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# Uptake of Albumin is Coupled with Stretch-Induced Hypertrophy of Skeletal Muscle Cells in Culture

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**ABSTRACT**—Hypertrophy is induced in skeletal muscle when mechanical overload, for example repetitive stretching, is presented. This is a well-known phenomenon and the molecular mechanism involved has been investigated from various aspects. In this study, with a system that enables periodic stretching of cultured skeletal muscle cells, myotubes, along the long cellular axis uni-directionally at a constant frequency, we examined the effects of stretching on skeletal muscle using mouse C2 myotubes in culture as a model. Significant hypertrophy was observed in the myotubes after several days of periodic stretching and this was accompanied by the accumulation of a protein of about 67kDa. This protein was identified with albumin, which was present in the culture medium, based on its antigenicity, size and pI. When bovine serum albumin tagged with biotin was added to the culture medium, it became detectable in the cytoplasm of the stretched myotubes. mRNA encoding albumin was not detectable in the myotubes by northern blotting irrespective of their stretching or non-stretching, indicating that transcription of the albumin gene was not induced in the stretched muscle cells. From these results, we conclude that the accumulation of albumin in stretched myotubes was due to uptake of the protein from the culture medium not to *de novo* synthesis of the protein in myotubes. We suggest that albumin uptake may be involved in skeletal muscular hypertrophy.

**Key words:** skeletal muscle, muscle cell culture, albumin, muscle hypertrophy, mechanical stress

## INTRODUCTION

Many types of cells respond to mechanical stress in a variety of ways. For example, endothelial cells of blood vessels are aligned in the direction of blood flow in response to hydrodynamic shear stress (White *et al.*, 1983). Under culture conditions, dynamic reorientation of cells and reorganization of stress fibers are induced in a dramatic manner under mechanical stress provided by periodic stretching (Shirinsky *et al.*, 1989; Takemasa *et al.*, 1998; Hayakawa *et al.*, 2000, 2001). It is known that muscle is sensitive to mechanical stress; for example, mechanical overload leads to hypertrophy of cardiac as well as skeletal muscle tissues *in vivo*. Vandeburgh and Kaufman (1979) have devised an *in vitro* model for stretching of cultured skeletal muscles and have shown that stretching of myotubes leads to hypertro-

phy and stimulates synthesis of muscle structural proteins like myosin heavy chain (Vandeburgh *et al.*, 1989). Recently, it has been reported that IGF-1 stimulates muscle hypertrophy (Adams 2002). IGF-1 promotes hypertrophy by activating PI3K/Akt/mTOR or /GSK3 pathways (Rommel *et al.*, 2001) and is increased by stretching (Yang *et al.*, 1996; Bamman *et al.*, 2001). It is also known that calcineurin has a role in skeletal and cardiac muscle hypertrophy (Dellus *et al.*, 2000; Olson and Sanders Williams 2000). Furthermore, it has been demonstrated that soon after stretching, the expression of several immediate-early genes such as *c-fos*, *c-jun*, *c-myc* is enhanced in cardiac cells in culture, followed by increased synthesis of fetal cardiac muscle proteins (Komuro *et al.*, 1991; Sadoshima *et al.*, 1992). In addition, increased expression of *c-ski* gene was reported to lead to hypertrophy of skeletal muscle fibers (Leferovich *et al.*, 1995). As a factor that suppresses muscle hypertrophy, myostatin, a protein belonging to the TGF- $\beta$  super family, has been characterized (McPherron *et al.*, 1997).

Albumin is a protein primarily synthesized in the liver, and it is a major protein component in serum. However, several investigators have also described the existence of this protein in muscle tissue. The presence of an albumin-like

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protein in muscle tissues, especially at a higher level in slow muscle than in fast muscle, was reported by Crepax (1952). Later, albumin was detected in muscle by various methodologies. However, its exact location within muscle and whether it is an intrinsic muscle protein or is of extraneous origin have been the subjects of argument. Muller and Heizmann (1982) reported that albumin is localized at the A/I junction regions of myofibrils in chicken skeletal muscle by use of immunoelectron microscopy. In contrast, Yokota (1982) demonstrated that albumin is localized predominantly in the extracellular space of rat skeletal muscle and in addition, that it is present within the lumen of T-tubules and in small subsarcolemmal vesicles in skeletal muscle fibers by immunocytochemical methods.

Accumulation of albumin in skeletal muscle under compensated hypertrophy was first demonstrated by Yamada *et al.* (1984). They further suggested that albumin could be synthesized by muscle cells, because  $^{35}\text{S}$ -methionine was incorporated into albumin in the culture of minced muscles. Later, however, Heilig and Pette (1988) reinvestigated the location of albumin in normal and chronically stimulated muscle by immunohistochemical methods and albumin synthesis in skeletal muscle by administration of radio-active methionine into muscle tissue and they reached different conclusions. They claimed that albumin is accumulated in the stimulated muscle but is distributed mainly in the interstitial space of muscle and in addition, that *de novo* synthesis of albumin in skeletal muscle is unlikely. On the other hand, Chiesi and Guerini (1987) isolated a soluble protein from skeletal muscle, which they identified as albumin, and they suggested that this protein functions as a protective agent of the  $\text{Ca}^{++}$  pump of the sarcoplasmic reticulum. However, it must be argued that such function of albumin would not be realistic, if it were not present in the muscle cytoplasm. Therefore, it is necessary to make a definite determination of whether or not albumin could be located in the muscle cell cytoplasm.

In this study, we investigated the response of cultured muscle cells to mechanical stress, namely periodic stretching, with particular focus on the phenomena coupled with hypertrophy, using the myotubes formed by C2 myoblasts, a cell line derived from mouse skeletal muscle. When the myotubes were periodically stretched for several days under our culture conditions, their size increased significantly. We found that albumin present in the culture medium became incorporated into the myotubes during the hypertrophic process. Our observations indicate definitely that albumin could be in the cytoplasm of skeletal muscle cells.

## MATERIALS AND METHODS

### Cell culture and stretching

Mouse C2 myogenic cells (Yaffe and Saxel, 1977) were propagated in DMEM (GIBCO Laboratories, Gland Island, NY) containing 10% fetal bovine serum (FBS) and 60  $\mu\text{g}/\text{ml}$  each of streptomycin and kanamycin in a humidified atmosphere with 5%  $\text{CO}_2$  and

95% air at 37°C. For stretching, the cells were removed from the culture dish with 0.25% trypsin and plated at a density of  $1.8 \times 10^3$  cells/ $\text{cm}^2$  on a silicone membrane with fine grooves running in parallel and coated with 0.05% porcine Type I collagen (Koken, Tokyo). Preparation of the silicone membrane was previously described (Hayakawa *et al.*, 2000). The culture medium was replaced with a differentiation medium containing 5% horse serum in place of FBS and the cultures were maintained for 3 to 4 days until myotubes were formed. When bovine serum albumin (BSA) or glutathione-S-transferase (GST) was added to the stretching cultures, horse serum was not supplemented. The membrane was then stretched along the direction of the long cell axis reciprocally up to 1.1 times its original length at a frequency of 10 cycles/minute in the previously described stretching apparatus (Hayakawa *et al.*, 2000).

### Northern blotting

Total RNA was prepared from mouse tissues and C2 myotubes by the rapid one-step method (Chomczynski and Sacchi, 1987). 20  $\mu\text{g}$  of RNA was applied to each lane separated on 0.8% agarose-formamide gel and transferred to nitrocellulose filters, that were then cross-linked by UV cross-linker (Stratagene, La Jolla, CA). Hybridization was carried out by the procedure of Thomas (1980), with a  $^{32}\text{P}$ -labeled cDNA probe for mouse albumin by the method of Feinberg and Vogelstein (1983). The cDNA probe used was the cDNA fragment corresponding to the base #315-#1568 of mouse albumin cDNA sequence that was described by Strausberg (NIH accession number BC024643). We cloned this cDNA from our mouse cDNA library. The filters were finally washed in  $0.1 \times \text{SSC}$  (15 mM NaCl, 2.5 mM trisodium citrate) containing 0.1% SDS at 50°C. The washed filters were exposed to a BAS imaging plate (Fuji Film, Tokyo).

### Antibodies

The monoclonal antibody to troponin T (NT-302) has been described (Abe *et al.*, 1986). The monoclonal antibody to BSA, which also recognized horse albumin, was prepared by standard procedure (Galfré *et al.*, 1977). The polyclonal antibody to GST was prepared in rabbits with purified GST as immunogen. Other commercial antibodies used were alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (GAM), AP-conjugated goat anti-rabbit IgG (GAR) from Bio-Rad (Richmond, CA), and FITC-conjugated GAR, FITC-conjugated GAM and rhodamine-conjugated GAR from Tago (Burlingame, CA).

### Gel electrophoresis and Western blotting

Cells cultured on silicone membrane were rinsed carefully three times with PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.0) and collected with a cell scraper. For SDS-polyacrylamide gel electrophoresis (PAGE), proteins were extracted with an SDS-lysis buffer containing 50 mM Tris-HCl, pH 6.7, 2% SDS, 2% 2ME, and 10% glycerol. For two-dimensional PAGE (2D-PAGE), proteins were extracted with a urea-lysis buffer (8 M urea, 2% 2ME, 2% Triton X-100, ampholine pH 3.5–10, pH 5–7). Protein concentrations were determined by solid phase dye-binding assay (Minamide and Bamburg, 1990).

SDS-PAGE was carried out using 13.5% polyacrylamide gel in a discontinuous Tris-glycine buffer system (Laemmli, 1979). 2D-PAGE was performed by a combination of isoelectric focusing (IEF) in the first dimension (O'Farrell *et al.*, 1977) and SDS-PAGE in the second dimension using 13.5% gel and the protein spots on the gel were detected by silver staining according to Oakley *et al.* (1980). For Western blotting, proteins were electrophoretically transferred from SDS-polyacrylamide gel to nitrocellulose filter (Towbin *et al.*, 1979). After treating with 5% skim milk, the filters were incubated with primary and secondary antibodies for 1 hr respectively and then they were washed with TBS (0.5 M NaCl, 10 mM Tris-HCl, pH

7.5).

#### Detection of uptake of biotin-BSA by myotubes

Bovine serum albumin (BSA) (fraction V, SIGMA, St Louis, MO) was labeled with biotin (Pierce Chemical Co., Rockford, IL) as described by Harlow and Lane (1988). The biotin-labeled BSA was added to the culture medium, and stretching of C2 myotubes started soon after. Biotin-BSA in the myotubes was detected by epifluorescence microscopy with rhodamine-conjugated streptavidin (Biomed Co. Hayward, CA) and was detected by western blotting with AP-conjugated streptavidin (Zymed Laboratories, South San Francisco, CA).

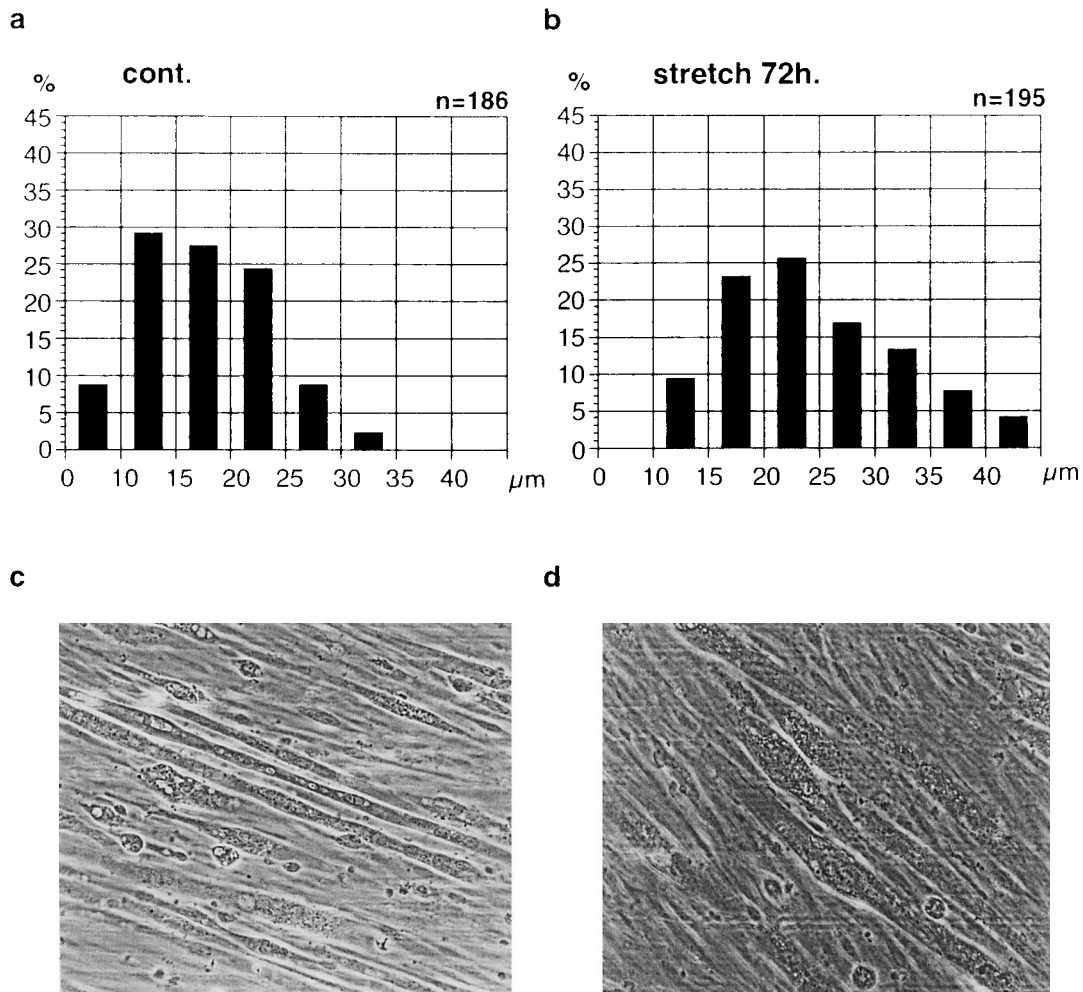
#### Fluorescence microscopy

Myotubes on the silicone membrane were embedded in a tissue-embedding compound (Tissue Tek II OCT Compound, Miles, Elkhart, IN) and quickly frozen in liquid nitrogen-cooled isopentane. After frozen, silicone membrane was peeled off the embedded myotubes. Transverse sections of myotubes in the frozen blocks were

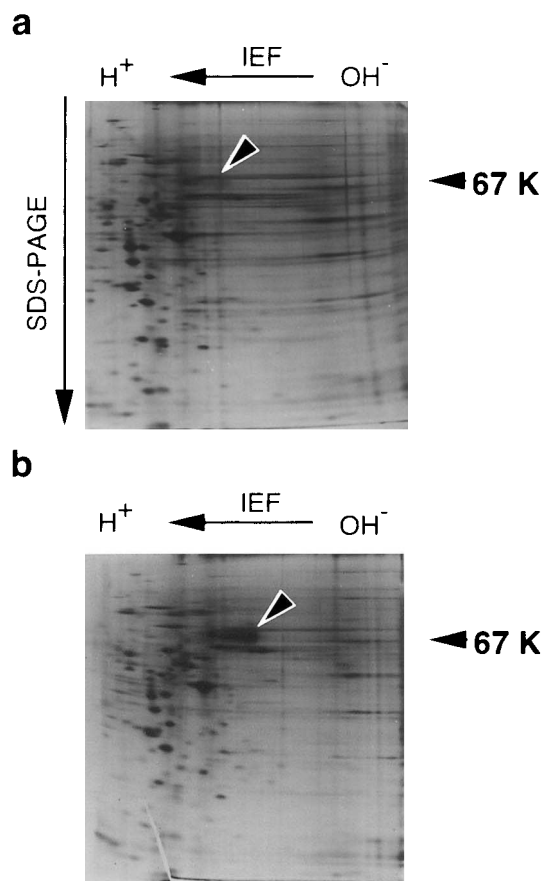
prepared at 8- $\mu$ m thickness with a cryostat microtome (Leica, Heidelberg, Germany) and fixed with 4% paraformaldehyde in PBS for 15 min. After rinsing with PBS, the specimens were treated with 1% BSA for 30 min and then reacted with the primary and secondary antibodies for 1 hr respectively. After the immunoreaction, the specimens were rinsed with PBS and mounted in a solution containing 50% glycerol, 75 mM KCl, 10 mg/ml paraphenylenediamine, and 50 mM sodium carbonate buffer, pH 8.0. The specimens were examined under a Zeiss epifluorescence microscope.

#### Other procedures

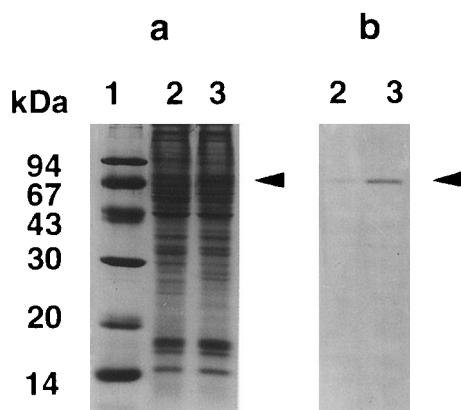
Recombinant GST was prepared with *E. Coli* strain XL1-Blue transfected with a GST expression vector, pGEX-2T (Pharmacia, Piscataway, NJ). The GST protein was expressed using standard procedures (Studier *et al.*, 1990: Pharmacia) and purified with glutathione beads according to the manufacture's directions (Pharmacia). Horse serum albumin was purified from commercial horse serum. Briefly, 70% saturation of ammonium sulfate was added to the serum and proteins in the supernatant were collected. Then,



**Fig. 1.** Stretch-induced hypertrophy of cultured C2 myotubes. The myotubes were cultured on silicone membrane. The membrane was stretched up to 1.1 times its original length at a frequency of 10 cycles/min for 3 days. Micrographs of phase-contrast images were taken and the size (diameters) of myotubes was measured. Populations of cells in each size range as shown in the abscissa were counted. The ordinate shows the percentage of cells in each size range. Total numbers of cells counted (*n*) are shown above the figure panels. In comparison with non-stretched cells (cont) (a), the size (diameter) of myotubes in stretching cultures (b) increased significantly. Typical examples of phase-contrast images of non-stretched and stretched myotubes are demonstrated in (c) and (d), respectively. Bar: 100  $\mu$ m.



**Fig. 2.** 2D-electrophoresis patterns of the proteins in non-stretched and stretched myotubes. (a) non-stretched myotubes; (b) myotubes stretched for 3 days as shown in Fig. 1. Arrowheads indicate the protein spots that differ significantly in amount between non-stretched and stretched myotubes. Size marker (67 kDa) is shown on the right.

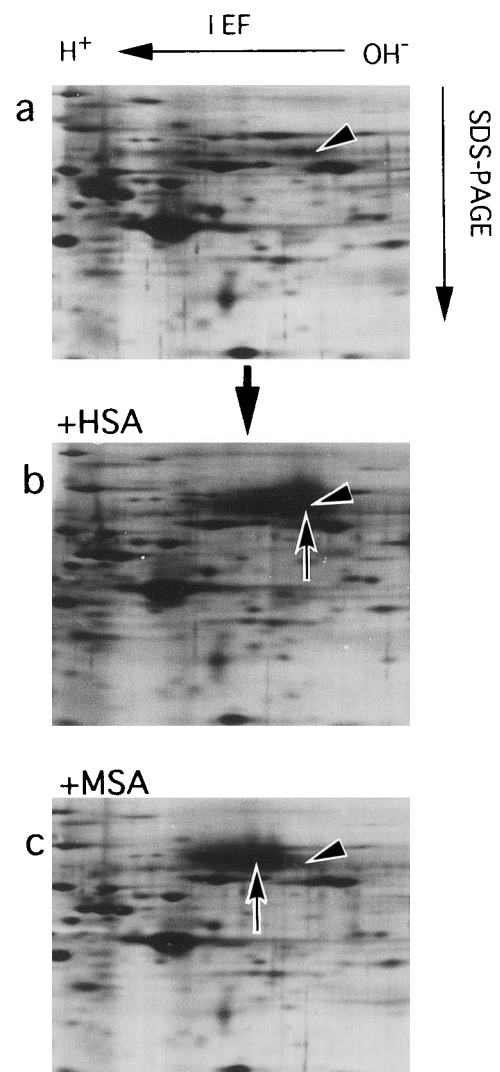


**Fig. 3.** Detection of albumin in non-stretching and stretching C2 myotube cultures by Western blotting. C2 myotubes were cultured as in Fig. 1 and the same amount of proteins from whole lysates of the non-stretching (lane 2) and stretching (lane 3) cultures were applied to electrophoresis and Western blotting. (a) CBB-staining patterns; (b) detection of the albumin band (marked by arrowheads) with anti-BSA monoclonal antibody. Size markers are shown in lane 1.

albumin was purified from them by DEAE-Sephadex A-25 (Pharmacia) with a sodium phosphate buffer gradient. Mouse serum albumin was purchased from Organon Teknika Corp. (Durham, NC)

## RESULTS

When C2 myotubes cultured on elastic silicone membranes were stretched periodically up to 1.1 times of their original length at a frequency of 10 cycles per minute for about 3 days, considerable increase in their width was observed. A typical example is shown in Fig. 1. The width of myotubes was measured at the regions where the diameter was largest. The average width was about 17  $\mu\text{m}$  before stretching but 24  $\mu\text{m}$  after stretching. Abnormal morphology such as disorganized myofibrils and vacuole forma-



**Fig. 4.** Co-migration of horse albumin with the 67-kDa protein in myotubes on 2D-electrophoresis gel. (a) The extract from myotubes stretched for 3 days as shown in Fig. 2; (b) horse albumin (HSA) was added to the extract from stretched myotubes; (c) mouse albumin (MSA) was added to the extract from stretched myotubes. Arrowheads indicate the 67-kDa protein spots in the myotubes. Arrows indicate the spots of HSA (b) and MSA (c).

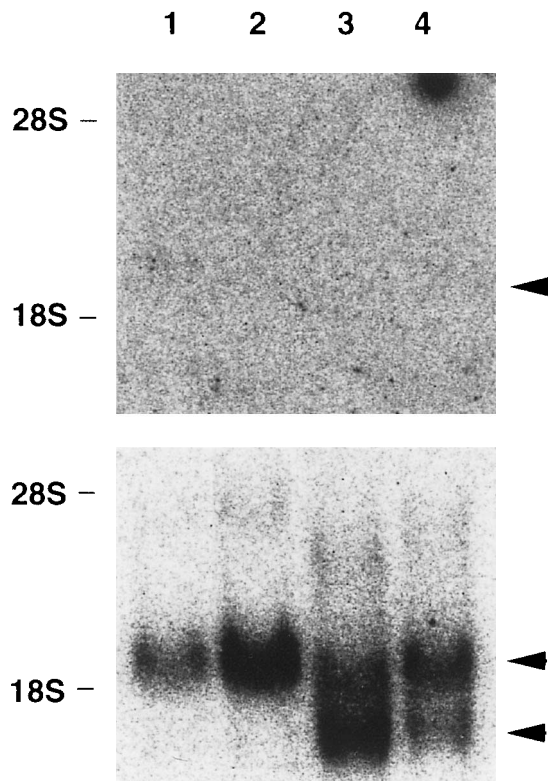
tion in the cytoplasm was scarcely observed in the stretched myotubes in comparison with non-stretched myotubes, suggesting that stretching under the current conditions did not cause any damage to the myotubes. Stretch-induced hypertrophy of myotubes was also observed in chicken primary muscle cultures; the cytoplasm of the stretched myotubes was filled with well-organized myofibrils (data not shown).

In order to examine whether the protein constituents in myotubes vary during stretching, 2D-electrophoresis was carried out with the myotubes both before and after stretching. As shown in Fig. 2, we observed that a protein spot of about 67 kDa increased in amount after stretching, although the overall electrophoresis pattern did not differ markedly between stretched and non-stretched myotubes. Since albumin is a protein of about 67 kDa, we carried out Western blotting with anti-albumin antibody. The 67 kDa spot was recognized by the antibody, although the spot was broad in the Western blot (data not shown). To further clarify whether the protein recognized by anti-albumin antibody change in amount after stretching, the cell lysates were examined by

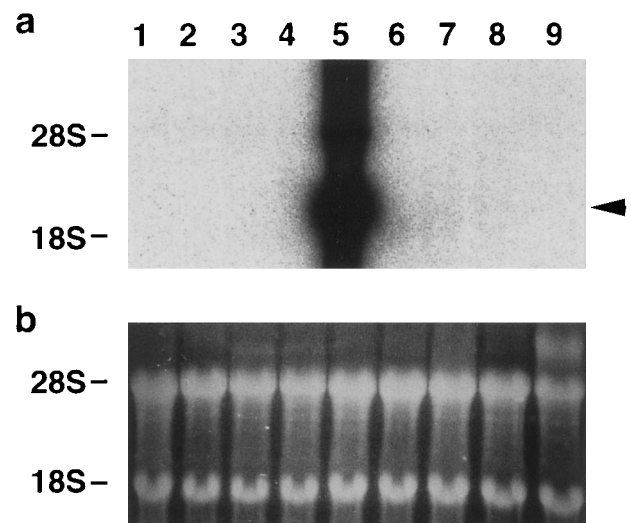
Western blotting combined with one dimensional SDS-PAGE. As shown in Fig. 3, a single protein band of about 67 kDa was recognized by anti-albumin antibody, and this band became more remarkable after stretching.

It became, then, a matter of interest of how the amount of albumin increased in the stretched myotubes. We entertained two possibilities; one was uptake of horse albumin from the culture medium, which had been abundantly added to the culture medium, and the other was the *de novo* synthesis of albumin by mouse C2 myotubes. To examine these possibilities, horse albumin (HSA) or mouse albumin (MSA) was added to the lysates of stretched C2 myotubes and the mixtures were subjected to 2D-electrophoresis to examine whether either horse or mouse albumin co-migrated with the 67 kDa protein (albumin) in the lysates of C2 myotubes. Horse albumin and mouse albumin were clearly distinguishable on the two-dimensional gel, since they were distinctly observed at different pH ranges. Horse albumin, present in the culture medium, co-migrated with albumin in C2 myotubes, as shown in Fig. 4. The results suggested that the increased amount of albumin was due to the uptake of horse albumin from the culture medium.

Next, in order to examine whether albumin expression was induced or enhanced in the stretched myotubes, 20  $\mu$ g of RNA extracted from control or the stretched myotube cultures was analyzed by northern blotting. As shown in Fig. 5, the message recognized by the mouse albumin cDNA probe was detected in neither control nor stretched myotubes at all. Positive messages were also not obtained from fibroblast cultures. Therefore, we conclude that the possibility of



**Fig. 5.** Northern blot analysis of expression of albumin mRNA in cultured fibroblasts (NIH3T3) and C2 myotubes as compared with that of actin. Top: Total RNA (20  $\mu$ g) from control NIH3T3 cells (lane 1), stretched NIH3T3 cells (lane 2), control C2 myotubes (lane 3), and stretched C2 myotubes (lane 4) was examined with albumin cDNA as a probe. Bottom: The same RNA samples as in the top panel were examined with actin cDNA probes. The positions of 28S and 18S ribosomal RNAs are indicated. Arrowheads indicate the position of albumin mRNA (Top) and actin mRNAs (Bottom). In myotubes, the message for  $\alpha$ -actin (lower band) was detected in addition to the message for  $\beta$ - and  $\gamma$ -actin (upper band).



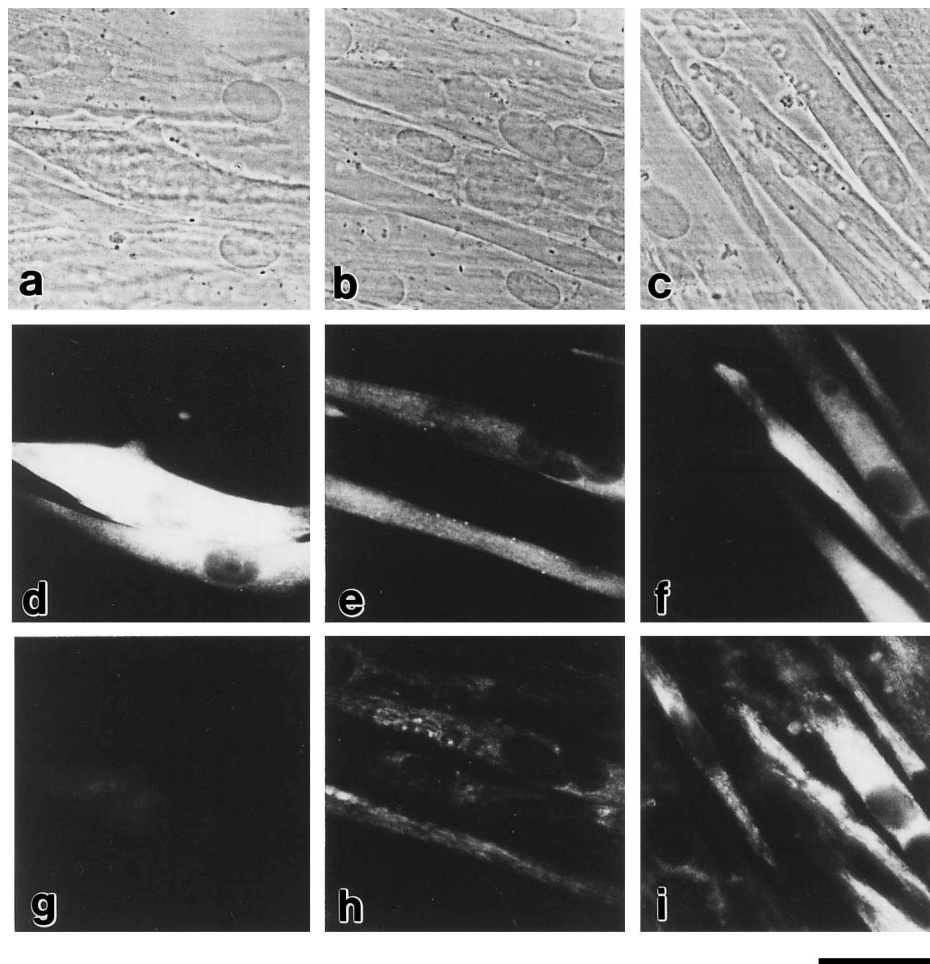
**Fig. 6.** Detection of albumin mRNA in mouse tissues by northern blotting. Top: Total RNA (20  $\mu$ g) from brain (lane 1), thymus (lane 2), lung (lane 3), heart (lane 4), liver (lane 5), spleen (lane 6), stomach (lane 7), kidney (lane 8), and skeletal muscle (lane 9) was examined with albumin cDNA as a probe. Arrowhead indicates the position of albumin mRNA. Bottom: Ethidium bromide staining of a parallel gel to confirm that equivalent amounts of RNA were loaded on each lane. The positions of 28S and 18S ribosomal RNAs are indicated.

*de novo* synthesis of albumin in myotubes can be ruled out. It should be noted that the cDNA probe reacted with the RNA from mouse liver, tissue known to be responsible for the synthesis of serum albumin, and give a strong positive signal, but not at all with RNA preparations from any other tissues (Fig. 6).

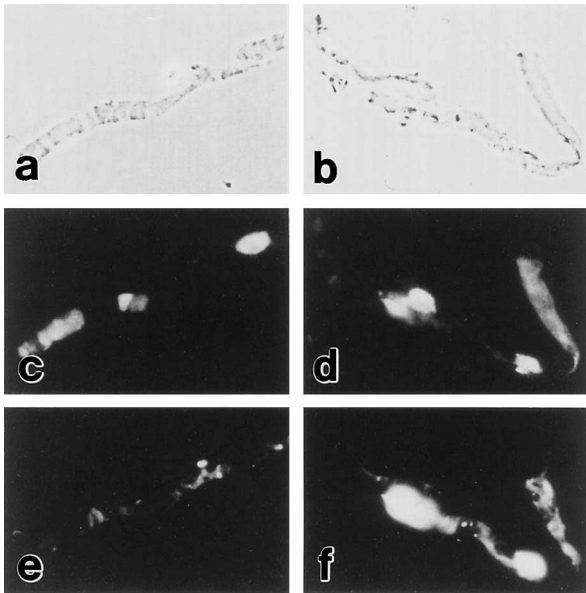
To further examine the stretch-dependent uptake of albumin by myotubes, periodic stretching of C2 myotubes was carried out in medium containing biotin-labeled BSA for 3 days and then, the myotubes were dually treated with rhodamine-labeled streptavidin and anti-troponin T antibody (NT-302), followed by treatment with FITC-GAM. The myotubes were examined by fluorescence microscopy. As shown in Fig. 7, biotin-BSA was clearly detected in the stretched myotubes (Fig. 7i), where troponin T, one of the muscle structural proteins, was detected by NT-302 (Fig. 7f). Biotin-BSA was scarcely detected in non-stretched myotubes (Fig. 7h). In order to clarify whether biotin-BSA was located in the cytoplasm of stretched myotubes, cryo-sections of the myotubes on silicone membrane were prepared

and stained with a combination of rhodamine-labeled streptavidin and NT-302. As shown in Fig. 8, biotin-BSA was clearly detected in the cytoplasm of stretched myotubes (Fig. 8f), just like troponin T (Fig. 8d), while in the case of non-stretched myotubes, the cytoplasm was stained with NT-302 but only faintly with rhodamine-labeled streptavidin (Fig. 8c, e). Of note is the fact that when the myotubes stretched in the medium containing biotin-BSA were subjected to SDS-PAGE, the biotin-BSA band detectable by AP-labeled streptavidin became more remarkable (data not shown). Thus, it is quite likely that albumin was incorporated into the myotube cytoplasm in a stretch-dependent manner.

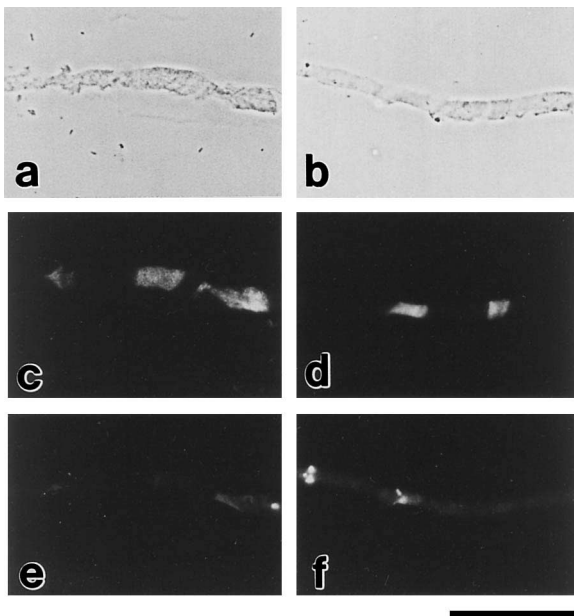
To clarify whether albumin was selectively incorporated into myotubes by stretching, GST was tested in place of albumin. We chose GST as a model, because the size of this protein, 27 kDa, is much smaller than that of BSA (68 kDa) and its *pI*, 6.1, is close to that of BSA, 5.6. GST was added to the culture medium and C2 myotubes were stretched periodically for 3 days. When the cryo-sections of the myotubes were treated with anti-GST antibody and



**Fig. 7.** Accumulation of biotin-labeled BSA in stretched myotubes. C2 myotubes were cultured in the presence of biotin-labeled BSA with or without stretching. Control myotubes cultured in non-labeled BSA without stretching (a, d, g), myotubes cultured in biotin-BSA without stretching (b, e, h) and myotubes cultured in biotin-BSA with stretching (c, f, i) were reacted with anti-troponin T antibody (NT-302), FITC-GAM and rhodamine-labeled streptavidin. Specimens were observed by phase-contrast microscopy (a, b, c), or fluorescence microscopy with an FITC channel (d, e, f) or a rhodamine channel (g, h, i). Bar: 50  $\mu$ m.



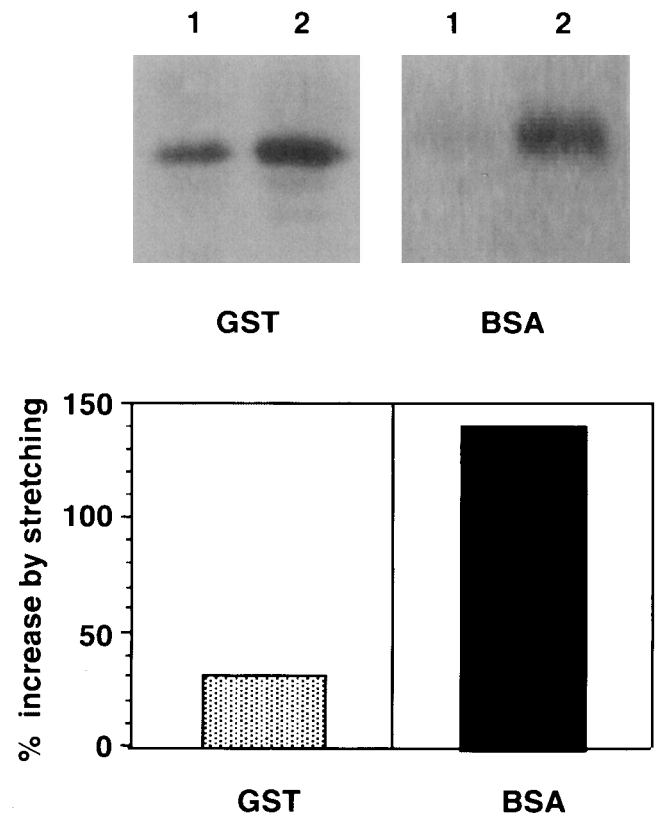
**Fig. 8.** Detection of biotin-labeled BSA in the cytoplasm of stretched myotubes. C2 myotubes were cultured in the presence of biotin-labeled BSA with or without stretching. Frozen-sections of control myotubes without stretching (a, c, e) and stretched myotubes (b, d, f) were reacted with anti-troponin T antibody (NT-302), FITC-GAM and rhodamine-labeled streptavidin. Specimens were observed by phase-contrast microscopy (a, b) or fluorescence microscopy with an FITC channel (c, d) or a rhodamine channel (e, f). Bar: 50  $\mu$ m.



**Fig. 9.** Analysis of myotubes stretched in GST. C2 myotubes were cultured in the presence of GST with or without stretching. Frozen-sections of control myotubes without stretching (a, c, e) and stretched myotubes (b, d, f) were reacted with the combination of anti-troponin T antibody (NT-302) plus FITC-GAM or anti-GST antibody plus rhodamine-GAR. Specimens were observed by phase-contrast microscopy (a, b) or fluorescence microscopy with an FITC channel (c, d) or with a rhodamine channel (e, f). Bar: 50  $\mu$ m.

observed by fluorescence microscopy, fluorescence of the myotube cytoplasm was very faint (Fig. 9), indicating that GST was scarcely taken up into myotubes.

On the other hand, when unlabeled GST or BSA was added to the stretching cultures and the cell lysates were subjected to Western blotting combined with SDS-PAGE using anti-GST antibody (or anti-BSA antibody), GST was detected in the lysates of the cultures regardless of stretching or non-stretching. GST seemed to be retained in the extra-cellular matrix of the cultures for some reason, in spite of the fact that they were rinsed with PBS before making the samples for electrophoresis. The GST level increased only slightly with stretching, becoming about 30% higher than in the non-stretched cultures. In contrast, when the myotubes were stretched in medium containing albumin (BSA), the BSA level in the stretched cultures was about 140% higher than in the non-stretched cultures. Thus, although the size of GST, 27 kDa, is much smaller than that of BSA, the stretch-dependent accumulation of GST is much less than that of BSA (Fig. 10). Therefore, we conclude that periodic stretching leads to the selective uptake of albumin into cul-



**Fig. 10.** Stretch-induced uptake of BSA and GST in myotubes. C2 myotubes were cultured in the presence of BSA or GST with or without stretching as in Fig 3 and whole extracts from cultures were prepared. BSA and GST in the extracts of non-stretching (lane 1) or stretching (lane 2) cultures were detected by Western blotting combined with SDS-PAGE using the antibodies for the respective proteins as shown in the upper panels. Stretch-induced increase of each protein in the cultures was estimated by densitometry from the immunoblot patterns.



tured myotubes.

## DISCUSSION

In this investigation, the myotubes cultured on elastic silicone membranes were aligned roughly in parallel. They were stretched reciprocally and periodically up to 1.1 times their original length at a frequency of 10 cycles/minute mostly along the long cell axis. The extent of mechanical stress from this stretching did not seem to be excessively severe for myotubes, as the cytoplasm of the myotubes of increased size were filled with complete myofibrils when mature myotubes were stretched and vacuole formation which occurs occasionally in unhealthy myotubes was not observed in the cytoplasm of these myotubes. Previously, we observed that dynamic reorganization of actin stress fibers and reorientation of cells were induced obliquely to the direction of stretching in A10 cells, a smooth muscle cell line, fibroblasts and myoblasts soon after the onset of stretching, when they were stretched up to 1.2 times their original length at a frequency of 60 cycles/minute in our system (Hayakawa *et al.*, 2000; 2001). However, under the current stretching conditions, reorganization of actin filaments in myofibrils and reorientation of myotubes obliquely to the direction of stretching were not induced. We think that myofibril reorganization and myotube reorientation did not take place as a result of the periodic stretching, because 1) myotubes were attached tightly to the substrate, 2) myofibril structures are much more stable than stress fibers, and 3) the mechanical stress under the current conditions was less severe.

The accumulation of albumin in skeletal muscle tissues *in vivo* under compensatory hypertrophy has been demonstrated (Yamada *et al.*, 1984). However, it was not revealed where and how albumin was accumulated in muscle tissue. Here, we have demonstrated that the amount of albumin in myotubes increased, when hypertrophy was induced by periodic stretching under culture conditions and in addition that this increase was due to the uptake of albumin from the culture medium not to *de novo* synthesis of the protein. Location of albumin in the cytoplasm of myotubes was clearly observed by immunocytochemical methods. It is unlikely that the localization of albumin to the cytoplasm of the stretched myotubes was a result of simple diffusion through damaged cell membrane, since neither Trypan blue nor Evans blue was able to enter the stretched myotubes (data not shown). Although a variety of proteins derived from horse serum and chicken embryo extract existed in the culture medium, the protein most markedly incorporated into the stretched myotubes was albumin. In addition, incorporation of GST, a protein of smaller size (MW: 27,000) than BSA (MW: 68,000) but with an isoelectric point (pI) in the acidic pH region just as BSA, was only slightly increased in a stretch-dependent manner, when added to the culture medium. These results indicate that the stretch-dependent uptake into myotubes is characteristic of albumin and label-

ing with biotin did not alter this property.

With regard to endocytosis of albumin, several albumin-binding glycoproteins, that are involved in albumin transcytosis, such as gp-60, gp-30, gp-10, have been characterized in endothelial cells of blood vessels (Schnitzer, 1992; Schnitzer *et al.*, 1992). Since these proteins were also detected in muscle tissues (Schnitzer, 1992; Schnitzer *et al.*, 1992), they might be involved in the stretch-induced albumin uptake in muscle cells as well. However, it has not yet been revealed how these proteins actually function in muscle.

It has been matter of argument whether or not albumin is an intrinsic muscle protein. Several reports demonstrated that albumin is localized predominantly in the extracellular space of skeletal muscle tissues (Yokota, 1982; Heilig and Pette 1988), while another described the location of albumin at the A/I junction regions of sarcomeres of muscle cells *in vivo* (Muller and Heinzman, 1982). Our findings show that albumin is scarcely detectable in the cytoplasm of myotubes that were maintained in the standard culture medium, suggesting that albumin could not be regarded as an intrinsic muscle protein. However, albumin could become located in the cytoplasm at least under certain conditions, for example when mechanical stress was provided to myotubes. Since cultured cells are excellent for examining cellular location of proteins, we carefully observed localization of albumin in the stretched myotubes which contained albumin in the cytoplasm; it was simply diffused in the cytoplasm and its association with striated structures was not observed.

The physiological implications of the uptake of albumin into stretched myotubes would be a matter of some interest. As a possible role of albumin in muscle cells, Chiesi and Guerini (1987) pointed out that albumin could act as a protective agent of the  $\text{Ca}^{++}$  pump of the sarcoplasmic reticulum. Since  $\text{Ca}^{++}$  is of vital importance for cell regulation, modulation of the  $\text{Ca}^{++}$  pump of the sarcoplasmic reticulum by albumin may cause profound effects on skeletal muscle cells. However, little is known as to the details. The manner of albumin involvement in muscle cell hypertrophy remains as an important subject for future studies.

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## REFERENCES

- Abe H, Komiya T, Obinata T (1986) Expression of multiple troponin T variants in neonatal chicken breast muscle. *Dev Biol* 118: 42–51
- Adams GR (2002) Autocrine/paracrine IGF-1 and skeletal muscle adaptation. *J Appl Physiol* 93: 1159–1167
- Bamman MM, Shipp JR, Jiang J, Gower BA, Hunter GR, Goodman A, McLafferty CL, Urban RJ (2001) Mechanical load increases muscle IGF-1 and androgen receptor mRNA concentrations in humans. *Am J Physiol* 280: E383–E390

- Chiesi M, Guerini D (1987) Characterization of heart cytosolic proteins capable of modulating calcium uptake by the sarcoplasmic reticulum. I. Isolation of a protein with protective activity and its identification as muscle albumin. *Eur J Biochem* 162: 365–370
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
- Crepax P (1952) Étude électrophorétique d'extraits de muscles doués de différentes propriétés morphologiques et fonctionnelles. *Biochim Biophys Acta* 9: 385–398
- Delling U, Tureckova J, Lim HW, De Windt LJ, Rotwein P, Molkentin JD (2000) A calcineurin-NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression. *Mol Cell Biol* 20: 6600–6611
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132: 6–13
- Galfre G, Howe SC, Milstein C, Butcher GW, Howard JC (1977) Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature* 266: 550–552
- Harlow E, Lane D (1988) Antibodies, a laboratory manual, Cold Spring Harbor Laboratory, New York, pp 340–341
- Hayakawa K, Hosokawa A, Yabusaki K, Obinata T (2000) Orientation of smooth muscle-derived A10 cells in culture by cyclic stretching: relationship between stress fiber rearrangement and cell reorientation. *Zool Sci* 17: 617–624
- Hayakawa K, Sato N, Obinata T (2001) Dynamic reorientation of cultured cells and stress fibers under mechanical stress from periodic stretching. *Exp Cell Res* 268: 104–114
- Heilig A, Pette D (1988) Albumin in rabbit skeletal muscle. Origin, distribution and regulation by contractile activity. *Eur J Biochem* 171: 503–508
- Komuro I, Katoh Y, Kaida T, Shibasaki Y, Kurabayashi M, Hoh E, Takaku F, Yazaki Y (1991) Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *J Biol Chem* 266: 1265–1268
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685
- Leferovich JM, Lana DP, Suttrave P, Hughes SH, Kelly AM (1995) Regulation of *c-ski* transgene expression in developing and mature mice. *J Neurosci* 15: 596–603
- McPherron A, Lawler AM, Lee S-J (1997) Regulation of skeletal muscle mass in mice by a new TGF- $\beta$  superfamily member. *Nature* 387: 83–89
- Minamide LS, Bamburg JR (1990) A sensitive dye-binding filter paper assay for the quantitative determination of protein in the presence of reducing agents and detergents. *Anal Biochem* 190: 66–70
- Muller G, Heizmann CW (1982) Albumin in chicken muscle. *Eur J Biochem* 123: 577–582
- Oakley BR, Kirsh DR, Morris NR (1980) A simplified ultra-sensitive silver stain for detecting proteins in polyacrylamide gel. *Anal Biochem* 105: 361–363
- O'Farrell PH (1975) High resolution two dimensional electrophoresis of proteins. *J Biol Chem* 250: 4007–4021
- Olson ER, Sanders Williams R (2000) Remodeling muscles with calcineurin. *Bioessays* 22: 510–519
- Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ (2001) Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* 3: 1009–1013
- Sadoshima J, Jahn L, Takahashi T, Kulik TJ, Izumo S (1992) Molecular characterization of the stretch-induced adaptation of cultured cardiac muscle. *J Biol Chem* 267: 10551–10560
- Schnitzer JE (1992) gp60 is an albumin-binding glycoprotein expressed by continuous endothelium involved in albumin transcytosis. *Am J Physiol* 262: H246–H254
- Schnitzer JE, Sung A, Horvat R, Bravo J (1992) Preferential interaction of albumin-binding proteins, gp30 and gp18, with conformationally modified albumins. Presence in many cells and tissues with a possible role in catabolism. *J Biol Chem* 267: 24544–24553
- Shirinsky V, Antonov A, Birukov K, Sobolevsky A, Romanov Y, Kabaeva N, Antonova G, Smirnov V (1989) Mechano-chemical control of human endothelium orientation and size. *J Cell Biol* 109: 331–339
- Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 185: 60–89
- Takemasa T, Yamaguchi T, Yamamoto Y, Sugimoto K, Yamashita K (1998) Oblique alignment of stress fibers in cells reduces the mechanical stress in cyclically deforming fields. *Eur J Cell Biol* 77: 91–99
- Thomas PS (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* 77: 5201–5205
- Towbin HT, Staehelin HM, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 76: 4350–4354
- Vandenburgh H, Kaufman S (1979) *In vitro* model for stretch-induced hypertrophy of skeletal muscle. *Science* 203: 265–268
- Vandenburgh H, Hatfaludy S, Karlisch P, Shansky J (1989) Skeletal muscle growth is stimulated by intermittent stretch-relaxation in tissue culture. *Am J Physiol* 256: C674–C682
- White GE, Gimbrone MJ, Fujiwara K (1983) Factors influencing the expression of stress fibers in vascular endothelial cells *in situ*. *J Cell Biol* 97: 416–424
- Yaffe D, Saxel O (1977) Serial passing and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* 270: 725–727
- Yamada S, Tomino S, Izumi S, Akino M (1984) Purification, molecular properties and biosynthesis of a specific protein component under compensatory hypertrophy in the rat skeletal muscle. *Biochim Biophys Acta* 798: 260–267
- Yang S, Alnaqeb M, Simpson H, Goldspink G (1996) Cloning and characterization of an IGF-1 isoform expressed in skeletal muscle subjected to stretch. *J Muscle Res Cell Motil* 17: 487–495
- Yokota S (1982) Immunoelectron microscopic localization of albumin in smooth and striated muscle tissue of rat. *Histochemistry* 74: 379–386

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