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mTORC1 and mTORC2 play different roles in regulating cardiomyocyte differentiation from embryonic stem cells

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ABSTRACT Mammalian target of rapamycin (mTOR) is a serine/threonine kinase and functions through two distinct complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2), with their key components Raptor and Rictor, to play crucial roles in cellular survival and growth. However, the roles of mTORC1 and mTORC2 in regulating cardiomyocyte differentiation from mouse embryonic stem (mES) cells are not clear. In this study, we performed *Raptor* or *Rictor* knockdown experiments to investigate the roles of mTORC1 and mTORC2 in cardiomyocyte differentiation. Ablation of *Raptor* markedly increased the number of cardiomyocytes derived from mES cells with well-organized myofilaments. Expression levels of brachyury (mesoderm protein), Nkx2.5 (cardiac progenitor cell protein), and α-Actinin (cardiomyocyte marker) were increased in *Raptor* knockdown cells. In contrast, loss of *Rictor* prevented cardiomyocyte differentiation. The dual ablation of *Raptor* and *Rictor* also decreased the number of cardiomyocytes. The two complexes exerted a regulatory mechanism in such a manner that knockdown of Raptor/mTORC1 resulted in a decreased phosphorylation of Rictor (Thr1135), which subsequently activated Rictor/mTORC2 in the differentiation of mES cells into cardiomyocytes. In conclusion, mTORC1 and mTORC2 played different roles in cardiomyocyte differentiation from mES cells *in vitro*. The activation of Rictor/mTORC2 was critical for facilitating cardiomyocyte differentiation from mES cells. Thus, this complex may be a promising target for regulating myocardial differentiation from embryonic stem cells or induced pluripotent stem cells.

KEY WORDS: *cardiomyocyte differentiation, mouse embryonic stem cell, Raptor/mTORC1, Rictor/mTORC2*

Introduction

Mammalian target of rapamycin (mTOR) has been reported to emerge in cardiac dystrophy protection and myocardium angiogenesis (Hwang *et al*., 2011; Aoyagi *et al*., 2012; Laplante *et al*., 2012). The mTOR forms two independent complexes, rapamycin-sensitive mTOR complex (mTORC1) and rapamycin-insensitive complex (mTORC2), which have distinct components. Association of Raptor within the mTOR complex defines mTORC1, whereas mTORC2 contains Rictor (Moschella *et al*., 2013; Oh *et al*., 2010). The mTORC1 involves in the diverse cellular processes, including cell growth, autophagy, and nutrient metabolism (Peng *et al*., 2002; Meijer *et al*., 2004; Parkhitko *et al*., 2014). The mTORC2 plays a pivotal role in cytoskeletal organization (Angliker *et al*., 2013), tumorigenesis (Tandon *et al*., 2014), embryonic development (Shiota *et al*., 2006), myocardial atrophy (Schips *et al*., 2006), and cardioprotection in myocardial ischemia-reperfusion injury (Volkers *et al*., 2013; Gurusamy *et al*., 2010). Previous studies reported that mTORC1 was associated with preosteoblast differentiation through Notch signaling pathway (Huang *et al*., 2015; Chen *et al*., 2015). Knockdown of *Rictor* suppressed differentiation of C2C12 myoblasts by interfering phosphorylation of Akt at Ser 473, a known downstream protein of mTORC2 (Shu *et al*., 2009). The two complexes, mTORC1 and mTORC2 played different roles in mesenchymal stem cell differentiation (Martin *et al*., 2015). However, limited information was available for the detailed function of mTORC1 and mTORC2 in the differentiation process of cardiomyocytes.

Abbreviations used in this paper: EB, embryoid body; mES, mouse embryonic stem cell; MTOR, mammalian target of rapamycin.

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The cardiomyocyte differentiation from mouse embryonic stem (mES) cells *in vitro* recapitulates the process *in vivo* (Anton *et al.*, 2007; Takahashi *et al.,* 2003). In this study, the differentiation system was employed to investigate the specific effects of *Raptor* (mTORC1) or *Rictor* (mTORC2) on the process of cardiogenesis. The research might contribute to discovery new targets for regulating mES cells to differentiate into cardiomyocytes. Lentivirus with Raptor short hairpin RNA (shRNA-*Raptor*) and shRNA-*Rictor* were applied during the mES cell differentiation. The assessment criteria for specific proteins of mesoderm and cardiomyocytes were measured. Meanwhile, the sarcomere structure integrity of cardiomyocytes from mES cells was observed. The results showed that shRNA-*Raptor* facilitated cardiomyocyte differentiation, while shRNA-*Rictor* had the contrary role. Knockdown of both *Raptor* and *Rictor* could suppress mES cells to differentiate into cardiomyocytes, which was similar to the effects of shRNA-*Rictor* in mES cells. The two complexes might be involved in a regulatory mechanism, in which Raptor/mTORC1 inactivated Rictor/mTORC2 through p70S6K-mediated phosphorylation of Rictor at Thr1135. The activation of Rictor/mTORC2 was critical for facilitating cardiomyocyte differentiation from mES cells, which might evolve into a promising target for regulating the myocardial differentiation and provide a useful reference for application of the induced pluripotent stem cell differentiation.

Results

Raptor knockdown accelerated cardiomyocyte differentiation from mouse embryonic stem (mES) cells

To investigate the role of mTORC1 in cardiomyocyte differentiation, we used shRNA to silence the expression of *Raptor*, the specific component of mTORC1. The levels of Raptor and p-p70S6K (Thr^{389}) were markedly reduced after lentivirus infection (Fig. 1A). The proliferation rate of *Raptor* knockdown mES cells was not altered in comparison to that of the control group detected at day 8 after infection by MTT assay (Fig. 1B). When mES cells were treated with shRNA-*Raptor*, the cardiomyocyte differentiation efficiency was significantly increased at day 7 and 8 (42±5% *vs.* 60±5% and 62±6% *vs.* 80±2%, respectively) (Fig. 1C). Furthermore, brachyury was detected to explore the role of Raptor on mesoderm development. The level of brachyury was significantly increased from day 3 to day 5, demonstrating that knockdown of *Raptor* could induce mesoderm differentiation (Fig. 1D). The expression levels of cardiac progenitor cell protein, Nkx2.5, and cardiomyocyte specific protein, α-Actinin, were also markedly increased in shRNA-*Raptor* treated mES cell group (Fig. 1D; Fig. 1E). Flow cytometry analysis of mES cell-derived cardiomyocytes at day 8 showed that cardiomyocyte differentiation efficiency was 11.6±2.2% in the control group, while the efficiency was increased to 15.8±2.9% when *Raptor* was knockdown (Fig. 1F). Furthermore, the strong red fluorescence and well-organized myofilaments were notably detected in the beating cells derived from the shRNA-*Raptor* mES cells by immunofluorescence analysis (Fig. 1G). The above data suggested that Raptor/mTORC1 exerted a negative role on mES cell differentiation into cardiomyocytes.

Rictor knockdown suppressed cardiomyocyte differentiation from mES cells

Knockdown of *Rictor* could markedly decrease the level of p-Akt (Ser^{473}), a known downstream effect protein of mTORC2 (Fig. 2A). MTT assay showed that the proliferation rate of mES cells infected with shRNA-*Rictor* did not change when compared to shRNA-*Con* cells (Fig. 2B). The proportion of beating embryoid bodies (EBs) showed a marked reduction by 21±6% and 42±6% in comparison to the control group (40±3% and 70±6%) (Fig. 2C). Meanwhile, the analysis of mesoderm specific protein showed that brachyury was significantly inhibited after shRNA-*Rictor* treatment (Fig. 2D). Nkx2.5 and α-Actinin also decreased in the shRNA-*Rictor* group, demonstrating a reduction in cardiomyocyte differentiation as compared to the shRNA-*Con* group (Fig. 2D; Fig. 2E). The flow cytometry analysis showed that the proportion of cardiomyocytes differentiated from mES cells was declined to 5.8±1.7% (Fig. 2F). Furthermore, the immunofluorescence analysis indicated that shRNA-*Rictor* treatment down-regulated the sarcomeric protein expression, accompanied by a disordered sarcomeric structure (Fig. 2G). These results indicated that *Rictor* knockdown in mES cells inhibited the cardiomyocyte differentiation, decreased the number of cardiomyocytes, and resulted in the unorganized sarcomeres.

Dual Raptor and Rictor knockdown inhibited differentiation of mES cells into cardiomyocytes

According to the above-mentioned different effects of *Raptor* and *Rictor*, we further investigated how both *Raptor* and *Rictor* double knockdown impacted the cardiomyocyte differentiation from mES cells. The efficiency of *Raptor* and *Rictor* double knockdown was validated by the decreased levels of p-p70S6K and p-Akt (Fig. 3A). At day 7 and 8, approximately 18±2% and 38±3% of EBs with both shRNA-*Raptor* and shRNA-*Rictor* treatment showed spontaneous contractions, respectively, while they were 42±5% and 70±6% in the control group (Fig. 3B). The western blot analysis further assessed the expression levels of mesoderm and cardiac marker genes during the process of differentiation. The specific protein of mesoderm layer brachyury was down-regulated in cells after the knockdown of *Raptor* and *Rictor* in comparison to their levels in the control group (Fig. 3C). Nkx2.5 and α-Actinin were also decreased by both shRNA-*Raptor* and shRNA-*Rictor* treatment (Fig. 3C; Fig. 3D). Then flow cytometry analysis further validated that the number of α-Actinin-positive cells decreased to 4.2±0.2% when compared to that of the control (11.6±2.2%) (Fig. 3E). The immunofluorescence analysis of the myofilaments in beating areas showed more unorganized sarcomeric myofilaments followed by the knockdown of *Raptor* and *Rictor* when compared with control group (Fig. 3F). These findings prompted that the effects of targeting both *Raptor* and *Rictor* were consistent with the influence of *Rictor* knockdown on mesoderm formation and cardiomyocyte differentiation.

The relationship between Raptor and Rictor during cardiomyocyte differentiation

To further illustrate the probable relationship of Raptor/mTORC1 and Rictor/mTORC2 on cardiomyocyte differentiation, we characterized the pathways between the two mTOR complexes. Proteins in downstream of Raptor (p70S6K and p-p70S6K), Rictor (Akt and p-Akt Ser^{473}), and p-Rictor Thr^{1135} were detected in *Raptor* or *Rictor* knockdown mES cells. The expression levels of Rictor and p-Akt (Ser^{473}) were significantly up-regulated after shRNA-*Raptor* treatment. Meanwhile, a marked reduction in the phosphorylation level of Rictor at Thr^{1135}, the downstream of p70S6K and upstream of Rictor, was detected in shRNA-*Raptor* cells (Fig. 1A). On the contrary, the expression for Raptor and p-p70S6K (Thr^{389}) did not change in shRNA-*Rictor* cells (Fig. 2A). Furthermore, the results showed that

p-Rictor (Thr[1135]) was clearly down-regulated with the decreased levels of Raptor, Rictor and downstream proteins (p-p70S6K and p-Akt) when both *Raptor* and *Rictor* were knockdown (Fig. 3A). These data were consistent with the effect of *Rictor* knockdown alone. The results demonstrated that the activation of Rictor was critical in regulating cardiomyocyte differentiation from mES cells. Knockdown of Raptor/mTORC1 resulted in decreased phosphorylation of Rictor (Thr1135), which subsequently activated Rictor/mTORC2 in the differentiation of mES cells into cardiomyocytes.

Discussion

The mTOR forms two independent complexes, mTORC1 and mTORC2, which have distinct partners (Moschella *et al.*, 2013).

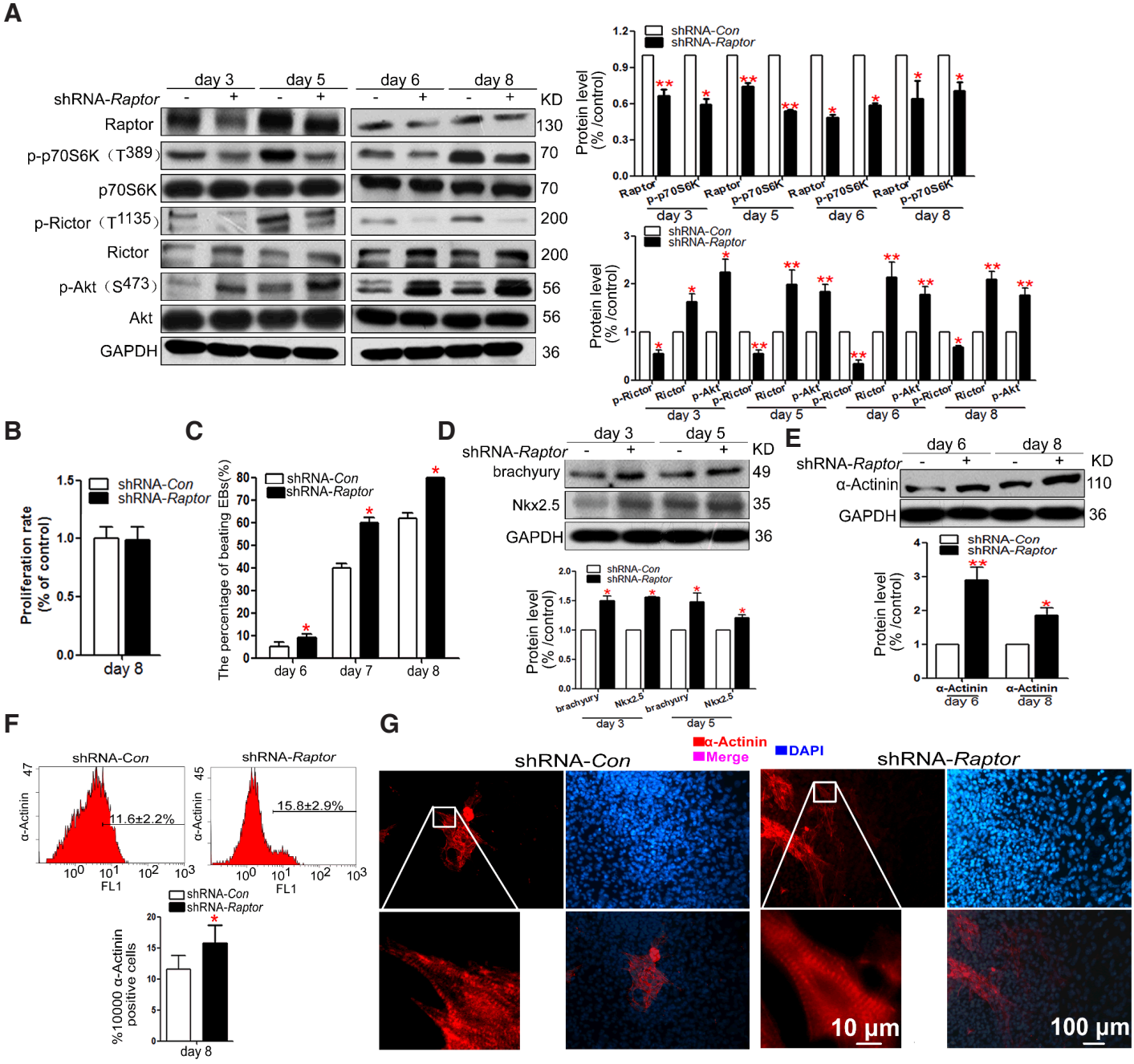

Fig. 1. The effect of *Raptor* knockdown on cardiomyocyte differentiation from mES cells *in vitro*. **(A)** Raptor *was knockdown in mES cells during the cardiomyocyte differentiation.* **(B)** *The proliferation rate of mES cells after infection by shRNA-*Raptor. **(C)** *The percentage of beating EBs from day 6 to 8.* **(D)** *The expression levels of mesoderm marker brachyury and cardiac progenitor cell marker Nkx2.5 during the early differentiation of mES cells.* **(E)** *The level of cardiac specific protein α-Actinin at day 6 and 8.* **(F)** *Quantification of sarcomeric protein (α-Actinin) by flow cytometry on day 8.* **(G)** *Immunofluoresence analysis of cardiac myofilament α-Actinin in EBs on day 8. DAPI staining indicated the location of cells. Bar=10 μm or 100 μm. Similar data was obtained from at least 3 independent experiments. *P<0.05, **P<0.01 (shRNA-*Raptor vs. *shRNA-*Con*).*

Raptor and Rictor are known as the core components of mTORC1 and mTORC2, respectively. As a molecular sensor of cell growth and proliferation, mTORC1 serves as a key signaling mediating protein synthesis (Goodman, 2014; Proud, 2009), lipid metabolism (Ricoult *et al.*, 2013), energy balance (Catania *et al.*, 2011), and cardiac hypertrophy (Balasubramanian *et al.*, 2009). The mTORC2 was related to diverse cellular processes, including cytoskeleton organization and cell migration (Jacinto *et al.*, 2006; Sarbassov *et al.*, 2014), cardiac structure, and function preservation (Zhao *et al.*, 2014). Cardiac *Raptor* deletion resulted in severe dysfunction, including dilated cardiomyopathy, reduced mitochondrial content, and increased apoptosis and autophagy (Shende *et al.*, 2011). The mTORC1 could improve cardiac viability and function through inhibiting the activity of 4E-BP1 (Zhang *et al.*, 2010). Study in murine embryonic fibroblasts and heart showed that the protein abundance and stability of Akt, PKCα, PKCβII, PKCδ, and PKCε, depended on the presence of active mTORC2 (Shende *et al.*, 2016; Ikenoue *et al.*, 2008; Facchinetti *et al.*, 2008). A recent study reported that cardiac specific mTORC2 disruption by *Rictor* knockout showed cardiac dysfunction and heart failure and attenuated adaption to pressure overload (Sciarretta *et al.*, 2015). In mice deficient for Raptor or Rictor, embryo died early during development after missing Raptor, while embryos could survive until E10.5 after loss of Rictor (Guertin *et al.*, 2006). Loss of Rictor/mTORC2 resulted in a suppressed differentiation of C2C12 myoblasts by interfering phosphorylation level of Akt (Ser^{473}) (Shu *et al.*, 2009). Previous

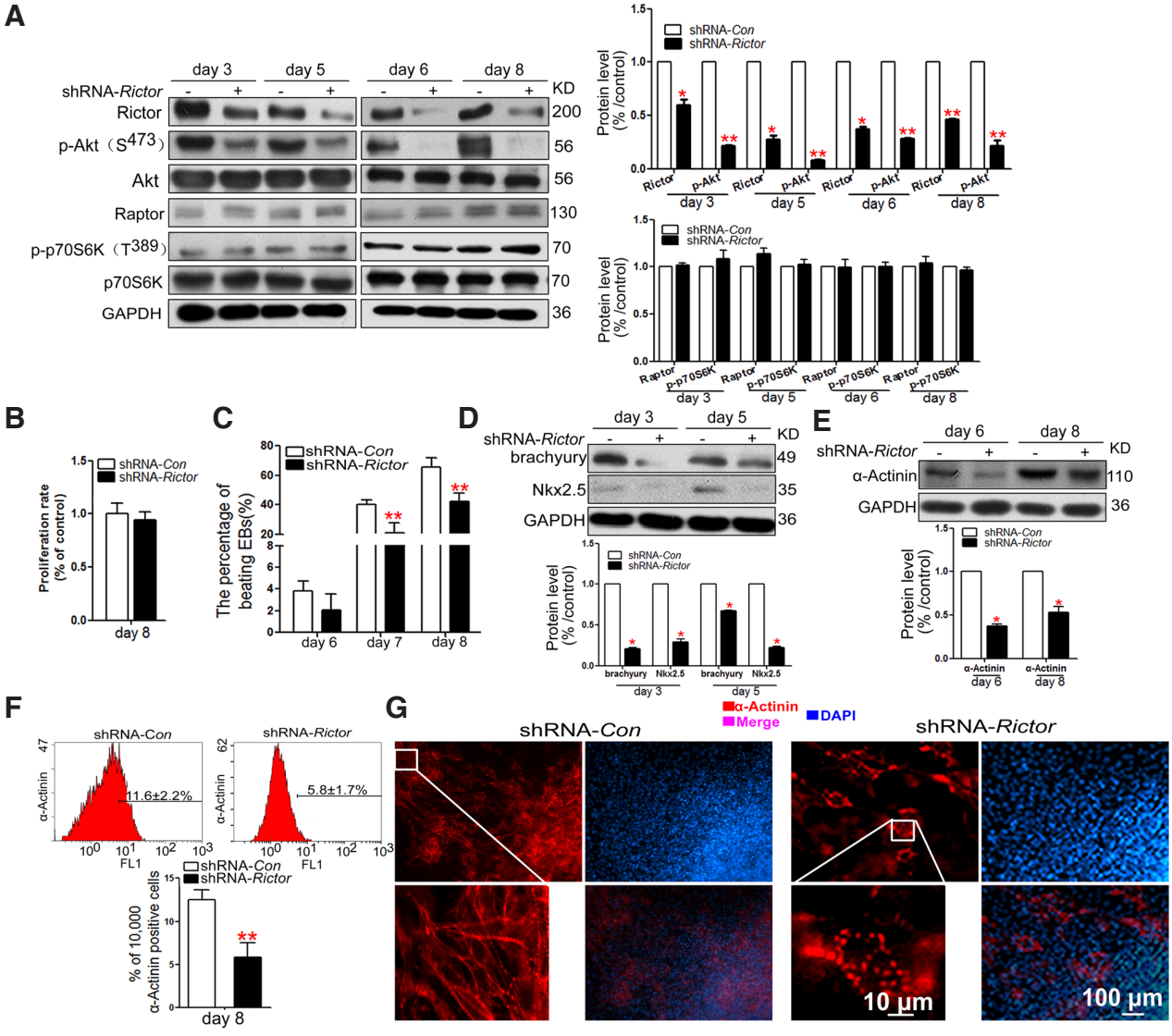

Fig. 2. The effect of *Rictor* knockdown on cardiomyocyte differentiation from mES cells *in vitro*. **(A)** Rictor *was knockdown in mES cells during the cardiomyocyte differentiation.* **(B)** *The proliferation rate of shRNA-*Rictor *and shRNA-*Con *mES cells.* **(C)** *The beating phenotype evaluated from day 6 to 8 after EBs plating.* **(D)** *The expression levels of mesoderm layer protein brachyury and cardiac progenitor protein Nkx2.5 during the early mES cell differentiation.* **(E)** *The expression level of sarcomeric protein detected by Western Blot at day 6 and 8.* **(F)** *The percentage of α-Actinin-positive cells by flow cytometry on day 8.* **(G)** *Analysis of the expression and distribution of sarcomeric α-Actinin in EBs on day 8 by immunofluoresence. DAPI staining indicated the location of cells. Bar=10 μm or 100 μm. Similar data was obtained from at least 3 independent experiments. *P<0.05, **P<0.01 (shRNA-*Rictor vs. *shRNA-*Con*).*

study also reported that mTORC1 and mTORC2 played opposite effects on the mesenchymal stem cell differentiation (Martin *et al.*, 2015). However, how Raptor/mTORC1 and Rictor/mTORC2 involved in cardiomyocyte differentiation have not been well explored. *In vitro* mES cell differentiation into cardiomyocytes was a unique model that could not only be used to study cardiac development, but also had therapeutic potential (Takahashi *et al.*, 2003; Wiese *et al.*, 2011). In our study, we employed mES cells stably infected with shRNA-*Raptor* or shRNA-*Rictor* to examine the possible role of mTOR complexes in controlling cardiomyocyte differentiation.

To characterize the cardiomyocyte differentiation of mES cells, we assessed specific protein of mesoderm layer brachyury (Xu *et al.*, 2015). Nkx2.5 and α-Actinin were indentified as the markers for the cardiac progenitor cells (Singla *et al.*, 2005) and the early differentiated cardiomyocytes (Ding *et al.*, 2008). Our results demonstrated that the proteins related to cardiomyocyte differentiation were significantly up-regulated after shRNA-*Raptor* treatment, while the cardiogenesis was suppressed in shRNA-*Rictor* treated mES

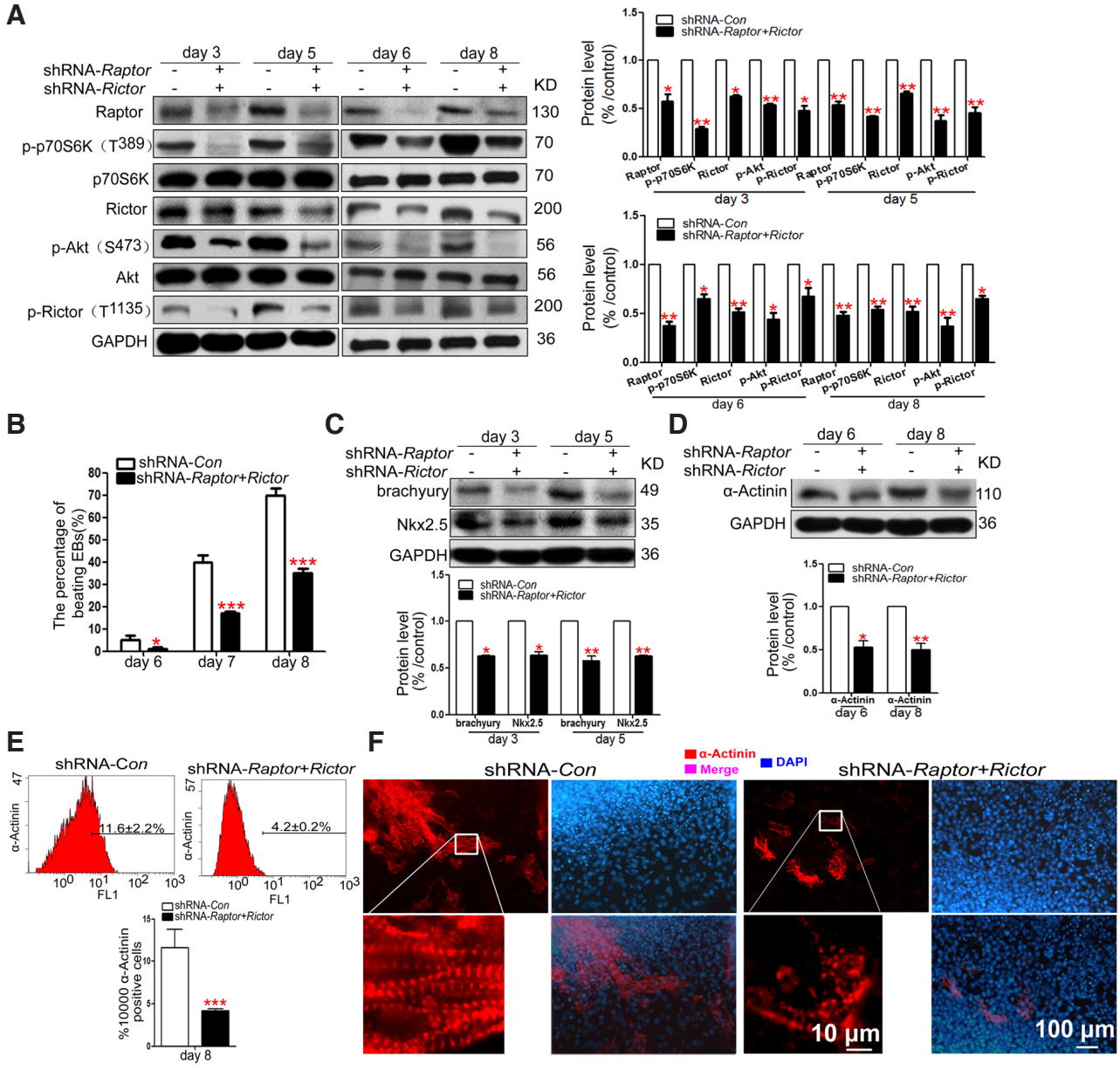

Fig. 3. Targeted knockdown of *Raptor* and *Rictor* inhibited mES cell differentiation into cardiomyocytes *in vitro*. (A) *Both* Raptor *and* Rictor *were knockdown in mES cells.* **(B)** *shRNA-*Raptor *and shRNA-*Rictor *have the effect of suppression in the cardiac differentiation. The beating activity from day 6 to 8 in EBs generated.* **(C)** *The expression levels of mesoderm layer protein brachyury and cardiac progenitor protein Nkx2.5 at day 3 and 5.* **(D)** *The level of cardiomyocyte specific protein α-Actinin analyzed by Western Blot at day 6 and 8.* **(E)** *shRNA-*Raptor *and shRNA-*Rictor *decreased the proportion of α-Actinin-positive cells by flow cytometry analysis.* **(F)** *Immunofluoresence analysis of α-Actinin in EBs on day 8 after infection. DAPI staining indicated location of cells. Bar=10 μm or 100 μm. Similar data was obtained from at least 3 independent experiments.* n*=3. *P<0.05, **P<0.01, ***P<0.001 (shRNA-*Raptor+Rictor vs. *shRNA-*Con*).*

cells. Knockdown of both *Raptor* and *Rictor* showed the similar results to the *Rictor*-knockdown cells. It suggested that Raptor and Rictor played different roles in the cardiomyocyte differentiation from mES cells.

To further investigate the possible relationship of Raptor/mTORC1 and Rictor/mTORC2 in cardiomyocyte differentiation from mES cells, we examined the pathways between mTORC1 and mTORC2. Akt was a well-known protein linking the signaling of the two complexes that could be phosphorylated by mTORC2 or PDK1 (Xie *et al.*, 2010). Furthermore, phosphorylation of Rictor (Thr1135) was shown to require the activation of mTORC1, specifically, the p70S6K, which was the downstream of mTORC1 as tested in HEK293 and Hela cells. The mTORC2 and Akt signaling were prevented in T1135A mutant cells (Treins *et al.*, 2010; Julien *et al.*, 2010). Therefore, we hypothesized that mTORC1 could negatively regulate the activity of mTORC2 through the phosphorylation of Rictor at Thr1135 by p70S6K in the cardiomyocyte differentiation from mES cells. Our investigation validated that *Raptor* knockdown decreased p70S6K-mediated phosphorylation of Rictor (Thr1135), inducing the activation of mTORC2, while the signaling of mTORC1 was not altered by knockdown of *Rictor*. It indicated that Rictor/mTORC2 signaling could be activated in *Raptor* knockdown mES cells. Rictor/mTORC2 pathway was the more critical mTOR complex in regulating mES cell differentiation into cardiomyocytes.

In conclusion, Raptor/mTORC1 and Rictor/mTORC2 played the different roles in the cardiomyocyte differentiation of mES cells. Knockdown of *Raptor* resulted in markedly increase in mesoderm protein brachyury, early cardiomyocyte protein Nkx2.5 and α-Actinin. Knockdown of both Raptor and Rictor could suppress mES cell differentiation into cardiomyocytes, which was similar to the effect of shRNA-*Rictor* in mES cells. Knockdown of *Raptor* decreased the expression level of p-p70S6K which subsequently downregulated the phosphorylation of Rictor at Thr1135, resulting in activation of Rictor/mTORC2 (Fig. 4). The activation of Rictor/mTORC2 was critical for facilitating cardiomyocyte differentiation from mES cells, which uncovered a promising target that regulates cardiogenesis from ES cells and might provide a useful reference for the potential application of the induced pluripotent stem cells.

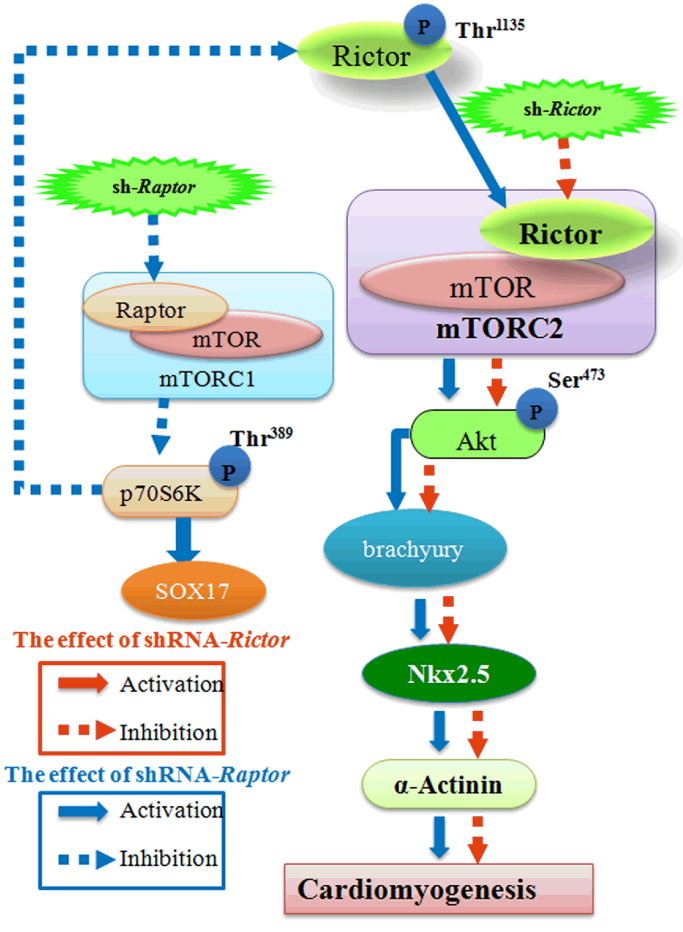

Fig. 4. The relationship of Raptor/mTORC1 and Rictor/mTORC2 in cardiomyocyte differentiation from mES cells. *Silencing mTORC1 by shRNA-*Raptor *could decreased the level of p-p70S6K (Thr389), which subsequently downregulated phosphorylation of Rictor at Thr1135, resulting in activation of Rictor/mTORC2. Red arrows represent the effect of shRNA-*Rictor *on its downstream signaling and blue arrows represent the influence of shRNA-*Raptor*. Solid and dashed arrows represent the effect of promotion and inhibition, respectively.*

Materials and Methods

Cell culture and cardiomyocyte differentiation

The mES cell line D3 (mES cell D3, purchased from American Type Culture Collection, USA) was cultivated in undifferentiated state on feeder layer of primary cultures of mouse embryonic fibroblasts. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Germany) supplemented with 10% Fetal Bovine Serum (FBS, Life Technologies, Germany), 1% non-essential amino acids (NEAA, Life Technologies, Germany), 0.1 mM β-mercaptoethanol (Sigma Aldrich, USA) and 10^6 units/L leukemia inhibitory factor (LIF, Chemicon, USA) in 5% CO_2 atmosphere at 37°C. Cells were routinely passaged every 2/3 days.

The mES cells were differentiated *in vitro* through the formation of EBs using the hanging drop method. EBs were generated at a density of 600 cells/droplet in differentiation medium (DMEM with 20% FBS, 0.1 mM β-mercaptoethanol and 1% NEAA). For 3 days of culture, EBs grew as single aggregates in hanging drops. After floating in the petri dishes for additional 2 days, EBs plated separately into gelatin (0.1%, Sigma Aldrich, USA)-coated 6-well plates. Morphology and beating behavior of EBs were monitored by light microscopy (Wo *et al.*, 2008).

Raptor and Rictor targeted shRNA infection

Lentivirus with Raptor short hairpin RNA (shRNA), Rictor shRNA or control shRNA were infected into mES cells. The lentiviruses labeled with GFP were constructed by Genepharma Company (Shanghai, China). The sequences were as follows: shRNA-*control*: TTCTCCGAACGTGTCACGTTC; shRNA-*Rictor*: GCCAGTAAGATGGGAATCATT; shRNA-*Raptor*: GCCCGAGTCTGTGAATGTAAT (Shu and Houghton, 2009).

For infection, mES cells were seeded into 12-well plates cultivated in medium supplemented with 10% fetal bovine serum and 5ng/ml LIF at a density of 10^4 cells per well. On the next day, the cells were infected with lentivirus at a mutiplicity of infection of 50 PFU/cell. After infected for 24 hours, cells were harvested for EB formation.

Cell proliferation assay

The infected mES cells of day 8 were seeded into 96-well plates at an initial density of 10^4 cells/well. At the experiment day, 100 μl DMEM containing 0.5 mg/ml MTT was added to each well and incubated at 37°C for 4 h after which 100 μl DMSO was added. Absorbance was measured at 560 nm using a microplate reader. Proliferation rate of cells was expressed as the ratio of shRNA-*Raptor* or shRNA-*Rictor* cells to shRNA-*Con* cells (Zhang *et al.*, 2015).

Western blot

Samples were lysed using RIPA buffer (1 mmol/L PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin) for 30 min and centrifuged. The protein concentration in the supernatant was determined using the Bio-Rad protein kit (Hercules, USA) and equal amount of proteins per sample were loaded on a sodium dodecyl sulphate (SDS)-polyacrylamide gel. Subsequently, the separated proteins were electrophoretically transferred to 0.45 μm pore size polyvinylidene difluoride membranes (PVDF, Millipore, USA) and blocked with 5% milk diluted in PBS with 0.1% Tween-20 at room temperature. Blots then were incubated overnight at 4.C with the appropriate primary antibodies. The antibodies included mouse monoclonal anti-GAPDH (Sigma Aldrich, G-8975, USA), mouse monoclonal anti-α-Actinin (Sigma Aldrich, A-7811, USA), rabbit polyclonal anti-p-Akt1/2/3 (Ser 473)-R (Santa Cruz, sc-7985-R, USA), rabbit monoclonal anti-brachyury (Santa Cruz, sc-20109, USA), rabbit polyclonal anti-Nkx2.5 (Abcam, ab-35842, USA), goat polyclonal anti-Rictor (Santa Cruz, sc-50678, USA), rabbit monoclonal anti-Raptor (Abcam, ab40768, USA), rabbit monoclonal anti-p-Rictor (Thr1135) (Cell Signaling, 3806, USA), rabbit monoclonal anti-p-p70S6K (Thr389) (Cell Signaling, 9234, USA), rabbit anti-p70S6K (Cell Signaling, 9202, USA). The membrane was washed with TTBS and incubated with corresponding secondary antibody (1:5,000 dilution) at room temperature (RT) for 1 hour. Chemiluminescence was detected with an enhanced chemiluminescent substrate (ECL, Pierce, USA) and developed. Images were acquired with a scanner and densitometry was determined using ImageJ software version 1.46s (Wo *et al.*, 2008).

Immunofluorescence analysis

Immunofluorescence was performed with mES cells derived cardiomyocytes (mES-CMs). The mES-CMs of day 8 were fixed by methanol at -20 °C for 15 minutes, followed by permeabilization with 0.05% Triton-100. Afterwards, cells were blocked with 10% FBS in PBS for 1 h at room temperature and stained with the primary antibody against α-Actinin (Sigma Aldrich, A-7811, USA) over night at 4 °C. The next day, cells were incubated for 2 h at room temperature using Alexa fluor 594-conjugated anti-mouse IgG antibody (Invitrogen, USA). Images were visualized with fluorescence inverted microscope or an Olympus IX81-FV1000 inverted multiphoton laser confocal microscope (Shen *et al.*, 2012).

Flow cytometry

Differentiated cell clusters obtained on day 8 were dissociated into single cells with 0.25% trypsin-EDTA. After this process, cells were fixed with 4% formaldehyde for 1 h and blocked by 3% BSA for another 1 h. Stained cells with the primary antibody against α-Actinin and stored for one night at 4°C. Cells were washed with PBS and incubated with Alexa fluor 488-conjugated anti-mouse IgG antibody (Invitrogen, USA) at 4 °C for 2h. For flow cytometry analysis of cardiac α-Actinin-positive cardiomyocytes, cells were centrifuged and suspended in 0.5% PBS until analysis. The fluorochrome was detected at 530 nm in the FL-1 and 575 nm in the FL-2 channel. All data analyses were carried out by using Cell Quest software (Zhu *et al.*, 2012).

Statistics

All experiments were performed at least 3 times to assure reproducibility and data were expressed as mean±standard deviation. Statistical significance was evaluated using one-way Analysis of Variance (ANOVA) with SPSS for Windows (version 13.0). A value of $P<0.05$ was considered statistically significant.

Acknowledgements

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