Abstract

Fibroblasts can be directly reprogrammed into induced cardiomyocytes (iCM) by expression of cardiac transcription factors Mef2c, Gata4, and Tbx5 (collectively called MGT). Since fibroblasts are readily available, this approach holds great potential for cardiac regeneration. However, the molecular basis underlying this conversion is still largely unknown. The starting fibroblasts must overcome epigenetic barriers, re-orchestrate chromatin organizations, and remodel cellular structures to obtain CM-like features. We hypothesized that autophagy, a highly conserved process for recycling cellular components, may play an important role in iCM reprogramming. Here, to test whether autophagy is induced during iCM reprogramming, we transduced fibroblasts with either MGT or lacZ as a negative control and quantified the resultant levels of p62, as well as the autophagosome marker LC3-II using Western blot and immunocytochemistry (ICC) imaging. Overexpression of MGT lead to LC3-II accumulation as well as p62 degradation, indicating that autophagy is activated in iCM reprogramming. To determine if transcription of autophagy-related genes changed significantly over the course of iCM reprogramming, cardiac fibroblasts (CFs) from neonatal mice were transfected with MGT. RNA was extracted at five time points, and qPCR was used to determine that transcription levels of autophagy related genes. Surprisingly, autophagy genes were not changed as a result of iCM reprogramming. Therefore, post-translational modifications may be responsible for the activation of autophagy during reprogramming. To determine the role of autophagy in iCM reprogramming, we depleted the key autophagy factor Beclin1 and its upstream regulators, Ulk1 and Ambra1, from mice CFs. In sum, we found that silencing these factors increased iCM reprogramming efficiency. Direct cardiac reprogramming will ultimately lead to therapies that can address the loss of contracting cardiomyocytes and restore heart function.

Introduction

Death of cardiac muscle cells, or cardiomyocytes, as a result of cardiac diseases such as heart failure (HF), myocardial infarction (MI) and ischemia/reperfusion (I/R) may lead to arrhythmias and chronic heart failure (Chiong et al., 2011). Cardiovascular diseases are a major cause of death for Americans, resulting in an average of one death every forty seconds (Mozaffarian, 2015). Many efforts have been made to therapeutically restore the loss of contracting cardiomyocytes in order to restore heart function.

Heart tissue is comprised of cardiac fibroblasts (CF), vascular cells, and cardiomyocytes, with CFs comprising a majority of the heart's total cells (Ieda et al., 2010). The heart's response to cardiomyocyte death is the formation of scar tissue by fibroblasts, which are structural cells that secrete signals but fail to restore the function of the lost cardiomyocytes (Chen & Qian, 2015; Ieda et al., 2010). Due to the heart's limited ability to regenerate cardiomyocytes, many efforts have been made to explore pathways to stimulate endogenous cardiac progenitor cells, embryonic stem cells, and induced pluripotent stem cells to differentiate and proliferate as cardiomyocytes (Chen & Qian, 2015). Other efforts have included direct reprogramming of mouse cardiac fibroblasts to induced cardiomyocytes (iCMs) through different transcription factor cocktails and epigenetic repatterning. Mef2c, Gata4, and Tbx5, collectively referred to as MGT, are together sufficient to convert mouse fibroblasts to iCM-like cells (Qian, Berry, Fu, Ieda, & Srivastava, 2013); however, the underlying molecular mechanisms of cardiac reprogramming are not completely understood.

To address the gap in knowledge about the mechanisms of cardiac reprogramming, other cellular pathways have been studied in conjunction with this process. For example, autophagy is a housekeeping mechanism that recycles intracellular materials for energy production and is used in emergency circumstances and stress responses. It is characterized by the formation of a double-membrane phagophore that seals to form an autophagosome, which can fuse with a lysosome to digest the encapsulated materials. During autophagy, p62, also known as Sequestosome-1, marks substances that are to be degraded with other substrates (Rusten & Stenmark, 2010). Given the fact that reprogrammed iCMs needs more metabolites to remodel the cell structure, like de novo formation of sarcomeres, and that iCMs require more energy to

support its beating function, we hypothesized that autophagy could play a role in reprogramming to help remodeling cellular structures.

To test whether autophagy plays a role in reprogramming and to characterize that role, autophagy markers p62 and LC3-II were analyzed using Western blot and immunocytochemistry imaging (ICC), respectively. Next, the transcriptional activity of a library of autophagy-related genes were analyzed using quantitative PCR (qPCR) to determine any changes in transcriptional activity throughout iCM reprogramming. Finally, fibroblasts from αMHC-GFP transgenic mice were transfected with MGT and Beclin1, an autophagy-related gene, was silenced to determine its specific role in iCM reprogramming.

Materials and Methods

Mouse Lines

Cardiac specific αMHC promoter driven-GFP transgenic mice were described previously (Ieda et al., 2010). Animal care was performed in accordance with the guidelines established by the University of North Carolina, Chapel Hill. All mouse protocols were approved by the Division of Laboratory Animal Medicine (DLAM), University of North Carolina, Chapel Hill.

Isolation of Fibroblasts

Fibroblast isolation was performed as previously described (Wang, 2015). Briefly, neonatal heart dissection was performed on day 0 to day 3 from αMHC promoter driven-GFP transgenic mice. GFP fluorescence was checked under a microscope, and 3-4 GFP positive hearts were minced into pieces less than 1 mm^3 in size in a 10 cm dish with 2 mL FB media. An additional 8 mL of FB media was added to the dishes containing hearts and were incubated for three days. Media was replaced every three days. On day 7, cells were washed and digested with trypsin. After digestion, cells were washed with FB and collected by gently scratching the plate

with a cell scraper. Cells were collected with strainers and pelleted by spinning at 200 xg for 5 min.

Viral Packaging and Transduction

Viral packaging and transduction were performed as previously described (Zhou et al., 2016). Retrovirus packaging was performed in platE cells, and lentivirus packaging was conducted in 293T. Both cell lines were maintained in DMEM growth media. platE cells were transfected with 20 μg pMXs-based retrovirus vectors for retrovirus packaging using 40 μl Lipofectamine 2000 (Life Technologies) or Nanofect (Alstem) according to manufacturer's instructions. Lentivirus packaging was accomplished using 10 μg shRNA viral vector, 7 μg psPAX2 and 3 μg pMD2.G in 293T cells.

iCM Reprogramming

Fibroblasts were transduced with MGT retroviruses and lentiviruses expressing shRNAs in iCM media (10% FBS of DMEM/M199 [4:1]). Media was changed every 2-3 days, and iCM media supplemented with puromycin was used for positive selection of transduced cells. Reprogrammed cells were harvested for analyses at indicated time points (d3, d5, d7 d10, d14, d21). GFP expression was observed under an inverted fluorescent microscope.

Quantitative RT-PCR

RNA extraction, reverse transcription, and qPCR were performed as previously described (Wang, 2015). The primers used are listed in *Table 1*.

Oligo Name	Forward Sequence	Reverse Sequence
Ambra1	CCAGAGAAGAATGCTGTACGAAT	TCCATCGAGTCTTATCCTCCAC
Atg16l	ATGGTGCGTGGAATGATA	CTGTATCTCCTTGTCCTTCT
Atg4b	TATGATACTCTCCGGTTTGCTGA	GTTCCCCCAATAGCTGGAAAG
mTOR	AGCAACAGTGAGAGTGAA	AGGAGATAGAACGGAAGAAG
Ulk1	AAGTTCGAGTTCTCTCGCAAG	CGATGTTTTCGTGCTTTAGTTCC
Bcn1	ATGGAGGGGTCTAAGGCGTC	TCCTCTCCTGAGTTAGCCTCT
VPS34	CAACCAAGCAACTCACATAT	CTTCAAGAACTTCGTCAGAG
Atg5	AGCCAGGTGATGATTCACGG	GGCTGGGGGACAATGCTAA
Atg7	GTTCGCCCCCTTTAATAGTGC	TGAACTCCAACGTCAAGCGG
Atg3	CACAAGGAAAATAGTGGAAGCC	TGACAGGGGAAACCCTACTAGA
LC3B	CAGTGATTATAGAGCGATACAA	GCCGTCTGATTATCTTGATG
UVRAG	ATGAGCTCCTGCGCCTCGCT	TCACTTGTCGGAACTCCTGC
Actc1	CTGGATTCTGGCGATGGTGTA	CGGACAATTTCACGTTCAGCA
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Table 1. List of primer sequences used in qPCR.

Western Blot

Cells were collected and lysed in 2x SDS loading buffer (Bio-Rad) and subjected to SDS-PAGE. After separation, proteins were transferred to nitrocellulose membranes and probed with the primary antibodies. In this experiment, LC3 (CST), GFP (Invitrogen), and p62 (Abcam) were used. Target proteins were detected by chemiluminescence (ECL, Thermo Scientific). The membranes were subject to stripping buffer (Sigma-Aldrich) and re-probed with a secondary protein or β-actin (Santa Cruz) as a loading control.

ICC Imaging

ICC analysis of reprogramming efficiency was performed as previously described (Wang, 2015). The primary antibody used to stain for GFP-LC3 was rabbit anti-GFP IgG (Invitrogen, 1:500) . The secondary antibody used to stain for GFP-LC3 was Alexa Fluor 7488 conjugated donkey anti-rabbit IgG. For all other cTnT, GFP, and nuclei staining, mouse anti-troponin T, cardiac isoform (Thermo Scientific, 1:200), rabbit anti-GFP IgG (Invitrogen, 1:500), Alexa Fluor 488 conjugated donkey anti-rabbit IgG, Alexa Fluor 647-conjugated donkey anti-

mouse IgG (Jackson ImmunoResearch Inc, 1:500), and Hoechst 33342 (Thermofisher). All secondary antibodies were used at a 1:500 dilution in 1% BSA/PBS.

Flow cytometry

FACS analysis of reprogramming efficiency was performed as previously described (Wang, 2015). Briefly, at day 10 post-transfection, reprogrammed cells were washed with PBS and dissociated with 0.05% trypsin (Life Technologies). Cells were washed with cold FACS buffer, fixed, and stained with Cell Fixation/Permeabilization Kids (BD Biosciences). Cells were incubated with antibodies at concentrations recommended by manufacturers. Cells were reconstituted in staining buffer and counted using BD Accuri™ C6 flow cytometer. FACS data was collected and analyzed by FlowJo software (Tree Star). The primary and secondary antibodies used for FACS analysis were the same as those used in ICC imaging.

Statistical analyses

Statistical analyses were performed with two-way t-test or two-way ANOVA. A P value of <0.05 was considered statistically significant (*), a P value of <0.01 was considered highly significant (**), and a P value of <0.001 was considered strongly significant (***).

Results

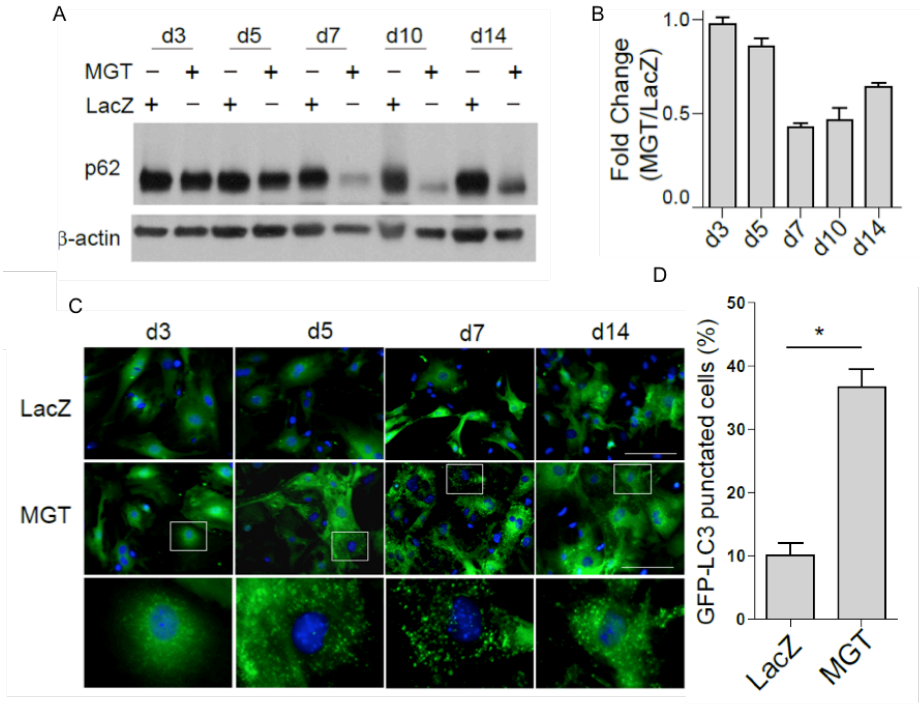

Figure 1. Autophagy is activated during reprogramming. (A) Western blot of fibroblasts with MGT overexpression. (B) Quantification of Western blot. (C) Representative images of GFP-LC3 puncta formation in fibroblasts transduced with either MGT or lacZ control virus on d7. Green = LC3-GFP; Blue = nuclei. (D) Quantification of C. Bars, mean ± s.e.m.; ***p<0.001, **p<0.01, *p<0.05

Wu et al. (2015) previously described the induction of autophagy during reprogramming of mouse fibroblasts into induced pluripotent stem cells (iPS) using the transcription factors Sox2, Oct4, Klf4, and c-Myc. They linked autophagy to p62 degradation, a protein that typically facilitates reprogramming. However, it has not been categorized how autophagy influences the direct reprogramming of mouse fibroblasts to cardiomyocytes. In order to determine the role of autophagy in direct reprogramming, freshly isolated cardiac fibroblasts were transduced with polycistronic MGT or a lacZ control vector. Fibroblasts were collected at different time points between day 3 and day 14 and probed for p62 and β-actin using Western blot analysis (*Figure 1A*). Starting at day 7 post MGT transfection, the cells showed signs of p62 degradation as indicated by the decrease in fold change compared to the lacZ control (*Figure 1B*).

The microtubule associated protein light chain 3 (LC3), a protein at the internal surface of growing autophagasomes, serves as an indicator of autophagy (Boya, 2013). To further analyze the role of autophagy in direct iCM reprogramming, ICC images were taken of MGT or lacZ transduced fibroblasts that were co-transduced with GFP-LC3 producing retroviruses on day 7 (*Figure 1C*). Quantification of the field images shows an increase in GFP-LC3 punctated cells in MGT transduced cells in comparison to the lacZ control.

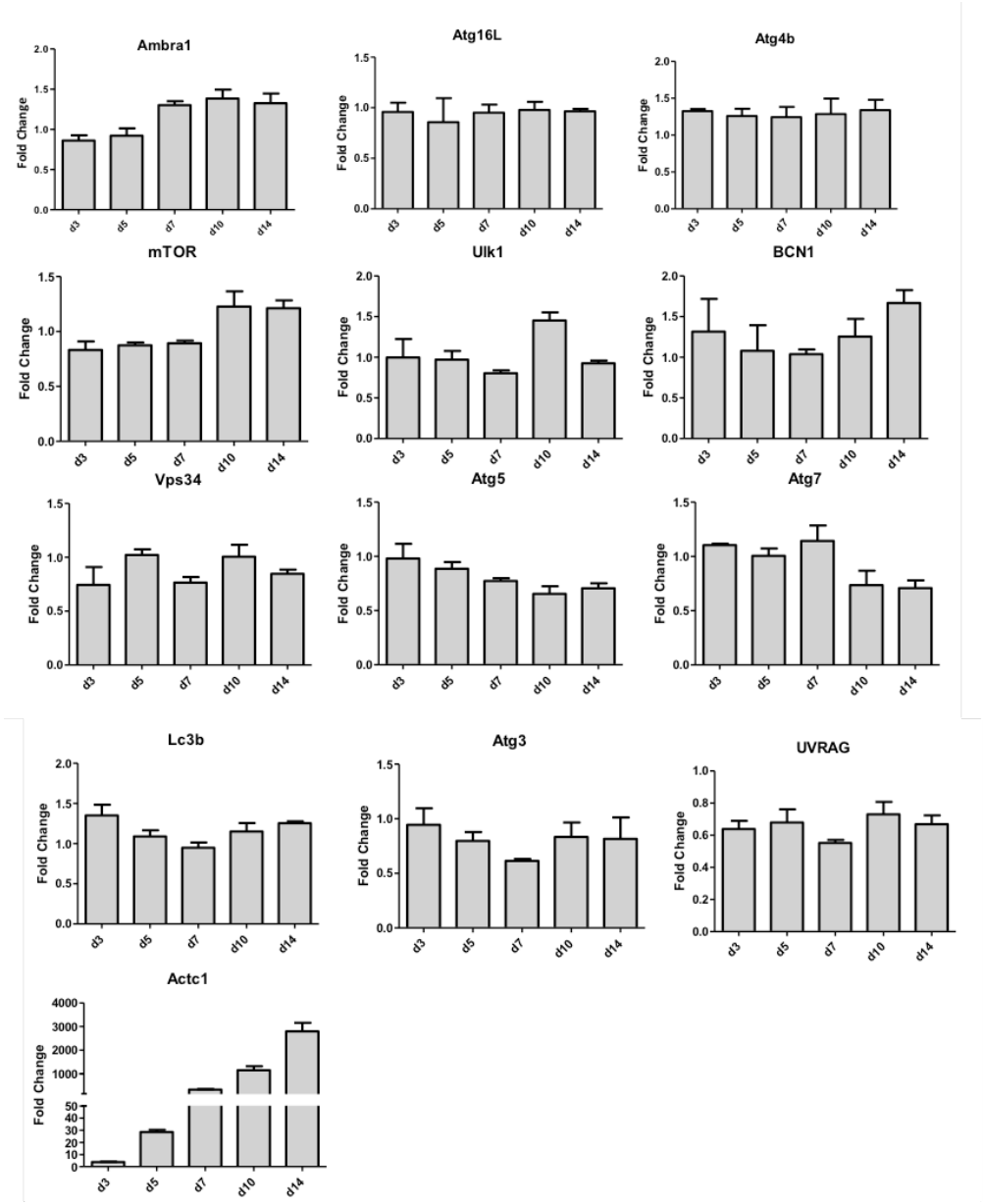

Figure 2. Transcription of autophagy-related genes does not change during iCM reprogramming. Histograms of transcriptional levels of autophagy related genes and cardiac marker, Actc1, normalized to housekeeping gene, Gapdh.

To assess whether the transcriptional level of these autophagy-related genes was affected by the induction of reprogramming, mouse fibroblasts transduced with MGT at day 0 were collected at various time points from day 3 to day 14. After RNA extraction and reverse transcription, they were subject to qPCR analysis with the autophagy-related gene primers

indicated (*Figure 2*). α-Actinin 1 (Actc1) was used as a cardiomyocyte marker representing a successful iCM conversion. Very surprisingly, we did not observe any transcriptional changes on autophagy-related genes from day 3 to day 14.

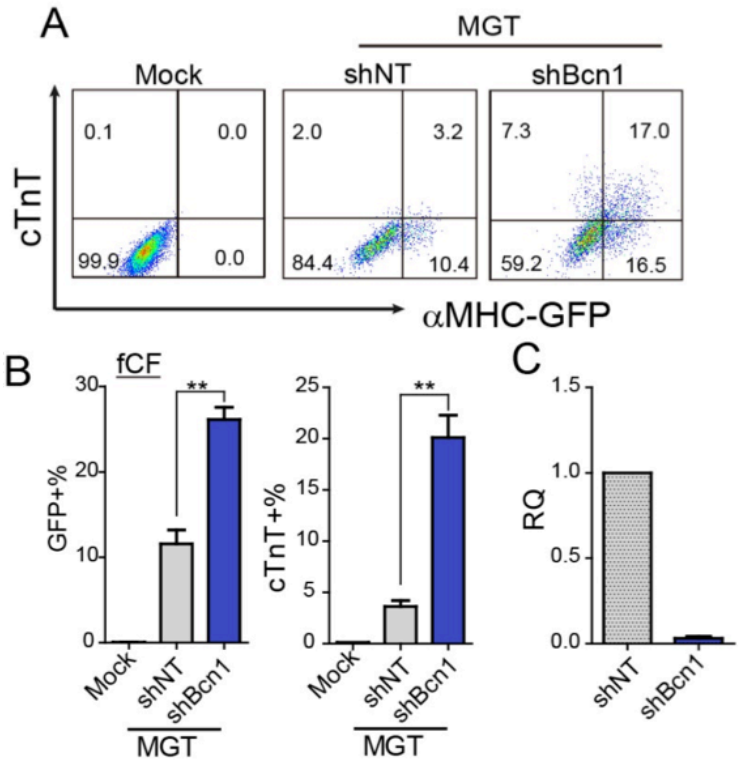

Figure 3. Beclin1 (Bcn1) knockdown increases iCM reprogramming efficiency. (A) Representative flow plots for αMHC-GFP+ and cTnT+ cells 10 days after transduction of MGT together with shBcn1 or shNT. (B) Quantification of flow data in fCFs. (C) Knockdown efficiency of shBcn1 compared to shNT control.

Beclin1 (Bcn1) has been previously described as "a platform protein which assembles an interactome consisting of diverse proteins which control the initiation of autophagocytosis and distinct phases in endocytosis" (Salminen, Kaarniranta, & Kauppinen, 2013). Additionally, it has been reported that phosphorylated Bcn1 was required for the induction of autophagy in amino-acid deficient cells (Russell et al., 2013). Because Bcn1 is the core protein of an important complex in a signaling cascade, the protein was subjected to further experimentation to determine its effects on direct reprogramming.

shRNA was used to deplete Bcn1 from fCFs of αMHC-GFP transgenic mice. Additionally, a non-targeting shRNA (shNT) vector and mock vector were used as controls. To evaluate the knockdown efficiency of shRNA against Bcn1, we performed real time qPCR analysis on viral infected cells. As shown in *Figure 1C*, the knockdown efficiency reached over 95%, suggesting the successful depletion of Bcn1. The tested shRNAs were applied to fCFs together with MGT retrovirus. Ten days after infection, reprogrammed cells were collected and expression of GFP and cardiac troponin T (cTnT) was used as indicators of reprogramming. FACS analysis (*Figure 3A*) showed an increase of cTnT and GFP expression in shBcn1 cells as compared to the non-targeting control. GFP expression increased from around 10% to approximately 26% of cells while cTnT expression increased from 4% to 20% (*Figure 3B*).

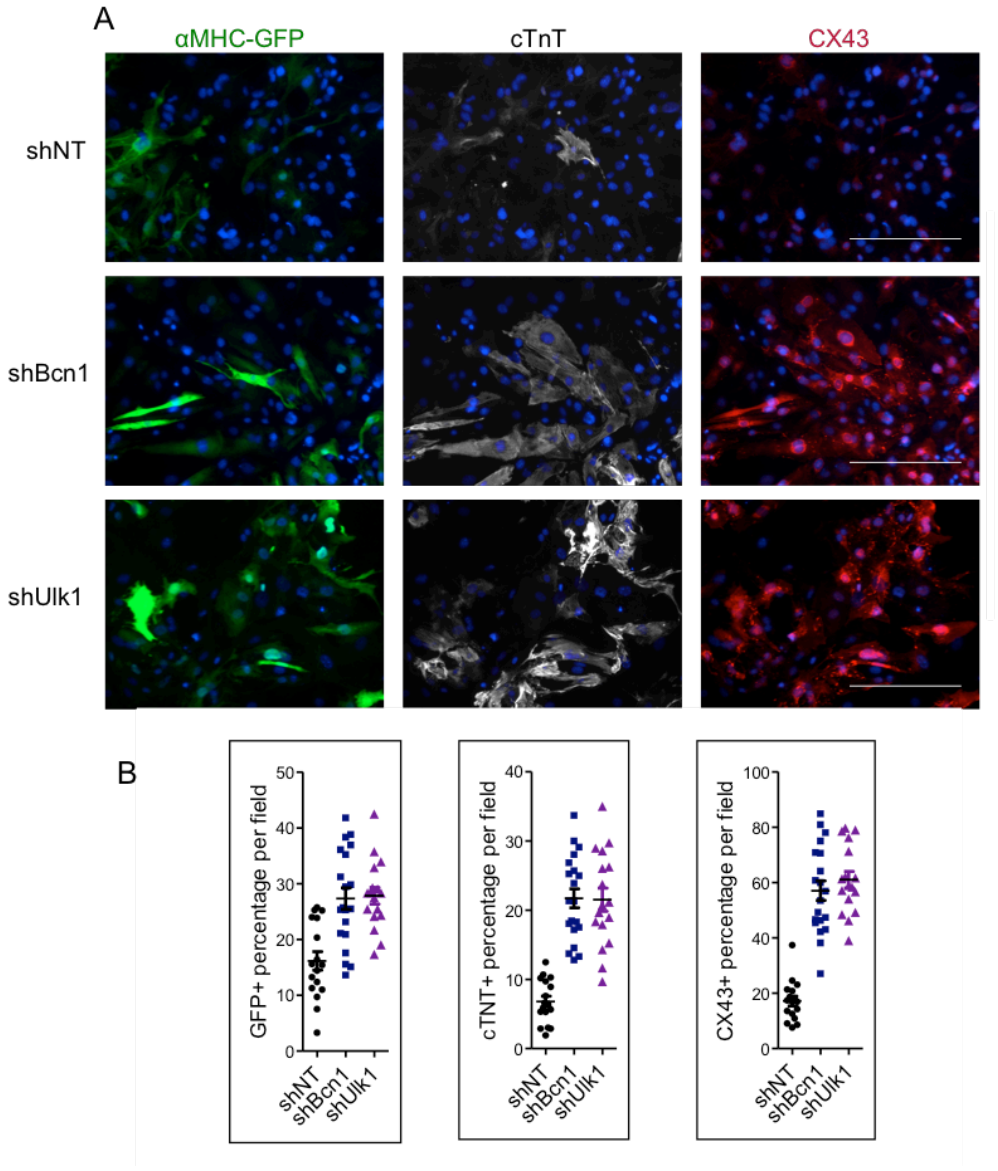

Figure 4. Knockdown of upstream regulator Ulk1 increases reprogramming efficiency. (A) Representative ICC images of MGT and either shNT, shBcn1, or shUlk1 transduced fCFs. (B) Quantification of ICC images.

Because Beclin1 is part of a signaling cascade, interfering with upstream regulators, such as Ulk1, could provide as to whether or not they are necessary for autophagy induction. Here, fCFs transduced with MGT were co-transfected with shRNAs for shNT, shBcn1, and shUlk1. Samples were imaged at day 7 and quantified in terms of GFP, cTnT, and Connexin43 (CX43)

expression (*Figure 4A*). CX43, a gap junction protein, is an indicator of iCM maturation. It is evident that GFP, cTnT, and CX43 expression increases in shBcn1 and shUlk1 knockdowns as compared to the non-targeting control.

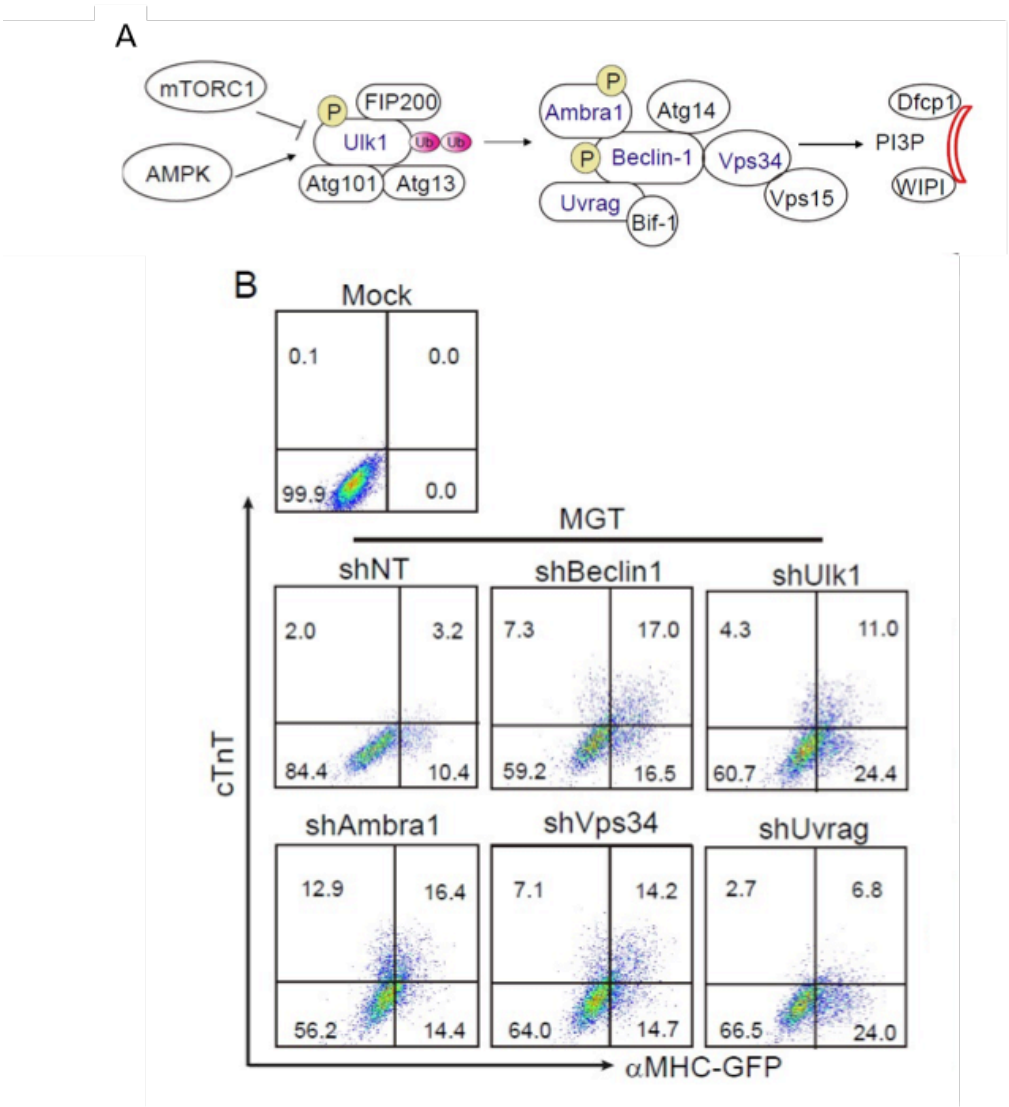

Figure 5. Regulation of Beclin1 pathway is complex. (A) Schematic description of Beclin1 complex in autophagy. (B) Representative flow plots for αMHC-GFP+ and cTnT+ cells 10 days after transduction together with shRNAs against Beclin1, Ulk1, Ambra1, Vps34, Uvrag or non-targeting (NT) control on fCFs.

Because ICC imaging indicated an increase in cardiac gene markers (GFP, cTnT, CX43), further knockdown analysis was performed on other members of the Beclin1 signaling complex. Russell et al. (2013) demonstrated that Uvrag binds Beclin1 to promote autophagosomal maturation as well as phosphorylation of Beclin1 by Ulk1. Therefore, Uvrag and Ulk1, in addition to Vps34 and Ambra1, were knocked down using shRNA to analyze whether the depletion of each individual protein enhanced or inhibited reprogramming efficiency. FACS analysis was performed on MGT transduced fCFs, and cTnT and GFP were used as indicators of reprogramming (*Figure 5B*). Knockdown of all of the Beclin1-associated proteins caused an increase in reprogramming efficiency compared to the mock and the non-targeting control. However, knockdown of Beclin1 showed the greatest increase in both cTnT and GFP expression (17.0) while the knockdown of Ambra1 was slightly less at 16.4. Knockdown of Uvrag appeared to show the lowest increase in cTnT and GFP expression. The schematic depicted in *Figure 5A* shows the interaction among the different proteins involved in the Beclin1 signaling pathway and how they interact to induce autophagosome formation.

Discussion

Autophagy is activated during iCM reprogramming

Western blot analysis indicated that p62 decreased at day 7 after MGT overexpression in MEFs (*Figure 1A*). Quantification showed that p62 decreased by approximately 50%. Although by day 14 p62 increased slightly, it is still less than days 3 and 5 post-transfection p62 levels (*Figure 1B*). The decrease in p62, an indicator of autophagic flux, demonstrated that autophagy was activated after reprogramming was induced with MGT. To further indicate autophagy activation, ICC images were taken of MGT transfected GFP-LC3 MEFs day 3, 5, 7, and 10 post-

transfection (*Figure 1C*). Autophagosome formation can be seen as early as day 3, as shown by the patchy or speckled green fluorescence as compared to the control.

We expected autophagy to be activated because of its role in cellular remodeling, and because this activation was also reported by Wu et al. (2015) when using iPS reprogramming factors.

Transcription of autophagy-related genes are not changed during iCM reprogramming

Although it has been demonstrated that reprogramming activates autophagy, the transcriptional activity of autophagy-related genes do not change throughout the course of iCM reprogramming. This indicates that the genes activated during reprogramming do not influence the transcription of autophagy genes. The increase in Actc1, a cardiomyocyte marker, transcriptional activity indicated that reprogramming had occurred; however, the transcriptional activity of the autophagy related genes remained around 1, indicating no fold change (*Figure 2*). Therefore, although autophagy is activated during reprogramming, they remain independent processes at the transcriptional level. This leads to the hypothesis that there may be post-translational modifications that induce the activation of autophagy.

Knockdown of Beclin1 significantly increased iCM reprogramming efficiency

We subjected Beclin1 to knockdown experiments because previous reports claimed that overexpression of Beclin1 enhanced autophagy (Wu et al., 2015). Using shRNA, Beclin1 was knocked down in MEFs. Knockdown of this core protein significantly increased GFP and cTnT expression, indicating an increase in reprogramming efficiency compared to the mock and non-targeting control (*Figure 3B*). The increase in reprogramming can also be visualized using ICC (*Fig 4A*), which showed the increase in GFP+, cTnT+, CX43+ cells as compared to the shNT. An additional knockdown experiment with upstream regulator Ulk1 demonstrated similar results

to the knockdown of Bcn1. Because the knockdown of Ulk1 was similar in quantification and morphology to Bcn1 knockdowns (Figure *4A,B*), Ulk1 must play an important role in the activation of the Beclin1 complex.

These results were surprising and contrary to our initial hypothesis. We believed that the knockdown of these autophagic factors would decrease overall reprogramming efficiency because we believed that the process of autophagy would assist in iCM reprogramming. However, these results indicate to us that the role of autophagy in iCM reprogramming is more complex and perhaps detrimental to the process. Wu et al. (2015) justifies this phenomenon by claiming that high oxidation levels in the early stages of reprogramming cause a stress response where the cells cease dividing. In this series of experiments, we did not investigate the oxidative levels within the cell, so this would be an interesting direction to explore. Another explanation for the counterintuitive role of autophagy in reprogramming is that previous demonstrations have shown that p62 knockdown decreases reprogramming efficiency (Wu et al., 2015). Therefore, it would be reasonable to assume that autophagy, which degrades p62, would also decrease reprogramming efficiency.

Knockdown of Beclin1 signaling cascade also enhances iCM reprogramming efficiency

Similarly to the knockdown of upstream regulator Ulk1, knockdown of proteins that associate with Beclin1 to form a multiprotein complex also result in the increase of reprogramming efficiency as demonstrated by FACS. Because depletion of Beclin-1 associated proteins, such as Vps34 results in the increase of GFP+ and cTnT+, they are likely required for the proper functioning of Beclin1 in recruiting the proteins necessary for autophagosome formation. Russell et al. (2013) previously characterized Vps34 binding to p150, which then associates with Beclin1 to induce autophagy during starvation. Here, it is also shown that Uvrag

and Ambra1 are also required for proper autophagosome formation. The findings of this paper are summarized in *Figure 6.*

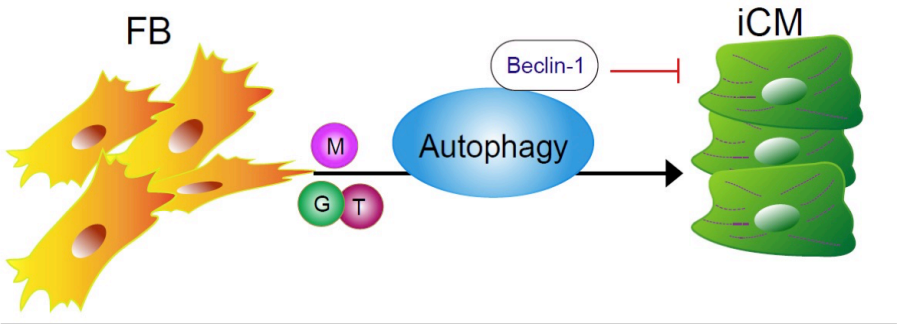

Figure 6. Summary of the role of Beclin1 in reprogramming.

As previously mentioned, the mechanism by which iCM reprogramming leads to autophagy activation is unclear. Further experimentation should address the potential post-translational mechanisms that may cause the activation of autophagic machinery. Additionally, although the knockdown of Beclin1 increased the efficiency of iCM reprogramming, it is not clear how it influences cardiomyocyte maturation and whether they become fully functional, beating cardiomyocytes. The results presented here were contrary to our initial hypothesis that autophagy would increase reprogramming efficiency; instead, the process appeared to decrease reprogramming efficiency. It would be beneficial to also explore other proteins that are involved in autophagy, separate from the Beclin1 pathway, to see how the knockdown of those proteins affects reprogramming efficiency. For example, ongoing investigation of the proteins Atg5 and Atg7, both of which are also involved in autophagy, seem to show that their knockdown decreases reprogramming efficiency, which would suggest that autophagy could indeed play a supportive role in iCM reprogramming. In induced pluripotent stem cells (iPSC), it has been shown that this process is mediated by mammalian target of rapamycin complex 1 (mTORC1), a regulator of cytoarchitecture and cell size (Wu et al., 2015). The role of mTORC1 in direct

reprogramming, especially in the context of induced cardiomyocytes, has not yet been characterized and should be subject to experimentation to determine the balance between the paradoxical roles that autophagy can play in reprogramming.

Acknowledgments

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