Novel 5'TOPmRNAs Regulated by Ribosomal S6 Kinase Are Important for Cardiomyocyte Development: S6 Kinase Suppression Limits Cardiac Differentiation and Promotes Pluripotent Cells Toward a Neural Lineage

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Moving stem cells from bench to bedside has been a challenging task. Undermining this task is comprehending and optimizing the underlying regulatory mechanisms that drive differentiation of stem cells into desired cell and tissue types. Here we present evidence that ribosomal S6 kinase (S6K) is among the proteins upregulated as embryonic stem cells (ESCs) and human induced pluripotent stem cells differentiate into beating cardiomyocytes. We hypothesized that S6K plays a pivotal role in cardiomyogenesis, primarily because it regulates the translation of 3 cardiac-involved genes recently shown to have 5' terminal oligopyrimidine (5'TOP) sequences: connexin 43 (*Cx43*), desmoplakin (*Dsp*), and phosphatase and tensin homolog (*PTEN*). Along with another independent laboratory, we confirmed that S6K is indeed upregulated in beating ESC-derived cardiomyocytes compared to the surrounding nonbeating, differentiated cells. S6K short interfering RNA-transfected stem cell cultures indicate that inhibition of S6K strongly hinders development of cardiomyocyte beating and translation of *Cx43*, *Dsp*, and *PTEN*; these cardiac 5'TOP mRNAs were only properly translated in cells with S6K, supporting our hypothesis. An unexpected discovery took the role of S6K one step further: S6K-knockdown stem cell cultures developed significantly more neurons than seen in embryoid bodies subjected to a typical cardiac differentiation protocol. These results introduced the novel idea that in addition to its critical cardiac roles, S6K may be a significant factor that prevents stem cells from pursuing a neuronal pathway. Overall, results have indicated the necessity of S6K for normal stem cell cardiomyogenesis, as well as lowered S6K expression for stem cell neurogenesis.

Introduction

USING A PROTEOMICS, kinase expression assay, we have identified and verified a number of signaling mechanisms that are important for stem cells to properly differentiate into cardiomyocytes and neurons [1–5]. One protein found in our proteomics assay, p70 ribosomal S6 kinase (S6K), has generated interest due to its role in regulating and initiating translation machinery [6–11]. More recently, S6K activation has been implicated in causing differentiation of human embryonic stem cells (ESCs), though specific differentiation pathways have not been examined [12].

Regulated upstream by the mammalian target of rapamycin (mTOR) [6,8–11] and phosphoinositide-3 kinase (PI3K) [13], S6K in turn has important roles in a number of downstream pathways. mTOR inhibition and subsequent inhibition of S6K reduces activation of elongation initiation

factor 4B (eIF4B) and phosphorylation of ribosomal protein S6 (rpS6), a substrate of S6K [14]. mTor/S6K is important for initiating translation and appears to be essential for cells to function normally [15]. Perhaps more importantly, inhibition of mTOR has been found to repress a subgroup of mRNAs that include those with 5' terminal oligopyrimidine (5'TOP) sequences, which function through their secondary structure [14]. Evidence suggests that S6K is an intermediary kinase responsible for this process [7,16,17].

While a single *S6K* gene is present in some genera such as *Drosophila*, in mammals there are 2 genes—p70 S6K (*S6K1*) and *S6K2*—which code for homologous S6Ks [10,18]. When sequentially phosphorylated, S6K1 is activated resulting in the shift of 5'TOP mRNAs into polysomes. This shift may be instigated by an increased rate of binding to ribosomes, caused, in part, by promoting general translation initiation by eIF4B [19]. An *S6K1*-activated allele also

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protects 5'TOP mRNAs from rapamycin, whose inhibitory effect on mTOR represses 5'TOP mRNA translation [20] and, with short-term treatment, promotes expansion—as opposed to differentiation—of pluripotent cells [12]. Both the shift into polysomes and the protection conferred by the S6K1-activated allele act to enhance 5'TOP mRNA translation.

The 5'TOP mRNA subgroup has been known for some time to include ribosomal proteins and elongation factors [7,19]. Interestingly, a 2008 study by Yamashita et al. [21] showed that the 5'TOP subgroup also included 3 genes involved in cardiac development. These genes were desmoplakin (*Dsp*), an essential protein for desmosomes [22,23]; connexin 43 (*Cx43*), a gap junction protein necessary for heart tube development [24] and hematopoiesis [25]; and phosphatase and tensin homolog (*PTEN*). The knockout phenotypes for each of these genes possess severe cardiac developmental defects [26–28].

Based on the above information, we hypothesized that S6K regulates differentiation of stem cells into beating cardiomyocytes by promoting translation of cardiac 5'TOP mRNAs. We found that knocking down S6K significantly delayed cardiac differentiation and, surprisingly, also increased incidence of neural differentiation.

Materials and Methods

ESC culture and cardiomyocyte production

Mouse CCE ESCs were maintained and differentiated similarly to our previously published protocols [1–5]. Briefly, ESCs were grown on 0.1% gelatin in 1,000 U/mL leukemia inhibitory factor (LIF). Differentiation was induced by removing LIF and converting colonies of ESCs into embryoid bodies (EBs) [3,5]. Briefly, for EB production, ES colonies were picked using a pipette tip and transferred to Petri dishes designed for growing cells in suspension. These EBs were incubated in DMEM with 15% fetal bovine serum for 2 days in the absence of LIF. At the end of incubation day 2, individual EBs were placed into a single well of a 24-well plate, where they adhered and spread. Rhythmic beating of EBs, indicating cardiac muscle differentiation, was monitored by daily inspection of cultures using phase microscopy. In our hands, beating usually commences ~day 9 after removal of LIF.

Beating areas were isolated from nonbeating areas following our previously published procedure in Refs. [1–3,5]. In brief, beating areas were isolated using a microscalpel (Fine Science Tools). Any nonbeating cells associated with the beating area were trimmed away. Ten beating and nonbeating areas of similar size were separated and briefly washed in DEPC-treated (RNase-free) phosphate buffered saline (PBS). Subsequent EBs were analyzed for cardiomyocyte (or alternative tissue) formation by reverse transcriptase-polymerase chain reaction, DIC microscopy, immunofluorescence, and Western blot, or prepared in Kinexus™ sample buffer, frozen, and sent to Kinexus, Inc., for kinase expression analysis. An explanation of how they analyze each protein can be found at www.kinexus.ca

Human induced pluripotent stem cell maintenance and differentiation

Human induced pluripotent stem cells (hiPSCs) were acquired from WiCell. They were maintained according to

their protocols. Differentiation was induced by following the protocol by [29].

Antibodies

Anti-S6K was purchased from Abcam. Cx43, PTEN, and β -tubulin (ubiquitously expressed) antibodies used in Western blot Figs. 1, 3, and 6T were purchased from Cell Signaling, Inc. Alexa Fluor® Labeled, neuronal class III β -tubulin (TUJ1) monoclonal antibody used for Fig. 6C–R was purchased from Covance, Inc. Anti-actin was purchased from BioLegend. Anti-cardiac troponin T was purchased from Neomarkers. Anti-Dsp was purchased from American Research Products. Antibodies for Nkx2.5 and Gata4 were purchased from Santa Cruz.

Confocal microscopy and cell counting

Beating and nonbeating areas were surgically spliced out of EBs using microdissection and prepared for confocal microscopy [1,2]. The protocol for confocal microscopy was performed similarly to [1–5] on these areas. Primary antibodies were diluted 1:50 and secondary AlexaFluor antibodies purchased from Invitrogen, Inc. were diluted 1:800. An Olympus Fluoroview 500 Laser Scanning Microscope (Olympus America, Inc.) was used to image the samples.

Flow cytometry

Mouse embryonic stem cells (mESCs) were differentiated according to [1]. Cells were washed in PBS, trypsinized (0.05%), and fixed in 2% paraformaldehyde. Primary antibodies used were either cardiac troponin, or Cx43. Secondary antibody used was AlexaFluor 488 antibody (Invitrogen, Inc.) against the appropriate primary antibody. Members of the Georgetown University Flow Cytometry Core Facility acquired the flow cytometry data. Analysis was done in FCS Express 4 Flow Research Edition (De Novo Software).

Short interfering RNA production and transfection

S6K short interfering RNA (siRNA) was ordered from Prologi. S6K siRNA was transfected into the mouse CCE ESCs to specifically inhibit S6K at the mRNA level, preventing translation of the S6K protein. Western blotting confirmed the downregulation of S6K. Addition of 1–3 μ L of Qiagen's HiPerfect Transfection Reagent per 300 μ L of CCE differentiating media in each well of cells served as a control. siRNA was transfected into EBs on day 6 post removal of LIF from the medium.

Statistical analysis

All experimental trials were repeated 3 times, minimum. Protein expression was measured using histogram analysis software that measured pixel intensity. Each experimental band representing a protein (eg, Dsp Fig. 3) was normalized to the control band (eg, actin). The density of control was measured against S6K siRNA-treated cells. We did not see any marked difference between the outcomes of each blot for each protein. Graphical data displays are plotted as means with error bars representing the standard error of the mean of the trials. Statistical significance was determined by unpaired *t*-tests.

Results

S6K is upregulated in beating versus nonbeating areas

Comparison of protein levels in beating and nonbeating areas in a proteomic screen (Kinexus, Inc.) showed that S6K concentration was significantly higher (69%) in nascent beating areas than in nonbeating areas (Fig. 1A–C). These results were independently confirmed by our laboratory, using Western blotting (Fig. 1D).

Confocal imaging revealed the cytosolic presence of S6K [10], which showed greater expression in cells within the newly beating areas (1–2 days after the onset of beating) than cells within nonbeating areas (Fig. 1E). Furthermore, areas with high levels of S6K often coincided with areas that developed into beating areas positive for cardiac troponin T (CnnT; Fig. 1F, G).

S6K is necessary for normal cardiomyogenesis

Mouse ESCs are an excellent model system for analyzing cardiomyogenesis because after removal of LIF and plating

FIG. 1. Comparison of nonbeating versus beating areas in ESC-derived cardiomyocytes. Graphs show raw and normalized data taken directly from the Kinexus protein kinase screen. Kinexus, Inc. (www.kinexus.ca), uses a highly sensitive imaging system with a 16-bit camera (Bio-Rad Fluor-S Max Multi-Imager) in combination with quantitation software (Bio-Rad Quantity One) to quantify and analyze the chemiluminescent samples. The resulting trace quantity for each band scanned at the maximum scan time is termed the raw data. As the relationship between scan time and band intensity is linear over the quantifiable range of the signal intensity, the raw data from the scans are normalized to 60 s (counts per minute) for uniformity. (A) Raw data from the Kinexus protein kinase screen comparing S6K concentration in nonbeating versus beating cardiomyocytes. Counts/mins are the units measured by the algorithm used by Kinexus, Inc., in their proteomics assay. (B) The raw data from Kinexus were converted to percentages of the control band intensity. (C) The bands used by Kinexus to obtain the raw data in (A). The darker B band shows that more S6K is present in B areas. (D) The Kinexus data were confirmed by our follow-up Westerns. Here, S6K concentration was 2.07 times higher in B (left lane) versus NB (right lane) areas. Data were normalized using β -tubulin. (E–G) Representative view from ~ 10 EBs showing confocal imaging of beating and nonbeating cells revealed that S6K [arrows in (E)] had far greater expression in cells populating B versus NB areas. Cardiac troponin identifies the beating cells [arrows in (F)]. Note, beating cells co-localize with high S6K concentrations shown in the merged image (G). Space bars = 10 μ m. NB, nonbeating; B, beating; S6K, S6 kinase; EBs, embryoid bodies. Color images available online at www.liebertonline.com/scd

EBs, beating typically occurs ~9 days in small areas that can then be quantified [1,2,5]. S6K expression was inhibited with siRNA 3 days before the commencement of beating. S6K-repressed EBs were monitored for beating over a 132-h period and compared to control, untreated EBs, or mock-treated siRNAs. The number of wells containing EBs that developed at least one beating area was significantly less at 72 and 96 h in those transfected with S6K siRNA when compared to those transfected with scrambled siRNA (Fig. 2A). The siRNA wells showed an increase in beating over time due to the degradation of the siRNA [2,30]. EBs at each time point in the graph in Fig. 2A were analyzed by Western blot (Fig. 2B), which showed the knockdown effect of S6K siRNA.

The numbers of beating areas in siRNA and control wells for each experimental trial were also counted. Pooling to-

gether the data sums for each time point revealed an obvious difference in number of beating areas between the control groups and the siRNA groups (Fig. 2C). Further analysis of the data made this discrepancy even clearer. Throughout the entire 132 h, the average number of beating areas per control well was always greater than that of the siRNA wells (Fig. 2D). Significance ($p < 0.05$) was reached at 72 and 96 h.

S6K regulates the translation of key cardiac 5'TOP genes

S6K has previously been implicated in the translation of 5'TOP mRNAs [17–19,31]. To date, of the 1,645 putative 5'TOP mRNAs [21], we have identified 3 to be important for cardiac development: Cx43, Dsp, and PTEN (Fig. 3A). We surmised that if S6K expression was suppressed using

FIG. 2. Treatment of ESCs with S6K siRNA strongly hindered differentiation of ESCs into beating areas. **(A)** Rhythmic beating was assessed at each time point in cells treated with S6K siRNA and compared to cells treated with scrambled siRNA or untransfected. About 72 h after transfection with 200 nM siRNA, fewer ESCs differentiated into beating areas than in controls. This inhibition persisted through 132 h after transfection, when previous work shows that the siRNA begins to be degraded. S6K expression was inhibited at 72 and 96 h. **(B)** Western blots representing each time point in **(A)** show the knockdown effects of S6K siRNA on S6K. **(C)** During differentiation, beating areas within EBs and can be counted. Combining all data at each time point reveals markedly fewer beating areas in EBs treated with 200 nM S6K siRNA. **(D)** The average number of beating areas per well in siRNA versus control cells was also measured. To measure significance, the mean number of beating areas $\pm$ the standard error of the mean was measured at each time point, which was subjected to a *t*-test. Significance ($*p < 0.05$) between 200 nM S6K siRNA and 200 nM control scrambled siRNA was acquired at the 72 and 96 h time points for % EBs beating **(A)** and average number of beating areas **(D)**. This method was similar to that explained in Refs. [1,2,5,44]. ESCs, embryonic stem cells; siRNA, short interfering RNA.

FIG. 3. siRNA to *S6K* results in down-regulation of cardiac genes with 5'TOP sequences. **(A)** Using the screening methods developed by Yamashita et al. [21], the TSSs of 13,717 human genes—specifically, the -4 to +7 positions in 87,397 TSSs—were analyzed. Based on the TSSs of 48 known 5'TOP genes, a position-specific weighted matrix was generated. A score was defined by the equation $\sum_{i=1}^l \log[(n_{if}+1)/(N_i+4)*(1/4)]$, where l = length of the matrix (11), n_{if} = number of base f (A, C, G, or T) observed at the i -th position, and N_i = number of all bases observed at the i -th position [30]. Identification of a gene as a candidate 5'TOP gene involved the fulfillment of 3 criteria: (i) a score of over 0.1 (the lowest score found in known 5'TOP genes was 0.12), (ii) a cytosine (C) in the +1 position, and (iii) pyrimidines in the -1 to +4 positions. One thousand six hundred forty-five genes (2772 TSSs) were identified—including cardiac genes *PTEN*, *Dsp*, and *Cx43*. **(B)** Translation of cardiac mRNAs with 5'TOP sequences was affected. Expression of *Dsp*, *PTEN*, and *Cx43* was strikingly reduced in *S6K* siRNA-treated ESCs, with down-regulation ranging from 30% to 70% of the control. The expression of *S6K*, acting as a positive control to ascertain *S6K* siRNA functionality, was also reduced, as would be expected. Actin was the loading control for all but the *Cx43* gel, which used β -tubulin. *PTEN* and *S6K* were assessed on the same membrane in this experiment. *Dsp* and *Cx43* were assessed on different membranes. **(C)** Non-5'TOP mRNAs such as *Nkx2.5*, *Gata4*, cardiac troponin, actin, and β -tubulin were not affected by *S6K* siRNA. C'–C''' are from the same sample. Note: all blots where the actin or β -tubulin controls are separated and placed above or below the experimental bands were done so because 2 different exposures had to be performed. For example, in the *Nkx2.5* figure the exposure time to resolve *Nkx2.5* resulted in over-exposure of the β -tubulin band. TSSs, transcriptional start sites; 5'TOP, 5' terminal oligopyrimidine; *Cx43*, connexin 43; *Dsp*, desmoplakin; *PTEN*, phosphatase and tensin homolog.

FIG. 4. FCM analyses confirm our Western blot, confocal, and morphological data. **(A)** A representative FCM analysis shows no difference in the number of cells expressing *CnnT* in mESCs treated with Mock siRNA or S6K siRNA; however, S6K siRNA treatment resulted in fewer cells expressing the 5'TOP mRNA protein product Cx43. This result was observed in 3 different experiments. **(B)** Quantifying these data revealed a significant difference between the number of cells expressing CnnT and Cx43 when treated with Mock siRNA or S6K siRNA. Very few undifferentiated mESCs expressed CnnT (or Cx43-data not shown). FCM, flow cytometry; mESCs, mouse embryonic stem cells; * $p < 0.001$ compared to mock and S6K siRNA. Color images available online at www.liebertonline.com/scd

siRNA, then the protein products of the three 5'TOP mRNAs involved in cardiac development would also be suppressed. EBs were transfected with control siRNA or siRNA to S6K 6 days after removal from LIF. This is equivalent to ~3 days before beating normally occurs. Four days after transfection, EBs were accumulated and analyzed by Western blot. Interestingly, knockdown of S6K markedly reduced the expression of all 3 mRNAs with 5'TOP sequences (Fig. 3B); protein levels of both isoforms of Dsp (Dsp I and Dsp II) showed decreased expression levels, as did Cx43 and PTEN. Non-5'TOP miRNA products were assessed to determine cardiomyocyte differentiation. Expression of NkX2.5, cardiac troponin (CnnT), Gata4, and actin and β -tubulin (ubiquitously expressed) were not decreased by S6K siRNA (Fig. 3C–C''), suggesting that S6K primarily affects specific components of cardiomyogenesis.

Subjecting cells to flow cytometry, we were able to quantify the number of mESCs affected by S6K siRNA (Fig. 4). We found that the percentage of cells expressing cardiac troponin was not significantly different between the Mock siRNA and S6K siRNA treatments; however, the number of differentiated mESCs expressing the 5'TOP mRNA product Cx43 was significantly reduced. This provided further evidence that S6K is necessary for normal cardiac development.

Similar to mESCs, suppression of S6K in hiPSCs results in aberrant cardiomyocyte differentiation

To verify the ubiquity, of S6K function during cardiomyogenesis, we transfected S6K siRNA into hiPSCs during differentiation into the cardiac lineage [12]. Four days after transfection, EBs were analyzed morphologically for their capacity to fully differentiate into beating areas. Figure 5 reveals that S6K siRNA markedly reduced the average and total number of beating areas derived from human iPS.

Inhibiting S6K unexpectedly shifts differentiation towards a neural fate

We consistently observed that after S6K siRNA degraded, the number of beating areas would approach, but not draw

even with, the number of beating areas within control EBs (Fig. 2). This observation continued in most experiments up to 21 days after transfection of siRNA. Surprisingly, 7–10 days after siRNA transfection, cell morphology within the spreading EBs was noticeably different. High-magnification

FIG. 5. To determine if S6K activity was restricted to mouse cardiac development or was conserved, hiPS cells were subjected to S6K siRNA. The average number **(A)** and total number from all experiments **(B)** of beating areas in hiPS cells was markedly reduced in S6K siRNA-treated cells compared to (–) siRNA. Differentiation was induced using the protocol from [29].

FIG. 6. ESCs treated with S6K siRNA and cultured for an extended time form neurons. Neurons were very easy to spot by simple morphological observations using phase-contrast microscopy (**A**, **B**). Arrowheads indicate axons. Arrows indicate neuron cell bodies. Uni-polar, bi-polar, and multi-polar neurons were all observed. (**C–R**) Dosage of siRNA confirmed our phase-contrast observations. >200 nM S6K siRNA resulted in hundreds of Tuj1-positive processes. Panels (**F**) and (**J**) show high-magnification views of Tuj1-positive cell bodies and processes (arrows). (**K–R**) Transfection with 100 nM siRNA resulted in few Tuj1-positive processes and even fewer in untreated EBs. (**S**) Using confocal images of S6K siRNA-treated EBs, dosages were quantitatively analyzed with Photoshop. Each image of cell nuclei (DAPI blue) was divided into 4 quadrants of comparable cell density, which were adjusted for maximal contrast between nuclei color and the black background. Relative and total fractions of cell confluency were calculated by comparison of cyan pixel to total pixel count. Analysis of the neuronal presence (green) was performed using the same quadrants and methods. We found significantly more Tuj1-positive cells in EBs treated with 200 nM and 1 μ M S6K siRNA when compared to 0 or 100 nM S6K siRNA. (**T**) Western blot analysis of EBs treated with S6K siRNA or scrambled siRNA revealed that neuron differentiation occurred more quickly in S6K siRNA-treated EBs (by day 7) when compared to treatment with scrambled siRNA. Given enough time in culture, day 14 post-siRNA treatment (21 days postremoval from leukemia inhibitory factor), NF-M was detectable in both siRNA treatments. Twenty-one days of differentiation is an average time for mESCs to form neurons [45]. Color images available online at www.liebertonline.com/scd

FIG. 7. In vivo analysis during development reveals an S6K localization pattern that mimics differentiation in vitro. (**A, B**) Low magnification of a frozen section from an E9.5 day embryos shows anti-S6K antibodies marking cardiomyocytes of the heart tube (*arrows*), which is identified using anti-cardiac troponin antibodies (CnnT). (**C–E**) Higher magnification of the heart tube provides clear evidence that S6K localizes to cardiomyocytes in the entire developing heart tube including cells within trabeculae, which at this stage are not contractile (as shown by the lack of CnnT staining), but will become contractile within 1–2 days of development. Panel (**C**) shows a merged view of (**D**) and (**E**) and the addition of the *blue* channel representing Dapi staining of nuclei. (**E'**) High magnification of striated cells are marked by the *arrow*. (**F–K**) A few distinct areas within the neural tube at this stage show a weak, but distinct band of staining of S6K in some cells comprising the neural tube (*arrows* in Fig. 6J). However, it is very clear that the staining in the heart tube is much more robust than that found in any cells populating the neural tube. Also, there are markedly more cells with levels of S6K staining that are virtually undetectable in the neural tube (represented by nestin staining). When compared to the heart tube. Bar in (**A**) = 100 μ m for (**A, B**), 25 μ m for (**C–E**), 10 μ m for (**E'**), and 50 μ m for (**F–K**). nc, notochord; v, ventricular zone; sv, subventricular zone. Color images available online at www.liebertonline.com/scd

views revealed that EBs subjected to S6K siRNA repeatedly resulted in areas containing numerous neurons compared to control EB cultures (Fig. 6A, B). N.b., no neural-differentiation media was used in these experiments. This result was further confirmed using different S6K siRNA concentrations, which revealed that the shift to a neural lineage was dependent upon the dosage of S6K siRNA (Fig. 6C–R). Quantifying multiple areas from multiple experiments provided further evidence that S6K siRNA pushes ESCs down the neural pathway (Fig. 6S). Finally, Western analyses clearly revealed that suppression of S6K promoted differentiation toward a neural fate as neurofilament M (NF-M) protein was detected as soon as 7 days after S6K siRNA transfection (Fig. 6T). In contrast, 7 days after Mock siRNA, NF-M was below the level of detection in mESCs treated with scrambled siRNA. As a control, given enough time in culture, day 14 post-siRNA treatment (21 days postremoval from LIF), as might be expected, NF-M was eventually detected in scrambled siRNA-treated EBs.

In vitro analysis of S6K expression mimics in vivo expression during early development

We surmised that if upregulation of S6K is important for cardiomyogenesis and its down-regulation is necessary for neurogenesis in vitro, then this scenario should be mirrored in vivo. E9.5 day embryos (Fig. 7) revealed that S6K is highly expressed in cardiomyocytes within the developing heart tube when compared to its expression in the neural tube.

Discussion

A number of studies have analyzed the functional necessity of S6K, and confirmed its role in 5'TOP mRNA translation [17,18,31]; however, few thus far have examined the implications of S6K activity in stem cell differentiation [12]. Here, we provide evidence in human and mouse pluripotent cells that during differentiation, S6K is up-regulated in newly beating areas isolated from EBs. Furthermore, inhibiting S6K abrogates development of beating areas in both mouse and human pluripotent cells. Surprisingly, down-regulation of S6K also appears to be important in neuronal developmental.

S6K plays a central role in 5'TOP mRNA translation because it was shown to enable more efficient translation of the mRNAs, including those necessary for generating cardiac development [21]. The S6K knockouts further accentuated the importance of this kinase in vivo. Of the 2 mammalian forms of S6K [10,18], S6K1 is the gene chiefly responsible for regulating cell size in mammals [32–40]. Single knockouts of S6K2 in mice (S6K2^{-/-}) resulted in no significant alteration of the normal phenotype [20,41]. The phenotypes of S6K1^{-/-} and S6K1^{-/-}/S6K2^{-/-} mice were much the same—with the exception of significantly higher incidence of lethality in double knockout mice [20] with complications indicative of heart developmental defects. Nonviable mice showed multiple areas of hemorrhaging within their myocardium [20].

Past experimentation has shown that S6K1 is responsible for the 5'TOP mRNA translation-promoting effects [17]. S6K2 is only able to compensate partially in the absence of S6K1 [18]. As a result, it is plausible that S6K1^{-/-}/S6K2^{-/-} mice and

their hearts were unable to develop normally because they were without the benefit of p70 S6K1 function, and thus of the translation of cardiac 5'TOP mRNAs Cx43, Dsp, and PTEN.

Of high interest is the alteration of EB phenotype from cardiac to neural 1 week after S6K siRNA treatment. The in vivo analysis supported the in vitro data as S6K expression was markedly lower in the neural tube versus the heart tube. The mechanism driving this shift is not known [42]; however, our work does corroborate with that of D'Aniello et al. [43], who showed that an association between S6K and a G-protein tilts differentiation toward the cardiac lineage. Understanding this mechanism will be a focus of future work.

Discovery of the key mechanisms involved in stem cell differentiation into tissue-specific cell populations is necessary before stem cell therapies can proceed safely. Comprehension of the kinetics of these critical factors will lead to the production of purer cell populations, more effective than shot-in-the-dark approaches heavily reliant on chance. Increased population purity will, in turn, facilitate improvement in stem cell therapy quality control and in so doing minimize the risks involved in such therapies.

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Author Disclosure Statement

All authors declare that no competing financial interests exist.

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