Human Erbb2-induced Erk Activity Robustly Stimulates Cycling and Functional Remodeling of Rat and Human Cardiomyocytes

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Abstract

Multiple mitogenic pathways capable of promoting mammalian cardiomyocyte (CM) proliferation have been identified as potential candidates for functional heart repair following myocardial infarction. However, it is unclear whether the effects of these mitogens are species-specific and how they directly compare in the same cardiac setting. Here, we examined how CM-specific lentiviral expression of various candidate mitogens affects human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) and neonatal rat ventricular myocytes (NRVMs) *in vitro*. In 2D-cultured CMs from both species, and in highly mature 3D-engineered cardiac tissues generated from NRVMs, a constitutively-active mutant form of the human gene Erbb2 (caErbb2) was the most potent tested mitogen. Persistent expression of caErbb2 induced CM proliferation, sarcomere loss, and remodeling of tissue structure and function, which were attenuated by small molecule inhibitors of Erk signaling. These results suggest transient activation of Erbb2/Erk axis in cardiomyocytes as a potential strategy for regenerative heart repair.

Introduction

The adult mammalian heart is composed primarily of post-mitotic CMs (Bergmann et al., 2009; Mollova et al., 2013). Due to an apparent lack of a resident stem cell population (van Berlo et al., 2014), the heart is unable to replace lost CMs following myocardial infarction (MI), and instead undergoes fibroblast-mediated scarring, resulting in a decline in cardiac function. One potential approach to regenerate heart tissue following MI is to stimulate the proliferation of survived CMs by a gene therapy targeting cell cycle regulation (Leach & Martin, 2018; Lin et al., 2014; Mohamed et al., 2018; Tzahor & Poss, 2017). While inducing adult CM proliferation is only the first step towards achieving efficient cardiac repair post-MI, it is arguably the most challenging and important one. Which of the proposed candidate genes and pathways would optimally induce human CM proliferation to eventually restore cardiac tissue function remains to be determined.

Several approaches involving increased expression of cell cycle-related proteins and modulation of the Hippo, neuregulin (Nrg1), or Wnt signaling pathways have been shown to generate robust proliferative responses in rodent and zebrafish CMs. Overexpression of cyclin D2 alone or a combination of cyclin-dependent kinase 1 (CDK1), CDK4, cyclin B1, and cyclin D1 promoted cell cycle activation in post-mitotic mouse, rat, and human CMs *in vitro* and in mouse hearts *in vivo* (C. Fan et al., 2019; Mohamed et al., 2018; Zhu, Zhao, Mattapally, Chen, & Zhang, 2017). Hippo pathway modulation via overexpression of constitutively active (ca) Yap variants (incapable of being phosphorylated and degraded) led to robust proliferative responses *in vitro* and *in vivo* through chromatin alterations surrounding proliferation-inducing genes (Byun et al., 2019; Monroe et al., 2019). The mitogen Nrg1 induced CM proliferation in zebrafish through activation of its co-receptor, Erbb2 (Bersell, Arab, Haring, & Kuhn, 2009; Gemberling, Karra, Dickson, & Poss, 2015), and overexpression of caErbb2 promoted CM proliferation in adult mice by inducing their de-differentiation (D'Uva et al., 2015), possibly via Yap activation (Aharonov et al., 2020). Wnt stimulation by small molecule inhibition of GSK3 β (Buikema et

al., 2020; Y. Fan et al., 2018; Richard J. Mills et al., 2019) or induction of β -catenin release from the cell membrane (Y. Fan et al., 2018) were shown to induce proliferation in human CMs *in vitro*. Yet, there have been no studies that directly compared mitogenic capacity of these different pathways in the same CM preparation.

In this report, we sought to directly compare manipulations of different signaling pathways for their ability to induce cell cycle activation and proliferation in CMs from various species and maturation levels, including 2D monolayer cultures of human induced pluripotent stem cell-derived CMs (hiPSC-CMs (I. Y. Shadrin, Khodabukus, A. & Bursac, N, 2016; Zhang et al., 2013)) and neonatal rat ventricular myocytes (NRVMs), as well as functional 3D engineered cardiac tissues ("cardiobundles") made from NRVMs (Helfer & Bursac, 2020; C. P. Jackman, Carlson, & Bursac, 2016). We then probed the mechanisms underlying the observed proliferative responses. We confirmed that Erk signaling is a major contributor to Erbb2-induced cell cycle activation (D'Uva et al., 2015), and found that the human caErbb2 ortholog (caErbb2) induces more potent proliferative effects in hiPSC-CMs and NRVMs than rat caErbb2 (carErbb2) (Aharonov et al., 2020; D'Uva et al., 2015), which was unable to activate Erk signaling in human CMs.

Results

Lentiviral Expression of Mitogens Promotes hiPSC-CM Proliferation without Inducing Apoptosis

Lentiviral vectors (LVs) were designed using the MHCK7 promoter (Salva et al., 2007) to drive muscle-specific expression of mitogens together with an mCherry reporter and applied to transduce hiPSC-CMs and NRVMs (Figure 1 – figure supplement 1). We used a flow cytometry strategy (Figure 1 – figure supplement 2) validated using an established hiPSC-CM mitogen (GSK3 inhibitor, CHIR99021) (Buikema et al., 2020; Richard J. Mills et al., 2019) to determine whether a LV-induced mitogen expression resulted in cell cycle activation or apoptosis specifically in mCherry-labelled CMs. Compared to LV-driven expression of mCherry only, hiPSC-CMs transduced with LVs (Figure 1A) coding for caCtnnb1 (ca β -catenin), Ccnd2 (Cyclin D2), carErbb2, or caErbb2, but not caYap8SA exhibited significantly higher incorporation of 5-ethynyl-2'-deoxyuridine (EdU), indicating greater DNA synthesis (Figure 1B). Increased numbers of CMs expressing phosphorylated histone H3 (H3P), an indicator of the mitotic (M) phase of the cell cycle, were also found with caErbb2 and Ccnd2 transduction (Figure 1C). Furthermore, we observed comparable percentage of diploid and polyploid hiPSC-CMs between control and LV-treated total or EdU⁺ CMs (Figure 1D,E). Collectively, these results showed that hiPSC-CMs with LV-mediated expression of caErbb2 and Ccnd2 were induced to enter the DNA synthesis and mitotic phases of the cell cycle at a higher rate than control hiPSC-CMs, and continued to undergo successful cytokinesis. Additionally, from cleaved caspase-3 (Cc3) analysis, transduced mitogens did not increase CM apoptosis, with caYap8SA having an anti-apoptotic effect (Figure 1F). In contrast, both increased DNA synthesis and mitosis as well as pro-apoptotic effects were found with the application of CHIR99021 (Figure 1 – figure supplement 1). Together, increased EdU incorporation and no change in CM polyploidy or apoptosis upon transduction with caCtnnb1, Ccnd2, carErbb2, or caErbb2 LVs indicated induced CM proliferation.

Lentiviral Expression of Mitogens in hiPSC-CMs Activates Negative Feedback Loops

We then assessed molecular effects of LV transduction and found that caYap8SA-transduced hiPSC-CMs exhibited significantly increased gene expression of *Yap* and its downstream targets *Ctgf* and *Cyr61* (Figure 1G). However, the expression of the total and active, non-phosphorylated Yap protein was significantly decreased (Figure 1H). Immunostaining of transduced hiPSC-CMs further revealed that the nuclear abundance of active Yap was unchanged by caYap8SA expression (Figure 1I,J). Because caYap8SA cannot be phosphorylated and degraded, these studies imply the existence of a

negative feedback loop that degraded endogenous Yap protein to compensate for caYap8SA overexpression. This compensatory mechanism was likely responsible for the lack of mitogenic effects in caYap8SA transduced hiPSC-CMs. We further assessed caCtnnb1-transduced hiPSC-CMs and found that the gene expression of *Ctnnb1* was unaltered, but noted an increased expression of *Lef1* and *Axin2* (Figure 1K). This suggested increased downstream Wnt pathway activity that may have resulted in a negative feedback loop to suppress endogenous and/or exogenous *Ctnnb1* expression (Bernkopf, Hadjihannas, & Behrens, 2015; Lustig et al., 2002). Supporting this hypothesis, we observed no difference in Ctnnb1 protein expression between control and transduced cells (Figure 1L), which suggested that endogenous Ctnnb1 was degraded or repressed while exogenous caCtnnb1 accumulated and entered the nucleus to upregulate downstream Wnt targets such as *Lef1* and *Axin2* and drive CM proliferation. Similar to caYap8SA and caCtnnb1, we also observed increased *Ccnd2* gene but not protein expression in Ccnd2-transduced hiPSC-CMs (Figure 1M,N). Still, immunostaining analysis revealed that Ccnd2-transduced hiPSCs exhibited higher nuclear abundance of the protein (Figure 1O,P), which likely contributed to the observed increase in CM cycling (Zhu et al., 2017). Overall, LV-mediated expressions of various mitogens in hiPSC-CMs appeared to induce negative feedback responses at a post-transcriptional level to limit protein overexpression, which in the case of caYap8SA prevented downstream proliferative effects.

caHesb2 Induces Cell Cycle Entry in NRVM Monolayers Associated with Sarcomere Disassembly

We then sought to determine whether LV-induced expression of the mitogens affected NRVMs, as these cells represent more mature CMs than the relatively immature hiPSC-CMs. NRVMs were transduced with the LVs the day of plating and analyzed by flow cytometry after two weeks of monolayer culture (Figure 2A). We found that LV transduction of caHesb2 but not other mitogens resulted in increased EdU incorporation in NRVMs, while both *Ccnd2* and caHesb2 yielded increased H3P expression (Figure 2B,C). However, all transduced mitogens resulted in significantly decreased fraction of diploid and increased fraction of polyploid NRVMs (Figure 2D), indicating that LV-induced cycling events led to polyploidization rather than cytokinesis, in contrast to the results in hiPSC-CMs (Figure 1D). This inference was supported by the finding that compared to control LV case, the diploid fraction of EdU⁺ NRVMs was significantly decreased and polyploid fraction significantly increased in all LV-treated groups (Figure 2E). Together, these results revealed that *Ccnd2* and caHesb2 promote NRVM cell cycle entry and mitosis, while all tested mitogens induce NRVMs to become binucleated or mononuclear and polyploid, rather than completing cytokinesis. In both NRVMs and hiPSC-CMs, LV expression of caHesb2, but not other mitogens, also induced sarcomere disassembly (Figure 2G). While, expectedly, gene expression of *Hesb2* was upregulated in caHesb2-transduced cells, expression of sarcomeric genes was unaltered (Figure 2F), suggesting that post-transcriptional changes at a protein level were responsible for the loss of sarcomeric organization. Furthermore, increased expression of *Runx1* along with the loss of sarcomers implied that caHesb2 induced CM dedifferentiation in addition to proliferation (D'Uva et al., 2015; Kubin et al., 2011).

caHesb2 Induces Cell Cycle Activation, Growth, and Contractile Deficit in NRVM Cardiobundles

We further tested whether LV delivery of mitogens affected CM proliferation and function in 3-dimensional NRVM cardiobundles, currently representing the most mature *in vitro* model of the postnatal myocardium (C. Jackman, Li, & Bursac, 2018b; C. P. Jackman et al., 2016). NRVMs were transduced at the time of cardiobundle making and assessed after two weeks of culture (Figure 3A), first ensuring that no studied structural or functional properties differed between control LV-transduced and untransduced tissues (Figure 3 – figure supplement 1). From cardiobundle cross-sectional stainings, EdU⁺ nuclei were observed in both vimentin⁺ cardiac fibroblasts (C. Jackman et al., 2018b; C. P. Jackman et al., 2016) predominantly residing at the tissue periphery and in F-actin⁺ vimentin⁻ CMs (Figure 3B, top, middle). Similar to hiPSC-CM and NRVM monolayers, all studied mitogens except

caYap8SA increased EdU incorporation in NRVM cardiobundles, with caHrbb2 showing the strongest effect (Figure 3C). We then assessed morphological and functional characteristics of cardiobundles and found that caHrbb2 transduction uniquely increased both the total cross-sectional area (CSA, Figure 3D) and F-actin⁺ (CM) area of cardiobundles, leading to the formation of a necrotic core (devoid of Hoechst-positive nuclei; Figure 3E), likely caused by limited diffusion of oxygen into the center of the tissue (Suvannapathaki, Wu, Lantigua, Nguyen, & Camci-Unal, 2019). We also observed increased vimentin⁺ CSA, indicating increased fibroblast abundance (Figure 3F). By measuring the contractile force generation in cardiobundles, we found a reduced twitch amplitude in tissues transduced with caHrbb2 but not other mitogens (Figure 3G). This loss of active force generation was accompanied by significant increase in passive tension (Figure 3H), indicating increased tissue stiffness. The caHrbb2-transduced cardiobundles also showed a near-complete loss of sarcomere structure, consistent with the findings in CM monolayers (Figure 2G), while the NRVM alignment was maintained (Figure 3B, bottom). Taken together, among the LV-expressed mitogens, caHrbb2 induced the most potent proliferative effects in both rat and human CMs, which involved the sarcomere loss characteristic of CM dedifferentiation observed in caHrbb2-expressing mice (D'Uva et al., 2015). Furthermore, in engineered NRVM cardiobundles, caHrbb2 proliferative effects were uniquely associated with the increase in tissue size, loss of sarcomeres and contractile force, and tissue stiffening.

Human but not Rat caHrbb2 Activates Erk Signaling in hiPSC-CMs

We were intrigued by the finding that human but not rat caHrbb2 exerted significant pro-proliferative effects in hiPSC-CMs and NRVMs, and have decided to further probe the mechanisms of caHrbb2 action. Previously, expression of rat caHrbb2 in mouse CMs led to sarcomere disassembly *in vitro* and *in vivo*, while downstream Erk and Akt signaling were found to be the primary drivers of caHrbb2-induced CM cell cycle activation (D'Uva et al., 2015). In hiPSC-CMs in our study, caHrbb2 but not caHrbb2 expression resulted in increased pErk and Erk abundance (Figure 4A). Moreover, previously reported pAkt increase was not observed for either rat or human caHrbb2 expression, with caHrbb2 expression reducing total Akt and pAkt (Figure 4A). We then measured the relative gene expression of Erk downstream targets indicative of increased Erk activity (Wagle et al., 2018). Whereas caHrbb2 expression robustly increased transcription of multiple Erk targets, caHrbb2 expression caused no such increase (Figure 4B). Consistent with the caHrbb2-induced Erk activation, we also observed an increase in nuclear localization of Erk protein (Figure 4C), and further confirmed Erk activation by analyzing expression of additional gene targets (Figure 4D). In contrast to Erk, Akt localization was unaffected by caHrbb2 expression (Figure 4E). Interestingly, caHrbb2 but not caHrbb2 also increased the abundance of phosphorylated ribosomal protein S6 (pS6) in Western blots (Figure 4F), which was further confirmed by immunostaining (Figure 4G). pS6 is usually considered as a downstream target of mTOR, which can be activated by stimulation of Akt or Erk pathway (Roux et al., 2007; Warfel, Niederst, & Newton, 2011). Curiously, pmTOR expression was unaffected by caHrbb2 (Figure 4F) and Akt pathway was not upregulated (Figure 4A,B); thus, the human caHrbb2-induced pS6 increase likely resulted from an mTOR-independent, Erk-dependent mechanism previously associated with Ser235/236 phosphorylation of pS6 (Roux et al., 2007).

Erk or Mek Inhibition Attenuates caHrbb2-induced Effects in hiPSC-CMs and NRVMs

To determine whether upregulated Erk signaling was required for the observed effects of caHrbb2 expression on hiPSC-CMs and NRVMs, we performed flow cytometry analysis in cells treated with the Mek inhibitor (Meki) PD0325901 (Mirdametinib) or the Erk inhibitor (Erki) SCH772984 applied for 48 hours before sample collection; EdU was applied during the final 24 hours to capture DNA synthesis that only occurred after adding the inhibitors (Hashimoto et al., 2014). We simultaneously measured Cc3 abundance because excessive Erk inhibition was expected to interfere with homeostatic Erk activity required to promote cell survival (Lu & Xu, 2006). As expected, in both control and caHrbb2-

treated hiPSC-CMs, Meki or Erki treatment resulted in a dose-dependent decrease in EdU incorporation and a dose-dependent increase or an increasing trend in apoptotic events (Figure 5 – figure supplement 1). The increased Erk activity in caErbb2-transduced CMs appeared to both protect against the Erki/Meki-induced apoptosis and necessitate higher inhibitor doses to maximally block EdU incorporation (Figure 5 – figure supplement 1). We then tested whether the inhibition of Erk signaling pathway can prevent caErbb2-induced effects in NRVM cardiobundles by applying 100nM Erki or 100nM Meki between days 8 and 14 of culture (Figure 5A). We found that both inhibitors reduced CM EdU incorporation in control and caErbb2 LV-transduced cardiobundles, but the caErbb2-expressing tissues still showed higher rates of cycling upon inhibition compared to the control (Figure 5B, middle, 5C). Strikingly, the Erk/Mek inhibition also prevented caErbb2-induced changes in tissue morphology, which was evident from the Erki- and Meki-reduced total, F-actin⁺, and vimentin⁺ CSA in caErbb2-transduced but not control cardiobundles (Figure 5B,D,E,F), as well as the appearance of better-organized sarcomeric structure, especially with the Erki treatment (Figure 5B). Furthermore, the inhibitors did not affect force generation in the control cardiobundles, but partially prevented loss in active force generation (Figure 5G) and increase in passive tension (Figure 5H) in caErbb2-transduced but not control tissues. Collectively, these results suggested that the baseline CM cycling in NRVM cardiobundles is Erk-dependent and that caErbb2-induced morphological and functional deficits can be attenuated by Erk or Mek inhibition, further providing the evidence that Erk pathway is a major effector of caErbb2 expression in CMs.

Discussion

Development of methods to induce transient proliferation of endogenous or transplanted CMs could lead to therapeutic strategies to restore lost muscle mass and function of the injured heart (Karra & Poss, 2017). In this *in vitro* study we sought to directly compare a panel of lentivirally-delivered mitogens for the ability to induce the most potent and conserved pro-proliferative effects in CMs from different species, maturation stages, and culture environments. We found that constitutively active human Erbb2 induced a robust and more consistent proliferative response in hiPSC-CMs and NRVMs than rat caErbb2, human caCtnnb1, human Ccnd2, or human caYap8SA. Interestingly, we found apparent negative post-transcriptional feedbacks on expression of mitogen proteins following transduction with caYap8SA, caCtnnb1, or Ccnd2 LVs. This feedback appeared strongest in caYap8SA-transduced rat and human CMs, where we did not find increased EdU incorporation, while simultaneously observing a decrease in both total and active (non-phosphorylated) Yap protein. A negative feedback mechanism in the Hippo pathway mediated by Lats1/2 upregulation was previously reported when constitutively active Yap was expressed or Sav1 was deleted in mice and cultured cells (Kim et al., 2020; Moroishi et al., 2015; Park et al., 2016). Regardless, our findings appear to contradict potent proliferative effects of constitutively active Yap mutants observed in previous studies (R. J. Mills et al., 2017; Monroe et al., 2019; von Gise et al., 2012), which may be attributable to differences in species and mechanical environment of studied CMs (Aragona et al., 2013; Benham-Pyle, Pruitt, & Nelson, 2015; Meng et al., 2018; Panciera et al., 2020; Zhao et al., 2007).

We were surprised to find that only the human but not rat ortholog of caErbb2 was able to activate Erk signaling in hCMs and NRVMs, especially considering the rat Erbb2-V663E and human Erbb2-V659E activating mutations are both located in the transmembrane domain and known to promote homo- and hetero-dimerizations of the receptor (Ou et al., 2017). Previously, rat caErbb2 has been shown to drive CM dedifferentiation, proliferation, and hypertrophy when inducibly expressed in transgenic mice (Aharonov et al., 2020; D'Uva et al., 2015). In our study, the Erk activation downstream of caErbb2 but not caErbb2 expression induced not only CM cycling, but also sarcomere disassembly accompanied by reduced contractile force generation and increased tissue stiffness and fibroblast-occupied area,

which in several aspects resembled pathologic cardiac hypertrophy mediated by hyperactive Erk signaling (Gallo, Vitacolonna, Bonzano, Comoglio, & Crepaldi, 2019). While we noticed increased *Runx1* expression in caErbb2-transduced hiPSC-CMs, we did not observe a gene expression pattern characteristic of sustained CM de-differentiation as *Myh6*, *Myh7*, and *Tnnt2* levels remained unchanged. Although the studied *in vitro* systems lack the complexity of an *in vivo* cardiac milieu (e.g. cellular diversity and related signaling cross-talks), it is possible that the effects of caErbb2 expression in CMs may be species or maturation-dependent. Further studies will be needed to determine whether temporally-controlled overexpression of human caErbb2 or its downstream effectors can generate comparable or more potent therapeutic effects to those of rat caErbb2 in both small and large animal studies.

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Author Contributions

Conceptualization, N.S., S.H., R.G., and N.B.; Methodology, N.S., S.H., R.G., and N.B.; Validation, N.S. and S.D.; Investigation, N.S., S.D., and G.J.C; Data Curation, N.S. and S.D.; Writing – Original Draft, N.S., S.D., and N.B.; Writing – Review & Editing, N.S., S.D., and N.B.; Visualization, N.S. and N.B.; Supervision, N.S. and N.B.; Project Administration, N.B.; Funding Acquisition, N.B.

Declaration of Interests

The authors declare no competing interests.

Lead Contact

Further information and requests for resources and reagents should be directed to the Lead Contact, Dr. Nenad Bursac (nenad.bursac@duke.edu).

Materials Availability

Plasmids generated in this study are available from the corresponding author upon reasonable request.

Data and Code Availability

Source data for figures in the paper will be available upon request.

Figure 1. LV-delivered Mitogens Drive hiPSC-CM Proliferation in Monolayers. (A) Schematic of experimental design in hiPSC-CM monolayers. (B-F) Flow cytometry analysis of mCherry⁺ hiPSC-CMs showing (B) fold-change (FC) in EdU incorporation relative to control LV-transduced hiPSC-CMs, (C) percentage of H3P⁺ CMs, (D,E) percentage of 2N and >2N cells in all CMs (D) and EdU⁺ CMs (E), and (F) percentage of apoptotic Cc3⁺ CMs. (G,H) Analysis of relative (G) gene expression of *Yap* and its targets *Ctgf* and *Cyr61*, and (H) total and active Yap protein abundance in caYap8SA-transduced vs. control hiPSC-CMs. (I,J) Representative immunostaining images (I) and quantified nuclear Mean Fluorescence Intensity (MFI, J) of YAP in caYap8SA-transduced vs. control hiPSC-CMs. (K-L) Analysis of relative (K) expression of *Ctnnb1* and Wnt-signaling genes *Lef1* and *Axin2* and (L) *Ctnnb1* protein abundance in caCtnnb1-transduced vs. control hiPSC-CMs. (M,N) Analysis of relative *Ccnd2* (M) gene and (N) protein expression and (O,P) Representative immunostaining images (O) and quantified nuclear MFI (P) of *Ccnd2* in Ccnd2-transduced vs. control hiPSC-CMs. Data: mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001 vs. Ctrl LV). See Table 1 for sample numbers and complete statistical information for all Figures.

Figure 2. *cahErbb2* Induces NRVM Cycle Entry in Monolayers and Promotes Sarcomere Disassembly in NRVMs and hiPSC-CMs. (A) Schematic of experimental design in NRVM monolayers. (B-E) Flow cytometry analysis of mCherry⁺ NRVMs showing (B) fold-change (FC) in EdU incorporation relative to control LV-treated NRVMs, (C) percentage of H3P⁺ CMs, and (D,E) percentage of 2N and >2N cells in all CMs (D) and EdU⁺ CMs (E). (F) Relative expression of *Erbb2*, sarcomeric genes (*Myh6*, *Myh7*, *Tnnt2*), and dedifferentiation marker *Runx1* in *cahErbb2*-transduced vs. control hiPSC-CMs. (G) Representative immunostaining images of sarcomeric α -actinin (SAA) showing sarcomeric structure in LV transduced NRVMs and hiPSC-CMs. Data: mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Ctrl LV).

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Figure 3. cahErbb2 Induces NRVM Cycle Entry in Cardiobundles and promotes Sarcomere Disassembly and Contractile Dysfunction. (A) Schematic of experimental design in NRVM cardiobundles. (B) Representative immunostaining images of cardiobundle cross-sections showing morphology (top), EdU incorporation (middle), and whole-mount tissue showing sarcomere structure (bottom). (C-F) Quantification of immunostained cardiobundle cross-sections for (C) NRVM EdU incorporation and (D) total, (E) F- actin⁺, and (F) vimentin⁺ cross-sectional area (CSA). (G-H) Force analysis in LV-transduced cardiobundles showing (G) representative twitch traces and quantified maximum twitch amplitude and (H) passive force-length and force amplitude at 12% stretch. Data: mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001 vs. Ctrl LV).

Figure 4. cahErbb2 but not carErbb2 Activates Erk Signaling to Drive Proliferation in CMs. (A-B) Representative Western blots and quantified relative protein (A) and Erk target gene expression (B) in carErbb2- or cahErbb2-transduced vs. control hiPSC-CM monolayers. (C) Representative immunostaining images and quantified nuclear MFI of Erk in cahErbb2-transduced vs. control hiPSC-CMs. (D) Quantified relative Erk target gene expression in cahErbb2-transduced vs. control hiPSC-CM monolayers. (E) Representative immunostaining images and quantified nuclear MFI of Akt in cahErbb2-transduced vs. control hiPSC-CMs. (F) Representative Western blots and quantified relative phosphorylated mTOR (pmTOR) and ribosomal protein S6 (pS6) expression in carErbb2- or cahErbb2-transduced vs. control hiPSC-CMs. (G) Representative immunostaining images and quantified nuclear MFI of pS6 in cahErbb2-transduced vs. control hiPSC-CMs. Data: mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Ctrl LV).

Figure 5. Erk or Mek Inhibition Attenuates *cahErbb2*-induced Effects in NRVM cardiobundles. (A) Schematic of experimental design in *cahErbb2* and Ctrl LV NRVM cardiobundles. (B) Representative immunostaining images of cardiobundle cross-sections showing morphology (top), EdU incorporation (middle) and whole-mount tissue showing sarcomere structure (bottom). (C-F) Quantification of immunostained cardiobundle cross-sections for (C) NRVM EdU incorporation and (D) total, (E) F- actin⁺ (shown in Figure S5B), and (F) vimentin⁺ cross-sectional area (CSA). (G-H) Force analysis in cardiobundles showing (G) representative twitch traces and quantified maximum twitch amplitude and (H) passive force-length and force amplitude at 12% stretch. Data: mean $\pm$ SEM (*p < 0.05, **p < 0.01, ***p < 0.001 vs. Ctrl LV; ###p < 0.01, ####p < 0.001 vs. *cahErbb2*).

Methods

NRVM Isolation and 2D culture

All animal procedures were performed in compliance with the Institutional Animal Care and Use Committee at Duke University and the NIH Guide for the Care and Use of Laboratory Animals. Neonatal rat ventricular myocytes (NRVMs) were isolated as previously described (C. Jackman, Li, & Bursac, 2018a; C. P. Jackman et al., 2016; Li, Asfour, & Bursac, 2017). Briefly, ventricles were harvested from P2 male and female Sprague-Dawley rat pups, minced finely, and pooled before overnight trypsin incubation at 4°C. The following day, the minced ventricular tissue was subjected to several collagenase digestion and filtering steps to yield single cell suspension. Cells were pre-plated for 1 hour to remove non-myocytes and enrich the NRVM population. The non-adherent cells were resuspended in 2D cardiac medium (DMEM, 10% FBS, penicillin (5 U/ml), vitamin B12 (2 µg/ml)) and plated onto fibronectin-coated Aclar® coverslips at a density of 5×10^5 cells per well of a 12-well plate. Twenty-four hours following plating the medium was changed to only include 5% FBS and full media changes were performed every other day.

Cardiobundle Fabrication and 3D Culture

NRVM cardiobundles were prepared as previously described (Helfer & Bursac, 2020; C. Jackman et al., 2018a; C. P. Jackman et al., 2016). Briefly, 6.5×10^5 freshly isolated NRVMs were mixed with a fibrin-based hydrogel (2.5 mg/ml fibrinogen, 1 U/ml thrombin, 10% v/v Matrigel) and cast in PDMS tissue molds with two 2 mm x 7 mm troughs and a porous nylon frame. The molds containing the hydrogel-cell mixture were incubated at 37°C for 45 minutes to allow the hydrogel to fully polymerize and attach to the nylon frame. Tissues were then immersed in 3D cardiac medium (Low Glucose DMEM, 10% horse serum, 1% chick embryo extract, aminocaproic acid (1 mg/ml), ascorbic acid 2-phosphate sesquimagnesium salt hydrate (50 µg/ml), penicillin (5 U/ml), vitamin B12 (2 µg/ml)). The following day, the cardiobundles on frames were carefully removed from the molds and cultured in free-floating dynamic conditions on a rocker. Full media changes of 2 mL per well were performed every other day for 14 days.

hiPSC maintenance and CM differentiation

BJ fibroblasts from a healthy male newborn (ATCC cell line, CRL-2522) were reprogrammed episomally into hiPSCs at the Duke University iPSC Core Facility and named DU11 (Duke University clone #11) following verification of pluripotency as described previously (I. A. Shadrin, BW; Qian Y; Jackman, CP; Carlson, AL; Juhas, ME; Bursac, N, 2017). hiPSCs were maintained as feeder-free cultures on ESC-Matrigel in mTeSR Plus medium and colony-passaged as small (10–20 cells) clusters every 3 days using 0.5 mM EDTA (1:10 split ratio). All hiPSC-CM experiments were performed using DU11 hiPSCs between passages 24 - 45.

hiPSCs were differentiated into CMs via small-molecule-based modulation of Wnt signaling, as previously described (Lian et al., 2012). Briefly, DU11 hiPSCs were dissociated into single cells using Accutase and plated into ESC-Matrigel coated dishes at $4 \times 10^5/\text{cm}^2$ with 5 µM Y27632 (ROCK inhibitor). Maintenance media was changed daily prior to differentiation. To induce cardiac differentiation (day 0), cells were incubated in 6 µM CHIR99021 in RPMI-1640 with B27(-) insulin.

Exactly 48 h later, CHIR was removed and replaced with basal RPMI/B27(−) medium. On day 3, the cells were incubated in RB- containing 5 uM IWR-1, which was switched to basal RPMI/B27(−) medium on day 5. From day 7 onward, cells were cultured in RPMI/B27(+)-insulin with media changed every 2–3 days. Spontaneous contractions of hiPSC-CMs started on day 7–10 of differentiation. Differentiating CM cultures were purified via metabolic selection between days 10 and 14 (Tohyama et al., 2013), by incubation in “no glucose” medium (glucose-free RPMI supplemented with 4 mM lactate, 0.5 mg/mL recombinant human albumin, and 213 µg/mL L-ascorbic acid 2-phosphate) for 48h. At the end of the selection period, cultures were dissociated into single cells using 0.05% trypsin/EDTA followed by quenching with stop buffer (DMEM, 20% FBS, 20 µg/mL DNase I (Millipore 260913)) and replated onto fresh Matrigel-coated dishes to remove dead cells and debris. hiPSC-CM cultures with greater than 90% cTnT positive cells as measured by flow cytometry were used for all experiments.

Cloning of Mitogen Constructs

For generation of mitogen constructs caYap8SA, caCtnnb1, Ccnd2 OE, carErbB2, and cahErbB2, plasmid containing the gene sequence was used as PCR template for amplification of inserts prior to cloning (see Key Resources Table for plasmids). PCR primers were designed to add complementary restriction site overhangs to the gene inserts which were also present in the MHCK7-MCS-P2A-mCherry backbone used for cloning the constructs. Standard restriction cloning was used to insert the gene fragments. Sanger sequencing was performed to ensure maintenance of reading frame and correct sequence.

Preparation of Lentivirus Vectors

Lentiviral vectors were prepared as previously described (Rao, 2018). Briefly, Hek293T cells were cultured in high glucose DMEM containing 10% FBS and 1% Penicillin/Streptomycin. Plasmids (Construct plasmid, Pax2, and VSVG) were purified using midiprep before transfection into Hek293T cells at 65–75% confluence using Jetprime transfection reagent. Medium was changed 16 hours following transfection, and medium containing virus was harvested 3–4 days following initial transfection. Virus was purified by precipitation using 3 volumes medium to 1 volume LentiX-Concentrator at 4°C overnight, then pelleted by centrifugation at 1500xg for 45 minutes at 4°C. Precipitated virus was aliquoted and stored at -80°C before use.

For experiments investigating the effects of the LVs on hiPSC-CM monolayers, cells were transduced with the LVs 17–20 days following the initiation of differentiation and were maintained for one week to allow the LVs to reach maximal expression before terminal analysis. For NRVM monolayer experiments, viral suspension was added at the time of cell plating. For NRVM cardiobundle experiments, lentiviral vectors were added to the hydrogel-cell mixture at the time of cardiobundle fabrication to yield transduction efficiency between 45–80%.

Flow Cytometry

NRVM or hiPSC-CM monolayers were rinsed with PBS then dissociated using 0.05% Trypsin-EDTA at 37°C for 3 minutes, upon which monolayers were triturated several times to yield a single cell

suspension. Trypsin was quenched with DMEM/F12 containing 20% FBS and 20µg/mL DNase I. The cell suspension was centrifuged at 300xg for 5 minutes, then resuspended in 4% PFA diluted in PBS. Cells were incubated in PFA for 10 minutes at RT, centrifuged again, then resuspended in PBS containing 5% FBS for storage at 4°C.

Cells were stained for flow cytometry after centrifugation at 300xg for 5 minutes. If EdU staining was performed, cells were incubated with the EdU flow cytometry staining cocktail as per manufacturer protocol (ThermoFisher) and incubated in the dark for 30 minutes, then washed 2X by addition of PBS followed by centrifugation. Antibody staining was performed after EdU staining. For antibody staining, cells were resuspended in FACS buffer (PBS with 0.5% BSA, 0.1% Triton-X 100, 0.02% Azide). Primary antibodies including an isotype control were diluted in FACS buffer and incubated for 1 hour on ice. Cells were washed 2X with FACS buffer before addition of secondary antibodies and Hoechst diluted in FACS buffer. Secondary antibodies were incubated for 30 minutes at RT. Samples were run on a BD Fortessa X-20.

Immunostaining and Imaging

Cell monolayers were fixed with 4% v/v PFA at room temperature for 15 minutes, then blocked in antibody buffer (5 w/v donkey serum, 0.1% v/v Triton X-100, in PBS) for two hours at room temperature and incubated with primary antibodies for 2 hours in antibody buffer. Primary antibody dilutions are indicated in the Key Resources Table. The monolayers were washed with PBS before incubation with Alexa Fluor-conjugated secondary antibodies at 1:1000 and Hoechst at 1:200 in antibody buffer for 1 hour. Monolayer samples were mounted using Fluoromount-G™ Mounting Medium and imaged using an Andor Dragonfly spinning disk confocal microscope.

Engineered cardiobundles were fixed with 2% v/v PFA on a rocking platform at 4°C overnight. For cross-sectional analysis, the fixed tissues were suspended in OCT and flash frozen in liquid nitrogen until solidified. The frozen tissue blocks were sectioned using a cryostat (Leica) into 10µm sections. Cardiobundle cross-sections were blocked in antibody buffer for two hours at room temperature. Whole bundles for longitudinal images were blocked overnight at 4°C. All samples were incubated with primary antibodies 4°C overnight in antibody buffer. Primary antibodies were used at the indicated dilutions in the Key Resources Table. Samples were incubated with Alexa Fluor-conjugated secondary antibodies at 1:1000 and Hoechst 1:200 in antibody buffer for 2.5 hours at room temperature for cross-sections and overnight at 4°C for whole bundles. Cross-sections and un-sectioned whole bundles were mounted with hard-set mounting medium (Antifade Glass) and imaged using an Andor Dragonfly spinning disk confocal microscope.

qPCR

RNA was extracted using RNeasy Plus Mini Kit according to the manufacturer's instructions (Qiagen). Total RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad). Standard qPCR reactions with 5 ng cDNA per reaction were performed with iTaq Universal SYBR Green Supermix (Bio-Rad) in the CFX Connect Real-Time PCR Detection System. All primers used are listed in Antibody Table in Key Resources Table.

Contractile Function

After 14 days of culture, cardiobundle force generation was measured using custom-made force measurement setup consisting of an optical force transducer and linear actuator as previously described (C. P. Jackman et al., 2016). In 37 °C Tyrode's solution, the cardiobundle was pinned to chamber at one end and a PDMS float connected to a linear actuator controlled by Labview software at the other end. Using platinum electrodes, a 90V electrical pulse was applied for 5 ms at 2 Hz rate to induce contraction. The force measurements were performed at the ends of 4% stretch steps lasting 45 seconds, until 16% stretch was reached. Maximum twitch amplitude and passive force-length curves were calculated as previously described using custom Matlab software (Madden, Juhas, Kraus, Truskey, & Bursac, 2015).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed with GraphPad Prism software. Statistical details can be found in Supplemental Table 1. Outliers were identified and removed using GraphPad Prism 8.3.0 ROUT method, Q=1%, normality testing was done using the Shapiro-Wilk test. All experiments were carried out in multiple cell batches.

Image Analysis

Image analysis was performed using custom CellProfiler (Carpenter et al., 2006) and FIJI (Schindelin et al., 2012) macros. Briefly, CellProfiler was used to determine nuclear number as well as vimentin⁺ and EdU⁺ cells. To quantify nuclear number and EdU⁺ nuclei, the identify primary objects function was used with global minimum cross entropy thresholding. To quantify vimentin⁺ cells, vimentin signal was smoothed using Gaussian filter, thresholded using the minimum cross entropy function, holes were removed using the remove holes function, and the watershed function was applied for segmentation. Colocalization analysis using the relate objects function between vimentin signal and nuclei as well as EdU signal and nuclei was performed to exclude proliferative fibroblasts from cardiomyocyte EdU quantification. FIJI was used to determine cardiobundle F-actin⁺ area and total cross-sectional area (CSA). For measuring F-actin⁺ CSA, the Huang method for auto-thresholding the F-actin signal intensity was used (Huang, 1995). For measuring total CSA, the Huang method for auto-thresholding vimentin signal intensity was used, followed by the Fill Holes function.

Materials

Reagent	SOURCE	IDENTIFIER
Antibodies		
Anti-Cardiac Troponin T antibody	Abcam	ab45932

Anti-Sarcomeric Alpha Actinin antibody	Abcam	ab137346
Cleaved Caspase-3 (Asp175) Antibody	Cell Signaling Technology	9661
GAPDH Antibody	Santa Cruz Biotechnology	sc-47724
HER2/ErbB2 (29D8) Rabbit mAb	Cell Signaling Technology	2165
Mouse IgG1, kappa monoclonal [15-6E10A7] - Isotype Control	Abcam	ab170190
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody	Cell Signaling Technology	9101
p44/42 MAPK (Erk1/2) Antibody (Total Erk)	Cell Signaling Technology	9102
Phospho-Histone H3 (Ser10) (6G3) Mouse mAb	Cell Signaling Technology	9706
Phospho-mTOR (Ser2448) Antibody	Cell Signaling Technology	2971
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody	Cell Signaling Technology	9101
Phospho-S6 Ribosomal Protein (Ser235/236) Antibody	Cell Signaling Technology	2211
Rabbit IgG, polyclonal - Isotype Control	Abcam	ab37415
Recombinant Anti-active YAP1 antibody [EPR19812]	Abcam	ab205270
Recombinant Anti-AKT1 (phospho S473) antibody [EP2109Y]	Abcam	ab81283
Recombinant Anti-AKT1 + AKT2 + AKT3 antibody [EPR16798]	Abcam	ab179463
Recombinant Anti-Cyclin D2 antibody	Abcam	ab207604
Recombinant Anti-Vimentin antibody [EPR3776] - Cytoskeleton Marker	Abcam	ab92547
YAP (D8H1X) XP® Rabbit mAb (Total Yap)	Cell Signaling Technology	14074
β-Catenin Antibody (Carboxy-terminal Antigen)	Cell Signaling Technology	9587
Biological Samples		
Horse Serum (for 3D medium)		
C3999 Chick Embryo Extract, Ultrafiltrate, Liquid (CEE)	US Biological	C3999

Bovine fibrinogen	Sigma	F8630-1G
Thrombin from bovine plasma	Sigma	T6634-1KU
Chemicals, Peptides, and Recombinant Proteins		
ICG-001	Cayman Chemical	16257
SCH772984	Cayman Chemical	19166
PD0325901	Sigma	PZ0162-5MG
Critical Commercial Assays		
Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit	Thermo Fisher	C10419
Click-iT® EdU Alexa Fluor® 488 Imaging Kit	Thermo Fisher	C10337
Experimental Models: Cell Lines		
DU11 iPSC Line	Duke University Stem Cell Core	
Hek293T	ATCC	
Experimental Models: Organisms/Strains		
P2 Sprague-Dawley Rat Pups	Charles River	
Oligonucleotides		
See Supplementary Table 1 for qPCR primers	This paper (or published)	
Recombinant DNA		
pLV-beta-catenin deltaN90	pLV-beta-catenin deltaN90 was a gift from Bob Weinberg	Addgene plasmid # 36985
HER2 CA (V659E)	HER2 CA (V659E) was a gift from Mien-Chie Hung	Addgene plasmid # 16259
R777-E020 Hs.CCND2-nostop	R777-E020 Hs.CCND2-nostop was a gift from Dominic Esposito	Addgene plasmid # 70304
pSV2 neuNT	pSV2 neuNT was a gift from Bob Weinberg	Addgene plasmid # 10918
pCMV-flag YAP2 5SA	pCMV-flag YAP2 5SA was a gift from Kunliang Guan	Addgene plasmid # 27371
Pax2	psPAX2 was a gift from Didier Trono	Addgene plasmid # 12260
VSVG	pMD2.G was a gift from Didier Trono	Addgene plasmid # 12259
Control Lentivirus (MHCK7-mCherry)	This manuscript	
Cyclin Lentivirus (MHCK7-Ccnd2-P2A-mCherry-NLS)	This manuscript	
B-catenin Lentivirus (MHCK7-Ctnnb1(Δ90)-P2A-mCherry-NLS)	This manuscript	

Human Erbb2 Lentivirus (MHCK7-Erbb2(V659E-P2A-mCherry))	This manuscript	
Rat Erbb2 Lentivirus (MHCK7-Erbb2(V663E-P2A-mCherry-NLS))	This manuscript	
Yap Lentivirus (MHCK7-Yap8SA-P2A-mCherry-NLS)	This manuscript	
Software and Algorithms		
CellProfiler	Mcquin et al., 2018	www.cellprofiler.org
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
FlowJo™ Software	Becton, Dickinson and Company; 2019	
GraphPad Prism		www.graphpad.com
Matlab		

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qPCR Primers

Oligonucleotides		
Yap1	TAGCCCTGCGTAGCCAGTTA TCATGCTTAGTCCACTGTCTGT	PrimerBank ID: 303523510c1
Myh6		Shadrin
Myh7		Shadrin
Ctgf	CAGCATGGACGTTTCGTCTG AACCACGGTTTGGTCCTTGG	PrimerBank ID: 98986335c1
Cyr61	CAGCATGGACGTTTCGTCTG AACCACGGTTTGGTCCTTGG	PrimerBank ID: 197313774c3
Ctnnb1	CATCTACACAGTTTGATGCTGCT GCAGTTTTGTCAGTTCAGGGA	PrimerBank ID: 148233337c2
Lef1	AGAACACCCCGATGACGGA GGCATCATTATGTACCCGGAAT	PrimerBank ID: 260656055c1
Axin2	CAACACCAGGCGGAACGAA GCCCAATAAGGAGTGTAAGGACT	PrimerBank ID: 195927058c1
Ccnd2	TTTGCCATGTACCCACCGTC AGGGCATCACAAGTGAGCG	PrimerBank ID: 209969683c3
Erbb2	TGTGACTGCCTGTCCCTACAA CCAGACCATAGCACACTCGG	PrimerBank ID: 54792097c2
Epha2	AGAGGCTGAGCGTATCTTCAT GGTCCGACTCGGCATAGTAGA	PrimerBank ID: 296010835c2
Epha4	GCAAGGAGACGTTTAACCTGT CTTGGGTGAAGCTCTCATCAG	PrimerBank ID: 45439363c3
Fn1	AGGAAGCCGAGGTTTTAACTG AGGACGCTCATAAGTGTCACC	PrimerBank ID: 47132556c2
Dusp5	GCCAGCTTATGACCAGGGTG GTCCGTGCGGAGACATTCAG	PrimerBank ID: 62865889c2
Dusp6	GAAATGGCGATCAGCAAGACG CGACGACTCGTATAGCTCCTG	PrimerBank ID: 42764682c1

Spry4	TCTGACCAACGGCTCTTAGAC GTGCCATAGTTGACCAGAGTC	PrimerBank ID: 188595696c1
Etv5	TCAGCAAGTCCCTTTTATGGTC GCTCTTCAGAATCGTGAGCCA	PrimerBank ID: 194018465c1
Phlda1	GAAGATGGCCCATTCAAAAGCG GAGGAGGCTAACACGCAGG	PrimerBank ID: 83977458c1
Spry2	CCTACTGTCGTCCCAAGACCT GGGGCTCGTGCAAGAAGT	PrimerBank ID: 22209007c1
Dusp4	GGCGGCTATGAGAGGTTTTCC TGGTCGTGTAGTGGGGTCC	PrimerBank ID: 325651887c1
Etv4	CAGTGCCTTTACTCCAGTGCC CTCAGGAAATTCCGTTGCTCT	PrimerBank ID: 118918427c2
Fos	GGGGCAAGGTGGAACAGTTAT CCGCTTGAGTGTATCAGTCA	PrimerBank ID: 254750707c2
Dab2	GTAGAAACAAGTGCAACCAATGG GCCTTTGAACCTTGCTAAGAGA	PrimerBank ID: 349585059c1
Pdgfa	GCAAGACCAGGACGGTCATTT GGCACTTGACACTGCTCGT	PrimerBank ID: 197333758c1
Runx1	CTGCCCATCGCTTTCAAGGT GCCGAGTAGTTTTCATCATTGCC	PrimerBank ID: 169790826c1
Hprt1	CCTGGCGTCGTGATTAGTG AGACGTTTCAGTCCTGTCCATAA	PrimerBank ID: 164518913c1

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Antibody Dilutions

Immunofluorescence:

Target	Dilution
SAA	1:200
Phalloidin	1:200
Ccnd2	1:100
Yap	1:200
Active Yap	1:500
Vimentin	1:500
Akt	1:100
Erk	1:200
pS6	1:100
Hoechst	1:100

Western Blot:

Target	Dilution
Yap	1:1000
Active Yap	1:1000
GAPDH	1:1000
B-catenin	1:1000
Cyclin D2	1:1000
Akt	1:10000
pAkt	1:5000
Erk	1:1000
pErk	1:1000
pmTOR	1:1000
pS6	1:1000

Flow Cytometry:

Target	Dilution
cTnT	1:200
H3P	1:1000
Cc3	1:800
Rabbit Isotype	1:2000
Mouse Isotype	1:2000
Hoechst	1:200

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