Differentiation of glutamatergic neurons from mouse embryonic stem cells requires raptor S6K signaling

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Abstract Although the mammalian target of rapamycin complex 1 (mTORC1) functions as an important signaling complex in many cellular processes, the role of mTORC1 in neurons derived from embryonic stem cells (ESCs) has been less explored. Here, using a modified protocol to differentiate mouse ESCs (mESCs) into almost uniform glutamatergic neurons, we explored the importance of raptor/mTORC1 in the differentiation of mESCs. Raptor gene-trap mESCs, and raptor-knockdown mESCs formed smaller-sized embryonic bodies than the wild type and failed to undergo neuronal differentiation. Treatment with 1 μM rapamycin starting at the point when neuronal precursors began to differentiate from mESCs caused the gradual loss of neurites, shrinkage of soma, and a decreased ratio of neurite length to cell number over 48 to 72 h of treatment. This change was accompanied by activation of caspase-3 and S6 kinase (S6K), but not 4E-binding protein 1 (4EBP1). Knockdown of raptor during neuronal differentiation from mESCs also resulted in gradual loss of neurites and shrinkage of cell bodies. Loss of neurite density resulting from rapamycin treatment could be reversed by overexpression of S6K T389E. Taken together, these data demonstrate that raptor/mTORC1/S6K plays a critical role in the differentiation and survival of neurons derived from mESCs.

Introduction

In recent years, considerable research efforts have been directed towards the generation of neurons from human and mouse embryonic stem cells (ESCs). Examples include motor neurons (Chipman et al., 2012), dopaminergic neurons (Kriks et al., 2011), GABAergic cerebral cortical interneurons (Goulburn et al., 2012) and glutamatergic neurons (Bibel et al., 2004; Chuang et al., 2011; Hubbard et al., 2012). Because of their physiological relevance and potential applications, in addition to cell lines or primary neurons, neuronal cells derived from ESCs have gradually emerged as one focus (Ban et al., 2007; Erceg et al., 2009). Currently, however, two obstacles still hinder the application of the in vitro ESC model in translational medicine. First, differentiation protocols result in the simultaneous production of heterogeneous cell populations, thus constraining studies on selected subsets of cells; and second, relatively little is known about differentiation pathways in culture and how these pathways compare with those in developing embryos (Wobus and Boheler, 2005).
The serine-threonine protein kinase, mammalian target of rapamycin (mTOR) is a central regulator of many cellular processes including growth, survival, metabolism, and many diseases (Dazert and Hall, 2011). In mammalian cells, mTOR forms complexes, termed mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 consists of mTOR, mLST8, DEPTOR, PRAS40, and raptor. It controls the translation by directly phosphorylating the downstream S6 kinase (S6K) at the Thr389 residue and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) at the Thr37/46 residues. mTORC2 consists of mTOR, mLST8, DEPTOR, PROTOR, mSin1, and rictor. It phosphorylates Akt, PKCα, and SGK1, and regulates actin dynamics (for reviews see Appenzeller-Herzog and Hall, 2012; Laplante and Sabatini, 2012)). Knockout of mTOR gene in mice resulted in embryonic lethality (Murakami et al., 2012). Like mice null for mTOR, embryos lacking mLST8 and rictor die around E10.5 (Guertin et al., 2006). In the same study, raptor-deficient embryos were also found to die early in development. The role of raptor/mTORC1 in neurons differentiated from mouse ESCs (mESCs) is unknown.

Several studies using cell lines or primary cells have reported that mTOR plays a role in neuronal growth. mTOR contributes to increases in filopodia/spine number (Kumar et al., 2002), and synaptic transmission (Weston et al., 2005; Tavazoie et al., 2005), dendritic arbor formation (Jaworski et al., 2005), long-lasting long-term potentiation (St. Louis, MO, USA) unless indicated. Therefore, the cultivated mESC medium contained Glasgow Minimum Essential Medium (GMEM; Sigma) supplemented with 0.05 mM β-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, 1× non-essential amino acids, 10% fetal bovine serum (FBS), and 500–1000 U leukemia inhibitory factor (LIF; Millipore ESGRO). As the confuency of cell growth reached 90%–95%, cells were split using trypsin/EDTA and continually cultivated in a 37 °C, 5% CO2 incubator to maintain the mESCs. The procedures used for EB dissociation and neuronal differentiation, as well as partial characterization of neurons, have been described previously (Chuang et al., 2011; Lin, 2012). Briefly, we cultivated mESCs in 10-cm bacterial dishes containing mESC medium but no LIF. The mESCs gradually proliferated and aggregated to form spherical shapes called embryonic bodies (EBs). The medium was changed every two days by passing cells through a 40-μm nylon cell strainer and placing them in a new bacterial dish. To initiate neuronal differentiation, we directly added a final concentration of 5 μM all-trans retinoic acid to the medium on day 4 after EB formation started. Four more days later, EBs were dissociated for around 8 to 10 min in a trypsinization buffer, which was freshly prepared from 0.05% powdered trypsin and 0.02% EDTA dissolved in a phosphate buffered saline (PBS) solution. To collect cells, the mixture was gently pipetted up and down and centrifuged for 5 min at 1000 rpm at room temperature. The pellet in the tube was resuspended in N2 medium (DMEM/F12 supplemented with 8.6 mM glucose, 0.5 mM L-glutamine, 20 mM NaHCO3, and 1× N2 supplement) and filtered through a 40-μm cell strainer. An optimal cell density of around 1.35 × 10^6/cm² was determined and seeded onto poly-DL-ornithine/laminin-coated plates. The medium was changed to fresh N2 medium at 2 and 24 h after plating. At 48 h after plating, we changed the N2 medium to Neurobasal-B27 medium (Neurobasal medium supplemented with 0.5 mM L-glutamine and 1× B27 supplement). Thereafter, the Neurobasal-B27 medium was partially replaced with fresh medium every other day to maintain the neuronal culture.

Feeder-independent mESC culture, embryoid body dissociation, and neuronal differentiation

The mESC clone ES-E14TG2a was purchased from American Type Culture Collection and cultivated in a feeder-independent pattern by which the culture plates were coated with 0.1% gelatin for at least 30 min before seeding mESCs onto them. The methodology was originally based on the protocol of Bibel et al. (2004). They developed the method and reported that essentially all neurons were positive for glutamate vesicular transporter 1. After 1–3 weeks in culture, the synaptic activity observed in their cultures can be accounted for by the action of majority glutamate and minor GABA (5%) on postsynaptic cells. Initially, after all of the procedures were deliberately repeated, we found that a homogeneous population was still not always obtained (Chuang et al., 2011). After many attempts, we modified some procedures and found that it is crucial to dissociate the embryoid bodies (EBs) with appropriate timing rather than completely trypsinize EBs. Eventually, we consistently established almost completely uniform neurons derived from mESCs in our laboratory.

Materials and methods

Reagents

Antibodies included type III beta-tubulin, synaptotagmin, AMPA receptor (AMPA) (Abcam, Cambridge, UK), PSD-95, Oct4 (BD Falcon, Franklin Lakes, NJ, USA), MAP2 (Millipore, Billerica, MA, USA); vGLUT1/2 (SYSY, Gottingen, Germany), Akt Thr308, S6K Thr389, 4EBP1 Thr37/46, p44/42 MAPK, p44/42 MAPKThr202/Tyr204, cleaved caspase-3 Asp175 (Cell Signaling Technology, Danvers, MA, USA), pro-caspase-3, S6K (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt, Akt Ser473, 4EBP1, 4EBP1 Thr37, S6, S6 Ser235/236 (Epitomics, Burlingame, CA, USA), and horseradish peroxidase (HRP) anti-rabbit and anti-mouse secondary antibodies (Jackson, West Grove, PA, USA). Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12), N2 supplement, Neurobasal medium, B27 supplement, Neurobasal A medium, and geneticin/G418 were obtained from Invitrogen/Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Thermo Scientific/Hyclone (Logan, UT, USA). All other chemicals came from Sigma (St. Louis, MO, USA) unless indicated.
Raptor gene trap mESC clone selection, three-primer PCR, and Southern blot

We obtained the raptor gene trap mESC clone RRO611 containing pGT2Lxf gene trap vector (~8.6 kb) integrated into the intron sequence (~51 kb) between exon 1 and exon 2 of the raptor gene from BayGenomics/Mutant Mouse Regional Resource Center (MMRRC, San Francisco, CA, USA), a product of the International Gene Trap Consortium. This clone was from mouse strain 129/Ola and its parental mESCs were the E14TG2a clone. To obtain the homozygote raptor gene trap clone, we applied geneticin 1.75–2.25 mg/ml to the culture of RRO611 mESCs. After drug selection for 9 to 12 days, we selected several clones grown from a single cell and verified them by three-primer PCR. We mapped the insertion site of the trapping vector pGT2Lxf around 31 kb downstream of exon 1 on the raptor gene. Based on this, the primers designed to verify the genotype were forward primer 5′-GAAGAGGTCACAATGTCC-3′ within the intron, reverse primer 5′-CCTCCGGCAAA-3′ within the trapping vector, and another reverse primer 5′-CTGATCCAGCGCAGGAAG-3′ within the intron, by which a PCR with short extension time would produce around 800 and 1400 base pair fragments if the genotype is heterozygote. For Southern blot analysis, mESC genomic DNA was lyzed in a buffer that contained 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% (w/v) N-Lauroylsarcosine, and 1 mg/ml proteinase K. After phenol/chloroform extraction, 5 M NaCl precipitation, and ice-cold ethanol purification, the DNA was subjected to restriction enzyme BamHI digestion, run onto agarose gel, and transferred to nitrocellulose paper. A DNA fragment of around 570 base pairs was originally generated by PCR with forward primer 5′-ATCCTGGCTACTTGCCGCTCCAG-3′ and reverse primer 5′-CTGTCGCAACAGCCCA-3′. This was further used as a template to obtain a single strand probe with radioisotope labeling, by performing PCR containing single primers, dATP, dTTP, dGTP and 32P dCTP. The outcome shows two bands with 6200 and 4500 base pairs if the genotype is heterozygote.

Primary cortical neurons cultured from mice

In the experiments using primary cortical neurons as a positive control, cerebral cortices were dissociated from BALB/c mice on postnatal day 2 (P2). The protocol was adopted from Brewer and Torricelli (2007) with modifications. Briefly, about 85 mg of dissociated cortex tissue was maintained temporarily in cold Neurobasal A medium. We transferred cell pieces to a tube for papain digestion by shaking for 20 min. The residues after gravity precipitation were incubated in fresh Neurobasal A medium and centrifuging at 1250 rpm for 5 min. They were plated onto poly-D-lysin coated culture dishes at a density of 1.04 × 10^5/cm². The medium was subsequently changed every three days using Neurobasal A medium.

Immunocytochemistry of neuronal cells and neurite density analysis

Differentiated neurons were seeded on glass coverslips coated with poly-DL-ornithine/laminin or poly-D-lysine/laminin (Roche, Mannheim, Germany). At a given time after differentiation, cells were washed with PBS, fixed in 4% paraformaldehyde prepared in PBS for 30 min, and permeabilized in 0.3% Triton X100 in PBS for 10 min. The sample was further blocked in 1% bovine serum albumin made in PBS for 1 h at room temperature. A corresponding primary antibody was added for 2 h, followed by washing with PBS. A secondary antibody such as Alexa 488-conjugated anti-mouse (Invitrogen), Alexa 488-conjugated anti-rabbit (Invitrogen), DyLight 549-conjugated anti-rabbit IgG (Invitrogen), or rhodamine-conjugated anti-mouse (Jackson ImmunoResearch, West Grove, PA, USA) was incubated for 45 min followed by a PBS wash. Nuclei of cells were stained with 4′,6′-diamidino-2-phenyindole (DAPI) for an additional 15 min, followed by another PBS wash. The coverslip was then mounted on a glass slide using Fluoromount G (Southern Biotech, Birmingham, AL, USA). Images were collected using a Leica SP2 confocal microscope connected to a CCD camera (Leica, Wetzlar, Germany) or using a Zeiss Axio Observer D1 microscope (Carl Zeiss, Jena, Germany). We defined density of neurites as neurite length divided by neuron number measured by Metamorph software or Image-Pro Plus software. The images were usually taken as three different fields in each experiment, which was repeated three to four times. Therefore, a total of around 9 to 12 images were captured. Each image typically contained more than 50 counted neurons, so a total of around 500 neurons were evaluated. Figures were prepared using Adobe software.

Construction of overexpression plasmids and RNAi viral particle preparation

In order to make proteins that overexpress in mESCs or neurons differentiated from mESCs, we replaced the U6 promoter in the pLKO.1 puromycin construct with cytomegalovirus (CMV) promoter, a constitutive promoter. 56K WT or a constitutive active form, 56K T389E, was engineered to fuse with green fluorescent protein (GFP) in the plasmid. By taking advantage of the pLKO.1 backbone, the infection rate, selection, and preparation of viruses will be the same as the one using RNAi. For RNAi viral particle preparation, bacterial clones or plasmids containing short hairpin RNA oligonucleotides of the target genes were obtained from the National RNAi Core Facility (Genomic Research Center, Academia Sinica, Taipei, Taiwan). Functional clones and corresponding effective target sequences of control, raptor, and rictor were verified and are listed below: luciferase (control), 5′-CGCTAGTACTTGCAAGATGTC-3′, raptor sequence, 5′-CTCATGCTCAAGTCTCCTTCAA-3′, and rictor sequence, 5′-GCACGTAAGATGGAAATCATT-3′. RNAi plasmids were co-transfected with two other viral packaging plasmids, pCMV8.91 and pMD.G, into HEK 293T cells. The virus-containing medium was collected after transfection for...
40 h and concentrated by ultracentrifugation at 25,000 rpm for 2 h. Pellets were concentrated and stored at −80 °C until use. We followed a protocol provided by the RNAi Core Facility in Academia Sinica, Taipei, Taiwan to measure the RNAi titer (http://rnai.genmed.sinica.edu.tw/file/protocol/4_1_EstimationLentivirusTiterRIUV1.pdf). To test the virus titer, we also modified this protocol using primary culture of mice cortical neurons, a cellular model that is much more sensitive to virus infection, instead of using A549 cells.

Cell lysate and immunoblot assay

Cells used for the immunoblot assays were processed as previously described (Lin et al., 2002). Briefly, one 3.5-cm dish of frozen cells was scraped into 0.1 ml of lysis buffer (50 mM Tris base, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 mM calyculin A, 0.5% Triton X-100, 1 tablet/50 ml of protease inhibitor (Roche)). When processing EB lysate, EBs were collected at 1000 rpm centrifuge for 5 min, lysed in lysis buffer, and transferred into a 2 ml Dounce homogenizer and homogenized with 25–35 strokes. Lysates were centrifuged at 13,500 rpm for 10 min. Aliquots of the supernatants containing equal amounts of protein were measured by Bradford assay (Bio-Rad). For low-molecular-weight proteins such as 4EBP1, cell lysates were prepared as described above and subjected to tricine sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Schagger’s (2006) method. Briefly, the separation gel contained an additional 0.1% glycercerol in a regular format. The cathode buffer (0.1 M Tris, 0.1 M Tricine, and 0.1% SDS; pH 8.25) and anode buffer (0.1 M Tris and 0.0225 M HCl; pH 8.9) were loaded onto a gel apparatus, separated, and transferred. Blots were visualized using a horseradish peroxide (HRP)-conjugated secondary antibody followed by chemiluminescence as outlined by the manufacturer (Thermo Scientific/Pierce, Rockford, IL, USA).

Statistical analysis

Data are presented as the mean ± SE. Treatment effects were evaluated using a two-tailed Student’s t-test. A P value of less than 0.05 was considered statistically significant.

Results

Characterization of mESCs derived neurons

We followed the basic protocol with caution to appropriately dissociate EBs rather than completely trypsinized EBs (Supplemental Fig. S1). Almost completely uniform neurons were derived from mESCs. The neuron type was confirmed by Immunostaining with a vesicular glutamate transporter (vGLUT1/2) antibody, a marker of glutamatergic neurons (Fig. 1A). More than 86% of cells were stained with both vGLUT1/2 and DAPI. Also, neurons/neurites were confirmed by staining with class III β-tubulin (red), a neuronal marker, as shown in Fig. 1B. Within the same field, synaptotagmin (green), which functions as a calcium sensor in the regulation of neurotransmitter release, also stained these neurons seven days after differentiation. We further verified the neurons by western blot analysis. As shown in Fig. 1C, AMPA receptor (AMPA), an ionotropic transmembrane receptor for glutamate in the central nervous system, began to be expressed on day 3 after differentiation, and was expressed at a higher level on day 7. PSD-95, a protein that is almost exclusively located in the post synaptic density of neurons and is involved in anchoring synaptic proteins, was also expressed with a similar temporal pattern to AMPAR and synaptotagmin. The lysates from HEK 293T cells and primary neuronal cultures of mice cerebral cortex functioned as negative and positive controls, respectively, for all the antibodies used. Within the same blot, Oct-4 and type III β-tubulin, which are markers of mESCs, and differentiated neurons, respectively, demonstrated that mESCs were properly maintained and neurons were well developed, and also indicated that synapses could potentially be constructed within these neurons.

Raptor gene-trap mESCs exhibited stunted growth and formation of EBs

We next examined the loss-of-function effect of raptor knockout on mESCs and neuronal differentiation. Homozygous mESC clones were selected from heterozygous clones using a high dose of genetin/G418 2.25 mg/ml. The genetic composition of these clones was verified. As shown in Fig. 2A, both three-primer PCR and Southern blot confirmed that homozygote clones were produced. However, trace amounts of protein were still detectable in western blot analysis, as loading more total lysate (Fig. 2B). We also performed raptor immunoprecipitation within these clones and sent them for MS-SPEC analysis (data not shown). The results showed that there was indeed residue of raptor protein. Therefore, the EB formation that resulted from these mESCs was smaller than that from wild type (Fig. 2C). We further characterized the alteration of signaling molecule phosphorylation in these clones. As shown in Fig. 2D, phosphorylation of S6K Thr389 still responded to insulin-like growth factor 1 (IGF-1) stimulation, while the raptor gene trap mESCs showed trace raptor expression. Among the signaling surveys, phosphorylation of MAPK, but not Akt, showed the most significant change with serum withdrawal and the re-addition of IGF-1.

Knockdown of raptor in mESCs by raptor RNAi also retarded EB formation

Above we demonstrated that homozygote mESCs derived from raptor gene-trap formed EBs were smaller than the wild type and often resulted in unsuccessful neuronal differentiation. To study the role of raptor in neurons differentiated from mESCs from a different perspective, we next established raptor knockdown mESCs using raptor RNAi infection, followed by puromycin selection. Rictor RNAi was used as an additional control for comparison. We verified that raptor and rictor protein levels and phosphorylation of the corresponding downstream signaling molecules such as S6K Thr389 and Akt Ser473 were decreased in knockdown mESCs (Fig. 3A). The phosphorylation of 4EBP1
Thr37/46 was not examined at this point as decreased phosphorylation in either S6K Thr389 or 4EBP1 Thr37/46 is sufficient to indicate that the raptor knockdown RNAi is effective. During neuronal differentiation, EBs formed from raptor-knockdown mESCs were smaller than those formed from luciferase- or rictor-knockdown mESCs (Fig. 3B). EB size was quantitated by integrating the area of images using Image Pro Plus. As shown in Fig. 3C, after six days, rictor RNAi also decreased EB size, although not to the same extent as raptor RNAi. This EB growth was similar to that in raptor gene-trap mESCs and also resulted in unsuccessful differentiation. Overall, this result confirmed that both raptor and rictor RNAi exhibited efficient target-specificity and are functional; it also indicated that both mTORC1 and mTORC2 may play a role in EB formation.

The decreased EB size formed from raptor gene-trap mESCs and raptor-knockdown mESCs may result from an imbalance between cell growth and/or cell death. To investigate this possibility, we examined S6K, 4EBP1 and caspase 3, which are related to cell growth and death, respectively (Fingar et al., 2002; Scott et al., 2007; Shima et al., 1998). We found that phosphorylation of S6K, but not 4EBP1 or cleaved caspase-3, is altered in EBs derived from raptor-knockdown mESCs during the formation of EBs (Fig. 3D). The results shown in Fig. 3E indicate that phosphorylation of S6K was significantly decreased in raptor knockdown EBs in comparison with the control at all time points examined. Conversely, although there was a slightly increased tendency for cleaved caspase-3 over the period of 2 to 6 days in the control luciferase RNAi group, there was no change in cleaved caspase 3 in EBs derived from raptor knockdown mESCs when compared to the luciferase knockdown group. These results may partially explain why the EBs are smaller in the raptor gene knockdown mESCs.

Rapamycin impaired neuronal differentiation of mESCs and/or impeded neuronal maturation at day 3, which may be associated with caspase and S6K activation, but not 4EBP1

Next, we studied the role of raptor/mTORC1 in the neurons differentiated from wild-type mESCs. Neuronal precursors trypsinized from EBs and plated on a culture matrix were
immediately treated with rapamycin (a TORC1 inhibitor) or wortmannin (a PI3K inhibitor). At 24 h, neurites of mESC-derived neurons treated with 1 μM rapamycin had spread out to a density similar to that of the control group (Fig. 4A); but at 72 h, neurite loss and soma shrinkage had begun to appear. This observation could also be interpreted to imply that rapamycin induces neurite retraction so as to delay neuronal maturation. The same phenomenon was observed when 0.2 μM rapamycin was used (data not shown). Neurite length divided by neuronal cell number is plotted in Fig. 4B. The effect of rapamycin treatment on this parameter was significant at 72 h after plating. After inhibitors treatment for 60 h, the effects on the corresponding downstream signaling molecules were examined (Fig. 4C). Rapamycin treatment only resulted in a decrease in phosphorylation of S6K Thr389, whereas wortmannin treatment decreased the phosphorylation of S6K Thr389, Akt Thr308, and Akt Ser473. The phosphorylation of 4EBP1 Thr37/46 at this point was not examined as decreased phosphorylation of either S6K Thr389 or 4EBP1 Thr37/46 is sufficient to examine whether the rapamycin treatment is effective. It is worth noting that 1 μM wortmannin significantly impeded neurite growth from the start of treatment (Fig. 4B, column 3).

Figure 2 Raptor gene trap homozygote mESC clones form smaller embryoid bodies (EBs). A. The heterozygous raptor gene trap mESC clone (RRO611) purchased from the International Gene Trap Consortium was subjected to treatment with 1.75–2.25 mg/ml of genetin for selection. A few surviving clones were chosen to be further characterized by three-primer PCR analysis. The three-primer PCR product and Southern blot analysis of heterozygote clones were around 800/1400 base pairs of DNA fragments and 6200/4500 base pairs, respectively. B. Thirty-five micrograms per lane of protein from each clone was analyzed by blotting with anti-raptor and anti-mTOR antibodies. Actin was used as the loading control, along with inclusion of HEK293T as an additional control. As 50 μg/lanes was used, the trace of raptor signals appeared in the raptor gene trapped clones on the western blots (bottom panel). C. We used inverted phase microscopy to record the morphology of EB formation from wild-type and raptor gene trap homozygote clone #46 mESCs on days 2 and 8. Scale bar, 100 μm. D. Alteration of signaling molecules responding to IGF-1 stimulation as indicated was examined in control and a raptor gene trapped clone, with serum or after serum starvation, at different points in time. These blots are representative of one experiment that was repeated three times.
apoptosis could be one of the mechanisms of neuron/neurite loss after rapamycin treatment, we examined caspase3, a protein that functions in the convergence of apoptotic cascades. Promotion of caspase-3 cleavage in rapamycin-treated neurons began to appear at 24 h after plating cells on culture plates, and had become more dramatic by 72 h (Fig. 4D). Interestingly, phosphorylation of S6K Thr 389 was almost completely inhibited by rapamycin treatment at 24 h and inhibition persisted up to 72 h, while phosphorylation of 4EBP1 Thr 37/46 showed no change over the period of rapamycin treatment. Mouse C2C12 cell lysates treated with TNF-α plus cyclohexamide (CHX) or with insulin were also included to verify the efficacy of the antibodies. Quantitated results are shown in Fig. 4E. Cleaved caspase 3 was significantly increased at the time points analyzed. In summary, rapamycin treatment significantly decreased the phosphorylation of S6K Thr 389, but did not change the phosphorylation of 4EBP1 Thr37/46 over time.

Raptor knockdown after neuronal differentiation of wild-type mESCs impeded growth of neurites

We further explored the role of raptor in the neuronal differentiation of wild-type mESCs. After differentiation of mESCs for three days, neurons were infected with raptor RNAi, control luciferase RNAi or received no treatment. Three days after virus infection, neuron/neurite length was very similar among the various groups as shown by type III β-tubulin staining (Fig. 5A). However, neurite networks with raptor RNAi infection began to appear severely discontinuous and fibrous on day 6 or 7 after virus infection, a phenomenon not seen in the control RNAi group or group

Figure 3  Knockdown of raptor in mESCs retards EB formation and results in unsuccessful neuronal differentiation. A. mESCs were infected with viruses containing RNAi of luciferase, raptor, or rictor followed by puromycin selection. Western blot analysis was performed on cell lysates to detect the corresponding proteins and related signaling molecules. These blots are representative of one experiment that was repeated four times. B. Puromycin-selected mESCs containing raptor knockdown clones were cultivated and began to form EBs. The morphology of EBs on days 2, 4, and 6 was recorded by inverted phase microscopy. For comparison and clarity of various EBs sizes and shapes, local and magnified morphologies are shown. This set of images is representative of experiments that were repeated four times. Scale bar, 100 μm. C. EB size was quantitated by integrating the cross-area of images using Image Pro Plus software. The images were usually taken from three different fields in each experiment which was repeated three to four times. Each image typically contains around 10–20 EBs counted so that a total of around 100–200 EBs were evaluated. The y-axis is the mean cell dimension, and the x-axis is the timing of different treatments. Data are shown as the mean ± S.E. *P < 0.05. D. The lysate from each mESCs group derived EBs at days 2, 4, and 6 was collected and run onto SDS-PAGE. These samples were further processed by Western blot analyses to detect proteins as indicated. These blots are representative of an experiment that was repeated three times. E. Quantitation of cleaved caspase-3, phospho-S6K, phospho-4EBP1, and raptor abundance were respectively normalized to the individual control which is luciferase RNAi at day 2. Data are shown as the mean ± S.E. (n = 3). *P < 0.05.
with no RNAi. On day 7 (i.e., 10 days after plating), some neurons in all the groups developed varicosity, including those treated with raptor RNAi, control RNAi, and even in the group with no RNAi. However, only neurites in the raptor RNAi treatment group developed severe discontinuity and fibrous morphological appearance, whereas there was not much difference between the varicosity in the control RNAi or no RNAi groups. This result suggests that the change in appearance in the neurons was not due to virus infection, but to the loss of function of raptor. The ratio of neurite length to number of cells is shown in Fig. 5B. This parameter was significantly decreased at seven days after raptor RNAi viral infection, phosphorylation of S6K Thr389 was decreased, whereas phosphorylation of 4EBP1 Thr37 was unchanged (Fig. 5D). These results indicate that mTORC1 downstream molecules could function differently in neurite survival/network maintenance in glutamatergic neurons differentiated from mESCs.

Overexpression of S6K T389E prevents rapamycin-induced neuron/neurite loss

In order to further investigate the role played by raptor/mTORC1/S6K in the neuronal differentiation of mESCs, we next constructed a vector that functionally mimics active S6K and overexpresses in mESCs. To test if this plasmid is functional, the lentiviral backbone vector encoding green
fluorescent protein (GFP) tagged S6K T389E and driven by
the CMV promoter was transfected into HEK293T cells
(Fig. 6A). Cells transfected with GFP alone or S6K wild type
constructed in the same lentiviral backbone were used as
controls. The cells containing S6K T389E could still resist
the inhibition of mTORC1 by rapamycin as evidenced by
sustained S6 phosphorylation, an indicator of S6K activation.
As viral particle-containing constructs were used to infect
mESCs and puromycin selection was performed, the cells
that expressed the relevant proteins were confirmed by the
GFP images and S6K antibody blotting (Fig. 6B). When these
mESCs began to differentiate into neurons, they were also
treated with 1 μM rapamycin. The difference in neuron/
neurite morphology became obvious at day 4 after plating.
As shown in Fig. 6C, rapamycin treatment resulted in a
neuronal loss in S6K wild type or control group. However, the
neuronal loss caused by rapamycin was prevented by S6K
T389E overexpression. Initially, we also performed rescue
experiments by which the wild-type mESC-derived neurons
were infected with the abovementioned constructs at 12 h
after rapamycin treatment. The results showed that the
neurons almost completely die within 3–4 days in all groups
except those overexpressing S6K T389E (data not shown).
However, because so few cell lysates could be collected to
clearly demonstrate the expression of relevant proteins, we
adopted the strategy of overexpressing abovementioned

Figure 5  Knockdown of raptor in neurons differentiated from wild-type mESCs impedes morphology of neurites. A. Viral particles
containing raptor or luciferase RNAi began to infect neurons when they were differentiated from wild-type mESCs for three days. A
set of neurons derived from wild-type mESCs with no RNAi infection was also included for comparison. Cultures were observed for 10
more days. These samples were fixed and immunocytochemistry was performed with class III β-tubulin (green) as the primary
antibodies and Alexa 488-conjugated anti-rabbit IgG as the secondary antibodies. The same field was stained with DAPI. Images shown
here are representative of days 3 and 7 after RNAi infection. Local and magnified morphologies are shown. White arrows
representatively indicate some discontinuous neurites. Scale bar, 20 μm. B. The ratio of neurites to number of cells was analyzed by
MetaMorph software. The images were usually taken from three different fields in each experiment, which was repeated three to four
times. More than the 50 neurites were typically counted in each image, so that a total of around 500 neurites were evaluated in all.
The y-axis is the ratio, and the x-axis is the time of infection. Data are shown as the mean ± S.E. *P < 0.05. C. Neurons were harvested
six days after viral infection with raptor or luciferase, or after no viral infection, and subjected to a western blot analysis. Cell lysates
were immunoblotted with different antibodies as indicated. These blots are representative of one experiment that was repeated
three times. D. Phosphorylation of S6K Thr389 and 4EBP1 Thr37, and raptor abundance were quantitated using the freeware, NIH
Image J. Quantitation was respectively normalized to the individual control with no RNAi treatment. Data are shown as the mean ±
S.E. (n = 3). *P < 0.05.
constructs before rapamycin treatment to show the role of S6K in neurons derived from mESCs. Neurite length divided by neuronal cell number was significantly decreased in cells overexpressing GFP or GFP S6K wild type treated by rapamycin in comparison with the ones treated by DMSO (Fig. 6D). The parameter was slightly decreased in the group of cells overexpressing GFP S6K T389E. However, loss of neurite density resulting from rapamycin treatment in cells overexpressing GFP or GFP S6K wild type could be greatly prevented by overexpression of S6K T389E. Together, the results suggest that the raptor/S6K signaling pathway mediates the neuronal differentiation of mESCs.

Discussion

The central finding of this study is that raptor/S6K is critical in neurogenesis of mESCs, as measured in a predominantly glutamatergic population. We also found that S6K, but not 4EBP, correlates with this process. Furthermore, the role of
S6K in the morphological change of neurons/neurites may also be time dependent.

An interesting discovery was that mTORC1 did not affect neurite density, as measured by neurite length divided by number of neurons, in the initial period in neuronal differentiation. Considering that the number of neurons did not change over time but the neurons shrank in size in rapamycin-treated mESCs, the decreased parameter observed at 48–72 h could be primarily due to the loss of neurite length. It thus can be inferred that rapamycin did not destroy the distribution of neurites, as S6K Thr389 had already significantly decreased at 24 h after EB dissociation (Fig. 4). This phenomenon seems to differ from many cell types such as HEK 293T, HeLa, and MEF cells, in which cell death occurs a few hours after treatment with high concentrations of rapamycin. Similarly, the neurite network was significantly disrupted within 24–72 h of treatment with wortmannin. Differences in the effects of rapamycin and wortmannin may simply be attributed to different efficacies of the drugs or dosages. Furthermore, comparing morphological and signal differences between rapamycin and wortmannin treatment, phosphorylated Akt might also play a role in neurite outgrowth. Nevertheless, raptor/mTORC1 might be a specific regulator by which neuronal differentiation may become more mTORC1-dependent after differentiation for 48–72 h. The time-dependence of the importance of S6K and rapamycin in development of neurons in vivo has been reported. During differentiation of extending neural processes in mice and Drosophila, S6K was reported to play an increasingly important role in the increase in cell size (Bateman and McNeill, 2004; Shima et al., 1998; Zhang et al., 2000). Hence, we suggest that neurons derived from mESCs rely on raptor/mTORC1, and this process may be development dependent.

Another interesting observation is that phosphorylation of S6K, but not 4EBP1, was decreased in rapamycin- or raptor RNAi-treated neurons derived from mESCs, and this decrease was associated with the loss of neurons/neurites. Both S6K and 4EBP are direct downstream molecules of mTORC1, but their differing functions in vivo systems of cell differentiation have been reported. In undifferentiated photoreceptor cells of the ommatidia in Drosophila, mutation of TSC leads to a profound impairment in the timing of differentiation in these cell clusters. S6K, but not 4EBP-1, found to act downstream of TORC1 in this pathway (McNeill et al., 2008). Furthermore, Mainwaring and Kenny demonstrated a novel mechanism whereby eIF4E and S6K are differentially regulated in proliferating cerebellar neural precursors by SHH (Mainwaring and Kenney, 2011). Therefore, the observation of a difference between S6K and 4EBP1 phosphorylation in neurons either in vivo or in vitro such as in our mESC-derived neuronal model implies that additional regulation might be involved.

The homozygote raptor gene trap clones verified by PCR and Southern blot analysis demonstrated that the gene is indeed being “trapped,” so that no proteins are expressed. Despite the genotype, the faint raptor band seen in the western blot, as well as subsequent immunoprecipitation for MS-SPEC analysis (data not shown), precludes us from claiming that these clones are knockouts. Since the intron-trapping vector contained a splice acceptor, it is highly possible that these clones developed a "leak" in the gene-trap approach due to a skip in splicing. Even a low level of raptor expression resulting either from the gene trap or RNAi knockdown caused a decrease in EB size, which might subsequently result in the failure of neuronal differentiation. Raptor knockout mice die early in development (Guertin et al., 2006). Nevertheless, Drosophila mutants or mice containing inactivated S6K, one of mTORC1 direct substrates, survived development and exhibited a normal body shape, but a 50% decrease overall in body size due entirely to a decrease in cell size, while the overall number of cells was unaltered (Montagne et al., 1999; Shima et al., 1998). The differences caused by raptor and its downstream effector S6K on development suggest that raptor could have a broader effect on biological function, in addition to cell growth/size.

In conclusion, in this study we differentiated mESCs into almost completely uniform glutamatergic neurons and demonstrated that loss-of-function of raptor/mTORC1 impeded their neurogenesis from mESCs. Equally important, a difference between the activation of mTORC1 downstream molecules S6K and 4EBP1 was observed during the differentiation of glutamatergic neurons from mESCs. Considering the importance of mTORC1 in cell growth, neurogenesis from mESCs in addition to measuring predominantly glutamatergic neurons like our model could also require mTORC1/raptor/S6K signaling.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.08.003.

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