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DEVELOPMENTALLY REGULATED EXPRESSION OF CALPONIN ISOFORMS AND THE EFFECT OF H2-CALPONIN ON CELL PROLIFERATION

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(Abstract)

H2-calponin is found in both smooth muscle and non-muscle cells and its function remains to be established. Western blots using specific monoclonal antibodies detected significant expression of h2-calponin in the growing embryonic stomach and urinary bladder and the early pregnant uterus. While the expression of h1-calponin is up-regulated in the stomach and bladder during postnatal development, the expression of h2-calponin is decreased to low levels in quiescent smooth muscle cells. To investigate a hypothesis that h2-calponin regulates the function of the actin cytoskeleton during cytokinesis, a smooth muscle originated cell line (SM3) lacking calponin was transfected to express either sense or antisense h2-calponin cDNA and the effects on the rates of cell proliferation were examined. Both stable and transient sense cDNA-transfected cells had a significantly decreased proliferation rate compared to the antisense cDNA-transfected or non-transfected cells. Immunofluorescence microscopy showed that the force-expressed h2-calponin was associated with actin-tropomyosin microfilaments. The number of bi-nuclear cells was significantly greater in the sense cDNA-transfected culture, in which h2-calponin was concentrated in a nuclear ring structure formed by actin filaments. The results suggest that h2-calponin may regulate cytokinesis by inhibiting the activity of the actin cytoskeleton.

(Key words)

h2-calponin; smooth muscle development; cytokinesis; tropomyosin; actin cytoskeleton; monoclonal antibody; transfective expression

INTRODUCTION

Calponin is a family of actin filament-associated proteins. Three isoforms of calponin, h1- (**9, 33, 43**), h2- (**42**) and acidic (**1, 45**), have been identified. Isoelectric points (pIs) of these three calponin isoforms show that h1-calponin is basic (pI = 8.5 - 9.2), h2-calponin is neutral (pI = 7.2 - 7.6), and acidic calponin is, expectedly, acidic (pI = 5.5 - 5.8). The extensively investigated chicken gizzard calponin has been found to be equivalent to mammalian h1-calponin, the major calponin found in smooth muscle cells. This calponin has been shown to inhibit actin-activated myosin ATPase, which has led to a model wherein it functions as a modulator of smooth muscle contractility (**30, 41, 48**).

Actin-myosin interaction-based motility is essential for cytokinesis, a process in which the membrane and cytoplasm of a cell are partitioned through the ingression of a cleavage furrow to form two daughter cells (**8, 12 14**). Cleavage furrow ingression requires a contractile cortical ring of actin and myosin (**38**, **39**); thus, the activity of the actin cytoskeleton has an effect cell division (**15**). Actin-myosin interaction also powers cell proliferation by driving cytoplasmic streaming, which may contribute to the division of the cytosolic components of the cell during cytokinesis. Accordingly, through the inhibition of actin-myosin interaction, calponin may play a role in regulating the functions of the actin cytoskeleton, such as coordinating changes in cell shape and intracellular molecular trafficking, both of which are critical events in cytokinesis (**15**). Indeed, forced expression of chicken gizzard calponin in cultured smooth muscle cells and fibroblasts showed an inhibition of cell proliferation (**19)**. Therefore, calponin, through its regulation of actin-myosin interaction, and possibly actin filament stability, may function as a controlling factor for cytokinesis and the rate of cell proliferation.

The conservation in primary structure between the h1 and h2 isoforms of calponin indicates that they most likely function through similar molecular mechanisms. However, the extensive sequence diversity and differences in physical properties between the two isoforms suggest that they have adapted to divergent biological activities. Since expression of h1-calponin in smooth muscle is up-regulated during differentiation and development (**7, 11, 13, 32, 46**), it

may have a role in the functional maturation of smooth muscle myofilaments. On the other hand, the tissue distribution, developmental regulation, and functional significance of h2-calponin are not well understood. While h1-calponin may play a modulator role in tuning smooth muscle contractility (**48**) as previously discussed, the potential role of h2-calponin in regulating the function of the actin cytoskeleton needs to be investigated.

In the present study, we investigated the expression of h2-calponin during development and its effect on cell proliferation. Using an immortalized vascular smooth muscle cell line (SM3) (**37**) with no calponin expression, we examined the effects of transfective expression of h2-calponin on the function of the actin cytoskeleton and cell proliferation. We found that this forced expression of h2-calponin significantly decreased the rate of cell proliferation. The expressed h2-calponin associated with actin-tropomyosin thin filaments and caused an increased number of bi-nuclear cells in which h2-calponin was concentrated in a nuclear ring structure formed by actin filaments. The data suggest that h2-calponin suppresses cytokinesis by inhibiting the activity of actin cytoskeleton. Further supported by its regulated expression in uterus smooth muscle during pregnancy, h2-calponin may play a role in modulating cell proliferation during tissue growth and remodeling.

MATERIALS AND METHODS

Specific antibodies against calponin isoforms. Two monoclonal antibodies (mAb) raised against chicken gizzard calponin (CP1 and CP3) (**21**) which react to mammalian h1-calponin but not h2-calponin (Fig. 1) were used in the present study to detect the expression of mouse h1-calponin.

A polyclonal antiserum (RAH2) raised against mouse h2-calponin with a weak cross-reaction to h1-calponin (Fig. 1) was first used to examine the expression of h2-calponin in cell cultures.

To develop mAbs specific to h2-calponin, mouse h2-calponin (**32**) was used to immunize 8-week-old female Balb/c mice in a short-term immunization protocol (**47**). The mice were injected intraperitoneally with 50 μg of purified h2-calponin antigen in 100 μl PBS mixed with an equal volume of Freund's complete adjuvant. Ten days later, the mouse was intraperitoneally boosted two times with 200 μg each of the antigen in 200 μl PBS without adjuvant on two consecutive days. Two days following the last boost, spleen cells were harvested from the immunized mouse and fused with SP2/0-Ag14 mouse myeloma cells (ATCC) using 50% polyethaglycol$_{1500}$ containing 7.5% dimethyl sulfoxide as described previously (**21**). Hybridoma colonies were selected by HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal bovine serum and screened by indirect enzyme-linked immunosorbant assay (ELISA) using horseradish peroxidase-labeled, goat anti-mouse total immunoglobulin (Sigma) second antibody. The anti-h2-calponin antibody-secreting hybridomas were subcloned three or four times by limiting dilution method using young Balb/c mouse spleen cells as feeder to establish stable cell lines. The hybridoma cells were then cultured to produce high titer supernatant and introduced into 2,6,10,14-tetramethyl pentadecane (pristane, Sigma)-primed peritoneal cavity of Balb/c mice to produce mAb-enriched ascites fluids (**21**). The specificity of the mAbs was verified by Western

blot analysis (Fig. 1). The anti-h2-calponin mAbs, showing no cross-reaction to h1-calponin, was used in Western blots to examine the expression of h2-calponin.

Construction of expression vectors. The coding region of mouse h2-calponin cDNA cloned into the pAED4 prokaryotic expression plasmid by reverse transcription-coupled polymerase chain reaction (RT-PCR) (**25**) was first subcloned into the pBluescript KS(-) plasmid for the isolation of an *Eco*RV-*Sma*I restriction fragment with two blunt ends. The cDNA coding template was then cloned into the *Eco*RV site of the G418-resistant pcDNA3 eukaryotic expression vector (Invitrogen) down stream of the cytomegalovirus (CMV) promoter in sense or anti-sense orientations. The recombinant pcDNA3 plasmids encoding sense and antisense h2-calponin cDNA were identified by *Apa*I and *Pst*I restriction enzyme mapping and verified by DNA sequencing using the dideoxy chain termination method as described previously (**18**). The sense expression construct encodes a non-fusion full-length mouse h2-calponin protein for authentic functional characterization and the antisense construct provides a transfection control in the present study. The recombinant pcDNA3 plasmid DNA was prepared from transformed JM109 *E. coli* in large quantities using an alkaline lysis method followed by ion-exchange chromatography.

SM3 cell culture and transfection. SM3 is an immortalized cell line derived from rabbit aortic smooth muscle cells (**37**). The SM3 cells were cultured in DMEM containing 10% FBS, penicillin (100 μg/mL) and streptomycin (100 μg/mL) at 37˚C in 5% CO_2.

Transfection of SM3 cells was carried out using the DOTAP liposomal transfection reagent (Boehringer Mannheim) following the manufacturer's instructions. SM3 cells were seeded on Corning 10-cm culture dishes at 2 x 10^6 cells per dish and grown until the monolayer cells reached 60-80% confluent. Twenty μg of the recombinant supercoil plasmid DNA in 50 μl TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) was mixed with 100 μl DOTAP in 20 mM HEPES buffer (pH 7.3) and incubated at room temperature for 20 min. The DOTAP/DNA

mixture was then gently mixed with 5 ml DMEM containing 10% FBS and add to the dish after removing the old culture medium. The SM3 cell monolayer was incubated with the DOTAP/DNA medium for 18 hours at 37˚C in 5% CO_2 before the change to fresh culture media.

In transient transfection experiments, the cell cultures were continued in DMEM containing 10% FBS, penicillin (100 μg/mL) and streptomycin (100 μg/mL) at 37˚C in 5% CO_2 and the cells were harvested at a series of time points for characterization. In the establishment of stable transfection of SM3 cells, the transfected cells were cultured in DMEM containing 10% FBS plus G418 (500 μg/ml, ICN Biomedical, Inc.). Results from testing the tolerance of non-transfected SM3 cells to G418 showed that this cell line is highly sensitive to G418. In culture media containing 20 μg/ml G418, all cells died after 9 days of culture. The pcDNA3 transfected SM3 cell colonies resistant to G418 were individually picked up from the culture dish by trypsin digestion in small cylinders greased to the dish. The cells were expanded for extracting DNA to verify the transfection by PCR as described previously (**18**). The expression of h2-calponin in the sense cDNA transfected cells was examined on total cellular protein extract by Western blotting using the RAH2 antibody. The SM3 cell lines stable-transfected with the sense or antisense h2-calponin cDNA expression constructs were expanded and stored in DMEM containing 30% FBS and 10% DMSO in liquid nitrogen for later phenotype characterization.

SDS-polyacrylamide gel electrophoresis and Western blotting. To examine h2-calponin expression in the transfected SM3 cells, as well as h1- and h2-calponins in smooth muscle tissues from New Zealand White rabbits and C57B6 mice, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as described previously (**47**).

The smooth muscle layer of the tissue samples were homogenized in SDS gel electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 0.1% bromophenol blue, 10% glycerol) using a Polytron-type high speed tissue homogenizer (PRO Scientific Inc., Monroe, CT) to extract total cellular proteins. The h2-calponin sense and anti-sense cDNA transfected SM3 cells were suspended from the culture dishes using the Versene solution (0.537 mM EDTA,

136.8 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) and washed three times with phosphate-buffered saline (PBS), pH 7.2. The elimination of trypsin digestion from the collection of cells avoided enzymatic degradation of the cellular proteins. SDS gel sample buffer was added to lyze the cells and the total protein was extracted by vortexing.

After heating at 80 °C for 5 min and clarification by centrifugation, the tissue or cell samples were applied on a 12% gel with an acrylamide/bisacrylamide ratio of 29:1 prepared in the Laemmili discontinuous buffer system. After electrophoresis, the SDS-gels were fixed and stained with Coomassie Blue R250 to confirm sample integrity and optimize the amount of loading. The loading amounts of different samples were normalized by the area and intensity of the actin band. Protein bands in duplicate gels were electrophoretically transferred to a nitrocellulose membrane using a BioRad semi-dry transfer apparatus at 4-5 mA per cm^2 for 30 min. The blotted membranes were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS, 150 mM NaCl and 50 mM Tris-HCl, pH 7.5) before the incubation with anti-calponin primary antibodies. After washes with TBS containing 0.05% Tween-20, the membranes were further incubated with alkaline phosphatase-labeled anti-rabbit IgG or anti-mouse IgG second antibody (Sigma). After final washes of the Western blot membrane, the expression of calponin isoforms was revealed by incubation in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chromogenic substrates. Purified mouse h2- and h1-calponin expressed in *E. coli* (**32**) were used as positive controls in the SDS-PAGE and Western blot experiments.

Densitometry analysis of the Western blots was done on images scanned at 600 dpi and the NIH Image program version 1.61 was used to quantify the levels of calponin isoform expression. The calponin bands detected in Western blots were normalized against the actin band in the parallel SDS-gel to correct for the minor differences in the total protein concentration among the samples.

Measurement of cell numbers in culture. A number of different methods are currently in use for direct or indirect measurements of cell numbers in culture to monitor cell proliferation. Crystal Violet staining is a rapid and sensitive method for the cell number measurement in monolayer cultures (**10, 23**). In this method, cell nuclei are stained with the Crystal Violet dye and the excess dye is washed out before the Crystal Violet absorbed to the cell nuclei is extracted for optical density (O.D.) measurements which reflect the number of cells in the sample.

To investigate the effects of h2-calponin on cell proliferation, we have adopted the Crystal Violet method to measure the number of SM3 cells in culture. Cells in 96-well culture plates containing 200 µl of medium per well were fixed by adding 20 µl of 11% glutaraldehyde solution. After gentle shaking at room temperature for 15 min, the plates were washed three times with double distilled water and air-dried. The plates were then stained with 100 µl of 0.1% Crystal Violet (Sigma) in 20 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer (pH 6.0). After gentle shaking at room temperature for 20 min, excess dye was removed by extensive washing with double distilled water and the plates were air-dried prior to extracting the bound dye with 100 µl of 10% acetic acid. Optical density of the dye extracts was measured at 595 nm using an automated microtiter plate reader (Benchmark, BioRad Labs).

To evaluate the accuracy of this method for measuring different types of cell cultures, we first tested the procedure on uniformly seeded SP2/0Ag14 mouse myeloma cells. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS), penicillin (100 µg/mL) and streptomycin (100 µg/mL) at 37˚C in 5% CO_2. Cells in log phase growth were harvested by gentle blowing with a Pasture pipette. The cell numbers were counted in a hemacytometer before seeding in 96-well culture plates in DMEM containing 10% FBS. Six hrs after seeding, the cells were fixed and processed for Crystal Violet staining as above. The results, shown in Fig. 2A and Fig. 2B, demonstrate a very good linear relationship between the $O.D._{595nm}$ values of Crystal Violet nuclear staining and the wide range of cell numbers seeded in the culture plate (2×10^2 - 8×10^4/well).

SM3 cells growing as an attached monolayer were then examined. The cell numbers were counted for seeding in 96-well culture plates. After incubation for 6 hr in DMEM containing 10% FBS at 37 °C in 5% CO_2, the monolayer SM3 cells were fixed and processed for Crystal Violet staining. The results in Fig. 2C and Fig. 2D also show a very good linear relationship between the $O.D._{595nm}$ values Crystal Violet nuclear staining and the wide range of cell numbers (3.12×10^2 - 5×10^4 cells per well).

Monitoring the proliferation rate of SM3 cells in culture. We then established the seeding cell density for a reliable measurement of the proliferation rate of SM3 cells. Non-transfected SM3 cells were harvested from pre-confluent cultures by digestion with 0.025% trypsin in 0.02% EDTA solution and seeded into 96-well culture plates at 500, 1000 and 1500 cells per well in DMEM containing 10% FBS. Five identical sets of cultures were started on 5 consecutive days and were stopped altogether to obtain 6, 30, 54, 78 and 102 hr cultures. The plates were processed for Crystal Violet staining. Cell proliferation curves were plotted to demonstrate the relationship to the initial seeding cell density. The results in Fig. 3 show that the SM3 cells cultured in 96-well plates from all of the three initial densities had linear growth curves up to 102 hours without changing media. Accordingly, the proliferation rates of the transfected SM3 cells were examined using these conditions, except that the dispersion of the transiently transfected SM3 cells was done by using the Versene solution to avoid enzymatic damage of the membrane proteins which may affect the initial rate of cell proliferation.

Immunofluorescence microscopy. Pre-cleaned glass cover slips were coated with 0.1% gelatin and dried under UV radiation before being placed in the culture dish. The transfected SM3 cells were seeded to grow monolayers on the cover slips. The cover slips with monolayer SM3 cells were collected before confluent and washed with PBS. The cells were fixed with cold acetone for 30 minutes. Immunofluorescence microscopy was carried out as described previously (**20**) to examine the cellular localization of the transfectively expressed h2-calponin. After

blocking with 1% BSA in PBS at room temperature in a humidity box for 30 minutes, the cover slips were incubated with the rabbit anti-h2-calponin antibody RAH2 and a mouse mAb against tropomyosin (CG3, provided by Dr. Jim Lin, University of Iowa) (**25**), alone or in combination, at room temperature for 2 hr After washes with PBS containing 0.05% Tween-20, the cover slips were stained with TRITC-conjugated goat anti-rabbit IgG and/or FITC-conjugated goat anti-mouse IgG second antibodies (both from Sigma) at room temperature for 1 hr. After final washes with PBS containing Tween-20, the cover slips were mounted on glass slide and examined under a Zeiss Axiovert 100H phase contrast-epifluorescence microscope. A Plan-Neo phase fluorescence 100X objective lens (oil, N.A. 1.30) was used for the photography of both phase-contrast and fluorescence images. The TRITC and FITC fluorescence images representing the localization of calponin and tropomyosin, respectively, were selectively viewed through two sets of filters (CZ915 and CZ909).

To determine the frequency of bi-nuclear cells in the non-transfected and transfected SM3 cell cultures, cover slips with pre-confluent monolayer cells were fixed and directly examined by phase contrast microscopy as above.

Statistical analysis. The quantitative data of cell proliferation are presented as mean ± SD. Regression coefficients were calculated using the Microsoft Excel computer program. Paired comparisons were carried out by Student's *t* test to examine the significance of difference.

RESULTS

Differentially regulated expression of h1- and h2-calponins during the postnatal development of mouse stomach and urinary bladder. The expression of h1- and h2-calponins in the stomach and urinary bladder smooth muscles of C57B6 mice during postnatal development was examined by Western blot analysis. The results in Fig. 4 show that h1-calponin is only expressed at low levels in the stomach and bladder muscles of neonatal mice but is up-regulated during postnatal development to high levels in adult stomach and bladder smooth muscles. Contrary to the postnatal up-regulation of h1-calponin, h2-calponin is expressed at high levels in the neonatal mouse stomach and urinary bladder smooth muscles and is down-regulated during postnatal development. Only a small amount of h2-calponin is present in the adult tissues (Fig. 4). Furthermore, the levels of each of the two calponin isoforms differ within the two types of smooth muscles. Although the expression of h1- and h2-calponin is regulated in a complementary way, the quantitative relationship does not make up a constant level of total calponin in the smooth muscle tissues. The separate regulations of the h1 and h2 isoforms of calponin suggest that they may play differentiated functions. These results are consistent with the previous studies that h1-calponin is expressed at a high level in adult phasic smooth muscles (**21, 32**). On the other hand, the high level expression of h2-calponin in neonatal stomach and bladder may indicate its role in tissue growth.

The CP21 mAb raised against cloned mouse h2-calponin showed no cross reaction to h1-calponin (Fig. 1 and Fig. 4). Interestingly, the Western blot in Fig. 4B detected an additional protein band with a higher molecular weight than that of h2-calponin in both the 1-month and 6-month mouse urinary bladder. The amount of this protein seems to be increased in the 6-month versus 1-month bladder. Further studies are underway to determine whether this protein is another calponin isoform or a phosphorylation variant of h2-calponin, both of which may be functionally significant in the development and activity of urinary smooth muscle.

Regulated expression of h1- and h2-calponins in uterus smooth muscle during pregnancy. Western blots using the anti-h1-calponin mAb CP3 and the anti-h2-calponin mAb CP21 showed high level h1-calponin expression in the non-pregnant and late-term uterus smooth muscle versus high level h2-calponin expression in the rapidly growing uterus of mid-term pregnancy (Fig. 5). The high level expression of h1-calponin in pre-labor uterus smooth muscle is consistent with the potential role of h1-calponin in modulating the contractility of smooth muscle. On the other hand, the high level expression of h2-calponin in rapidly growing uterus smooth muscle suggests its role in regulating the actin cytoskeleton during smooth muscle growth and cell proliferation.

Transfective expression of h2-calponin and inhibition of the rate of cell proliferation. The Western blots in Fig. 6A show that while both h1- and/or h2-calponin are expressed in rabbit vascular smooth muscle, the immortalized SM3 cells derived from rabbit aorta have ceased the expression of calponin in pre-confluent, confluent and differentiated cultures (**37**). This provides a useful system to study the effects of calponin on cellular functions. The role of h2-calponin in cell proliferation was investigated in SM3 cells through the transfective expression of h2-calponin. The Western blots in Fig. 6B show that the h2 sense, but not antisense, cDNA stable-transfected SM3 cells showed significant amounts of h2-calponin expression.

The cell proliferation curves in Fig. 7 demonstrate a significantly decreased proliferation rate in the h2 sense versus h2 antisense cDNA stable-transfected cells ($P < 0.001$). The effect of h2-calponin was independent of the presence or absence of G418 in the culture media. Initiated at the same number of cells, the number of h2 sense cDNA transfected cells were only 33-42% of that of h2 antisense cDNA transfected cells after 5 days of culture.

Significant amounts of h2-calponin expression were also obtained in SM3 cells transiently transfected with the h2 sense, but not antisense, expression vector (Fig. 8, inset). The cell proliferation curves in Fig. 8 demonstrate a significantly decreased proliferation rate in the h2-sense versus h2 antisense cDNA transfected cells ($P < 0.001$). The Western blot detected a

transient expression of h2-calponin in SM3 cells from 24 hrs to 96 hrs after transfection with h2 sense cDNA. In contrast to the continuously inhibited proliferation rate in the stable-transfected SM3 cells (Fig. 7), the inhibition of cell proliferation was transient in the transiently transfected SM3 cells following the expression of h2-calponin (Fig. 8). After the cells ceased h2-calponin expression at 120 hrs post-transfection, their proliferation rate returned to a level similar to that of both the h2 antisense cDNA transfected and non-transfected SM3 cell controls (Fig. 8). In the stable transfection experiments, the inhibitory effect of h2-calponin was seen as early as 6 hrs after re-plating the cells (Fig. 7). This more predominant effect seen in stable as opposed to transient expression may be due to higher levels of h2-calponin as well as the homogeneous expression exhibited in stable transfection in contrast to the heterogeneous transient transfection. Nevertheless, these results clearly demonstrate a direct relationship between h2-calponin expression and decreased cell proliferation rate. Most importantly, the results from transient transfection experiments represent a population phenotype, excluding any potential effects from non-specific changes in individual stable-transfected cell lines.

It is worth noting that the proliferation rates of non-transfected and h2 antisense cDNA-transfected SM3 cells began to accelerate 90 hrs post re-plating (the cell number doubling time shortened from 32-33 hrs to 24 hrs). In h2 sense transfected SM3 cells, the decrease in cell proliferation rate was not seen until 96 hrs post-transfection (90 hrs after re-plating and at least 72 hrs after the expression of h2-calponin was detectable). Thus, the expression of h2-calponin seems to have prevented the acceleration of proliferation rate in SM3 cell culture. It is possible that the expression of h2-calponin inhibited only fast, but not slow or moderate, cell proliferation when transiently expressed in the SM cells. This selective effect may suggest that h2-calponin plays a role as a negative balancing factor to maintain a physiological rates of cell proliferation. The inhibitory effect remained until 120 hrs post-transfection, even though h2-calponin already had dramatically decreased expression by this time (Fig. 8). This time lag in resuming proliferation rate may reflect the time needed for the cells to recover from the effects of forced h2-calponin expression.

The initial seeding density of the cells did not affect the amount of proliferation inhibition by h2-calponin. Experiments starting with 500, 1,000 or 1,500 cells per well yielded comparable results in both stably and transiently transfected cultures (only the 1,000 cells per well data are shown in Fig. 7 and Fig. 8). In all experiments, the proliferation rates of the h2 antisense cDNA-transfected cells and non-transfected cells were almost identical (Fig. 8), indicating that the transfection procedure and the integration of the vector DNA did not have significant non-specific effect.

Association of h2-calponin to the actin-tropomyosin filaments in the transfected SM3 cells. Immunofluorescence microscopy using anti-h2-calponin antibody demonstrates that the force-expressed h2-calponin localizes in the stress fiber structures (Fig. 9). By taking advantage of that the rabbit anti-h2-calponin antiserum and the anti-tropomyosin mAb are recognized by different second antibodies with FITC or TRITC labels which can be distinguished by viewing through different filter sets, double-staining immunofluorescence microscopy clearly showed the co-localization of h2-calponin and tropomyosin in the stress fibers (Fig. 9C). Tropomyosin is a known actin filament-associated protein (**26**) and the results demonstrate the association of h2-calponin with the actin filaments. The results also show a highly selective targeting of the force-expressed h2-calponin to the actin stress fibers since very little background staining was observed. The association of h2-calponin with the actin cytoskeleton suggests that its inhibitory effects on the rate of cell proliferation may be based on an inhibition of actin activity during cytokinesis. No other protein in SM3 cell had significant reaction with the anti-calponin RAH2 antibody and the anti-tropomyosin CG3 antibody used in the immunofluorescence localization (Fig. 6C).

Increased number of bi-nuclear cells in the SM3 cultures force-expressing h2-Calponin. The number of bi-nuclear cells was significantly increased in the h2 sense cDNA transfected cells (25.08±0.30%) versus the h2 antisense cDNA transfected (9.85±0.44%) and

non-transfected (9.83±0.30%) SM3 cultures (Fig. 10) ($P < 0.001$). This increase in the number of bi-nuclear cells indicates that the forced expression of h2-calponin does not directly reduce the rate of DNA replication to decrease cell proliferation rate, but rather inhibits the function of the actin cytoskeleton during cytokinesis to in slow cell division and proliferation. This hypothesis is consistent with the results shown in Fig. 8 in which a time lag was present between the expression of h2-calponin and the decrease of cell proliferation rate as detected by the nucleus staining method.

Nuclear division is not commonly seen in mammalian cell division. The increased frequency of cells with dividing nuclei in the cultures force-expressing h2-calponin suggests that the suppression of actin cytoskeleton function may prevent cytokinesis after chromosome replication in the nucleus. Immunofluorescence microscopy showed that in h2 sense cDNA transfected bi-nuclear SM3 cells, h2-calponin was enriched to surround the partially divided nuclei, often forming a nuclear ring structure (Fig. 11). This actin filament-based nuclear ring structure is similar to the plasma membrane contractile ring, suggesting that the actin cytoskeleton may play a compensatory role promoting nuclear division when cytokinesis is suppressed. In contrast to the broad stress fiber distribution of tropomyosin (Fig. 11A), the enriched association of h2-calponin with the nuclear ring structure (Fig. 11B) may imply its regulatory role in the nuclear division function of actin.

DISCUSSION

Independently regulated expression of h1- and h2-calponin. The observation that the developmental expression of h1- and h2-calponin in the bladder and stomach is regulated in opposite directions indicates their differentiated function. Up-regulated expression of h1-calponin has been observed during smooth muscle differentiation and development (**7, 11, 13, 32, 46**), suggesting that it is involved in the functional maturation of myofilaments. A previous study has observed increased expression of caldesmon in pregnant uterus smooth muscle, possibly playing a role in suppressing contractility for the maintenance of pregnancy (**49**). Thus, the decreased expression of h1-calponin in the mid-term pregnant uterus (Fig. 5) may also contribute to the suppression of uterus smooth muscle contractility. Western blots using the anti-h2-calponin mAb CP21 demonstrated that h2-calponin is expressed at high levels in rapidly growing tissues such as the embryonic stomach and bladder and down-regulated during postnatal development (Fig. 4). High levels of h2-calponin were also found in uterus smooth muscle during early pregnancy (Fig. 5). The expression patterns of h2-calponin may reflect its function in tissue growth and remodeling. The higher levels of h2-calponin in rapidly growing and remodeling tissues support its cytoskeletal function relating to cell proliferation. Therefore, while h1-calponin may play a modulator role in tuning smooth muscle contractility (**29, 48**), h2-calponin may play a regulatory role in the function of the actin cytoskeleton in smooth muscle and non-muscle cells.

The potential role of h2-calponin in regulating the rate of cell proliferation. H1-calponin's function as a regulatory protein for smooth muscle contractility has been extensively investigated. However, the absence of h1-calponin in rat aortic smooth muscle does not abolish contractility (**32**). In fact, h1-calponin knockout mice remain normal in many physiological activities (**29, 44**). Therefore, calponin is not an essential smooth muscle contractile protein, but rather a *tuning* element in smooth muscle contractility. The specific function of h2-calponin, on

the other hand is not yet known. Its presence in both smooth muscle and non-muscle cells indicates that it may have a cytoskeletal function. Considering calponin's inhibitory activity on actin-myosin interactions, h2-calponin may also play an inhibitory role in regulating the functions of the actin cytoskeleton, such as coordinating changes in cell shape and intracellular molecular trafficking, both of which are critical events in cytokinesis (**15**). Therefore, h2-calponin may act as a balancing mechanism to maintain the physiological levels of actin filament activity in both smooth muscle and non-muscle cells. In the present study we demonstrated that the expression of h2-calponin inhibits cell proliferation, suggesting its regulatory role in cytokinetic activities. The gene expression and activity regulation of h2-calponin may contribute to normal organ development and the physiological growth and remodeling of tissues. This hypothesis is supported by the observations that significant amounts of calponin are associated with the non-contractile actin cytoskeleton (**34**), and forced expression of chicken gizzard (h1) calponin in cultured smooth muscle cells and fibroblasts inhibits cell proliferation (**19**). Also, h1-calponin knockout mice displayed enhanced ectopic bone formation when they were stimulated by recombinant human bone morphogenetic protein-2, once again suggesting calponin's function as a suppressor of cell proliferation. The mice also had an early onset of cartilage formation and ossification and accelerated healing of bone fractures (**51**). Interestingly, calponin is expressed notably less in leiomyosarcoma cells than in normal smooth muscle cells (**16**). Transfective expression of calponin in leiomyosarcoma cells significantly reduced anchorage-independent growth and *in vivo* tumorigenicity, indicating its function as a tumor suppressor (**17**). In addition, calponin has been detected in the cytoplasm of human osteosarcoma cells, and the survival rate of patients whose tumors exhibit calponin are significantly higher than in those whose tumors do not express calponin (**50**).

H2-Calponin in the function of actin cytoskeleton. Actin-myosin interaction-based cell motility is essential for cytokinesis. The formation and function of a contractile ring during the cell division is a clear of example of this fact (**3, 4, 36, 39, 50**). The contraction of the contractile

ring is most likely generated by the interaction between the actin and myosin (**2, 6, 22, 27**). The actin cytoskeleton has been demonstrated to participate in anchorage-dependent cell division (**15**), and actin-myosin interactions have been shown to power cell proliferation by driving cytoplasmic streaming. *In vitro* experiments have shown that calponin inhibits the relative movements of actin and myosin (**40**). H2-calponin's association with the tropomyosin-actin filament also suggests that it may inhibit the organization and motility of the actin cytoskeleton. . Thus, calponin's function in regulating actin-myosin interaction and actin cytoskeleton function may affect cytokinesis and the rate of cell proliferation.

During eukaryotic cell division, the nuclear membrane disintegrates to allow for the mitotic separation of chromosomes. Although nuclear division is often seen in cell cultures, the significantly increased number of bi-nuclear cells in h2-calponin expressing cultures indicates an inhibition of cytokinesis post chromosome replication. H2-calponin in the binuclear cells was concentrated around the nuclei, specifically in a "nuclear ring" structure which, like the contractile ring, is formed by actin filaments (Fig. 10). The association of h2-calponin to the nuclear ring suggests that h2-calponin may inhibit the process of nuclear division to prevent multiploidy in cells in which cytokinesis was suppressed. While the actin-tropomyosin stress fibers are broadly distributed in the cell, the concentrated localization of h2-calponin around the dividing nuclei indicates the presence of a specialized domain of the actin cytoskeleton (Fig. 10) that is regulated by h2-calponin. We have observed that h2-calponin selectively binds low molecular weight non-muscle tropomyosin, suggesting a potential functional correlation (unpublished results). Therefore, the enrichment of h2-calponin in the nuclear ring may indicate that the regulatory activity of h2-calponin may be targeted through the cellular distribution of tropomyosin isoforms. Since calponin has been observed to participate in the protein kinase C signaling pathway (**24**), the function of h2-calponin in regulating the activity of the actin cytoskeleton may play an important role in maintaining physiological tissue growth and remodeling and deserves further investigation.

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(Figure Legends)

Fig. 1. Specific antibodies against h1- and h2-calponins. (A) Cloned mouse h1- and h2-calponins expressed in and purified from *E. coli* culture were analyzed by Western blotting with the CP1 and CP3 mAbs and the RAH2 antiserum using alkaline phosphatase-labeled anti-mouse IgG or anti-rabbit IgG second antibodies, respectively. The SDS-PAGE resolved the size difference between the two calponin isoforms and the immunoblots demonstrate that the CP1 and CP3 mAbs are specific to h1-calponin while the anti-h2-calponin RAH2 antiserum showed the expected weaker cross-reaction to h1-calponin. (B) Western blots demonstrate that mAb CP21 is specific to h2-calponin while mAb CP23 has a weak cross reaction to h1-calponin and mAb CP11 recognizes both h1 and h2-calponins.

Fig. 2. Measurement of cell numbers by Crystal Violet staining. (A) and (B): SP2/0Ag14 mouse myeloma cells were seeded in 96-well tissue culture plates in DMEM containing 10% FBS at low and high series of numbers, respectively, and stained with the Crystal Violet method after incubation at 37 °C in 5% CO_2 for 6 hr. (C) and (D): SM3 cells were seeded in 96-well culture plates at low and high series of numbers, respectively, and cultured in DMEM containing 10% FBS at 37 °C in 5% CO_2 for 6 hr before the monolayer cells were stained by Crystal Violet. The results from quartet experiments demonstrate excellent linear relationships between the $O.D_{.595nm}$ values and the cell numbers for both cell types over a wide range of cell numbers.

Fig. 3. Growth curve of SM3 cells at various seeding densities. SM3 cells were seeded in 96-well culture plates at 500, 1000 and 1500 cells per well. The cells were cultured in DMEM containing 10% FBS at 37 °C in 5% CO_2 and measured for cell numbers by Crystal Violet staining at a series of time points. The results from triplicate experiments show the duration of log phase growth of SM3 cells under the cultural conditions, which are used in the examination of h2-calponin's effects on the rate of cell proliferation.

Fig. 4. Developmentally regulated expression of h1- and h2-calponins. Total protein extracts from mouse stomach (A) and urinary bladder (B) at a series of time points during postnatal development were analyzed by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting using the anti-h1-calponin mAb CP3 and the anti-h2-calponin mAb CP21. Purified mouse h1- and h2-calponins were used as controls. The Western blots show a developmental up-regulation of h1-calponin in both organs. The expression of h2-calponin is down-regulated during postnatal development with different patterns in the stomach and urinary smooth muscles. An additional band with a molecular weight slightly higher than that of h2-calponin was detected by the anti-h2-calponin mAb CP21 in the 1-month and 6-month mouse bladder. The curves from densitometry quantification of multiple Western blots (mean ± SD, n = 3-4) clearly demonstrate the changes in calponin isoform expression.

Fig. 5. Regulated expression of h1- and h2-calponins in uterus smooth muscle during pregnancy and involution. Total protein extracts from the smooth muscle layer of mouse uterus were analyzed on SDS-PAGE and transferred to nitrocellulose membrane for Western blotting using the anti-h1-calponin mAb CP3 and the anti-h2-calponin mAb CP21. (A) The blots show high level expression of h1-calponin in the uterus smooth muscle prior to labor versus high level expression of h2-calponin in the rapid growing mid term uterus. Purified h2- and h1-calponins were used as controls. (B) The curves from densitometry quantification of multiple Western blots (mean ± SD, n = 3-4) clearly demonstrate the changes in calponin isoform expression.

Fig. 6. Transfective expression of h2-calponin in SM3 cells. (A) Total protein extract from rabbit blood vessels and SM3 cells before and after *in vitro* differentiation in DMEM containing 0.5% horse serum for five days were examined by Western blot using the anti-h1- and anti-h2-calponin mAbs. The results show that while both h1- and h2-calponins are expressed in rabbit vascular smooth muscle, the SM3 cell line derived from rabbit aorta has ceased calponin expression. (B) h2-calponin was expressed at significant amounts in the h2-calponin sense, but

not anti-sense, cDNA stable-transfected SM3 cells. (C) Western blot analysis on total cellular proteins extracted from SM3 cells confirmed the specificity of the RAH2 and CG3 antibodies.

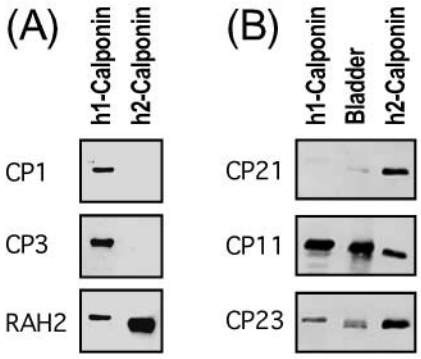
Fig. 7. Inhibition of cell proliferation by stable transfective expression of h2-calponin. SM3 cells stable-transfected with the sense or antisense h2-calponin cDNA were seeded in 96-well culture plates in DMEM containing 10% FBS and cultured at 37 °C in 5% CO_2 the presence (A) or absence (B) of G418 (500 μg/ml). The cultures were stopped at a series of time points and the cell numbers were measured by Crystal Violet staining. The cell growth curves were plotted from data of four experiments. The results demonstrated a decreased proliferation rate in the h2 sense transfected versus h2 antisense transfected cells. * $P < 0.001$.

Fig. 8. Inhibition of cell proliferation by transient transfective expression of h2-calponin. SM3 cells were transiently transfected with the h2 sense and h2 antisense expression vectors and cultured in DMEM containing 10% FBS at 37˚C in 5% CO_2 for 18 hours before re-plated in 96-well culture plates at 1000 cells per well in fresh media. The cultures were stopped at a series of time points and the expression of h2-calponin was examined by Western blot using the anti-h2-calponin antibody RAH2. The results in the inset show transient expression of h2-calponin in the h2 sense, but not h2 antisense, cDNA transfected SM3 cells. The cell numbers were measured at these time points by Crystal Violet staining. The cell growth curves were plotted from the results of four experiments. The dashed arrow lines outline the doubling time of the cultures. The growth curve of h2 antisense cDNA transfected cells is identical to that of the non-transfected cells. Although the initial proliferation rate of the h2-calponin expressing cells was not different from the controls when the growth was moderate, the accelerating growth as that seen in the non-transfected and h2 antisense cDNA transfected cells was significantly delayed (* $P < 0.001$). After a ~24-hr lag, the cell proliferating rate of the h2 sense transfected cells resumed after h2-calponin ceased expression.

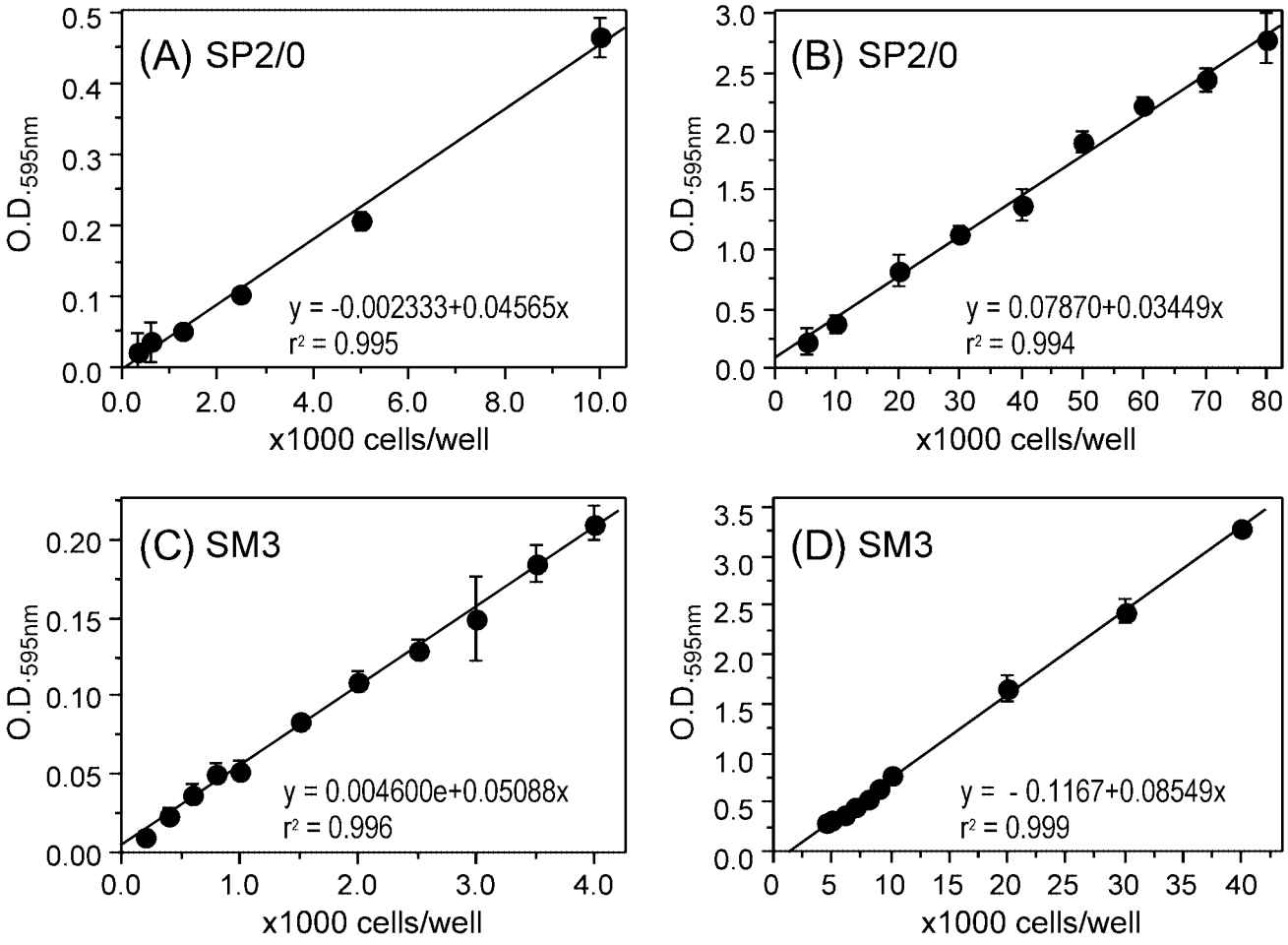
Fig. 9. Association of h2-calponin with the actin-tropomyosin filaments in the transfected SM3 cells. SM3 cells stable-transfected with h2 sense or h2 antisense cDNA were cultured on gelatin-coated cover slips. Pre-confluent monolayer cell samples were examined by immunofluorescence microscopy using the rabbit anti-h2-calponin antibody RAH2 and the mouse anti-tropomyosin mAb CG3, alone or as a mixture. TRITC-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG second antibodies, alone or as a mixture, was used to selectively detect the localization of h2-calponin and tropomyosin, respectively. Phase contrast and fluorescence microscopic images were photographed. (A) The TRITC and FITC fluorescence images can be selectively obtained by using appropriate filter sets. (B) Stress fiber association of both h2-calponin and tropomyosin was seen in the SM3 cells. (C) Double-antibody staining demonstrates a co-localization of h2-calponin and tropomyosin.

Fig. 10. An increase of bi-nuclear cells in h2-calpsonin expressing cultures. Stable h2 sense and h2 antisense cDNA transfected and non-transfected SM3 cells were cultured on gelatin-coated cover slips. Pre-confluent monolayer cell samples were collected and fixed with acetone for microscopic examination. 2,000 cells were examined on each cover slip to calculate the rate of bi-nuclear cells. The results summarized from 3 cover slips in each group demonstrated a significantly increased number of bi-nuclear cells in the h2 sense-transfected cultures (* $P < 0.001$).

FIG. 11. Nuclear ring structure formed in the transfected SM3 cells by h2-calponin-containing stress fibers. Pre-confluent monolayer cultures of h2-sense cDNA transfected SM3 cells on the cover slips were fixed with acetone and examined by immunofluorescence microscopy using the anti-tropomyosin CG3 mAb or the anti-h2-calponin antibody RAH2 and TRITC-conjugated second antibody. The phase contrast and immunofluorescence images of binuclear cells were examined and photographed. In contrast to the extensive stress fiber association of tropomyosin (A), h2-calponin was enriched around the nuclei and participates in the formation of a nuclear ring structure (B).



Hossain et al., Fig. 1



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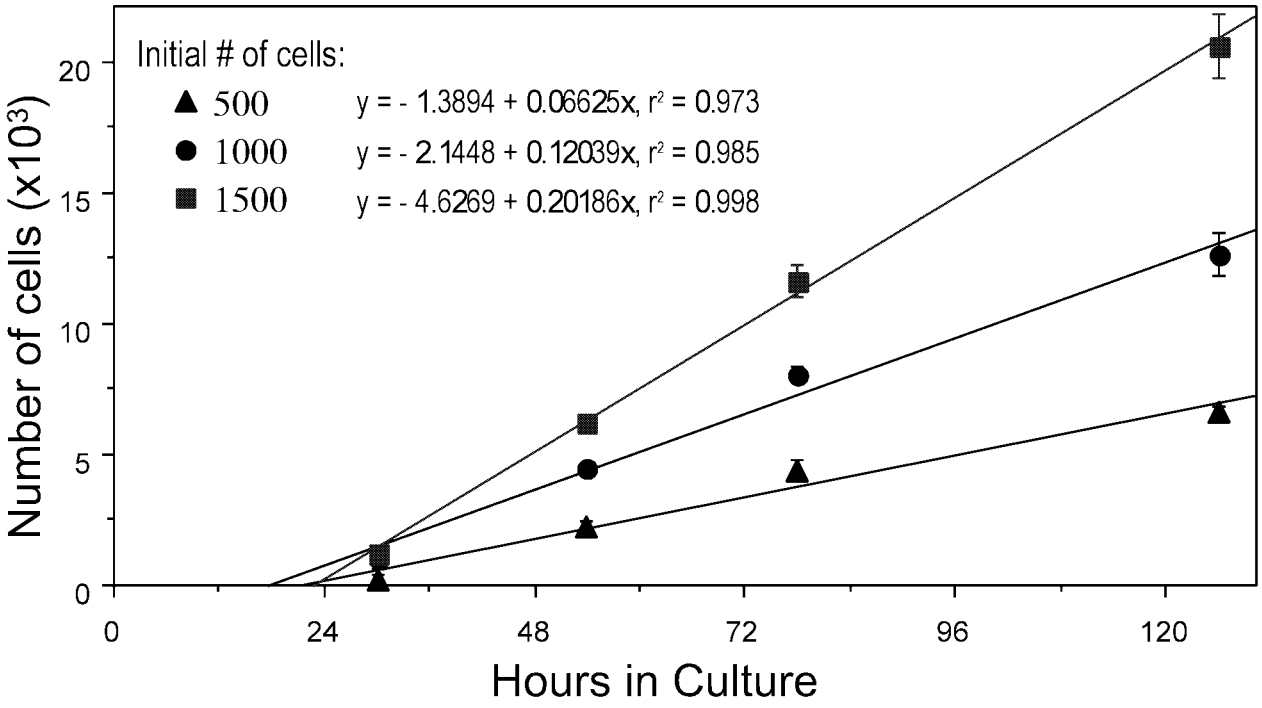

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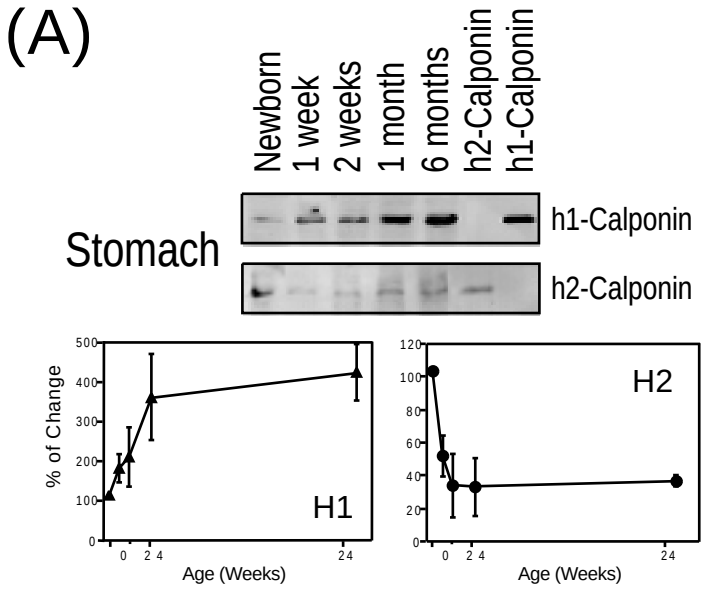
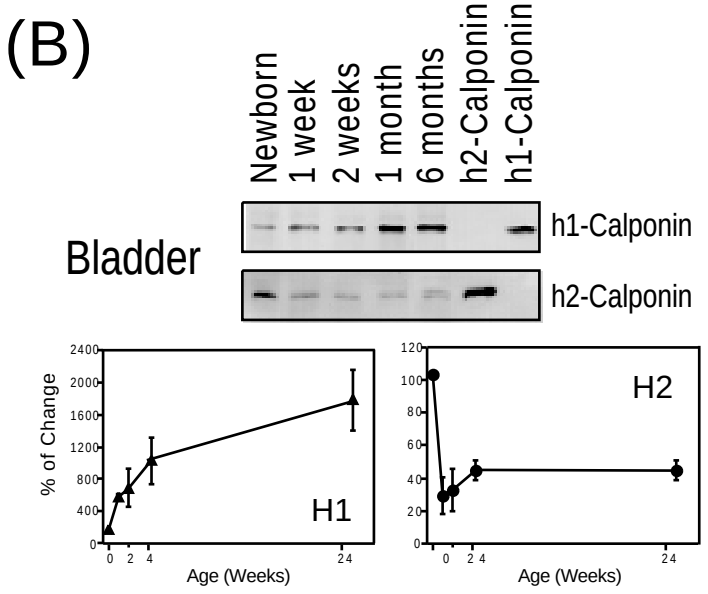
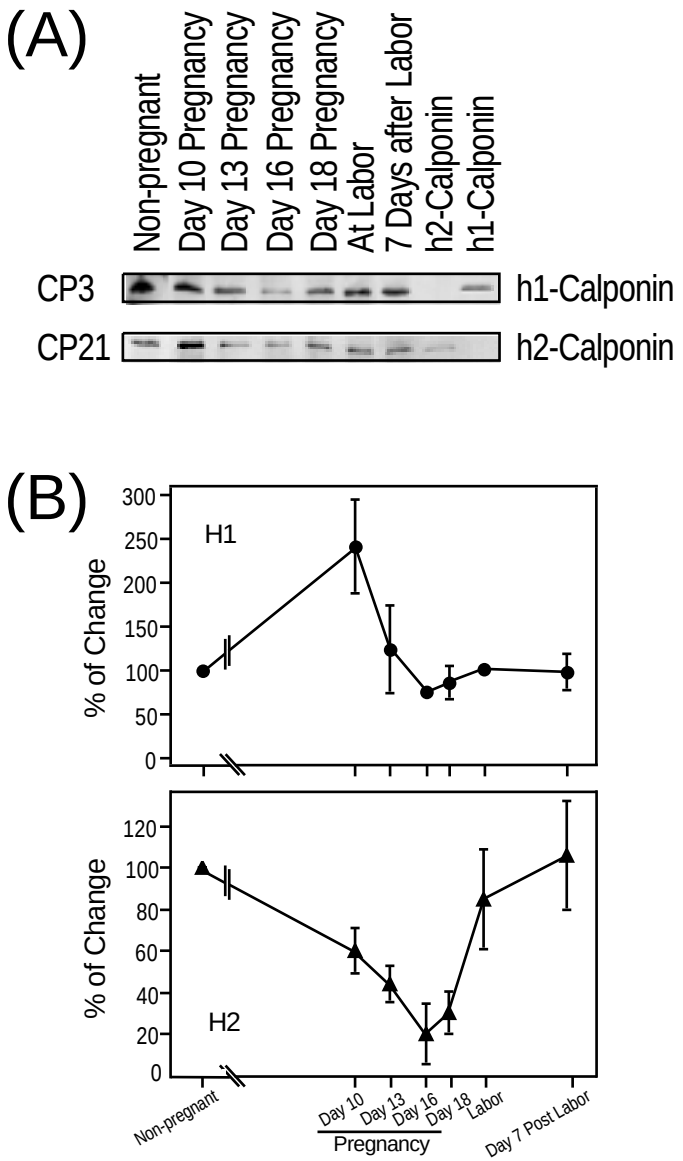

(A)
Newborn
1 week
2 weeks
1 month
6 months
h2-Calponin
h1-Calponin
Stomach
h1-Calponin
h2-Calponin
% of Change
H1
H2
Age (Weeks)
Age (Weeks)


(B)
Newborn
1 week
2 weeks
1 month
6 months
h2-Calponin
h1-Calponin
Bladder
h1-Calponin
h2-Calponin
% of Change
H1
H2
Age (Weeks)
Age (Weeks)



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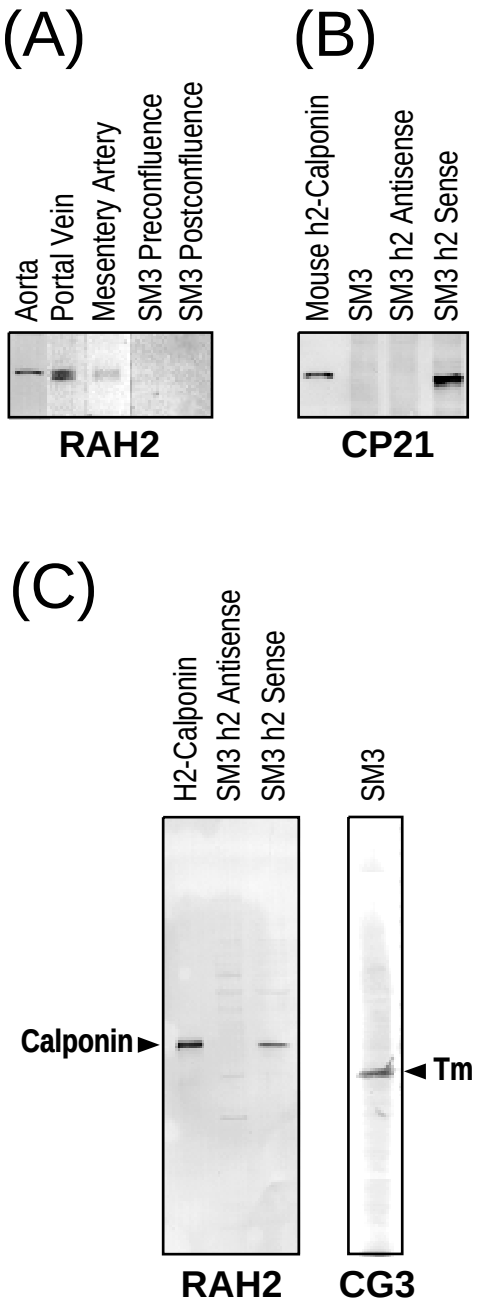

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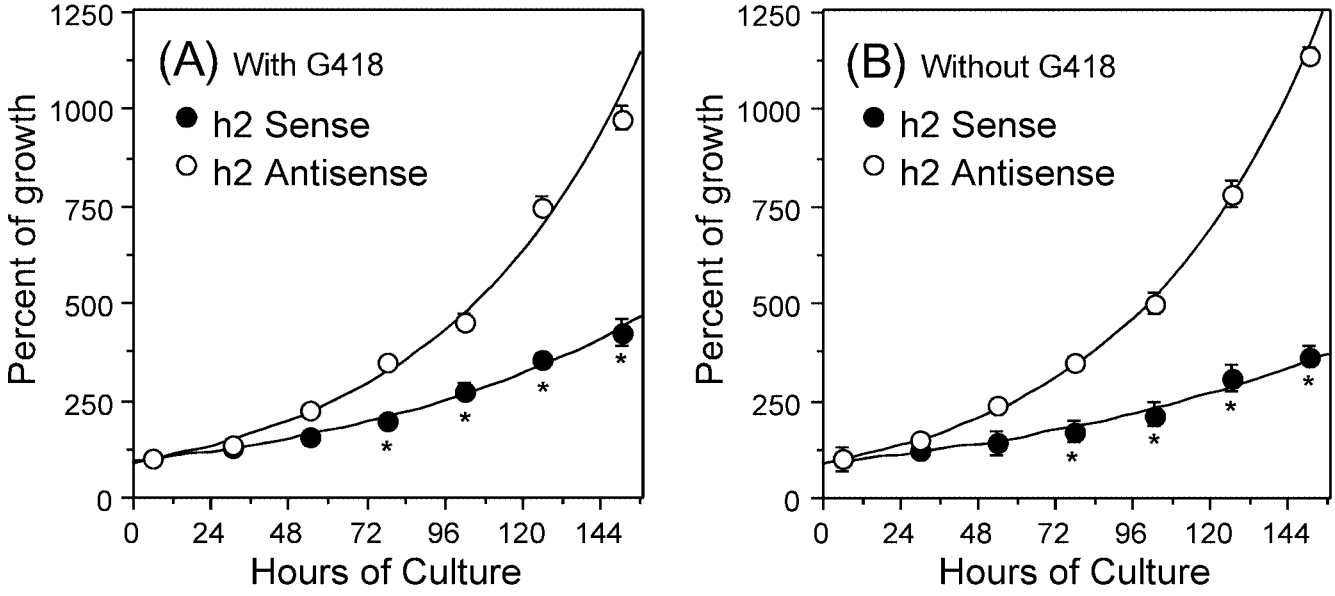

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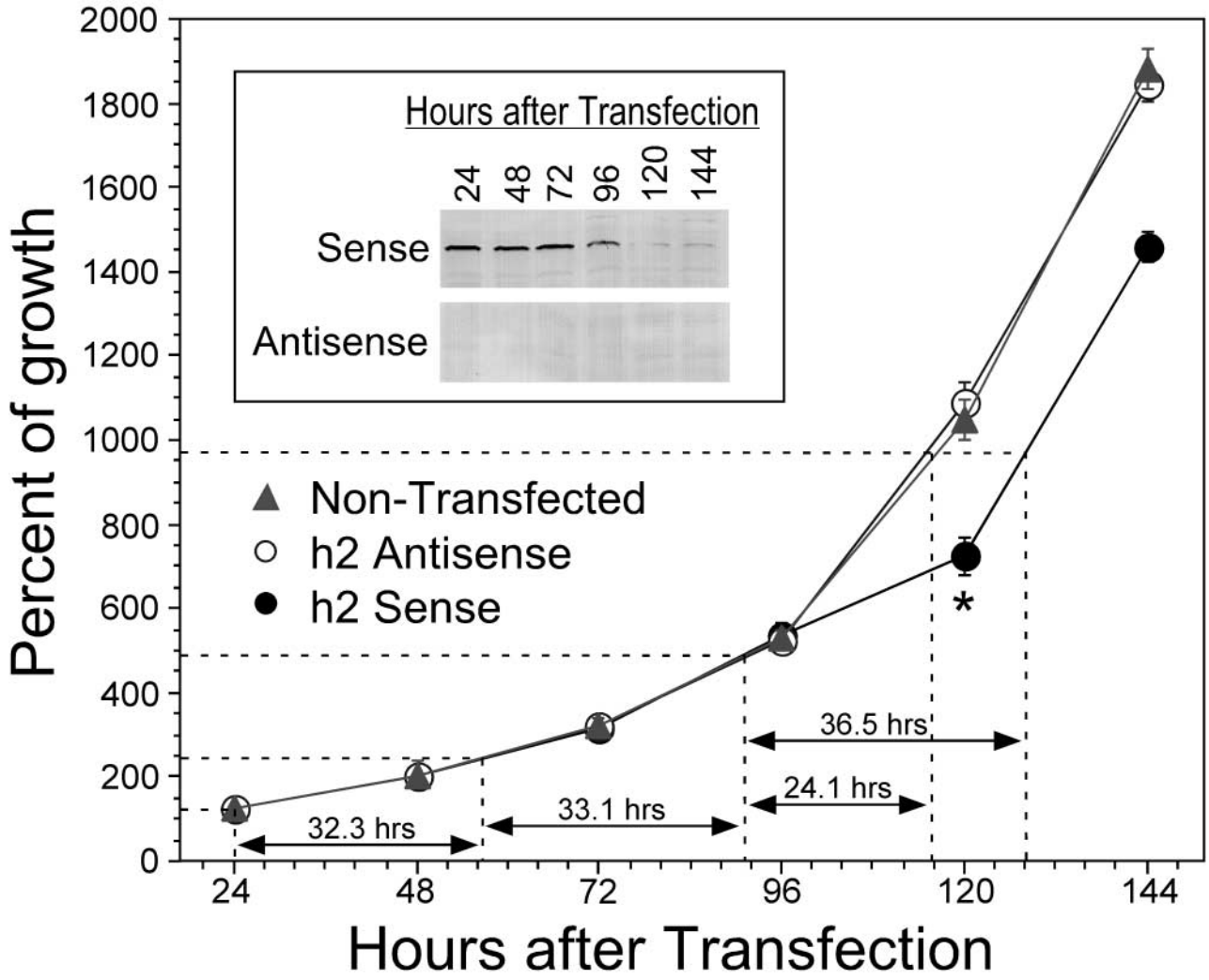

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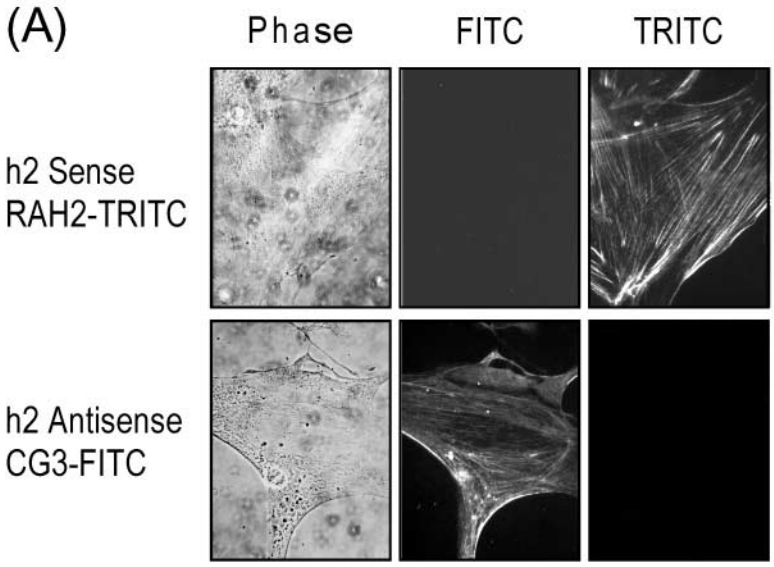

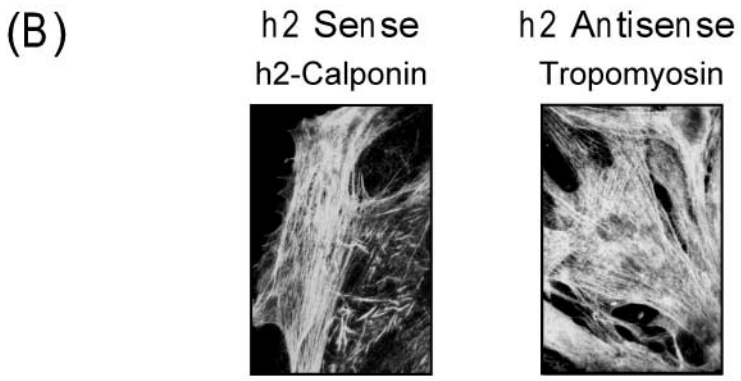

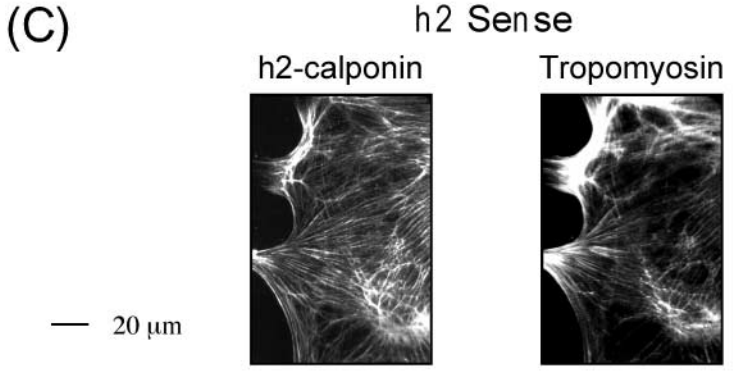

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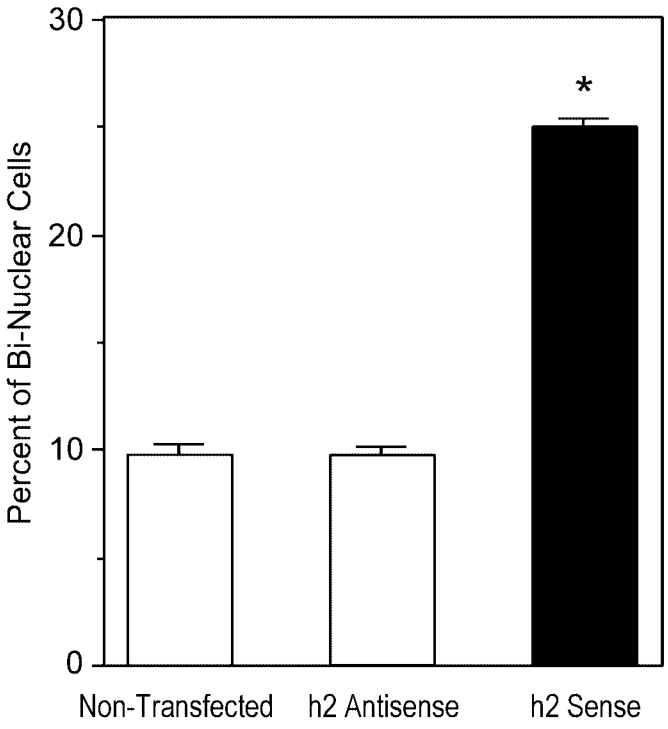

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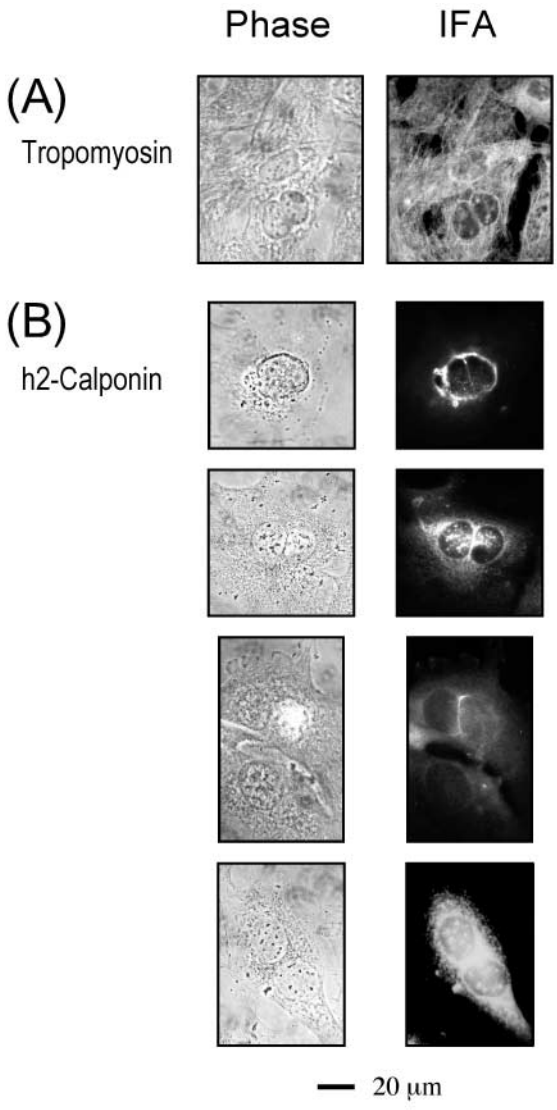

Hossain et al., Fig. 11