: n = 6 hearts for WT, n = 3 hearts for DKO). (E) Immunostaining and quantification of M phase cardiomyocytes (n = 4 hearts per group) in vivo at P4. (F) Immunostaining and quantification of binucleated cardiomyocytes generated by knockdown of Ect2 in cultured neonatal cardiomyocytes from β-AR DKO mice (P2, n = 3 cell isolations). Sc, scrambled siRNA (see fig. S12) for Ect2 siRNA validations. Scale bars, 20 μm. Statistical significance was tested with two-way ANOVA with Bonferroni’s multiple comparisons (C and D) and Student’s t test (A, B, E, and F).
Fig. 6. Pharmacologic alterations of β-AR signaling regulate cardiomyocyte abscission and endowment. (A) Ect2 mRNA expression in cultured NRVMs treated with Fsk (n = 5 cardiomyocyte isolations). (B) Immunostaining and quantification of multinucleated cardiomyocytes after Fsk administration in vivo (1 μg/g body, one intraperitoneal injection per day in newborn mice, n = 6 hearts per group). (C) Immunostaining and quantification of multinucleated cardiomyocytes (PBS: n = 4, prop: n = 3 hearts for P4; n = 4 hearts per group for P8) and (D) total number of cardiomyocytes (quantified by counting fixation-digested hearts; PBS: n = 7, prop: n = 6 hearts for P4; n = 4 hearts per group for P8) after propranolol administration (prop, 10 μg/g body, two intraperitoneal injections per day in newborn mice). (E) Immunostaining and quantification of cell cycle entry (n = 5 hearts per group for P8) and (F) M phase activity (n = 4 hearts per group for P8). (G) Immunostaining and quantification of multinucleated cardiomyocytes and (H) total number of cardiomyocytes (quantified by counting fixation-digested hearts) after alprenolol administration (alp, 10 μg/g body, two intraperitoneal injections per day in newborn mice, n = 6 hearts per group). Scale bars, 20 μm. Statistical significance was tested with Student’s t test (A, E, and F) and two-way ANOVA with Bonferroni’s multiple comparisons (B to D, G, and H).
Fig. 7. Propranolol-induced increase in the cardiomyocyte endowment in the neonatal period improves adult cardiac function and remodeling after MI. Mice received propranolol (prop, 10 μg/g body, two intraperitoneal injections per day, P1 to P12) or PBS. (A) Quantification of cardiomyocytes in adult hearts at baseline (fixation-digested hearts, n = 6 hearts per group for P42). (B) Ejection fraction of adult hearts at baseline (P60, n = 11 hearts for PBS, n = 6 hearts for prop). (C) Diagram of experimental design. MI was induced by permanent ligation of the left anterior descending coronary artery between 6 weeks (P44) and 2 months after birth (P60). MRI was performed in the acute phase (1 to 3 days post injury (dpi)) and recovery phase (10 to 12 dpi). (D) MRI of late gadolinium enhancement (LGE) in both groups in the acute and recovery phases. MI size is indicated in purple and quantified (n = 6 mice per group for the acute phase; PBS: n = 7, prop: n = 6 mice for the recovery phase). (E) MRI images and quantification of EF in the acute and recover phases in propranolol- or PBS-treated mice (PBS: n = 4, prop: n = 5 mice for the acute phase; PBS: n = 7, prop: n = 6 mice for the recovery phase). (F) MRI images and analysis of stretched myocardial wall (PBS: n = 5, prop: n = 4 mice for the recovery phase). (G to I) MRI images and analysis of systolic myocardial thickening in the acute phase (G and I; PBS: n = 4, prop: n = 5 mice) and the recovery phase (H and I; PBS: n = 7, prop: n = 6 mice). (J) Scar size quantified by AFOG staining (PBS: n = 5, prop: n = 4 hearts for the recovery phase) at 12 dpi. (K) Number of cardiomyocytes (determined by stereology, n = 5 hearts per group) and (L) heart weight–to–body weight ratio (PBS: n = 5, prop: n = 6 hearts). Statistical significance was tested with Student’s t test (A, B, F, and J to L) and one-way ANOVA with Bonferroni’s multiple comparisons (D, E, and I).
proportion of binucleated cardiomyocytes, and propranolol inhibited the dobutamine-stimulated increase completely (Fig. 8E). In conclusion, \(\beta\)-ARs regulate cytokinesis failure in cardiomyocytes from infants with ToF/PS, and propranolol decreases this effect.

DISCUSSION
To increase myocardial regeneration, conventional approaches stimulate cardiomyocyte proliferation in all phases of the cell cycle: cell cycle entry, progression to M phase, and cytokinesis. Here, we demonstrated that the number of cardiomyocytes can be increased simply by preventing cytokinesis failure. We show that \(\beta\)-ARs control the decision point of whether cardiomyocytes accomplish cytokinesis and divide, or fail and become binucleated (Fig. 8F). We therefore identified a function of \(\beta\)-AR signaling in regulating growth of cardiomyocyte endowment during development. Two major lines of evidence support this conclusion: (i) Cardiomyocytes have decreased expression of the critical cytokinesis gene \(Ect2\) when they become binucleated,
and (ii) blocking β-AR signaling disinhibits Ect2 transcription, which increases division in cycling cardiomyocytes and increases their numbers (endowment). We show that the higher endowment confers benefit after MI in adult mice and that the molecular mechanisms are also operative in human cardiomyocytes.

By identifying that β-ARs regulate Ect2 gene transcription, we were able to conduct experiments demonstrating that cardiomyocyte cytokinesis can be manipulated without altering cell cycle entry or progression. First, Ect2 gene inactivation or expression of the Ect2 cDNA altered cardiomyocyte cytokinesis but did not change cell cycle entry or progression. Second, increasing Ect2 gene transcription with β-AR gene knockout or β-blockers also did not alter cardiomyocyte cell cycle entry or progression. Prior studies (47–49) examining the mechanisms of formation of binucleated cardiomyocytes did not distinguish cytokinesis failure from regulation of the other phases of the cell cycle, indicating this finding is an important advance.

A variety of G protein–coupled receptors are required for RhoA activation during cytokinesis in cell lines (50). Our results differ in that reducing β-AR signaling indirectly increases RhoA activation by derepressing transcription of the RhoA-GEF Ect2. We identified the Hippo pathway as a critical signaling pathway connecting β-AR signaling to Ect2 gene regulation. Although direct genetic manipulations of the central Hippo pathway kinases and scaffolds, as well as the dystroglycan/agrin complex (18), induced cardiomyocyte proliferation by increasing cell cycle entry, M phase, and cytokinesis, we show that regulating the Hippo pathway via β-ARs alters only cytokinesis. This apparent contradiction may be explained by the different approaches taken to regulate the Hippo pathway, genetic manipulation of intracellular signaling nodes versus regulation by a transmembrane receptor. A prior study showed that β-blocker administration in neonatal rats decreased BrdU uptake (30). We demonstrate that genetic inactivation of β-AR or β-blockers increase the cardiomyocyte endowment but do not alter cardiomyocyte cell cycle activity. This apparent discrepancy may be explained by the lack of use of a cardiomyocyte marker by Tseng et al. (30).

Failure of cytokinesis is also an early event in cancer formation, leading, via entrapment of lagging chromosomes in the cleavage furrow, to aneuploidy (51). In this process, chromosome entrapment lowers RhoA activity in the cleavage furrow, leading to relaxation of the contractile ring and cytokinesis failure. However, we have no evidence for chromatin entrapment in cardiomyocyte cytokinesis failure. In cancer cells, cytokinesis failure activates the Hippo tumor suppressor pathway (52); however, we found that Hippo pathway activation triggers cytokinesis failure in cardiomyocytes. Cytokinesis failure is also a step in platelet formation, also induced by repression of Ect2 (53). This suggests a generalizable molecular mechanism of Ect2 repression in cytokinesis failure in somatic cells.

A common question is “How does cardiomyocyte cytokinesis failure affect the heart?” Our results imply that cytokinesis failure stops further cardiomyocyte division. To test this hypothesis, we performed a back-of-the-envelope calculation of cardiomyocytes predicted to be generated due to propranolol administration in mice from P4 to P8, for which initial and final data are available. Between P4 and P8, the cardiomyocyte endowment in propranolol-treated pups increased by 0.35 × 10^6 and in phosphate-buffered saline (PBS)–treated pups by 0.17 × 10^6 cardiomyocytes. Thus, the propranolol-induced increase between P4 and P8 was 0.18 × 10^6 cardiomyocytes. How does this compare to decreased cytokinesis failure, calculated from the decrease in the percentage of binucleated cardiomyocytes in propranolol-treated pups? At P8, propranolol-treated pups had 13.6% fewer cardiomyocytes undergoing cytokinesis failure than PBS-treated pups. Taking the endowment of PBS-treated pups at P4 (1.69 × 10^5 cardiomyocytes), this corresponds to 0.22 × 10^6 additional cardiomyocytes generated in propranolol-treated pups at P8. Thus, the number of cardiomyocytes predicted to be generated by enabling completion of cytokinesis with propranolol administration (0.22 × 10^6) corresponds to the number of cardiomyocytes calculated from the counts (0.18 × 10^6).

We show that cardiomyocyte cytokinesis failure is significant for patients with ToF/PS, where our results predict that it reduces the cardiomyocyte endowment by 25%. These changes develop in the first 6 months after birth, before any surgical intervention, and persist in older patients with ToF/PS. Because infants with ToF/PS are hypoxic until surgical repair, our results relate to the proposition that the oxygen surge at birth induces cardiomyocyte differentiation and cell cycle arrest (54, 55). Under this proposition, cardiomyocyte differentiation should be decreased and cell cycle activity should be increased in the infants with ToF/PS that we examined because they did not achieve normoxia. However, our presented results show increased differentiation (binucleation), and our prior results show decreased cell cycle activity (56). This difference could be explained by the more severe degree of hypoxia used in the published mouse studies (54, 55).

Our results suggest that β-blockers could rescue cytokinesis failure and increase cardiomyocyte division in infants with ToF/PS. The results in mice demonstrate that promoting the progression of cytokinesis to abscission in the postnatal period increases the endowment, which improves remodeling after MI. This suggests that formation of a higher or lower cardiomyocyte endowment during development connects to outcomes in adult human patients. This could be tested with β-blocker administration in human infants with ToF/PS to increase the endowment, followed by measuring clinical outcomes, such as myocardial function and risk of heart failure development. β-Blockers have been used sporadically to treat and prevent cyanotic spells in patients with ToF/PS (57). Although those studies demonstrated that β-blockers are safe in this population, an effect on myocardial growth mechanisms was not assessed. Further, our results predict that administration of β-blockers should produce the largest effect on cardiomyocyte cytokinesis during the first 6 months after birth, which is in line with our previously demonstrated effectiveness of stimulating cardiomyocyte cell cycling in the same period (56). The duration of this period should also be assessed in types of CHD other than ToF/PS.

We acknowledge certain limitations of our study. First, our results show that regulating the activity of β-AR signaling alters cardiomyocyte cytokinesis but does not change cardiomyocyte cell cycle activity. As such, the effectiveness of β-blocker administration for preventing cytokinesis failure requires cardiomyocytes to be in the cell cycle. Considering the fact that cardiomyocyte cell cycle activity stops within 2 weeks after birth in mice (58) and between a few months and 10 years after birth in humans (56), administration of β-blockers with the goal of reducing cardiomyocyte cytokinesis failure should happen before cardiomyocyte cell cycle activity ceases. Second, our results demonstrate that the molecular and cellular mechanisms of cytokinesis failure are operative in cardiomyocytes from infants with ToF/PS. However, before extending this concept to other types of CHD, its applicability should be formally examined. Last, although we tested the endowment concept in a mouse model of MI, its applicability for ToF/PS in vivo has to be tested in humans since animal models of ToF/PS do not exist.
We should point out an advantage of our stable isotope labeling approach that could be applied in a variety of research studies. Birthdating at the single-cell level has been notoriously challenging in humans. Our use of stable isotope–labeled thymidine with MIMS as readout is an innovative, highly effective approach that should be applicable for studying cellular proliferation in other organs.

The findings we report address a persistent challenge in studies of myocardial regeneration: Low cardiomyocyte proliferation in adult mammals continues to be a barrier for heart regeneration (18). To overcome this, molecular interventions to stimulate cardiomyocyte cell cycle entry in adults have been proposed: increasing positive cell cycle regulators [cyclins A2 and D2 (59, 60) and combinations (61)], removing negative cell cycle regulators [p53, pocket proteins (62, 63)], administering mitogenic growth factors [fibroblast growth factor, neuregulin 1, oncostatin M, and follistatin-like 1 (56, 64–66)], and regulating micro-RNAs (67). All of these approaches require increasing cell cycle entry, which could increase the risk of uncontrolled proliferation. Here, we support targeting the final stage of the cell cycle (abscission) as a viable strategy that could synergize with other interventions to increase cardiomyocyte generation.

**MATERIALS AND METHODS**

**Study design**

The goal of this project was to determine if and how formation of binucleated cardiomyocytes is altered in ToF/PS. The study design, including the number of animals and the numbers of cells counted, was predefined by the investigators. The genotypes of all mice were recorded throughout the entire period of the project. For studies involving human tissue, the number of tissue samples was determined according to the availability of the samples. The investigators were blinded for the quantification of samples. The number of biological and technical replicates is provided in table S3. Animal experiments were approved by the Institutional Animal Care and Use Committee. Research involving human material was approved by the Institutional Review Board (IRB). Cardiomyocytes from patients with ToF/PS were collected as part of standard care during surgical repair. Human fetal myocardial samples were collected from abortions at 18 to 23 weeks’ gestation.

**Quantification of cardiomyocyte endowment (number of cardiomyocytes) of mouse hearts**

**Counting of cardiomyocytes after fixation and digestion**

All fractions from the digestion of a heart were combined in 2 of PBS, and cardiomyocytes were quantified using a hemocytometer. The study design, including the number of animals and the numbers of cells counted, was predefined by the investigators. The genotypes of all mice were recorded throughout the entire period of the project. For studies involving human tissue, the number of tissue samples was determined according to the availability of the samples. The investigators were blinded for the quantification of samples. The number of biological and technical replicates is provided in table S3. Animal experiments were approved by the Institutional Animal Care and Use Committee. Research involving human material was approved by the Institutional Review Board (IRB). Cardiomyocytes from patients with ToF/PS were collected as part of standard care during surgical repair. Human fetal myocardial samples were collected from abortions at 18 to 23 weeks’ gestation.

**Quantification of cardiomyocytes with unbiased stereology**

Mouse hearts were washed in cardioplegia solution (PBS, 25 μM KCl), weighed, and fixed with 3.7% formaldehyde at room temperature overnight. The hearts were then placed in 30% sucrose at 4°C for 48 hours. The atria were cut off, and the ventricles were embedded in optimal cutting temperature (OCT) compound and sectioned on a Leica CM1950 cryostat in cross-sectional orientation (thickness, 15 μm), resulting in 75 to 80 slides per heart with four sections each. For random systematic sampling, we used a random number generator ranging between one and six to determine the first slide and then selected every 17th slide for staining. Myocardial volume and scar were quantified by the point count method on acid fuchsin–orange G (AFOG)–stained sections. Briefly, tissue sections were stained with AFOG (56), and photomicrographs were taken on a Leica MZ26 dissection microscope (objective lens 10×). Areas of myocardium (red after AFOG staining) and scar (blue after AFOG staining) were quantified using ImageJ (version 1.51 s). The image was overlaid with a grid to determine area per point. The distance between selected sections was calculated (17th slide × 4 sections per slide × 15-μm section thickness). The LV myocardial volume was measured by counting the number of grids on both LV myocardium and the scar region. We used the optical dissector method to determine the volume density of cardiomyocyte nuclei (fig. S15). Briefly, the immunofluorescence stained sections were imaged using a Nikon A1R confocal microscope. We selected four random spots on the technically best section from each slide and analyzed 20 random samples from each heart. The total number of positive cardiomyocyte nuclei per heart was counted, and the mean per sample volume was calculated. Total number of cardiomyocytes (endowment) was calculated as follows: number of cardiomyocytes = number of cardiomyocyte nuclei/([Mono% + 2 × Bi% + 3 × Tri% + 4 × Tetra%]).

**15N-thymidine labeling of human cardiomyocytes in vivo and analysis**

The clinical study protocol was approved by the IRB, and informed consent was obtained from the parents. On the basis of prior human MIMS studies (31, 68), the patient received 15N-thymidine [50 mg/kg per os (po), Cambridge Isotope Laboratories, on five consecutive days] at 3.5 weeks of age. The patient underwent surgery at 7 months of age. A discarded piece of right ventricular myocardium was obtained, fixed in 4% paraformaldehyde, embedded in LR White, and 500-μm sections were mounted on silicon chips. MIMS was performed on myocardial sections using the NanoSIMS 50L (CAMECA) and previously described analytical methods (32). 15N-thymidine labeling was measured by quantification of the 12C15N−/12C14N− ratio (fig. S2), obtained in parallel with mass images used for histological identification (12C14N−, 31P−, and 32S−). Quantitative mass images were then analyzed using OpenMIMS version 3.0, a customized plugin to ImageJ (NIH) that is available at https://github.com/BWHCNI/OpenMIMS (69). An observer blinded to the ratio images identified nuclei and assigned cellular identity using the 14N (12C15N−), 31P−, and 32S− images as previously described (32). Cardiomyocyte nuclei were identified by their close association with sarcomeric structures. The total number of both 15N-thymidine–positive and 15N-thymidine–negative cardiomyocytes in both mono- and binucleated cardiomyocytes was counted. The percentage of 15N-thymidine–positive cardiomyocytes in both mono- and binucleated cardiomyocytes was calculated.

**Quantification of mono- and binucleated cardiomyocytes**

**Intact cardiomyocytes in suspension**

Cardiomyocytes were isolated from fresh or frozen (human or mouse) ventricle myocardium using the fixation-digestion method, followed by immunostaining as described above. The cells were then imaged and quantified with a Nikon A1R microscope for mono-/bi-/multinucleation.

**Cardiomyocytes cultured on glass surfaces**

Primary (mouse, rat, or human) cardiomyocytes were cultured with BrdU (30 μM) and fixed with 3.7% formaldehyde. The BrdU-positive nuclei, cell-cell boundaries (pan-cadherin antibody), and cardiomyocyte markers (α-actinin or troponin I antibody) were labeled by immunofluorescence. The stained cells were imaged under Nikon confocal microscope. The binucleated cardiomyocytes and all BrdU-positive cardiomyocytes were quantified using Fiji software.
Cultured ToF/PS myocardium
Cardiomyocytes were isolated from cultured myocardium with the fixation-digestion method. After denaturing the DNA with 2N HCl and neutralization with 2N NaOH, immunofluorescence antibody labeling was performed in solution. The proportion of binucleated and BrdU-positive was quantified with a Nikon A1R microscope.

Quantification of ploidy of cardiomyocyte nuclei

Intact cardiomyocytes in suspension
The intact cardiomyocytes were isolated from frozen or fresh (human ToF/PS or mouse with Ect2 inactivation) myocardial samples using the fixation-digestion method. Then, the cardiomyocytes were labeled with α-actinin antibody, and the nuclei were stained with Hoechst. The stained cardiomyocytes were imaged using an epifluorescence microscope with a CMOS (complementary metal-oxide semiconductor) camera. The Hoechst fluorescence intensity of the nuclei of mononucleated cardiomyocytes was measured by Imagej software after correction for background fluorescence. The fluorescence intensity of the mononucleated cardiomyocyte nuclei was then normalized by that of the noncardiomyocytes to obtain the ploidy of the cardiomyocytes.

Ploidy of 15N-thymidine–positive cardiomyocyte nuclei
Adjacent sections of 15N-thymidine–positive nuclei of interest were selected, fixed, and stained with Hoechst (fig. S1). The DNA contents of the nuclei in mononucleated cardiomyocytes were evaluated by measuring the Hoechst fluorescence intensity. Ploidy was determined by normalizing the measured DNA content by that of noncardiomyocytes, after elimination of background fluorescence.

Statistical analyses
Statistical testing was performed with Student’s t test, Fisher’s exact test, and analysis of variance (ANOVA), followed by Bonferroni post hoc testing, as indicated. A two-sided P value ≤0.05 was accepted as statistically significant. Statistical analyses were performed with GraphPad Prism, version 6.

SUPPLEMENTARY MATERIALS
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Methods
Fig. S1. Approach for determining the ploidy of 15N-thymidine–labeled nuclei.
Fig. S2. The two nuclei in binucleated cardiomyocytes have the same 15N-thymidine intensity, as statistically significant. Statistical analyses were performed with hoc testing, as indicated. A two-sided P value ≤0.05 was accepted as statistically significant. Statistical analyses were performed with a Nikon A1R microscope.

REFERENCES AND NOTES


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Control of cytokinesis by β-adrenergic receptors indicates an approach for regulating cardiomyocyte endowment


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Divide and defend
Surgery can correct tetralogy of Fallot, a form of congenital heart disease; however, patients risk cardiac complications and morbidity as adults. Liu et al. found decreased cardiomyocyte cell division in pediatric patients, suggesting that a reduction in endowment (number of cells) could contribute to adult-onset cardiac dysfunction. In mouse models, inactivating the β-adrenergic receptor (β-AR) improved expression of Ect2 and decreased the number of binucleated cardiomyocytes. Propranolol (β-AR blocker) treatment increased cardiomyocyte proliferation in patient cells and in neonatal mice, which led to improved cardiac function and remodeling after myocardial infarction in adult mice. This study suggests that early-life β-blocker treatment could rescue cell division defects, with potential benefit to long-term cardiac health.