Heterotopic Cardiac Transplantation Decreases the Capacity for Rat Myocardial Protein Synthesis

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Heterotopic cardiac isografts are vascularly perfused hearts that maintain structural and functional integrity for prolonged periods of time. When placed in an infrarenal location, the heart is hemodynamically unloaded and undergoes negative growth, leading to cardiac atrophy. At 7 and 14 days after transplantation, the transplanted heart was decreased in size compared with the in situ heart \( (p < 0.001) \). To assess the possible mechanism(s) to account for this reduction in size we studied in vivo rates of total left ventricular (LV) protein synthesis, total LV RNA content, and 18S ribosomal RNA content by nucleic acid hybridization. The LV protein synthetic rate was 4.7 and 5.3 mg/day in the in situ heart and was significantly decreased to 2.9 and 2.7 mg/day in the transplanted hearts at 7 and 14 days, respectively. LV RNA content of the transplant declined to 53% and 48% of the in situ value at 7 and 14 days, respectively. Hybridization studies revealed that LV 18S ribosomal subunit content was reduced proportionally to total RNA in the heterotopic hearts. As a result of these changes, there was no significant difference in the efficiency of total LV protein synthesis between the in situ and transplanted hearts. The present studies demonstrate that the hemodynamic unloading and cardiac atrophy that is characteristic of heterotopic cardiac transplantation is accompanied by a decrease in LV total RNA content and 18S RNA, resulting in a decreased capacity for myocardial protein synthesis. \( \text{(Circulation Research 1991;68:1100–1107)} \)

The postnatal growth of the heart, similar to other specialized and well-differentiated tissues, is accomplished primarily by cellular hypertrophy.\(^1\) Previous studies\(^2\)–\(^5\) have demonstrated that cardiac work is a major determinant of the growth of the heart and maintenance of cardiac muscle mass. Various conditions that augment the work of the heart lead to rapid changes in cardiac protein synthesis and cardiac RNA content that mediate this growth process. In contrast, the process of cardiac atrophy (negative cardiac growth), such as accompanies heterotopic cardiac transplantation and hemodynamic unloading, has not been well studied.\(^6\)–\(^9\)

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Heterotopic cardiac isografts placed in an infrarenal location maintain coronary perfusion and structural and functional integrity for prolonged periods.\(^10\)\(^,\)\(^11\) When transplantation is performed as previously described,\(^7\)\(^,\)\(^10\) the aortic valve is maintained competent, left ventricular filling is decreased, and the heart performs a reduced amount of work. This hemodynamic unloading is associated with a marked decrease in total heart mass, left ventricular protein content, and left ventricular myosin content.\(^7\)\(^,\)\(^8\)\(^,\)\(^11\) To characterize the changes in protein synthesis that accompany cardiac atrophy, we have studied the in vivo rates of protein synthesis, protein content, and RNA and DNA content, as well as the content of 18S RNA of the left ventricle, at 7 and 14 days after transplantation. Reduced rates of protein synthesis accompanying cardiac atrophy could result from either a decreased synthetic efficiency or capacity. The transplanted heart model allows us to simultaneously measure and compare these parameters in both the growing, working heart and in the hemodynamically unloaded heart undergoing atrophy.

**Materials and Methods**

**Cardiac Transplantation**

Infrarenal heterotopic cardiac isografting was performed on inbred male Lewis rats weighing between
145 and 155 g (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), as previously described.7,10 The period of warm ischemia was less than 22 minutes. The procedure entailed anastomosis of the end of the donor heart aorta to the side of the recipient abdominal aorta and of the donor pulmonary artery to the recipient inferior vena cava to return myocardial blood flow. The aortic valve of the transplanted heart was maintained competent.11

Rats were weighed daily and killed 7 and 14 days after transplantation. Unoperated rats were killed at day 0 to serve as controls.

Protein Synthesis

In vivo protein synthesis was estimated according to the method of Garlick et al.12 On the day of death, the rats were lightly anesthetized with methoxyflu- rane by inhalation. They were then injected via the femoral vein with 2 ml of a “flooding” infusion tracer consisting of 100 μCi [3H]phenylalanine and 300 μmol unlabeled phenylalanine. After 10 minutes, the rats were decapitated, and blood was collected. The hearts were excised, quickly rinsed in phosphate buffered saline (3°C), and blotted; the left ventricle (including septum) was removed and weighed, and a portion was immediately frozen in liquid nitrogen.

Blood samples were centrifuged (10,000g for 5 minutes). A portion of the plasma (10 μl) was removed for scintillation counting; the remainder was deproteinized by addition of an equal volume of 10% (wt/vol) trichloroacetic acid. The amino acids were isolated from the supernatant fraction by cation exchange chromatography,13 and plasma phenylalana- nine specific radioactivity (dpm/nmol) was deter- mined by the method of Airhart et al.14 Plasma phenylalanine concentration (μM) was derived as follows: plasma 3H radioactivity (dpm/ml)/plasma phenylalanine specific radioactivity (dpm/nmol).

Left ventricular protein synthetic rates were esti- mated from the specific radioactivity of phenylala- nine in the tissue-free and in the trichloroacetic acid–precipitable total protein pools from both in situ and transplanted hearts. Biosynthetically labeled left ventricular tissue (100 mg) was homogenized in 19 vol buffer containing 100 mM KCl and 250 μM thioglycolate, pH 6.8. A portion of the whole-tissue homogenate (50 μl) was removed for determination of total protein content by the method of Lowry et al.15 Another portion (250–500 μl) was removed and added to an equal volume of 10% trichloroacetic acid at 3°C for 30 minutes. These samples were centri- fuged, and free amino acids in the supernatant fraction were isolated by cation exchange chromatography.13 Tissue-free phenylalanine specific radioactivity (dpm/nmol) and tissue-free phenylalanine concentra- tion (nmol/g) were assessed as described above.

The trichloroacetic acid–precipitable protein pel- let was washed four times in 1.5 ml of 10% trichloro- acetic acid, followed by a wash in 1.5 ml ethyl ether. The precipitated proteins were hydrolyzed in 6N HCl (24 hours at 110°C). Protein-bound phenylalanine specific radioactivity in the hydrolysates (dpm/nmol) was then determined by isotope dilution radioassay14 as previously modified.16 The fractional rate of protein synthesis (Ks) was estimated using the following formula:

$$K_s = P^*/(F^* \times t)$$

where $P^*$ is protein-bound phenylalanine specific radioactivity and $F^*$ is tissue-free phenylalanine specific radioactivity. $K_s$ was converted to actual protein synthetic rates ($R_s$, mg/day) by multiplying $K_s$ by the left ventricular protein pool size. These calculations assume rapid equilibration of extracellular, intracel- lular, and phenylalanyl-tRNA specific radioactivities after administration of a flooding dose of phenylala- nine, as previously discussed.12

RNA Content

Aliquots of left ventricular myocardium weighing between 50 and 150 mg were rapidly removed and homogenized in a Potter-Elvehjem tissue homoge- nizer in 35 vol RNA extraction buffer (0.15 M NaCl, 50 mM Tris at pH 8.3, 5 mM EDTA, and 1% sodium dodecyl sulfate. Suspended proteinase K was added, and the total volume was adjusted to 4.0 ml with extraction buffer.17 The homogenate was incubated at room temperature for 10 minutes and then ex- tracted twice with a solution containing phenol- chloroform-isooamyl alcohol (25:24:1 vol/vol/vol) and once with chloroform-isooamyl alcohol (24:1). The final aqueous phase was adjusted to 0.2 M sodium acetate, and the nucleic acids were precipitated by the addition of 2.5 vol cold ethanol and kept over- night at −20°C. The precipitate was collected by centrifugation, and the final pellet was taken to dryness and dissolved in 1 mM EDTA, pH 7.6.

Total nucleic acid content was assayed by spectro- photometric absorbance at 260 and 280 nm. Only samples giving a 260/280-nm absorbance ratio of between 1.8 and 2.0 were considered sufficiently pure for analysis.

DNA content of the purified aliquots was assayed by fluorescent analysis using Hoechst Dye 33258 (Polysciences Inc., Warrington, Pa.).18 Samples of left ventricular nucleic acid extract were added to a solution containing a final concentration of fluores- cent dye of 0.1 μg/ml, 10 mM Tris, 1.0 mM EDTA, and 0.1 M NaCl, pH 7.4. The samples were incubated for 10 minutes in the dark. Relative fluorescence was determined using a fluorometer (Gilford Systems, Oberlin, Ohio) at an excitation wavelength of 365 nM and an emission wavelength of 458 nM. Calf thymus DNA was used to prepare a standard curve. The assay was linear over the range of 0.05–1.0 μg DNA/ml.

Total RNA concentration of the nucleic acid ex- tract was calculated from the measured total nucleic acid absorbance and measured DNA concentration using the following formula:
RNA $A_{260}=\text{total nucleic acid } A_{260} - \frac{\text{DNA } \mu g/ml}{50 \mu g/ml} A_{260}$

RNA $\mu g=\text{RNA } A_{260} \text{ units} \times 40 \mu g$

The extinction coefficient for RNA and for DNA at 260 nm ($A_{260}$) was taken to be 40 and 50 $\mu g/ml$, respectively.

RNA extraction efficiency was analyzed using purified rat liver RNA labeled with $^{32}P$ orthophosphate by T4 polynucleotide kinase end-labeling. When $^{32}P$RNA was added to the first homogenate, the recovery in the final nucleic acid pellet was 90% ± 1%. The intra-assay coefficient of variation for seven separate control RNA extractions was 7.4%.

18S Ribosomal RNA Quantitation

Serially diluted samples of left ventricular RNA extract containing from 0.1 to 10.0 $\mu g$ were applied to 1.2% agarose/3% formaldehyde gels and electrophoresed for 17 hours at 35 V using a 20 mM Na$_2$HBO$_3$/3% formaldehyde buffer, pH 8.3. The RNA was then transferred to a nylon membrane (Gene Screen, New England Nuclear, Boston) by the technique of Northern blotting. RNA species were identified by molecular hybridization using $^{35}P$CTP randomly primed 18S genomic rDNA fragments. The filter was washed stringently with 0.3 mM NaCl and 0.03 M sodium citrate (pH 7.0) containing 1% sodium dodecyl sulfate at 63°C and then with 0.1 x sodium citrate at 23°C. The blot was exposed to Kodak R-P X-omat film, and the autoradiogram was used to identify the location of individual RNA bands. The area corresponding to 18S RNA was excised from the membrane, and the radioactivity was measured by liquid scintillation spectrometry.

Additional 18S RNA quantitation was performed by dot blot hybridization using serial dilutions of total left ventricular RNA containing from 0.1 to 1 $\mu g$. Hybridization and washing was as described for Northern blotting. The RNA dots were cut out and Scintillation activity was quantitated by liquid scintillation counting. Molecules of 18S left ventricular RNA hybridized per aliquot of left ventricular RNA were calculated according to the following formula: molecules of 18S RNA hybridized = (amount probe hybridized/probe MW) x (6.02 x 10$^2$), where amount probe hybridized (ng) is counts hybridized divided by probe $^{35}P$ specific activity (cpm/ng probe), MW is molecular weight, and probe MW is 6.18 x 10$^2$. Total molecules of 18S RNA hybridized per left ventricle were then calculated based on the left ventricular RNA content.

Statistical Analysis

All results are expressed as mean ± SEM. Significance was assessed by paired t test between results for host and transplanted hearts.

| TABLE 1. Left Ventricular Weight, Protein Content, and Protein Synthesis Rate |
|-------------------------------|-----------------|---------------|
|                               | Day 0 (n=4)     | Day 7 (n=4)   | Day 14 (n=6) |
|-------------------------------|-----------------|---------------|
| In situ heart                 |                 |               |
| Weight (mg)                   | 355 ± 6         | 434 ± 5       | 506 ± 5      |
| Protein content (mg)          | 62 ± 3          | 71 ± 10       | 88 ± 2       |
| Protein synthesis rate (mg/day)| 4.0 ± 0.2      | 4.7 ± 0.6     | 5.3 ± 0.6    |
| Fractional protein synthesis (%/day)| 6.5 ± 0.4| 6.4 ± 0.7   | 6.1 ± 0.5    |
| Transplanted heart            |                 |               |
| Weight (mg)                   | ... 242 ± 3*    | 224 ± 6*      |             |
| Protein content (mg)          | ... 40 ± 8*     | 37 ± 1*       |             |
| Protein synthesis rate (mg/day)| ... 2.9 ± 0.2†  | 2.7 ± 0.1*    |             |
| Fractional protein synthesis (%/day)| ... 7.3 ± 0.5‡ | 7.2 ± 0.5‡   |             |

Values are mean ± SEM for the total left ventricle from each experimental group. Day 0, control study performed on unoperated rats; day 7, study performed on transplanted rats 7 days after surgery; day 14, study performed on transplanted rats 14 days after surgery.

*p<0.001, †p<0.05, and ‡p>0.30 compared with corresponding value for in situ hearts.

Results

The heterotopic isografts maintained spontaneous heart rates greater than 200 beats/min when measured 7 and 14 days after transplantation. Prior studies have demonstrated that when the period of warm ischemia is minimized, these hearts are histologically and electrophysiologically normal. When compared with the donor heart left ventricular weight of 355 ± 6 mg at the time of transplantation (day 0), the isograft had significantly decreased in size to 242 ± 3 and 224 ± 6 mg ($p<0.01$) after 7 and 14 days, respectively (Table 1). The degree of left ventricular atrophy (negative growth) of the transplant was even greater when contrasted with the weight of the in situ left ventricle (506 ± 5 mg; p<0.001), which was hemodynamically loaded, working normally, and growing. Similar differences were observed when total heart weight at both time points was compared (295 ± 5 versus 579 ± 14 mg and 279 ± 5 versus 671 ± 8 mg, transplant versus in situ heart, respectively; $p<0.001$).

The total left ventricular protein content declined in a proportionate fashion; the protein content of the transplant was 56% (day 7) and 42% (day 14) of the corresponding in situ value and 65% (day 7) and 60% (day 14) of the day 0 in situ value (Table 1). Thus, the heterotopically transplanted isograft demonstrated a prompt and marked reduction in size and protein content in the absence of functional or histological evidence of myocardial damage.

In Vivo Left Ventricular Protein Synthesis

Administration of a large intravenous dose of phenylalanine (150 $\mu mol/100$ g body wt) to both control (day 0) and transplanted rats rapidly expanded the...
plasma and the in situ and transplant tissue-free pools of this amino acid in the rats studied at 14 days (Table 2). Plasma phenylalanine concentration was increased approximately 15-fold at 10 minutes after the intravenous injection, as compared with normal plasma levels of this amino acid. Both the in situ and transplant tissue-free phenylalanine pools were also markedly expanded. Tissue-free phenylalanine concentrations were similar in both the in situ and transplanted hearts (Table 2). Similar results were obtained in the rats studied at 7 days.

Expansion of the phenylalanine tissue-free pools was also associated with equilibration of plasma and of tissue-free phenylalanine specific radioactivities in both the in situ and transplanted left ventricles. Although the specific radioactivities of phenylalananyl-tRNA, the immediate precursor for new protein synthesis, were not measured in the present studies, it was assumed that rapid and complete equilibration of this labile intracellular phenylalanine pool was also attained.

By using measurements of tissue-free and protein-bound phenylalanine specific radioactivities in individual tissue samples, left ventricular total protein synthesis was calculated for the in situ hearts at day 0 and for the in situ and transplanted hearts at days 7 and 14. Protein synthesis (Rn) was expressed as milligrams protein synthesized per day per left ventricle; values were 4.0±0.2, 4.7±0.6, and 5.3±0.6 (mean±SEM) mg/day for the in situ hearts at days 0, 7, and 14, respectively. In contrast, the rates of left ventricular protein synthesis were significantly lower at 2.9±0.2 and 2.7±0.1 mg/day in the transplanted left ventricle at 7 and 14 days, respectively (Table 1). The fractional rates of total cardiac protein synthesis (Ko, %/day) were not significantly different between the host and transplanted hearts (Table 1).

**RNA and DNA Content**

Left ventricular RNA and DNA content was assayed in each group of hearts. The left ventricular RNA concentration was 1.55±0.05 mg/g at day 0 and was unchanged at both day 7 and day 14 in the in situ heart (1.57±0.11 and 1.46±0.09 mg/g) and in the transplanted heart (1.51±0.11 and 1.59±0.09 mg/g). In contrast, the total left ventricular RNA content was significantly decreased to 360±27 and 357±19 μg in the transplanted hearts at days 7 and 14, respectively, compared with the in situ hearts at all time points (Table 3). There was a significant increase in left ventricular DNA content in the in situ heart between 0 and 14 days. The left ventricular DNA concentration rose from 1.49 mg/g (day 0) in the control hearts to 1.83 mg/g (day 7) and 2.06 mg/g (day 14) in the transplanted hearts; however, there was a significant decline in total left ventricular DNA from 529±6 μg (day 0) in the control hearts to 445±31 μg (day 7) and 461±14 μg (day 14) in the transplanted hearts (Table 3). When compared with the day 0 values, the percentage decrease in RNA content for the 14-day transplant was 35%, a value significantly greater than that for the 13% decline in DNA content (p<0.01).

Aliquots of left ventricular RNA from all experimental points were assayed by dot blot hybridization to determine the number of molecules of 18S RNA. As seen in Figure 1, using the technique of Northern blotting the 35P-labeled rRNA probe was specific for 18S RNA. The amount of probe hybridized as assayed by liquid scintillation spectrometry was proportional to the amount of left ventricular RNA extract applied, and this relation was linear over a range of 0.1–1.0 μg left ventricular RNA (Figure 2). Based on the measured specific activity of the 35P-labeled 18S rRNA probe, the total number of molecules of 18S RNA hybridized was calculated and expressed per left ventricle. The values measured for the transplants at days 7 and 14 were significantly less than the control values at day 0 and the in situ values at days 7 and 14 (p<0.001) (Table 3).
To assess a possible role for the altered left ventricular RNA content on the observed changes in the rate of protein synthesis, the ratio of these parameters was calculated. When protein synthesis was expressed as a function of both total left ventricular RNA and left ventricular 18S RNA, the results were not significantly different between the three groups of hearts (Table 4). This observation suggests that the decrease in total left ventricular protein synthesis that accompanies the cardiac atrophy of heterotopic transplantation is a result of a decrease in capacity rather than efficiency of total cardiac protein synthesis.

**Discussion**

Heterotopic cardiac isografts are histologically normal, spontaneously beating hearts that undergo rapid and predictable negative growth (atrophy). In contrast to the in situ heart, the transplanted heart is hemodynamically unloaded and does less work. Various prior studies have documented that cardiac work is a major determinant of the rate of cardiac protein synthesis leading to changes in cardiac size. The present studies address the changes, resulting from in vivo protein synthesis, that accompany the cardiac atrophy of heterotopic transplantation. We have also investigated the level of protein synthesis regulation to better understand the mechanisms by which hemodynamic changes lead to alterations in the growth of the heart.

Protein synthesis has been assessed both in vivo and in vitro by using a variety of previously described methods. These techniques all rely on the quantitative analysis of tracer amino acids incorporated into individual or mixtures of newly synthesized proteins. A potentially limiting factor in all of these methods is the accurate assessment of the specific radioactivity of the precursor pool used for new protein synthesis (aminoacyl-tRNA) during the entire course of the biosynthetic labeling period. In the present experiments, total cardiac protein synthesis was estimated using the flooding infusion method of Garlick et al, in which a large dose of intravenously administered, radiolabeled phenylalanine is used to flood all extracellular and intracellular pools of this amino acid. When this technique is used, it is assumed that the specific radioactivities of phenylalanine in the extra-

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**TABLE 4. Efficiency of Left Ventricular Protein Synthesis**

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<thead>
<tr>
<th></th>
<th>Protein synthesis rate (mg protein/day)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total LV RNA</td>
<td>Molecules LV 18S RNA</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In situ hearts</td>
<td>7.26</td>
<td>0.81</td>
</tr>
<tr>
<td>Transplanted</td>
<td>6.93</td>
<td>0.81</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In situ hearts</td>
<td>8.00</td>
<td>0.84</td>
</tr>
<tr>
<td>Transplanted</td>
<td>7.10</td>
<td>0.78</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In situ hearts</td>
<td>7.56</td>
<td>0.84</td>
</tr>
<tr>
<td>Transplanted</td>
<td>7.56</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Values expressed are the rates of the protein synthesis as a function of total left ventricular (LV) RNA content and LV 18S RNA. Day 0, control study performed on unoperated rats; day 7, study performed on transplanted rats 7 days after surgery; day 14, study performed on transplanted rats 14 days after surgery.
It is generally assumed that the kinetics of protein synthesis can best be described as a zero-order process, in which the rate of protein synthesis is independent of the amount of protein in the protein pool. In non-steady-state systems of muscle growth or atrophy, changes in the protein pool size can occur independent of changes in the fractional protein synthetic rates. This has been observed in the growing rabbit heart, in which the fractional rate of protein synthesis falls while the actual rate of protein synthesis rises between 4 days and 9 weeks of age.

In our prior in vitro protein synthesis studies, we demonstrated evidence for an increase in the rate of protein degradation during the course of cardiac atrophy. In the present study, the observation that the fractional rate of protein synthesis is not altered at 7 and 14 days also suggests that hemodynamic unloading may increase the rate of protein degradation as an additional mechanism to explain the very rapid fall in cardiac mass. An increase in degradation was also reported by Thomason et al. in the recent study of muscle atrophy in unweaned soleus muscle.

To explore the possible mechanisms for the decrease in the protein synthesis rate, we have measured total left ventricular RNA content as well as the specific content of 18S rRNA. The techniques used for left ventricular RNA quantitation were reproducible, and both the concentration (mg RNA/g left ventricular tissue) and the total content (µg RNA/left ventricle) compare favorably to previously reported values. The total left ventricular RNA content was markedly and significantly reduced in the heterotopic transplant. A similar reduction was observed for 18S RNA, which was quantitated by hybridization to a 32P-labeled 18S rDNA probe using both Northern and dot-blot analysis. This parallel decline in both total left ventricular RNA and 18S RNA was not surprising in view of the fact that ribosomal RNA constitutes greater than 85% of total mammalian cellular RNA.

The dot-blot hybridization assay used for 18S RNA quantitation was linear over the range of 0.1–1 mg left ventricular RNA extract. At higher concentrations of extract RNA, the binding was reduced. This is consistent with a prior report for hybridization using complementary DNA probes for other RNA species. Calculations for the total content of 18S RNA in control heart samples suggest that the number of molecules hybridized is only a small portion of the total number of molecules present.

The ratio of the total left ventricular protein synthetic rate to both total left ventricular RNA and 18S RNA content reflects the relative efficiency of myocardial protein synthesis. Prior reports have explored changes in the capacity and efficiency of protein synthesis as a mechanism for mediating the cardiac growth response to increased workload resulting from contraction, pressure overload, and hyperthyroidism. When compared with the in situ hearts, the efficiency of synthesis was not decreased in the transplanted heart at a time when total syn-
thesis was decreased by 50%. This finding can be interpreted as demonstrating regulation of negative cardiac growth (atrophy) via changes in the overall level of protein translation. However, since ribosomal RNA synthesis and content are at least in part mediated by changes in the rate of RNA transcription, changes in the latter may also occur as a mechanism for regulating the growth of the heart.35

In addition to the marked reduction in left ventricular RNA, there was a smaller (12%) reduction in left ventricular DNA. Possible explanations for these later changes would include myocyte damage or loss, but in view of the previously normal histology and the observation that the left ventricular DNA concentration (µg/g tissue) rises in the transplant, this postulate seems unlikely. It is possible that the atrophying transplanted heart does undergo some loss of nonmuscle heart cells, but this question cannot be resolved without cell counting or quantitative histomorphometry.

In the rapid and marked atrophy resulting from heterotopic cardiac transplantation, it is possible to study some of the mechanisms that mediate the growth of mammalian cardiac myocytes. In response to mechanical unloading, the heart reduces its mass and actual rate of protein synthesis accompanied by a decrease in the capacity for protein synthesis. Changes in the rate of protein degradation may also be important in reestablishing a new steady state.9,11 Further studies will be required to identify how hemodynamic changes are transduced into changes in protein synthesis at both the level of the cell nucleus and cytoplasm.

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