Enhanced Cardiomyocyte DNA Synthesis During Myocardial Hypertrophy in Mice Expressing a Modified TSC2 Transgene

Kishore B.S. Pasumarthi, Hidehiro Nakajima, Hisako O. Nakajima, Shaoliang Jing, Loren J. Field

Abstract—Tuberous sclerosis complex (TSC) is a rare genetic disorder characterized by the appearance of nonmalignant tumors in multiple organs, including the brain, kidney, lung, skin, and heart. The familial form of the disease exhibits an autosomal dominant pattern of inheritance, and 2 disease-causing loci (designated TSC1 and TSC2) have been identified. The TSC1 gene, which encodes a 130-kD protein called hamartin, is located at chromosome 9q34.1 The TSC2 gene, which encodes a 198-kD protein called tuberin, is located at chromosome 16p13.3.2 Molecular analyses have demonstrated that tumor formation in TSC patients is frequently associated with loss of heterozygosity at either locus, 3,4 confirming earlier predictions based on genetic analyses that the TSC genes function as tumor suppressors. This view is further supported by analyses of Eker rats, which carry a mutation at the TSC2 locus.5,6 Rats homozygous for the TSC2 mutation die in utero at embryonic day 13.5, whereas rats heterozygous for the TSC2 mutation are viable but highly prone to renal carcinoma. Tumor progression in the homozygous Eker rats is accompanied by loss of heterozygosity at the TSC2 locus, and expression of a transgene encoding a wild-type TSC2 cDNA blocks renal carcinoma in this model.7

Primary myocardial tumors in the general population are extremely rare.8 In contrast, >50% of TSC patients exhibit primary myocardial tumors.9 These results suggest that the TSC gene products play an important role in the regulation of the cardiomyocyte cell cycle. This view is supported by analysis of embryonic day 12.5 fetuses derived from intercrosses of heterozygous Eker rats.10 Spontaneously contractile cardiomyocytes were observed after multiple passages of whole-embryo cultures prepared from fetuses homozygous for the mutant TSC2 allele but not from cultures prepared from heterozygous or wild-type fetuses. Immune cytological and electron microscopic analyses confirmed that the contractile cells in the homozygous mutant cultures were cardiomyocytes. Tritiated thymidine incorporation analyses indicated that these cells exhibited sustained DNA synthesis after as many as 8 passages. Although disruption of the TSC2 gene resulted in embryo death in mice, histological analyses suggested the presence of enhanced ventricular myocardial proliferation in the homozygous mutant fetuses.11 Thus, the TSC2 gene product is important for cardiomyocyte terminal differentiation.

Molecular analyses have identified several structural motifs that appear to be important for tuberin activity. For example, a small 38-amino-acid motif near the C-terminus of tuberin exhibits homology to the GTPase-activating protein rap1GAP,2,12 a protein that negatively regulates rap1. Rap1, a member of the ras superfamily, was originally isolated by its ability to diminish the transforming activity of the Ki-ras
The observation that tuberin stimulated the GTPase activity of rap1a suggested that this motif is functionally active, and a view supported indirectly by the colocalization of these two proteins in the Golgi apparatus. Other studies identified a 59-amino-acid motif located at the C-terminus of tuberin that binds to rabaptin-5. Rabaptin-5 is a direct effector of the small GTPase Rab5, a rate-limiting component of the endocytotic membrane docking apparatus. Tuberin possesses potent Rab5 GAP activity, leading these authors to propose that rabaptin-5 functions as an adaptor protein to recruit Rab5 for tuberin-mediated GAP activity. In support of this, abnormally high levels of endocytosis were observed in embryonic fibroblasts lacking tuberin. More recently, tuberin amino acid residues 346 to 371 were shown to be sufficient for binding to hamartin, the product of the TSC1 gene. Finally, an alternatively spliced region encoding a leucine zipper located at the N-terminus of the protein has also been identified.

In this study, we generated modified TSC2 cDNAs in which one or more of these structural motifs were altered. The modified cDNAs were then screened for their ability to promote growth in NIH-3T3 cells, which express relatively high levels of the endogenous tuberin gene product. Cells expressing the modified cDNAs exhibited phenotypes typical of TSC2-deficient cells, including enhanced growth rates, increased endocytosis, and aberrant p27 trafficking. The cDNA exhibiting the greatest growth-promoting activity in cell culture was tested for its ability to alter cardiomyocyte cell cycle regulation in vivo. Although cardiac development in transgenic mice expressing the cDNA under the control of the α-cardiac myosin heavy chain (MHC) promoter appeared normal, the level of cardiomyocyte DNA synthesis during isoproterenol-induced hypertrophy was 35-fold greater than that observed in nontransgenic siblings. These results are discussed within the context of promoting regenerative growth in the adult myocardium.

Materials and Methods

**Recombinant DNA Protocols**

Isolation and characterization of the wild-type murine tuberin cDNA was described previously. All recombinant DNA manipulations used standard methodologies, and construct fidelity was confirmed by diagnostic DNA sequencing. All animal experimentation was in accordance with institutional and federal guidelines.

**Cell Culture Experiments**

For colony growth assays, TSC2 cDNAs were subcloned into pRcCMV and transfected into NIH-3T3 cells. Cells were selected in G418 for 13 days (0.3 mg/mL), and the dishes were then stained with 0.1% gentian violet. Stable cell lines were isolated by long-term selection from the G418-resistant cells. p27 immune reactivity signal was visualized with a Vectastain ABC kit from Vector Laboratories.

**Endocytosis Assay**

Fluid-phase endocytosis was quantified with a horseradish peroxidase (HRP) uptake assay. HRP uptake was determined by measuring the absorbency values at 455 nm and normalized to the protein content in the sample.

**p27 Immune Reactivity**

Cells plated in chamber slides were serum-synchronized, fixed in ice-cold acetone and methanol (1:1), and processed for anti-p27 immune cytology (antibody sc-528, Santa Cruz Biochemicals) at a 1:100 dilution. p27 immune reactivity signal was visualized with a Vectastain ABC kit from Vector Laboratories.

**Generation of the MHCΔRL Transgenic Mice**

The MHCΔRL transgene was constructed by use of the transcriptional regulatory sequences of the mouse MHC gene and the TSC2 ΔRl cDNA. The SV40 early-region transcription terminator/polyadenylation site (nucleotide residues 2586 to 2452) was inserted downstream from the ΔRl cDNA insert. Transgene insert DNA was microinjected into zygotes by standard methodologies. Transgenic animals were identified by diagnostic polymerase chain reaction amplification as described.

**Cardiomyocyte DNA Synthesis Assays**

Cardiomyocyte DNA synthesis was monitored with a thymidine incorporation assay and an MHC-nLAC reporter transgenic strain as described. Mice received a single injection of [3H]thymidine [200 μCi/ IP at 28 Ci/(mmol/L), Amersham] and were euthanized 4 hours later. Standard methods were used for cryosectioning. Cardiomyocyte DNA synthesis was scored by the colocalization of β-galactosidase (β-gal) activity (dark blue staining when visualized under bright-field illumination) and silver grains. For anti-tuberin immune cytology, heart sections were reacted with anti-tuberin antibody C-20, and signal was visualized with a Vectastain ABC kit from Vector Laboratories.

Myocardial Hypertrophy Model

Myocardial hypertrophy was induced by isoproterenol infusion with Aloxet minipumps in adult mice as described previously. An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Colonies Growth Screen for TSC2 Modifications With Growth-Promoting Activity**

Previous analyses have identified several conserved motifs in human, rat, and mouse tuberin and to a lesser degree in puffer fish and Drosophila tuberin. These include a leucine zipper near the N-terminus of the molecule, as well as a rap1GAP homology motif and a rabaptin-5 binding motif near the C-terminus (Figure 1A). The high level of conservation suggests that these motifs may be important for tuberin tumor suppressor activity, a prediction that has been partially born out by functional studies (see Introduction). A series of TSC2 cDNA expression constructs in which the rap1GAP, leucine zipper, or rabaptin-5 motifs were deleted individually or in combination were generated (see Figure 1A) and tested for their ability to promote growth when expressed in cells with high levels of the endogenous TSC2 gene product. The expression constructs used the cytomegalovirus (CMV) promoter and also carried a neo' expression cassette to facilitate selection of transfected cells.
A colony growth assay was used to monitor the impact of the modifications on cell growth. NIH-3T3 cells were transfected with the various constructs, and the resulting colonies were visualized by gentian violet staining after 13 days of G418 selection (Figure 1B). Small colonies were observed in dishes transfected with constructs encoding tuberin molecules lacking the leucine zipper (CMV-LR) and rap1GAP (CMV-RG) motifs (Figure 2B). Paradoxically, growth inhibition (similar to that obtained with the wild-type tuberin construct) was observed in cells transfected with a construct in which all 3 motifs were disrupted (CMVΔLRG). These results were reproduced in 4 independent experiments using a minimum of 3 independent DNA preparations; moreover, no significant difference in transfection efficiency was observed between the different constructs.

**Modified TSC2 cDNAs Promote Cell Growth In Vitro and In Vivo**

Stable NIH-3T3 cell lines harboring the growth-promoting constructs were generated to determine the consequences of long-term transgene expression. Cell lines with the CMV-null and CMV-WT constructs were generated as controls. Western blot analyses indicated that each construct expressed similar amounts of recombinant protein (Figure 2A: data from representative cell lines are shown; the level of recombinant protein expression was ~2- to 3-fold greater than that of the endogenous tuberin gene product). Cells from each group were plated in triplicate at a density of 10^4 cells/100-mm dish and cultured for 2, 4, 6, and 8 days. The cultures were then trypsinized and the cell numbers determined (Figure 2B). In agreement with the colony growth assay, cells expressing the CMVΔRL and CMVΔRG constructs exhibited the highest rate of proliferation. Similar results were obtained when 6 independent cell lines for each construct were analyzed (not shown).
A SCID mouse tumor assay was used to determine whether the enhanced growth observed with the CMV∆RL and CMV∆RG constructs in vitro persisted in vivo. Cells expressing these constructs were injected into NOD/SCID mice (10^6 cells/mouse; 8 mice per group), and the animals were sequestered and monitored for tumor formation. The CMV-null and CMV-WT cell lines were used as controls. Tumor formation was accelerated in cells expressing the CMV∆RL and CMV∆RG constructs compared with the CMV-null construct, confirming that the growth enhancement of these modified tuberin cDNAs persisted in vivo (Figure 2C).

Western blot analyses revealed sustained tuberin expression in tumor material recovered from the mice (Figure 2D). Interestingly, no tumors were detected in mice injected with cells expressing the CMV-WT construct, which provides compelling proof of the growth-suppressing activity of wild-type tuberin (the experiment was terminated after 125 days).

To determine whether this tumor suppressor activity was associated with apoptosis, CMV-null and CMV-WT cultures were processed for in situ end-labeling (ISEL) reactivity, which provides a sensitive assay for DNA fragmentation. Many cells in the CMV-WT cultures had markedly condensed chromatin (visualized via Hoechst staining, blue signal), which was also positive for ISEL reactivity (green signal, see Figure 3A). Further inspection under phase-contrast illumination confirmed that the cells were dying (Figure 3A). Quantitative analysis of the cultures revealed that cells expressing the CMV-WT construct were highly prone to apoptosis (Figure 3B).

Increased Endocytosis and Aberrant Protein Trafficking in Cells Expressing Modified TSC2 cDNAs
Recent studies have suggested that loss of tuberin activity results in increased endocytosis and aberrant trafficking of negative cell cycle–regulatory proteins. Endocytosis was measured in the CMV-null, CMV∆RG, and CMV∆RL cell lines with an HRP uptake assay. A 2- to 2.5-fold increase in endocytosis rates was observed in the CMV∆RL and CMV∆RG cell lines compared with the CMV-null control (Figure 4A). The subcellular localization of p27, a negative regulator of the S-phase cyclin-dependent kinases, was also monitored. Prominent nuclear p27 immune reactivity was seen in the CMV-null control cells (Figure 4B). In contrast, only weak and diffuse cytoplasmic p27 immune reactivity was observed in the CMV∆RL cells (Figure 4C). Cytoplasmic p27 immune reactivity was also observed in the CMV∆RG cells (data not shown).

Cardiac Development Is Normal in Transgenic Mice Expressing the TSC2∆RL cDNA in the Heart
We next determined whether targeted expression of a modified TSC2 cDNA would alter cardiac development in transgenic mice. The TSC2∆RL cDNA was tested, because this modification had the greatest effect on cell growth in NIH-3T3 cells. A transgene comprising the mouse α-cardiac MHC promoter and the ARl tuberin cDNA was produced (Figure 5A). The resulting transgene (designated MHC∆RL) was microinjected into 1-cell embryos to produce transgenic animals. Seven of 29 mice born from the microinjected embryos were transgenic. These mice gave rise to 3 independent lineages, and transgene expression was stratified via Western blot analysis. MHC∆RL line 1 expressed high levels of the transgene (Figure 5B) and was selected for subsequent analysis. Endocytosis was examined in primary cardiomyocyte cultures prepared from control and MHC∆RL mice. Endocytosis was increased 2.5-fold in the transgenic hearts (Figure 5C), consistent with the biological activity observed for the TSC2∆RL cDNAs in cultured cells.

To monitor the consequences of transgene expression on cardiomyocyte DNA synthesis, the MHC∆RL mice were crossed with MHC-NLAC transgenic mice, which express a nuclear localized β-gal reporter under the regulation of the MHC promoter. Reporter gene activity permits rapid and unambiguous identification of cardiomyocyte nuclei in histological sections by simple X-Gal staining (cardiomyocyte nuclei appear dark blue as a result of the nuclear β-gal activity). Progeny inheriting both the MHC-NLAC and MHC∆RL transgenes were used as experimental animals, and those inheriting only the MHC-NLAC transgene were used as controls. There was no evidence of altered cardiac growth.
Cardiomyocyte DNA Synthesis in MHCΔRL Mice

Cardiomyocyte DNA synthesis was also monitored during isoproterenol-induced cardiac hypertrophy. After 7 days of isoproterenol infusion, a similar hypertrophic response was observed in the control MHC-nLAC and experimental MHC-nLAC/MHCΔRL animals (36±4.3% increase in HW/TL for control mice, 40±4.3% increase in HW/TL for experimental mice, see Table). In addition, a similar induction in ventricular atrial natriuretic factor mRNA expression was observed for the isoproterenol-treated control and experimental animals (not shown). The tritiated thymidine incorporation assay failed to detect any cardiomyocyte DNA synthesis in the MHC-nLAC mice after isoproterenol-induced hypertrophy (see Table; ~100 000 nuclei were counted). In contrast, DNA synthesis was reactivated in the ventricular myocardium of the MHCΔRL transgenic mice during isoproterenol-induced hypertrophy (see Table). Examples of cardiomyocytes synthesizing DNA (as evidenced by colocalization of silver grains and nuclear β-gal activity) are shown in Figure 6A and 6B. An overall cardiomyocyte tritiated thymidine labeling index of 0.018% was observed in the ventricles of MHCΔRL transgenic mice after 7 days of isoproterenol infusion. Immune histological analyses confirmed that the cells synthesizing DNA were expressing the MHCΔRL transgene (Figure 6C, arrow). Moreover, the level of transgene expression appeared to be similar to that in cardiomyocytes not synthesizing DNA (Figure 6C, arrowheads). Isoproterenol treatment had no effect on endocytosis in the transgenic hearts (Figure 5C), suggesting that β-adrenergic stimulation did not directly alter transgene activity. Finally, infusion of phenylephrine failed to reactivate cardiomyocyte DNA synthesis in the MHCΔRL transgenic mice, despite a marked hypertrophic response (52.0% increase in HW/BW ratio, 60 000 nuclei screened). This finding suggests that reactivation of cardiomyocyte DNA synthesis in MHCΔRL mice is dependent on β-adrenergic stimulation, rather than a simple response to hypertrophic myocardial growth.

<Figure 4> A, HRP endocytosis assay for CMV-null, CMVΔRG, and CMVΔRL cell lines. Results are normalized relative to the value for the CMV-null cells. Error bars indicate SEM. Each cell line was analyzed in triplicate. Endocytosis values were significantly different from the CMV-null controls (CMVΔRG, P<0.0005; CMVΔRL, P<0.0001; unpaired t test). B, Photomicrograph of a CMV-null cell processed for p27 immune reactivity (HRP-conjugated secondary antibody, black signal). Prominent nuclear p27 immune reactivity is seen. Of the cells expressing the CMV-null transgene, 97.1±0.52% exhibited nuclear p27 immune reactivity (n=500). C, Photomicrograph of a CMVΔRL cell processed for p27 immune reactivity (HRP-conjugated secondary antibody, black signal). Diffuse cytoplasmic p27 immune reactivity is seen; no signal is apparent in the nucleus. Only 4.2±0.34% of the cells expressing the CMVΔRL transgene exhibited nuclear p27 immune reactivity (n=500); the remainder of the cells had predominantly cytoplasmic immune reactivity.

<Figure 5> A, Schematic of the MHCΔRL transgene. The MHC promoter consisted of 4.5 kb of 5′ flanking sequence and 1 kb of the gene including exon 1, exon 2, and a portion of exon 3. SV40 refers to the SV40 early region transcription terminator and polyadenylation sequences. B, Western blot analysis demonstrating recombinant tuberin expression in the MHCΔRL transgenic heart. (−) indicates the level of tuberin expression in nontransgenic animals. C, HRP endocytosis assay of primary heart cultures prepared from nontransgenic and MHCΔRL transgenic hearts in the absence or presence of isoproterenol. Results are normalized relative to the value for the nontransgenic hearts in the absence of isoproterenol treatment. Error bars indicate SEM. Endocytosis values for the transgenic hearts were significantly different from the nontransgenic controls (MHCΔRL, P<0.0001; unpaired t test).
Discussion
The experiments described above were based on the premise that expression of TSC2 cDNAs in which one or more structural motifs were altered might antagonize the activity of the endogenous gene product and thereby mimic TSC2 deficiency. We selected 3 structural motifs for which there was either direct experimental evidence attesting to functional importance (the rap1GAP and rabaptin-5 binding motifs), or alternatively, evidence for splice variants that directly altered or deleted the motif (the leucine zipper). We have not yet tested the consequence of deleting the hamartin-binding domain, which has only recently been identified.18

Cells Expressing the ΔRG and ΔRL cDNAs Mimic TSC2-Deficient Cells
Loss of tuberin activity is associated with enhanced rates of proliferation.37 Similarly, enhanced proliferation was observed in the CMVΔRG and CMVΔRL cell lines. A 2-fold increase in endocytosis was reported in homozygous mutant Eker rat embryonic fibroblasts compared with wild-type fibroblasts.16 A similar increase in endocytosis was observed in CMVΔRL and CMVΔRG cells. Finally, aberrant subcellular localization of the cyclin-dependent kinase inhibitor p27 was observed in tuberin-deficient cells36 as well as in the CMVΔRG and CMVΔRL cells. Thus, cells expressing the ΔRG or ΔRL cDNAs share multiple phenotypic attributes with tuberin-deficient cells.

Given that tuberin clearly interacts with multiple proteins (including hamartin, raplα, and rabaptin-5), it is relatively easy to envision how the modified cDNAs could interfere with the activity of the endogenous gene product. For example, tuberin encoded by the ΔRL cDNA would lack the ability to bind rabaptin-5 and interact with other leucine zipper proteins but would nonetheless retain the capacity to interact with rap1 and potentially other G proteins. Expression of the ΔRL protein could effectively block the activity of the endogenous tuberin (and thereby mimic a loss of function mutation) by a simple titration mechanism. If a generalized scheme in which a disruption of protein/protein interactions underlies the observed cell cycle effects is correct, we would anticipate that some TSC2 mutations in TSC patients could act in a dominant negative manner (provided that the mutated protein was expressed at a high enough level to compete with the wild-type gene product). In that regard, it is of interest to note that a mutation encompassing an L717R substitution in tuberin resulted in the formation of multiple lung cysts in the absence of loss of heterozygosity, consistent with dominant negative activity.38

Cardiac Attributes in MHCΔRL Transgenic Mice

<table>
<thead>
<tr>
<th>Basic cardiac attributes (n=10)</th>
<th>MHC-nLAC (Control)</th>
<th>MHC-nLAC/MHCΔRL (Experimental)</th>
<th>Statistical Significance, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight, mg</td>
<td>152.3±6.01</td>
<td>147.3±5.81</td>
<td>NS</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>5.8±0.14</td>
<td>5.8±0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Heart weight/tibia length, mg/mm</td>
<td>8.5±0.27</td>
<td>8.5±0.28</td>
<td>NS</td>
</tr>
<tr>
<td>Cardiomyocyte DNA synthesis</td>
<td>0/100 692</td>
<td>0/105 342</td>
<td>NS</td>
</tr>
</tbody>
</table>

| After isoproterenol (n=5) | Heart weight, mg | 213.0±9.45 | 217.2±13.35 | NS |
| Heart weight/body weight, mg/g | 8.1±0.23 | 8.42±0.53 | NS |
| Heart weight/tibia length, mg/mm | 11.6±0.50 | 11.9±0.63 | NS |
| Cardiomyocyte DNA synthesis | 0/107 143 | 18/100 202 | <0.0001 |

Figure 6. A and B, Examples of cardiomyocyte DNA synthesis in sections prepared from MHC-nLAC/MHCΔRL transgenic animals with cardiac hypertrophy. After 7 days of isoproterenol infusion, the mice received a single injection of tritiated thymidine, and the hearts were harvested, sectioned, and processed for X-Gal staining and autoradiography. C, Analysis of tuberin immune reactivity and DNA synthesis in MHCΔRL cardiomyocytes. Tuberin immune reactivity was detected via HRP assay (reddish brown signal); cardiomyocyte nuclei were visualized by X-Gal staining. Similar levels of transgene expression were seen in cardiomyocytes synthesizing DNA (arrow) and cardiomyocytes that were not synthesizing DNA (arrowheads). The photographic emulsion used for the autoradiograph in C has finer grain, hence the difference in silver grain size compared with A and B.
cardiomyocyte terminal differentiation. Given the effects of TSC2\textit{ΔRL} cDNA expression in cell culture experiments, it was surprising that cardiac development was normal in the MHC\textit{ΔRL} mice. No apparent morbidity or mortality was associated with myocardial transgene expression, nor was there evidence for either abnormal cardiac hypertrophy or sustained cardiomyocyte DNA synthesis in unperturbed adult transgenic mice. Additional studies failed to detect any differences in cardiomyocyte nucleation or gross histological appearance in fetal and adult transgenic mice compared with their nontransgenic sibs (K.B.S.P. and L.J.F., unpublished observations). It would thus appear that expression of the TSC2\textit{ΔRL} cDNA did not perturb the normal process of cardiomyocyte terminal differentiation. This is in contrast to the situation in homozygous mutant Eker fetuses\textsuperscript{19} and in TSC2 patients,\textsuperscript{9} in whom there is clear evidence for increased cardiomyocyte proliferation in the absence of tuberin expression.

This observation suggests that expression of the TSC2\textit{ΔRL} cDNA in cardiomyocytes is not functionally equivalent to disruption of the tuberin gene. It is possible that the relative level of transgene expression was insufficient to completely antagonize the activity of the endogenous tuberin. It is nonetheless clear that the TSC2\textit{ΔRL} cDNA is biologically active, as evidenced by markedly increased cardiomyocyte endocytosis in the transgenic animals. The absence of a developmental phenotype might also be attributable to the timing of transgene expression. In the case of the Eker rat studies, the homozygous mutant Eker embryos never express tuberin. Consequently, tuberin deficiency preceded cardiomyogenic differentiation in that model. In contrast, the TSC2\textit{ΔRL} cDNA was under regulation of the MHC promoter, and as such, transgene expression was not induced until after cardiomyogenic differentiation in the atria and not until birth in the ventricles.\textsuperscript{24} If a developmental cardioproliferative phenotype requires compromised tuberin activity during cardiomyogenic differentiation or early cardiac development, the transgenic model could not recapitulate this phenotype. It is difficult to directly compare the transgenic mouse model with clinical observations, because it is not clear when in cardiac development myocardial cells (or their precursors) become tuberin-deficient in TSC patients. Finally, as with all transgenic mouse models, we cannot rule out the possibility that sustained transgene expression has altered the normal developmental program, thereby making the myocardium less sensitive to targeted expression of the TSC2\textit{ΔRL} cDNA.

**Potential Mechanism for the Reactivation of Cardiomyocyte DNA Synthesis After \(\beta\)-Adrenergic Stimulation in MHC\textit{ΔRL} Mice**

Given that cardiomyocyte terminal differentiation was normal in the MHC\textit{CARL} mice, it was surprising that isoproterenol-induced hypertrophy reactivated cardiomyocyte DNA synthesis in adult animals. A cardiomyocyte DNA synthesis rate of 0.018\% was observed in the MHC\textit{ΔRL} mice after a single injection of isotope. Although no DNA synthesis was detected in the control MHC-nLAC hearts in the present study, previous experiments have established a maximum cardiomyocyte DNA synthesis rate of \(\approx 0.0005\%\) in both unperturbed adult mice\textsuperscript{28} and in isoproterenol-treated animals.\textsuperscript{31} It should be noted that there is some discrepancy in the literature regarding the rates of cardiomyocyte DNA synthesis in normal and injured adult hearts (reviewed in References 39 and 40). Regardless of the differences observed in different species and with different models of cardiac injury, the salient observation in the present study is that transgene expression does not affect cardiomyocyte DNA synthesis in normal adult hearts but results in a 35-fold increase in cardiomyocyte DNA synthesis during hypertrophic myocardial growth.

The persistent cytoplasmic sequestration of p27 in mutant Eker embryonic fibroblasts\textsuperscript{46} and M-phase cyclins in TSC2-deficient Drosophila embryos\textsuperscript{38} suggest that anomalous protein trafficking might underlie the altered cell cycle regulation observed in tuberin-deficient cells. Expression of the TSC2\textit{ΔRL} cDNA also perturbed p27 subcellular localization in transfected NIH-3T3 cells. Reactivation of cardiomyocyte DNA synthesis during cardiac hypertrophy in the MHC\textit{ΔRL} transgenic mice may result from a similar mechanism. In vitro, hypertrophic stimulation of cultured neonatal cardiomyocytes (for example, angiotensin II treatment or mechanical stretch) is associated with a transient increase in a number of positive cell cycle–regulatory proteins, including immediate early genes, proto-oncogenes, and such G\(_{1}/S\) regulatory proteins as D-type cyclins, CDK4, and hyperphosphorylated RB.\textsuperscript{41,42} Increased expression of many of these same gene products has been documented during cardiac hypertrophy in vivo.\textsuperscript{43} Although induction of these growth-promoting gene products during hypertrophy is insufficient to reactivate cardiomyocyte DNA synthesis in nontransgenic hearts (see Table),\textsuperscript{28} induction in a genetic background in which negative growth-regulatory pathways are compromised could easily result in reactivation of cardiomyocyte DNA synthesis. Importantly, the activities of many of these gene products are directly antagonized by p27 and other CDK inhibitors, which appear to be preferentially affected by anomalous protein trafficking in TSC2-deficient fibroblasts.

Expression of the MHC\textit{ΔRL} transgene resulted in a 35-fold increase in the level of cardiomyocyte DNA synthesis during isoproterenol-induced hypertrophy compared with the nontransgenic animals. However, the absolute number of cardiomyocytes synthesizing DNA was low. Regional variability in the level of expression of CDK inhibitors throughout the myocardium,\textsuperscript{44} coupled to a global increase in cyclin and CDK expression observed in some instances during myocardial hypertrophy,\textsuperscript{45} may explain why only a small fraction of the cardiomyocytes expressing the MHC\textit{ΔRL} transgene reactivate DNA synthesis. Importantly, isoproterenol had no effect on endocytosis in the transgenic cardiomyocytes, suggesting that \(\beta\)-adrenergic stimulation did not directly alter transgene activity. Although several studies\textsuperscript{46–49} have support a potential role for p27 in the cardiomyocyte DNA synthesis seen in hypertrophic MHC\textit{ΔRL} hearts, preliminary efforts have failed to detect cytoplasmic sequestration of this protein in the transgenic hearts (K.B.S.P., unpublished observations). Thus, if aberrant protein trafficking does underlie the phenotype in the MHC\textit{ΔRL} hearts, it most likely results...
from dysregulation of another protein(s). Given the pivotal role of Rab5 in regulating endocytosis and vesicle membrane docking, it is likely that intracellular protein trafficking is compromised to some degree in the MHC-ΔRL hearts. At present, it is not clear whether those cardiomyocytes synthesizing DNA ultimately undergo karyokinesis and/or cytokinesis. Finally, it appears that the rap1GAP activity encoded by TSC2 is not important for adult cardiomyocyte cell cycle reactivation, because no DNA synthesis was detected in normal or hypertrophic hearts from mice expressing an MHCARG transgene (K.B.S.P. and L.J.F., unpublished observations).

**Implications for Regenerative Myocardial Growth**

Although a limited number of cardiomyocytes in the MHC-ΔRL mice retained the capacity to reenter the cell cycle in response to specific stimuli, the absence of DNA synthesis in unperturbed transgenic hearts suggests that developmental cell cycle withdrawal proceeds normally in these animals. This is in contrast to other transgenic models (as exemplified by mice expressing cyclin D1 in the myocardium), in which adult cardiomyocyte DNA synthesis appeared to result from a failure of the terminal differentiation process. This distinction is extremely important if the gene (or pathway) under study is to be exploited for therapeutic intervention in diseased hearts. Many forms of cardiovascular disease are accompanied by cardiomyocyte loss, and it is generally accepted that the regenerative capacity of the adult myocardium is limited. The ability to reactivate the cell cycle in the adult heart, thereby effecting regenerative growth, could thus be of considerable therapeutic utility. Targeted expression of the ΔRL tiberin cDNA, in conjunction with isoproterenol stimulation, appears to have reactivated the cell cycle in a limited number of adult cardiomyocytes. Identification of the pathway(s) activated in the responsive cells or alternatively, synergistic interaction with other regulatory pathways might provide a means to increase cell cycle reactivation to a point sufficient for regenerative cardiomyocyte growth.

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