Dedifferentiation, Proliferation, and Redifferentiation of Adult Mammalian Cardiomyocytes After Ischemic Injury

BACKGROUND: Adult mammalian hearts have a limited ability to generate new cardiomyocytes. Proliferation of existing adult cardiomyocytes (ACMs) is a potential source of new cardiomyocytes. Understanding the fundamental biology of ACM proliferation could be of great clinical significance for treating myocardial infarction (MI). We aim to understand the process and regulation of ACM proliferation and its role in new cardiomyocyte formation of post-MI mouse hearts.

METHODS: β-Actin-green fluorescent protein transgenic mice and fate-mapping Myh6-MerCreMer-tdTomato/lacZ mice were used to trace the fate of ACMs. In a coculture system with neonatal rat ventricular myocytes, ACM proliferation was documented with clear evidence of cytokinesis observed with time-lapse imaging. Cardiomyocyte proliferation in the adult mouse post-MI heart was detected by cell cycle markers and 5-ethynyl-2-deoxyuridine incorporation analysis. Echocardiography was used to measure cardiac function, and histology was performed to determine infarction size.

RESULTS: In vitro, mononucleated and bi/multinucleated ACMs were able to proliferate at a similar rate (7.0%) in the coculture. Dedifferentiation proceeded ACM proliferation, which was followed by redifferentiation. Redifferentiation was essential to endow the daughter cells with cardiomyocyte contractile function. Intercellular propagation of Ca^{2+} from contracting neonatal rat ventricular myocytes into ACM daughter cells was required to activate the Ca^{2+}-dependent calcineurin-nuclear factor of activated T-cell signaling pathway to induce ACM redifferentiation. The properties of neonatal rat ventricular myocyte Ca^{2+} transients influenced the rate of ACM redifferentiation. Hypoxia impaired the function of gap junctions by dephosphorylating its component protein connexin 43, the major mediator of intercellular Ca^{2+} propagation between cardiomyocytes, thereby impairing ACM redifferentiation. In vivo, ACM proliferation was found primarily in the MI border zone. An ischemia-resistant connexin 43 mutant enhanced the redifferentiation of ACM-derived new cardiomyocytes after MI and improved cardiac function.

CONCLUSIONS: Mature ACMs can reenter the cell cycle and form new cardiomyocytes through a 3-step process: dedifferentiation, proliferation, and redifferentiation. Intercellular Ca^{2+} signal from neighboring functioning cardiomyocytes through gap junctions induces the redifferentiation process. This novel mechanism contributes to new cardiomyocyte formation in post-MI hearts in mammals.

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Clinical Perspective

What Is New?

- Terminally differentiated adult cardiomyocytes (ACMs) in coculture with neonatal rat ventricular myocytes can reenter the cell cycle and form new cardiomyocytes through a 3-step process: dedifferentiation, proliferation, and redifferentiation.
- ACMs of all sizes and nucleation are capable of proliferating and forming committed progeny.
- Dedifferentiated ACMs require electric connections with neonatal rat ventricular myocytes and gain Ca^{2+} signals from neonatal rat ventricular myocytes to redifferentiate into functional myocytes with sarcomeres.
- Enhancing electric coupling between ACMs in the border zone of a post–myocardial infarction heart increases the number of newly formed cardiomyocytes and enhances cardiac function.

What Are the Clinical Implications?

- Increasing the generation of new cardiomyocytes in the myocardial infarction border zone could reduce infarct size and improve post–myocardial infarction cardiac remodeling.
- New cardiomyocytes can be generated in the adult heart from preexisting ACMs.
- Understanding the fundamental aspects of this process could lead to new strategies to repair the injured heart.

Acute myocardial infarction (MI) is a leading cause of morbidity and mortality throughout the world. MI causes the rapid death of cardiomyocytes with subsequent myocardial remodeling, eventually leading to heart failure. Current clinical therapies are unable to reverse the fundamental problem of reduced cardiomyocyte number.[1] Approaches that could safely increase the number of adult cardiomyocytes (ACMs) in the post-MI heart would treat the actual cause of cardiac dysfunction.

Many studies have demonstrated that a very small number of new cardiomyocytes are generated in the normal adult hearts of animals and humans,[1,2] and that a few additional new myocytes are generated after cardiac injury.[3] The source of these new myocytes has not been clearly defined, but accumulating evidence suggests that proliferation of preexisting cardiomyocytes is largely responsible for endogenous cardiac regeneration.[3,4] Therefore, understanding the biology of ACM proliferation could be of great clinical significance for treating MI, heart failure, and other cardiac diseases in which reduced cardiomyocyte number is the principle reason for deranged cardiac function.

The mechanism by which ACMs proliferate in mammals remains poorly understood. What is clear is that most, if not all, ACMs have withdrawn from the cell cycle soon after birth.[5,6] Regeneration could be achieved by these cells reentering the cell cycle or from a small population of ACMs that have not permanently withdrawn from the cell cycle. We[7] and others[6,8–11] have indicated that small mononucleated ACMs have greater potential to proliferate than bi/multinucleated and large-sized ACMs. However, because of the limitation of the conventional methodology, these studies did not directly examine cardiomyocyte proliferation with cytokinesis. These studies mostly observed the presence of markers for DNA synthesis and cell cycle activation, which could be complicated by DNA repair, polyploidy, and multinucleation in ACMs. Lineage tracing and time-lapse imaging were used in the present study to document ACM proliferation with complete cytokinesis and to explore its biological features.

In lower vertebrate species such as the zebrafish, ACMs proliferate through a process involving dedifferentiation followed by proliferation and subsequent redifferentiation.[12] ACM dedifferentiation is characterized by the disassembly of sarcomeric structure, extrusion of mitochondria, electric uncoupling, and expression of regulators of cell cycle progression.[12,13] Redifferentiation is characterized by the restoration of cell morphology, sarcomeric reorganization, and contractile function.[14] It has been postulated that increased myocyte proliferation is linked to increased myocyte dedifferentiation.[15] However, there is little direct evidence for ACM dedifferentiation, proliferation, and redifferentiation (DPR) in mammalian hearts, and the putative role of these processes in cardiac regeneration is unclear.

In the present study, we developed an in vitro model system to observe ACM-DPR with time-lapse imaging. Genetically labeled ACMs (to reliably track their fate) were isolated from adult mouse hearts and cultured with neonatal rat ventricular myocytes (NRVMs). ACMs dedifferentiated, proliferated, and then connexin 43 (Cx43)–mediated coupling between isolated ACMs and NRVMs was found to be required for ACM redifferentiation. Our data suggest that Ca^{2+} signals that propagate from NRVMs into electrically coupled and dedifferentiated ACMs influence ACM redifferentiation. To determine the in vivo relevance of these mechanisms we used fate-mapping mice with ACM genetic labeling to study ACM-DPR after MI. These results support the hypothesis that ACM-DPR is an importance source of new ACMs after MI and suggest that enhancing Cx43-mediated cell-cell coupling after MI could increase new ACM formation and improve cardiac pump function.

METHODS

Genetic β-actin-green fluorescent protein (GFP) and fate-mapping Myh6-MerCreMer-lacZ or Myh6-MerCreMer-tdTomato mice (4 months of age) were used in the present study. Animal care and all experimental procedures were performed in strict accordance with the approved protocols and animal welfare regulations of the Animal Care and Use Committee at Third Military Medical University. An in vitro coculture system was developed to mimic the in vivo ischemia environment in which ACM undergoes DPR in the rodent heart. ACMs were cocultured onto NRVMs at a ratio of 1:20 and cultured for up to 7 days. A long-term time-lapse imaging analysis was performed to capture ACM division events in the coculture system with an Olympus IX83 inverted microscope. DRAQ5 Fluorescent Probe was applied to label ACM nuclei. The time-lapse images were taken at intervals of 1 hour for 7 days. Calcium transients of NRVMs and redifferentiated ACMs were measured using a protocol similar to our previous studies.[16] Permanent ligation of the left anterior descending coronary artery was performed in these mice to induce MI injury.[17] Immunostaining, echocardiography, and infarct size analysis were performed as previously reported.[18] Mouse Cx43 mutant with serines 325/328/330 replaced by phosphomimetic glutamic acids (S3E) or by nonphosphorylatable alanines were a gift from Dr Glenn I. Fishman at New York University School of Medicine.[19] Cx43-S3E were resistant to pathological gap junction remodeling induced by cardiac ischemia.[19] Adeno-associated virus serotype 9 (AAV9)-Cx43-S3E was administrated to treat MI heart.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.0 for Windows (GraphPad Software). All values were normally distributed (Kolmogorov-Smirnov test, $P>0.10$ for each data set). The Student *t* test was performed for comparisons of 2 groups. One-way or 2-way ANOVA (with or without repeated measures) followed by Bonferroni correction was performed for multiple group comparisons. Data are given as mean±SEM, and a P value of <0.05 was considered significant.

RESULTS

ACM DPR In Vitro

Isolated mouse ACMs are subject to $[Ca^{2+}]$ overload–induced cell death in primary culture with media containing normal $[Ca^{2+}]$. Their high $[Na^+]_i$ favors persistent Ca^{2+} entry via the Na^+/Ca^{2+} exchanger (NCX) and this Ca^{2+} entry leads to sarcoplasmic reticulum, and eventually mitochondrial Ca^{2+} overload, causing cell death.[16] In the present study we developed an in vitro system in which mouse ACMs are cocultured with NRVMs to promote ACM survival for long time periods without using drug treatments to reduce cell Ca^{2+} (such as 2,3-butanedione monoximine). Cytosolic Ca^{2+} overload is one of the key mechanisms of ACM death during ischemia and reperfusion.[20] Thus, this coculture system, containing Ca^{2+}-overloaded mouse ACMs together with electrically coupled spontaneously beating NRVMs, in some ways, simulates the MI border zone where there are ischemic ACMs that have uncoupled from the surviving myocytes and are at high risk of necrotic and apoptotic cell death. Because the MI border zone is the region where newly formed cardiomyocytes have been most frequently observed,[21] our hypothesis is that ACMs that uncouple from their neighbors might be a source of new cardiomyocytes in the post-MI heart.

ACMs from 4-month-old β-actin-GFP transgenic mice were cocultured with NRVMs at a ratio of 1:20. GFP^+/trypan $blue^-$ cells were counted as live cells. When cultured alone, mouse ACMs hypercontracted and >84% were trypan $blue^+$ within 3 days. Less than 0.1% of ACMs survived >7 days under these conditions.[22,23] In contrast, nearly 50% of mouse ACMs in coculture with NRVMs were alive through day 7 (online-only Data Supplement Figure I).

We investigated the properties of surviving ACMs during the first week in coculture. After 3 days almost all (98.5%) ACMs lost organized sarcomeric structure, and, among these surviving ACMs, 8.7% did not have cardiac troponin I (cTnI) present, suggesting a greater extent of dedifferentiation (Figure 1A). During this time, some ACMs were immunopositive for the dedifferentiation markers Runx1 and Dab2. The presence of these markers was observed as early as day 1 in coculture, reached a peak on day 3 (54.6±2.3% of $Runx1^+$ ACMs and 33.4±4.0% of $Dab2^+$ ACMs) and decreased thereafter (Figure 1A). Collectively these results support the idea that some ACMs actively dedifferentiate in coculture with NRVMs.

Some ACMs in coculture were immunopositive for proliferation markers Ki67 and PH3, indicating those cells had reentered the cell cycle (Figure 1B). The expression of these cell cycle indicators has also been recognized as a feature associated with ACM dedifferentiation.[12] The expression of Ki67 and PH3 were not detected in the freshly isolated ACMs (day 0), but first seen on day 3 in the coculture, reached a peak by day 5 (2.25±0.23% and 0.85±0.11%, respectively) and decreased thereafter (Figure 1B).

Dedifferentiated ACMs that had reentered the cell cycle were mechanically quiescent, without organized contractile proteins. However, with time, these ACMs appeared to develop an organized contractile apparatus and were contractile (began at day 3). As shown in Figure 1C, the percentages of $sarcomere^+$ ACMs and beating ACMs were significant on day 5, and reached a peak on day 7 (45.9±7.7% with organized sarcomeres and 30.7±4.3% were beating). These data support the hypothesis that ACMs DPR in this in vitro coculture system.

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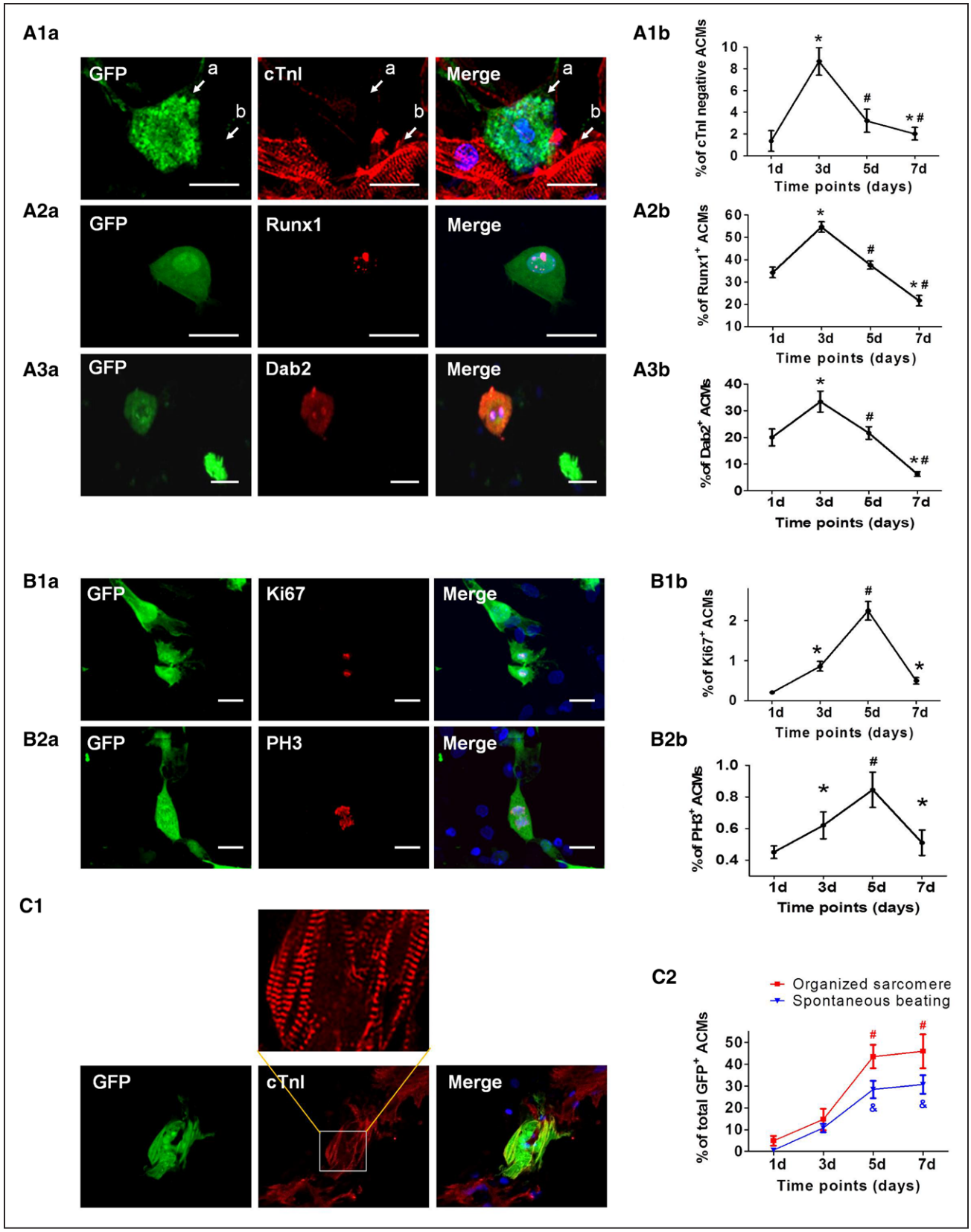

Figure 1. ACM dedifferentiation, proliferation, and redifferentiation in vitro.
Freshly isolated ACMs from β-actin-GFP mice were cocultured with NRVMs for 7 days, and the ACMs remodeled over time. **A,** Identification of ACM dedifferentiation. **A1,** Representative images (**A1a**) and quantification (**A1b**) of ACMs that lost the contractile protein cardiac troponin I at day 3 after coculture. Arrow a indicates an ACM without cardiac troponin I (*Continued*)

Terminally Differentiated Mouse ACMs Can Proliferate and Generate New Cardiomyocytes In Vitro

The results above were obtained in different ACM/NRVM coculture plates that were fixed and studied at specific time points after coculture. We next used time-lapse video microscopy to more definitively show the DPR process in individual ACMs. Mouse ACMs in coculture transformed from their normal rod shape to a spherical, hypercontracted form within 1 day. Over the next few days these cells dismantled their contractile apparatus (dedifferentiation) and assumed a flattened cell shape (Figure 2A). Proliferation was clearly documented with ACM cytokinesis into multiple progeny (online-only Data Supplement Movies I through IV). Only events with completed cytokinesis were counted as ACM proliferation, and a live cell nucleus labeling dye was used to label the nuclear number of ACMs. These studies showed that 7.0% of ACMs (of the 863 ACMs that were followed) proliferated (Figure 2B). Meanwhile, we repeated the time-lapse imaging experiments with ACMs isolated from fate-mapping transgenic Myh6-MerCreMer-tdTomato mice, with a red fluorescent reporter driven by a cardiomyocyte-specific promoter. The proliferation events with complete cytokinesis of these ACMs were also documented in coculture with NRVMs (online-only Data Supplement Figure IIA and IIB and online-only Data Supplement Movie V). The proliferation rate was similar to that of ACMs isolated from β-actin-GFP mice. These results unambiguously demonstrate that ACMs can proliferate in coculture with NRVMs.

Previous studies suggested that proliferating mammalian cardiomyocytes were predominantly mononucleated,[6–11] and only mononucleated cardiomyocytes could complete cytokinesis.[10] However, in our system, both mononucleated and bi/multinucleated ACMs were shown to proliferate with completed cytokinesis. Mononucleated ACMs had a tendency for higher proliferation rate than bi/multinucleated ACMs, but the difference was not significant (Figure 2C). Moreover, the average original cell size of the proliferated ACMs was comparable with other ACMs (Figure 2D**).** It is known that the majority of ACMs are bi/multinucleated in adult mouse heart (mononucleated, 20.1%; binucleated, 70.9%; and multinucleated, 9.0% in our system). The majority of the proliferated ACMs were bi/multinucleated (mononucleated, 26.3%; binucleated, 65.8%; and multinucleated, 7.9%) (Figure 2E). Multiple patterns of ACM proliferation were observed. Binucleated ACMs could divide into 2 mononucleated cells, 3 mononucleated cells, or 1 mononucleated cell and 1 bi/multinucleated cell (Figure 2F). Collectively, these findings indicate that terminally differentiated ACMs are able to reenter the cell cycle and proliferate in certain conditions, and the ACM proliferation can take place in mono- and binucleated myocytes.

On completion of time-lapse studies, ie, 7 days after coculture, the preparations were fixed and immunostained for cTnI to determine whether the ACM progeny had organized sarcomeric structures. Among the ACM-derived daughter cells, 22.7% were cTnI positive with organized sarcomere, an additional 15.9% were cTnI positive but without organized sarcomeres, and 61.4% were cTnI negative (Figure 2G and online-only Data Supplement Movies II through IV). These results indicate that dedifferentiated ACMs can proliferate, and some but not all of the progeny form new functional cardiomyocytes. The fact that after ACM proliferation most of the daughter cells were negative for cTnI suggests that redifferentiation is necessary for these cells to achieve functional regeneration.

To exclude cell fusion events, ACMs isolated from β-actin-GFP mice were cocultured with NRVMs infected with Adeno-RFP (online-only Data Supplement Figure IIIA). Approximately 8500 GFP^+ cells were counted and no GFP^+/RFP^+ cells were found during the 7-day coculture period, excluding the possibility that fusion of ACMs and NRVMs explains our results. To confirm these results, ACMs isolated from Myh6-MHC-MerCreMer-tdTomato mice were mixed with cardiac nonmyocytes isolated from β-actin-GFP mice, which were then cocultured with NRVMs (online-only Data Supplement Figure IIIB). More than 15 000 $tdTomato^+$ cells from 3 mice were counted, and 4 $GFP^+/tdTomato^+$ ACMs were found, indicating a fusion rate of <0.03% between mouse ACMs and nonmyocytes. This level is consistent with the previous in vivo study with Rosa26-mTmG mice, which reported cell fusion rates <0.005%.[24,25] Collectively, these results show that cell fusion does not explain ACM proliferation and the ACM-DPR process.

Figure 1 Continued. expression, whereas arrow b indicates a neighboring NRVM. **A2,** Representative images (**A2a**) and quantification (**A2b**) of ACMs expressing dedifferentiation marker Runx1. **A3,** Representative images (**A3a**) and quantification (**A3b**) of ACMs expressing dedifferentiation marker Dab2. **B,** Identification of ACM proliferation. **B1,** Representative images (**B1a**) and quantification (**B1b**) of ACMs expressing proliferation marker Ki67. **B2,** Representative images (**B2a**) and quantification (**B2b**) of ACMs expressing proliferation marker PH3. **C,** Identification of ACM redifferentiation. **C1**, Representative images of an ACM regained organized sarcomere at day 7 after coculture. **C2,** Quantification of ACMs with organized sarcomeres or spontaneous beating out of total survived ACMs. n=12. **P*<0.05 versus ACMs at day 1. #*P*<0.05 versus ACMs at day 3and *P*<0.05 versus % of spontaneous beating at day 3. Scale bars=20 μm. ACM indicates adult cardiomyocyte; cTnI, cardiac troponin I; GFP, green fluorescent protein; and NRVM, neonatal rat ventricular myocyte.

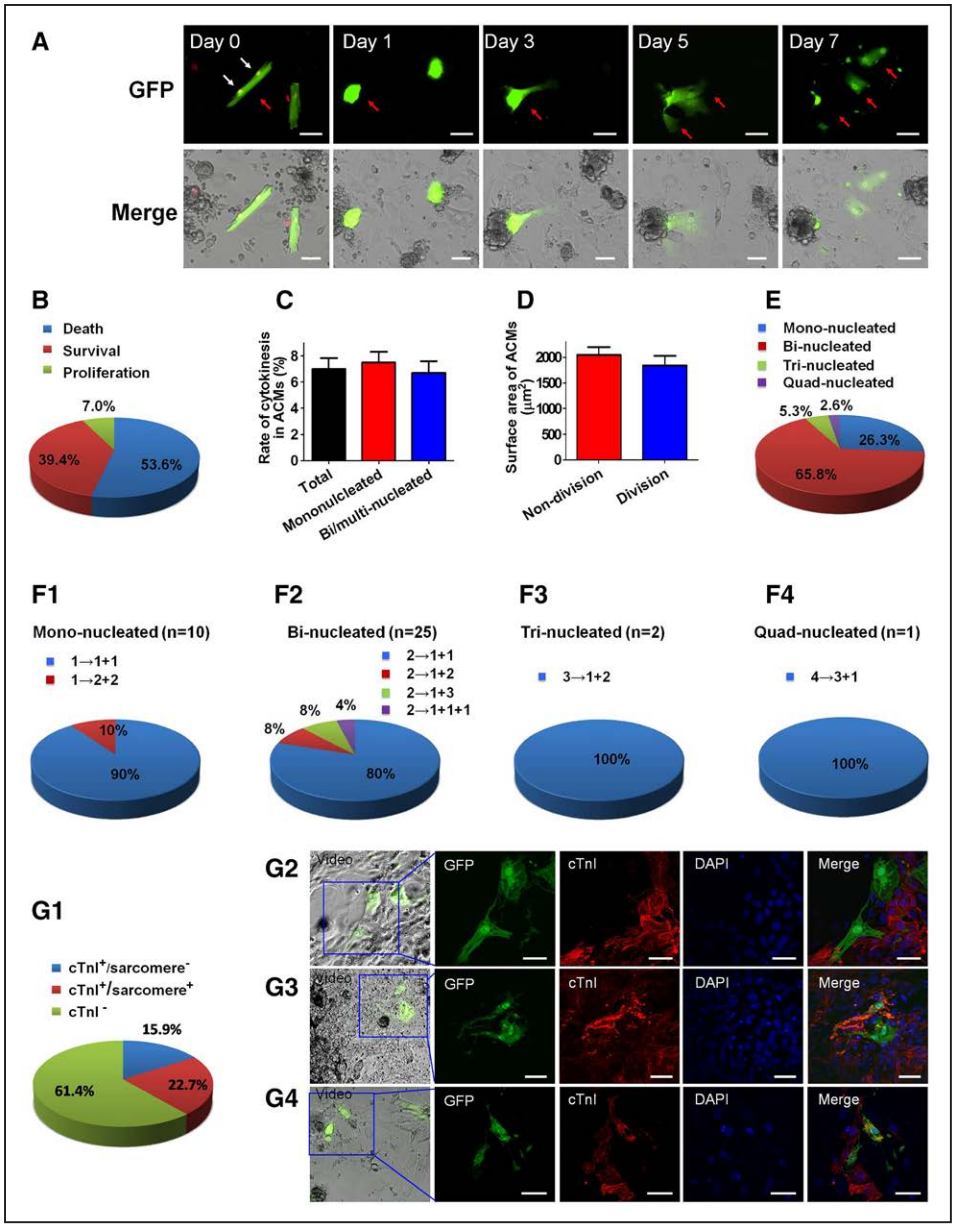

Figure 2. ACM proliferation with cytokinesis in vitro.
Freshly isolated ACMs from β-actin-GFP transgenic mice were cocultured with neonatal rat ventricular myocytes for 7 days. Every ACM was traced with a time-lapse video microscopy, and only the division events with completed cytokinesis were counted as ACM proliferation. **A,** The morphological remodeling of ACMs in the coculture system observed with a time-lapse video microscopy. A binucleated ACM indicated with a red arrow became spherical and lost organized contractile apparatus during the first 3 days of coculture, and then proliferated into several daughter cells that assumed a neonatal rat ventricular myocyte–like shape over the next few days. White arrow indicates the nuclei (labeled with DRAQ5 Fluorescent Probe) of the ACM that underwent cytokinesis at the beginning of coculture. The proliferation process of this ACM can be found in online-only Data Supplement Movie I. **B,** The cell fate of ACMs during the 7 days coculture with neonatal rat ventricular myocytes. The rates of cell death, survival, and proliferation were quantified. **C,** The proliferation rates of mononucleated and bi/multi-nucleated ACMs. **D,** The surface area of ACMs that underwent proliferation and those without proliferation. **E,** The percent composition of proliferated ACMs. A total number of 38 ACM proliferation events with clearly visible nuclei were quantified. **F,** The cell division patterns of ACMs with different nuclei number. **G,** The characteristics of the daughter cells after ACM cytokinesis immunostained with cTnI. **G1,** The percentage of ACM progeny with different properties. **G2**, Representative images showing that both of the ACM-derived daughter cells lost cTnI expression. **G3,** Representative images showing 1 ACM-derived daughter cell maintained sarcomeric structure, whereas the other lost cTnI. **G4,** Representative images showing both of the ACM-derived daughter cells regained sarcomeric structure. The proliferation process of the ACMs shown in **G2** through **G4** can be found in online-only Data Supplement Movies II through IV. For **A** through **G,** scale bars=50 μm. ACM indicates adult cardiomyocyte; cTnI, cardiac troponin I; DAPI, 4′,6-diamidino-2-phenylindole; and GFP, green fluorescent protein.

The Role of Gap Junction–Mediated Intercellular Ca^{2+} Signal Propagation in ACM Redifferentiation.

The determinants of ACM redifferentiation are not well known. In the coculture system, we found that ACMs needed to make direct contact with neighboring NRVMs to redifferentiate. When ACMs were physically separated from NRVMs in a transwell system, they dedifferentiated but did not proliferate and redifferentiate (online-only Data Supplement Figure IVA), suggesting the importance of physical contact for ACM proliferation and redifferentiation. We next tested the ability of NRVMs, fetal cardiomyocytes and cardiac fibroblasts to induce DPR. Fetal cardiomyocytes induced ACM-DPR similar to that seen with NRVMs. However, very rare ACM redifferentiation was observed in coculture with cardiac fibroblasts (online-only Data Supplement Figure IVB and IVC). With time-lapse imaging observation, the ACM proliferation rate (1.7±0.2%) and redifferentiation rate (reorganization of sarcomere, 3.3±0.5%) were both significantly lower when cocultured with nonmyocytes, in comparison with those cocultured with NRVMs (online-only Data Supplement Figure IVD and IVE). These results indicated that making contact with cardiomyocytes with spontaneous contractions (fetal or neonatal cardiomyocytes) rather than nonmyocytes is important for ACM proliferation and redifferentiation.

Cardiomyocyte contraction is triggered by Ca^{2+} influx through L-type Ca^{2+} channels and the release of Ca^{2+} from the sarcoplasmic reticulum. These and other Ca^{2+} signals are also known to regulate cardiomyocyte growth, gene expression, differentiation, and development.[26] We determined if the properties of the Ca^{2+} transients in NRVMs influence redifferentiation. NRVMs were pretreated with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*,*N*-tetraacetic acid or the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a) inhibitor thapsigargin to change the properties and frequencies of Ca^{2+} transients. Both treatments significantly decreased sarcomere reorganization of dedifferentiated ACMs (Figure 3A). The $[Ca^{2+}]_i$ transient amplitudes are influenced by Ca^{2+} regulatory proteins including SERCA2a and NCX1. NRVMs had increased $[Ca^{2+}]_i$ transient amplitude when infected with SERCA2a and decreased amplitude when infected with NCX1 (Figure 3B). Compared with control treatment, ACMs cocultured with $NRVM_{SERCA2a}$ had significantly higher redifferentiation rates, whereas ACMs cocultured with $NRVM_{NCX1}$ showed a lower redifferentiation rate (Figure 3C). These results suggest that the properties of the Ca^{2+} transient in NRVMs influence the redifferentiation of ACMs.

Hypoxia Hampers Cx43 Function, Thereby Inhibiting ACM Redifferentiation, Which Is Reversed by Ischemia-Resistant Cx43-S3E Mutant

As a direct cell-to-cell cytoplasmic pathway, gap junctions allow propagation of electric activity and other small molecules between cardiomyocytes.[27] Intercellular transfer of cytoplasmic contents through gap junction between ACMs and NRVMs was demonstrated by fluorescence recovery after photo bleaching analysis (online-only Data Supplement Figure VA). Furthermore, a Ca^{2+} imaging video showed the fluo-4–labeled spontaneous Ca^{2+} transients in a NRVM can propagate into a neighboring ACM (online-only Data Supplement Movie VI). These results were also confirmed by a line-scan image analysis (online-only Data Supplement Figure VB). Cx43 is the major isoform of gap junction proteins expressed in mammalian ventricular myocytes.[27] A mouse-specific Cx43–small interfering RNA (siRNA), designed to inhibit Cx43 expression in ACMs, reduced mRNA expression and decreased immunostaining of Cx43 in mouse ACMs but not in NRVMs (online-only Data Supplement Figure VC and VD). Cx43-siRNA significantly blocked the gap junction function (online-only Data Supplement Figure VA) and reduced ACM redifferentiation (Figure 3D). These results support the idea that gap junctions allow Ca^{2+} transients to propagate from NRVMs into electrically coupled, dedifferentiated ACMs to induce ACM redifferentiation.

Hypoxia/ischemia is known to cause cardiomyocyte electric uncoupling in vivo.[19] Phosphorylation at casein kinase 1δ sites at serines 325/328/330 on Cx43 is crucial for maintaining the function of gap junctions.[28] Hypoxia/ischemia induces dephosphorylation of these sites, thereby leading to a reduced activity of gap junction, relocation of Cx43 proteins, and enhanced degradation.[29] Cx43 mutants with these sites replaced by phosphomimetic glutamic acids (Cx43-S3E) are resistant to pathological gap junction remodeling induced by cardiac ischemia.[19] As shown in Figure 3E, hypoxia reduced ACM redifferentiation, and infection of NRVM with Cx43-S3E rescued the inhibitory effect of hypoxia on ACM redifferentiation. Conversely, a Cx43 mutant with serines 325/328/330 replaced by nonphosphorylatable alanines (Cx43-nonphosphorylatable alanines) enhanced the hypoxia-induced decrease of ACM redifferentiation. These data suggest that hypoxia reduced Cx43 function, thereby inhibiting ACM redifferentiation, which was reversed by ischemia-resistant Cx43-S3E mutant.

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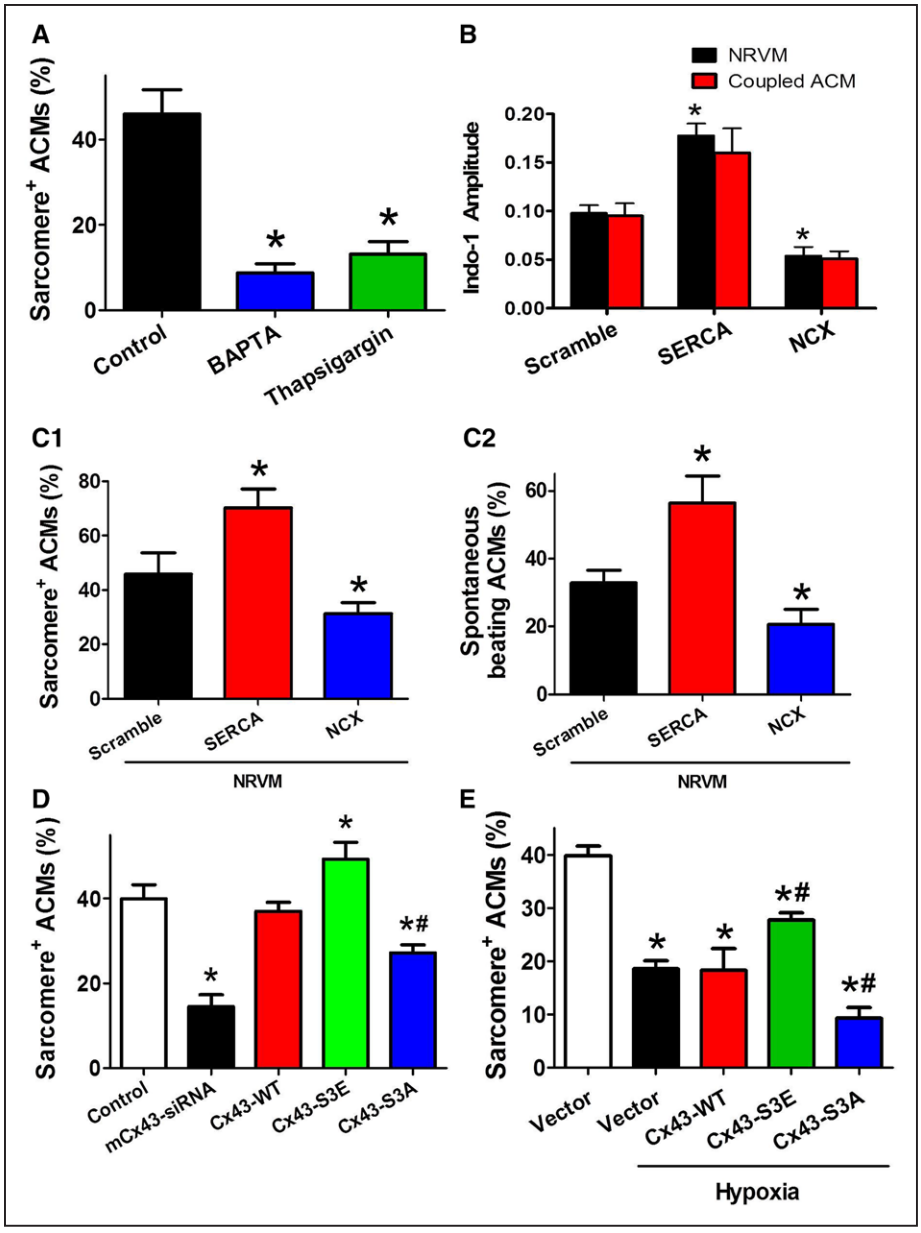

Figure 3. Connexin 43–mediated intercellular transmission of Ca^{2+} influences the ACM redifferentiation.
A, The percentage of sarcomere+ ACMs in coculture at day 7 with Ca^{2+} blocker 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid tetrakis (acetoxymethyl ester) and thapsigargin. **B,** The property of Ca^{2+} transients in ACMs cocultured with neonatal rat ventricular myocytes overexpressing Ca^{2+}-regulating protein SERCA2a or NCX1. The graph bars represent $[Ca^{2+}]i$ transient amplitude (change from diastolic $[Ca^{2+}]i$ to peak systolic $[Ca^{2+}]i$) of redifferentiated ACMs and their coupled neonatal rat ventricular myocytes. **C,** The percentage of ACMs with sarcomere+ (C1) or spontaneous beating (C2) in cocultures with neonatal rat ventricular myocytes overexpressing SERCA2a or NCX1 at day 7. **D,** The percentage of sarcomere+ ACMs in cocultures at day 7 with connexin 43 manipulation. **E,** The percentage of sarcomere+ ACMs in cocultures at day 7 with connexin 43 manipulation under hypoxia. n=8 to 10. **P*<0.05 versus control or scramble or vector. #*P*<0.05 versus vector+hypoxia. ACM indicates adult cardiomyocyte; Cx43, connexin 43; SERCA2a, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; NCX, Na^+/Ca^{2+} exchanger; and NRVM, neonatal rat ventricular myocyte.

Ca^{2+}-Dependent Calcineurin-NFAT Signaling Is Activated During ACM Redifferentiation

Changes in cytosolic Ca^{2+} can activate and regulate genes involved in cardiac growth and hypertrophy and can thereby induce changes in ACM phenotype.[30] Ca^{2+}-dependent activation of the phosphatase calcineurin/nuclear factor of activated T cells (NFAT)/myocyte-specific enhancer factor 2C (MEF2C) signaling pathway are centrally involved in many of these processes.[19,31] NFAT is also associated with myocardial remodeling,[32] includ-

ing myofibrillar reorganization. Knockdown of calcineurin, NFATc3 or MEF2C in ACMs with siRNAs or the calcineurin inhibitor cyclosporin A significantly reduced ACMs with reorganized sarcomeres and spontaneous beating (Figure 4A and 4B). These results suggest the involvement of these signaling molecules in ACM redifferentiation. In addition, knockdown of ACM Cx43 blocked the nuclear translocation of NFATc3 and the formation of organized sarcomeres (Figure 4C). Collectively, these data suggest that Ca^{2+} transients propagating into dedifferentiated ACMs from spontaneously beating NRVMs via Cx43 gap junction induce redifferentiation by activating calcineurin-NFAT signaling.

DPR of ACMs Occurs in the Post-MI Adult Mammalian Heart

Myh6-MerCreMer-lacZ mice were used to determine the origin of any myocytes regenerated after MI. Before MI, the majority of ACMs (>90%) were specifically and irreversibly marked with lacZ after 4-OH-tamoxifen treatment (online-only Data Supplement Figure VI). Labeled ACMs express lacZ even if they lose the expression of α-myosin heavy chain, whereas original noncardiomyocytes are lacZ negative (online-only Data Supplement Figure VIIA). Nuclear 5-ethynyl-2-deoxyuridine (EdU) incorporation was used to evaluate cellular proliferation. EdU^+/$tropomyosin^+$ cells were assumed to be newly formed cardiomyocytes. These cells were primarily found in the infarct border zone (online-only Data Supplement Figure VIIB). Over 2 million isolated $tropomyosin^+$ myocytes from 7 hearts were examined and 0.091±0.022% of them were EdU^+. It is important to note that 35% of these EdU^+/$tropomyosin^+$ cells were mononucleated, and all of these EdU^+ ACMs were $lacZ^+$. These results strongly support the idea that the new cardiomyocytes in the post-MI myocardium are derived from preexisting ACMs.

We next examined if any evidence for cardiomyocyte DPR could be shown in post-MI Myh6-MerCreMer-lacZ mice. The dedifferentiation markers Runx1 and Dab2 were found in a few $lacZ^+$ ACMs in the infarct border

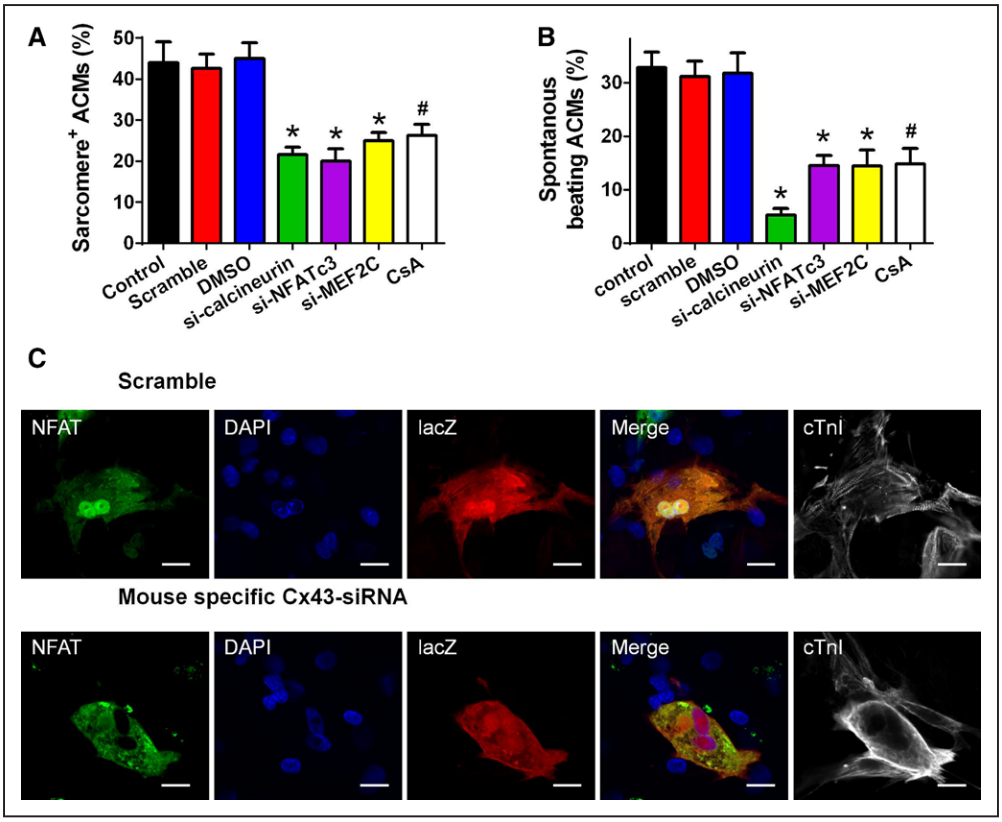

Figure 4. Intracellular Ca^{2+}-dependent calcineurin-NFAT signaling pathways regulate ACM redifferentiation. **A** and **B,** The role of Ca^{2+}-dependent calcineurin-NFAT signaling pathway in ACM redifferentiation. The percentage of ACMs with organized sarcomere (**A**) and spontaneous beating (**B**) were quantified at 7 days postculture. The cells were pretreated with mouse-specific small interfering RNAs against calcineurin, NFATc3 and myocyte-specific enhancer factor 2C, or calcineurin inhibitor cyclosporin A, respectively. n=6; * $P<0.05$ versus Scramble. $^{\#}P<0.05$ versus dimethyl sulfoxide. **C,** Representative images of nuclear translocation of NFATc3 in ACMs at day 5 postculture. The cells were pretreated with mouse-specific small interfering RNAs of Cx43 or scramble sequence as a control. Scale bars=20 μm. ACM indicates adult cardiomyocyte; CsA, cyclosporin A; cTnI, cardiac troponin I; Cx43, connexin 43; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; MEF2C, myocyte-specific enhancer factor 2C; NFAT, nuclear factor of activated T cells; and si-, small interfering.

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zone (Figure 5A). Some lacZ⁺/tropomyosin⁺ myocytes in the infarct border zone were also positive for the proliferation markers Ki67 and PH3 (Figure 5B). Mononucleated lacZ⁺/EdU⁺ ACMs with clear sarcomeric structure, indicating new ACMs formed after proliferation and redifferentiation, were found 3 weeks post-MI (Figure 5C). Among the EdU⁺ cardiomyocytes (trypomyosin⁺) isolated from post-MI mice, both sarcomere-negative and -positive subsets were identified (online-only Data Supplement Figure VIIIA). Only 35.2±4.5% of these EdU⁺

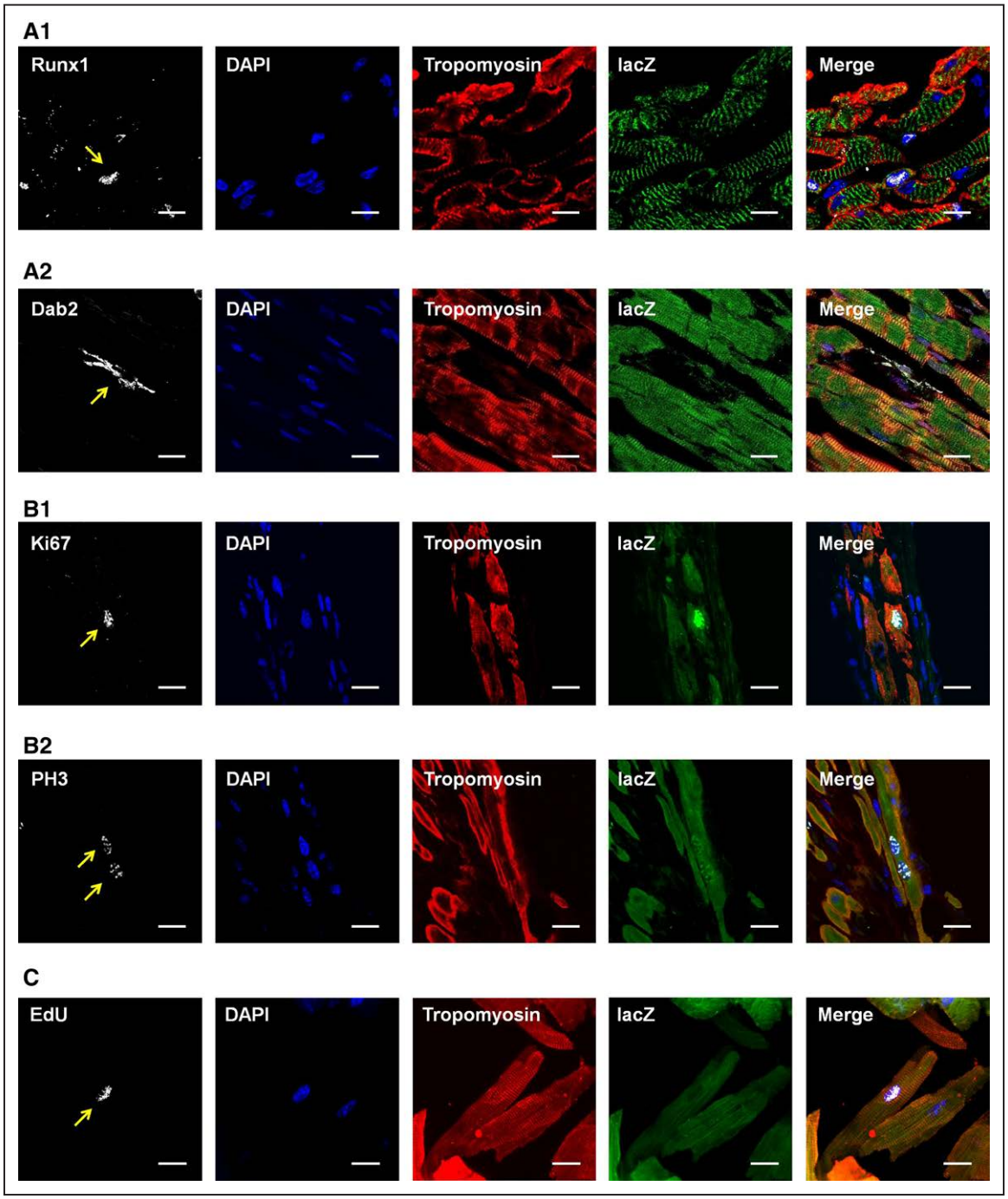

Figure 5. Dedifferentiation, proliferation, and redifferentiation of ACMs in Myh6-MerCreMer-lacZ mice subjected to myocardial infarction.

A, Representative images of ACMs expressing dedifferentiation markers Runx1 (**A1**) or Dab2 (**A2**) in the infarct border zone. **B**, Representative images of ACMs expressing proliferation markers Ki67 (**B1**) and PH3 (**B2**). **C,** Representative images of a mononuclear lacZ⁺/EdU⁺ ACM isolated from hearts 3 weeks after myocardial infarction. The organized sarcomere was clearly seen in the ACM. For **A** through **C**, scale bars=20 μm. ACM indicates adult cardiomyocyte; DAPI, 4′,6-diamidino-2-phenylindole; and EdU, 5-ethynyl-2-deoxyuridine.

cardiomyocytes were sarcomere positive with typical rod-shaped morphology. The remainder of the EdU+ cardiomyocytes had poorly organized or no detectable sarcomeric structure (online-only Data Supplement Figure VIIIB). It indicates that a large portion of the cells derived from ACM proliferation was still in a dedifferentiated state without significant contractile function. These results are consistent with the findings of ACM progeny in the coculture experiments (Figure 2E). These data collectively suggest that the few newly formed cardiomyocytes seen in the MI border zone are derived from ACMs that have undergone DPR, and that the poor redifferentiation rate of ACM progeny hinders the ACM-DPR–mediated new cardiomyocyte formation.

Ischemia-Resistant Mutant AAV9-Cx43 Promotes ACM Redifferentiation and Improves Cardiac Function in Post-MI Hearts

Ischemia-induced gap junctional uncoupling occurs in the MI border zone, and this protects the surviving regions of the heart by reducing the spread of proarrhythmic membrane depolarization and signals from dying myocytes into the surviving myocardium.[33] Our in vitro results suggest that ACMs that uncouple from their surviving neighbors can undergo DPR, but redifferentiation into functional myocytes requires recoupling to beating myocytes. If this is true, uncoupled myocytes that undergo dedifferentiation in vivo would need to recouple to myocytes that are part of the functional myocardium. To test this idea an AAV9 carrying ischemia-resistant Cx43-S3E mutant was used to enhance cell-cell electric coupling between the infarct border zone and surviving myocardium. AAV9-Cx43-S3E was injected directly into the MI border zone at 3 days post-MI. Cx43 phosphorylation assessed by Western blot analysis of total protein lysates from the post-MI hearts with empty AAV vector displayed a prominent P0 band and a faint P1 band, whereas those from the post-MI hearts with AAV-Cx43-S3E showed all (P1, P2, and P3) isoforms at a significantly higher abundance (Figure 6A). These results indicate that AAV9-Cx43-S3E administration mimicked Cx43 phosphorylation after MI.

MI caused left ventricular fractional shortening and ejection fraction to decrease in all groups of post-MI hearts. Hearts treated with AAV9-Cx43-S3E 3 days after MI had significantly decreased infarction size (Figure 6B), improved cardiac function (left ventricular ejection fraction and left ventricular fractional shortening), and less ventricular dilation 6 weeks post-MI (Figure 6C).

EdU+ cardiomyocytes were predominantly found in the infarct border zone at 3 weeks post-MI (EdU+ cardiomyocyte nuclei per total nuclei: infarct border zone, 1.181% versus remote area, 0.109%) (online-only Data Supplement Figure IX). Because misidentification of cardiomyocyte nuclei is common with conventional histology techniques, EdU incorporation into cardiomyocytes was also measured in enzymatically isolated ACMs from hearts with or without AAV9-Cx43-S3E administration. This method can unequivocally determine if an EdU+ nucleus resides within a cardiomyocyte.[34] As shown in online-only Data Supplement Figure VIIIB, Cx43-S3E did not change the number of EdU+ cardiomyocytes, but significantly increased the sarcomere-positive proportion within the total EdU+ cardiomyocytes. Conversely, Cx43-siRNA and calcineurin inhibitor cyclosporin A decreased the proportion. Significantly more lacZ+/EdU+/sarcomere+ ACMs were found in AAV9-Cx43-S3E–treated hearts (Cx43-S3E, 0.254% versus vector, 0.091%) (Figure 6D). Mononucleated lacZ+/EdU+/sarcomere+ ACMs were also found in significantly greater abundance in AAV9-Cx43-S3E–treated hearts than control (Cx43-S3E, 0.095% versus vector, 0.032%). Given the distinct distribution of EdU+ cardiomyocytes in post-MI heart, the percentage of lacZ+/EdU+/sarcomere+ ACMs in the infarct border zone could be much greater than the number obtained from isolated cardiomyocytes of the whole heart. These data show that ischemia-resistant Cx43 reduces infarct size, improves cardiac function, and increases the number of newly formed cardiomyocytes at the MI border zone.

DISCUSSION

What appears common to all post-MI studies is the presence of a few new cardiomyocytes in the infarct border zone.[4,6] Consistent with previous studies,[3] our data with a fate-mapping strategy support the view that the newly formed cardiomyocytes found in the post-MI heart are predominately derived from preexisting ACMs. It is unclear if these new myocytes are derived from a small pool of ACMs that retain their ability to proliferate or derived from mature ACMs. Our in vitro and in vivo data support the hypothesis that mature ACMs can dedifferentiate, and then proliferate and redifferentiate into new cardiomyocytes.

ACM Dedifferentiation

After an MI, injured myocytes at the infarct border uncouple from their neighbors.[33] These ACMs usually die over the next few days/weeks, and the infarct zone expands. Our studies suggest that some of these myocytes dedifferentiate after uncoupling from the parent myocardium, and they may be the source of newly forming myocytes in the post-MI heart. To test our idea, we established an in vitro system in which single and uncoupled ACMs are isolated from the adult heart and placed in coculture with NRVMs. This preparation

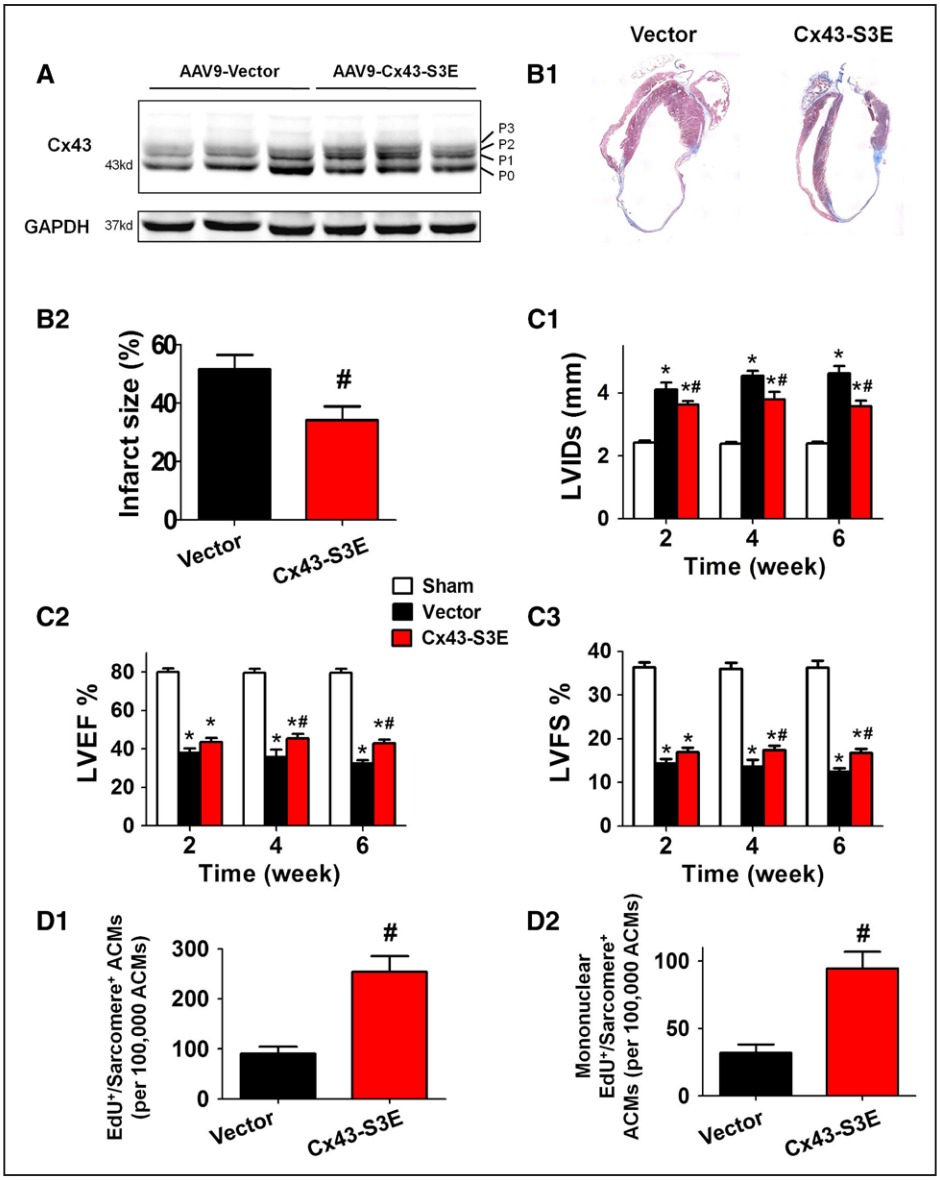

Figure 6. Cardiac AAV9-Cx43-S3E (ischemia-resistant Cx43 mutant) therapy promotes ACM proliferation and redifferentiation and improves cardiac function in the post-MI heart.
AAV9-Cx43-S3E or AAV9-vector was administrated 3 days after MI. **A,** Altered Cx43 phosphorylation in post-MI mice with AAV9-Cx43-S3E treatment. Western blot analysis of whole-cell lysates prepared from left ventricles of mice 6 weeks post-MI. Cx43 lysates are shown in various major phosphorylated forms of Cx43 (P0, P1, P2, P3) with the indicated treatments, probed with polyclonal pan-Cx43 antibody. **B,** The representative images (**B1**) and quantification (**B2**) of heart infarct size 6 weeks post-MI. **C,** The cardiac structure and function were assessed by serial echocardiography. Left ventricle internal diameter in systole (LVIDs, **C1**), left ventricular ejection fraction (LVEF, **C2**) and fraction shortening (LVFS, **C3**) at 2 to 6 weeks post-MI were presented. **D,** ACM proliferation and redifferentiation detected by EdU incorporation analysis at 3 weeks post-MI. Total EdU$^+$/sarcomere$^+$/lacZ$^+$ cells (**D1**) and mononuclear EdU$^+$/sarcomere$^+$/lacZ$^+$ cells (D2) were quantified. n=10 to 14; *P<0.05 versus Sham; #P<0.05 versus AAV9 vector. AAV9 indicates adeno-associated virus serotype 9; ACM, adult cardiomyocyte; Cx43, connexin 43; MI, myocardial infarction; and S3E, Cx43 mutant with serines 325/328/330 replaced by phosphomimetic glutamic acids.

contains spontaneously beating NRVMs together with isolated and Ca^{2+}-overloaded, electrically uncoupled ACMs.[16] This coculture system may mimic the in vivo ischemia environment where certain surviving myocytes in the infarct border zone have uncoupled from the myocardium and are at high risk of cell death. These uncoupled myocytes are neighboring healthy myocytes contracting in synchrony with the normal heartbeat.

Our studies show that coculture of ACMs with NRVMs promotes mouse ACM survival (online-only Data Supplement Figure I). ACMs that survived in coculture expressed dedifferentiation markers and dismantled their contrac-

tile apparatus. Dedifferentiation might serve as a preprogrammed survival mechanism for stressed ACMs,[35] protecting them from ischemia by reduction of ATP consumption attributable to changes in energy metabolism and inactivation of energy-intensive functions.[36] The dedifferentiation of mouse ACMs also might be induced by loss of cell-cell contact or by Ca^{2+}-overload stress.

ACM Proliferation

We used a variety of approaches to document ACM proliferation in coculture. Genetically modified or fate-mapping ACMs were used so that they and their progeny expressing the genetic marker could be easily followed. The strongest data supporting the proliferation of dedifferentiated ACMs were obtained by using time-lapse imaging followed by immunostaining of those regions of the cocultures that were observed over time. These experiments clearly showed that some ACMs proliferate during the first week in coculture with NRVMs. Cytokinesis of proliferating ACMs was directly observed to document that dedifferentiated ACMs can form committed progeny. Visualization of nuclei during live imaging showed that some cardiomyocytes may go through the whole mitosis process, ie, DNA replication, chromosome/nuclei separation, and cytokinesis, which is a significant finding. It also gave us the opportunity to determine if mononucleated ACMs are more likely to proliferate than binucleated ACMs. It is interesting to note that our data showed that mono- and bi/multinucleated myocytes are almost equally able to proliferate and form committed progeny. In addition, cell size did not appear to be a significant determinant for the potential of ACM proliferation. These findings suggest that proliferation, at least under the conditions used, is not limited to a small pool of so-called immature, small mononucleated ACMs as previously suggested.[6–11] Binucleated ACMs are the majority of cardiac muscle cells within the adult heart, and our results suggest that they are equally capable of DPR. Therefore, they are likely to be the major source of new myocytes in the adult heart. In addition, we are the first to define the dividing patterns of ACMs with different nucleation patterns. Binucleated ACMs could divide into 2 or 3 mononucleated cells, or 1 mononucleated cell and 1 bi/multinucleated cell. Furthermore, our data also suggest that cell-to-cell contact is important for ACM proliferation. Cx43 has been reported as a regeneration gene in zebrafish heart.[37] Additional mechanisms regulating ACM proliferation were not studied in detail and need further investigation.

Redifferentiation of ACMs

Redifferentiation of dedifferentiated ACMs into contractile myocytes is essential if they are to contribute to the pump function of the heart.[38] The coculture experiments document DPR and directly demonstrate new myocyte formation after ACM dedifferentiation and proliferation. In addition, our in vitro data show that less than half of the cells derived from dedifferentiated and proliferated ACMs go on to acquire a cardiomyocyte phenotype, at least during the time period of the current study. An important aspect of our results is that ACM redifferentiation was not observed unless the dedifferentiated ACMs made gap junction (Cx43)–mediated cell-cell connections with NRVMs (Figure 3 and online-only Data Supplement Figures IV and V).

Role of Cx43 and $Ca^{2\pm}$ Transients in ACM Redifferentiation

To our knowledge, there is currently no report in the literature on the regulation of ACM redifferentiation from a dedifferentiated state. Previous studies show that Ca^{2+} signaling is involved in embryonic cardiogenesis and stem cell cardiomyogenic differentiation.[39] Our in vitro data showed that the Ca^{2+} transients in NRVMs transiently elevate cytosolic Ca^{2+} in dedifferentiated ACMs via gap junctions to initiate redifferentiation. The entry of Ca^{2+} into ACMs appears to induce redifferentiation via a process that includes calcineurin-NFAT signaling pathways. In addition, the properties of the Ca^{2+} transient in NRVMs influenced ACM redifferentiation. Overexpression of SERCA2a (which increased the size of the Ca^{2+} transient) increased ACM redifferentiation. Overexpression of NCX1 (to decrease the amplitude of the Ca^{2+} transients) reduced ACM redifferentiation. In failing human hearts, the cardiomyocyte Ca^{2+} transient is reduced and prolonged because of impaired SERCA2a activity and increased expression of NCX1.[40,41] These changes might reduce ACM redifferentiation and reduce the small amount of new myocyte formation present in cardiac disease. We confirmed that the NRVM Ca^{2+} that influences ACM redifferentiation travels into ACM via gap junctions.[42] Ischemia impaired gap junction function and blocked Ca^{2+}-induced ACM redifferentiation. An ischemia-resistant mutant of Cx43 with stable phosphorylation at serine residues 325/328/330 rescued these effects.

Evidence for DPR After MI

The DPR process observed in our in vitro system may not exactly represent the DPR process in vivo. Our in vivo experiments using fate-mapping mouse models allowed for identification of new myocytes that were derived from preexisting ACMs ($lacZ^+$). Evidence of ACM dedifferentiation in the infarct border zone was observed at early times after MI, and $LacZ^+/EdU^+/sarcomere^+$ ACMs were found in the infarct border zone at later times. These results support the idea that ACMs have under-

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gone DPR. The ratio of lacZ$^+$/EdU$^+$/sarcomere$^+$ ACMs out of total ACMs isolated from the whole heart post-MI injury was ≈0.1%, consistent with previous reports that found evidence for new myocyte formation in the adult heart after ischemic injury.[34,43–45] Consistent with the findings of ACM progeny in the coculture experiments (Figure 2E), the in vivo data also suggest that most of the cells derived from ACM proliferation were in a dedifferentiation state without contractile function, which supported the DPR phenomenon in infracted hearts and the importance of redifferentiation (online-only Data Supplement Figure VIII). ACM redifferentiation requires Cx43-mediated recoupling of dedifferentiated ACMs to neighboring cardiomyocytes that have survived the MI and are beating in synchrony with the parent myocardium. Stabilization of Cx43 phosphorylation against ischemia with Cx43-S3E enhanced ACM redifferentiation and improved cardiac function in post-MI heart. Others have found that hypoxia (especially a certain concentration of O_2) enhances cardiomyocyte proliferation in adult post-MI hearts.[46] Therefore, hypoxia in the MI border zone could also be an important trigger for new myocyte generation. This is a complex issue that requires much more study.

In conclusion, the present study suggests that mouse ACMs, including bi/multinucleated and large-sized ACMs, can proliferate and form new myocytes through a DPR process. After MI, some ACMs in the infarct border zone uncouple from the surviving myocardium, dedifferentiate, proliferate, recouple to the surviving myocardium, and redifferentiate into functional ACMs (online-only Data Supplement Figure X), explaining the new cardiomyocytes observed in many previous studies.[3,10,15,21] Approaches that could safely enhance this process would be a significant step in regenerating those myocytes killed by ischemic injury.

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DISCLOSURES

None.

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FOOTNOTES

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