Am J Physiol Cell Physiol 284: C156–C167, 2003.
First published September 4, 2002; 10.1152/ajpcell.00233.2002.

Developmentally regulated expression of calponin isoforms and the effect of h2-calponin on cell proliferation

M. MOAZZEM HOSSAIN,[1] DAW-YANG HWANG,[1] QI-QUAN HUANG,[1]
YASUHARU SASAKI,[2] AND JIAN-PING JIN[1]
[1]*Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4970; and* [2]*Department of Pharmacology, Kitasato University, 5-9-1, Sirogane, Minatoku 108-8641, Japan*

Submitted 21 May 2002; accepted in final form 3 September 2002

Hossain, M. Moazzem, Daw-Yang Hwang, Qi-Quan Huang, Yasuharu Sasaki, and Jian-Ping Jin. Developmentally regulated expression of calponin isoforms and the effect of h2-calponin on cell proliferation. *Am J Physiol Cell Physiol* 284: C156–C167, 2003. First published September 4, 2002; 10.1152/ajpcell.00233.2002.—h2-Calponin is found in both smooth muscle and nonmuscle cells, and its function remains to be established. Western blots with specific monoclonal antibodies detected significant expression of h2-calponin in the growing embryonic stomach and urinary bladder and the early pregnant uterus. Although the expression of h1-calponin is upregulated 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 with the antisense cDNA-transfected or nontransfected cells. Immunofluorescence microscopy showed that the force-expressed h2-calponin was associated with actin-tropomyosin microfilaments. The number of binuclear 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.

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

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, as expected, acidic (pI = 5.5–5.8). The extensively investigated chicken gizzard calponin is equivalent to mammalian h1-calponin, the major calponin found in smooth muscle cells. The smooth muscle calponin has been shown to inhibit actin-activated myosin ATPase, which has led to a model in which 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 (28, 38, 39); thus the activity of the actin cytoskeleton has an effect on 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 (5). Because expression of h1-calponin in smooth muscle is upregulated 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. Whereas h1-calponin may play a modulator role in tuning smooth

Address for reprint requests and other correspondence: J.-P. Jin, Dept. of Physiology and Biophysics, Case Western Reserve Univ. School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106-4970 (E-mail: jxj12@po.cwru.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

muscle contractility as previously discussed (48), 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; Ref. 37) with no endogenous calponin, 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 binuclear 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 (MAbs) raised against chicken gizzard calponin (CP1 and CP3; Ref. 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-wk-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 of phosphate-buffered saline (PBS) mixed with an equal volume of Freund's complete adjuvant. Ten days later, one mouse was intraperitoneally boosted two times with 200 μg each of the antigen in 200 μl of PBS without adjuvant on two consecutive days. Two days after the last boost, spleen cells were harvested from the immunized mouse and fused with SP2/0-Ag14 mouse myeloma cells (American Type Culture Collection) with 50% polyethylene glycol 3400 containing 7.5% dimethyl sulfoxide (DMSO) 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 (FBS) and screened by indirect enzyme-linked immunosorbent assay (ELISA) with 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 a limiting dilution method with young BALB/c mouse spleen cells as feeders to establish stable cell lines. The hybridoma lines 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 MAb CP21, showing no cross-reaction to h1-calponin, was used in Western blots to examine the expression of h2-calponin.

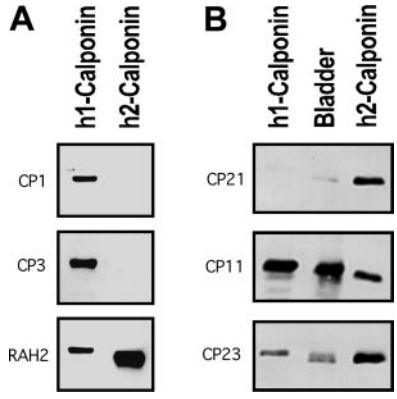

Fig. 1. Specific antibodies against h1- and h2-calponins. *A*: cloned mouse h1- and h2-calponins expressed in and purified from *Escherichia coli* culture were analyzed by Western blotting with the CP1 and CP3 monoclonal antibodies (MAbs) and the RAH2 antiserum and alkaline phosphatase-labeled anti-mouse IgG or anti-rabbit IgG second antibodies, respectively. SDS-polyacrylamide gel electrophoresis (PAGE) resolved the size difference between the 2 calponin isoforms, and the immunoblots demonstrate that the CP1 and CP3 MAbs are specific to h1-calponin, whereas 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, whereas MAb CP23 has a weak cross-reaction to h1-calponin and MAb CP11 recognizes both h1- and h2-calponins.

Construction of expression vectors. The coding region of mouse h2-calponin cDNA (32) 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) downstream of the cytomegalovirus (CMV) promoter in sense or antisense 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 with the dideoxy chain termination method as described previously (18). The sense expression construct encodes a nonfusion full-length mouse h2-calponin protein for authentic functional characterization, and the antisense construct provided a transfection control in the present study. The recombinant pcDNA3 plasmid DNA was prepared from transformed JM109 *Escherichia coli* in large quantities with 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 with the 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) liposomal transfection reagent (Boehringer Mannheim) following the manufacturer's instructions. SM3 cells were seeded on Corning 10-cm culture dishes at 2×10^6 cells per dish and grown until the monolayer cells reached 60–80% confluence. Twenty micrograms of the recombinant supercoil plasmid DNA in 50 μl of TE buffer (10 mM Tris·HCl, pH 8.0, and 1 mM EDTA) was mixed with 100 μl of 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 of DMEM containing 10% FBS and added to the culture dish to replace the old medium. The SM3 cell monolayer was incubated with the DOTAP-DNA medium for 18 h at 37°C in 5% CO_2 before the change to fresh medium.

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). Results from testing the tolerance of nontransfected SM3 cells to G418 showed that this cell line is highly sensitive to G418. In culture medium containing 20 μg/ml G418, all cells died after 9 days. The recombinant 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 with 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 35% 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 (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, 140 mM β-mercaptoethanol, 0.1% bromphenol blue, 10% glycerol) with a Polytron-type high-speed tissue homogenizer (PRO Scientific, Monroe, CT) to extract total cellular proteins. The h2-calponin sense and antisense cDNA-transfected SM3 cells were suspended from the culture dishes with Versene solution (in mM: 0.537 EDTA, 136.8 NaCl, 2.68 KCl, 8.1 Na_2HPO_4, 1.47 KH_2PO_4, pH 7.2) and washed three times with 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 lyse 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-to-bisacrylamide ratio of 29:1 prepared in the Laemmli 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 with a Bio-Rad semidry transfer apparatus at 4–5 mA/cm^2 for 30 min. The blotted membranes were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; in mM: 150 NaCl and 50 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 nitro blue 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 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 (OD) 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/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 before extraction of the bound dye with 100 μl of 10% acetic acid. Optical density of the dye extracts was measured at 595 nm (OD_{595}) with an automated microtiter plate reader (Benchmark; Bio-Rad 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% 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 Pasteur pipette. The cell numbers were counted in a hemacytometer before seeding in 96-well culture plates in DMEM containing 10% FBS. Six hours after seeding, the cells were fixed and processed for crystal violet staining as described above. The results, shown in Fig. 2, *A* and *B*, demonstrate a very good linear relationship between the OD_{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 cells/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 h 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. 2, *C* and *D*, also show a very good linear relationship between the OD_{595} values of crystal violet nuclear staining and the wide range of cell numbers (3.12×10^2–5×10^4 cells/well).

Monitoring 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. Nontransfected SM3 cells were harvested from preconfluent cultures by digestion with 0.025% trypsin in 0.02% EDTA solution and seeded into 96-well culture plates at 500, 1,000, and 1,500 cells/well in DMEM containing 10% FBS. Five identical sets of cultures were started on five consecutive days and were stopped altogether to obtain 30-, 54-, 78-, and 102-h cultures. The plates were processed for crystal violet stain-

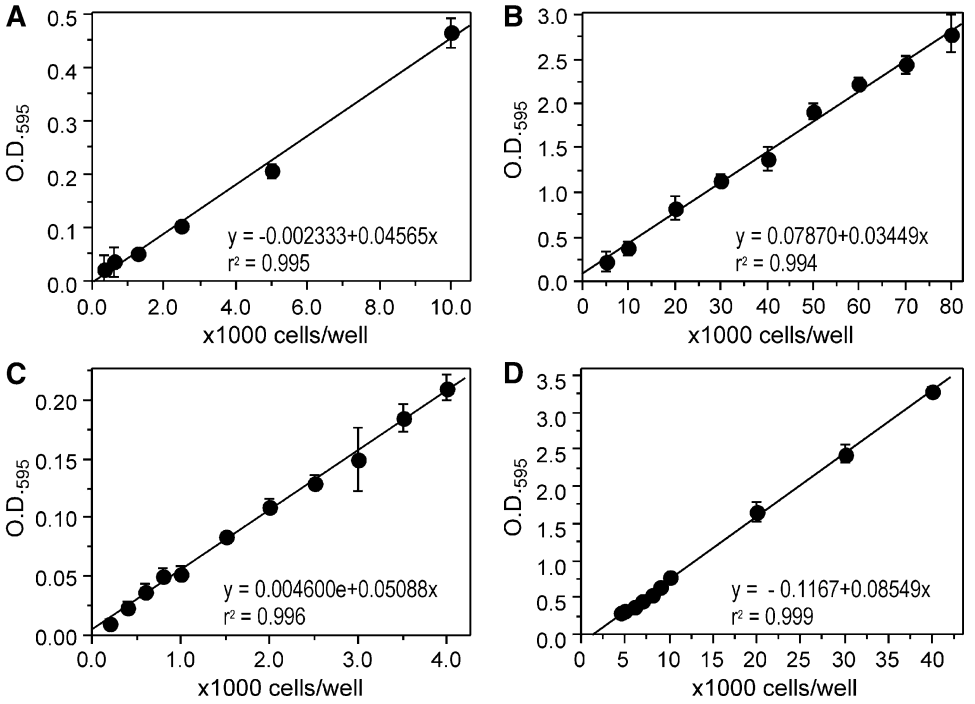

Fig. 2. Measurement of cell numbers by crystal violet staining. *A* and *B*: SP2/0Ag14 mouse myeloma cells were seeded in 96-well tissue culture plates in DMEM containing 10% FBS at low and high series of numbers, respectively, and stained with the crystal violet method after incubation at 37°C in 5% CO_2 for 6 h. *C* and *D*: SM3 cells were seeded in 96-well culture plates at low and high series of numbers, respectively, and cultured and stained by crystal violet as in *A* and *B*. Results plotted from means ± SD of quartet experiments demonstrate excellent linear relationships between the optical density values at 595 nm (OD_{595}) and the cell numbers for both cell types over a wide range of cell numbers.

ing. 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 h without changing media. Accordingly, the proliferation rates of the transfected SM3 cells were examined under these conditions, except that the dispersion of the transiently transfected SM3 cells was done by using Versene solution to avoid enzymatic damage of the membrane proteins that may affect the initial rate of cell proliferation.

Immunofluorescence microscopy. Precleaned glass coverslips 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 coverslips. The coverslips with monolayer SM3 cells were collected at ~70% confluence and washed with PBS. The cells were fixed with cold acetone for 30 min. 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 min, the coverslips were incubated with the rabbit anti-h2-calponin antibody RAH2 and a mouse MAb against tropomyosin (CG3; provided by Dr. Jim J.-C. Lin, University of Iowa; Ref. 25), alone or in combination, at room temperature for 2 h. After washes with PBS containing 0.05% Tween-20, the coverslips were stained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG and/or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG second antibodies (both from Sigma) at room temperature for 1 h. After final washes with PBS containing 0.05% Tween 20, the coverslips were mounted on glass slides and examined under a Zeiss Axiovert 100H phase contrast-epifluorescence microscope. A Plan-Neo phase fluorescence ×100 objective lens (oil; NA 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 different sets of filters (CZ915 and CZ909, respectively).

To determine the frequency of binuclear cells in the nontransfected and transfected SM3 cell cultures, coverslips

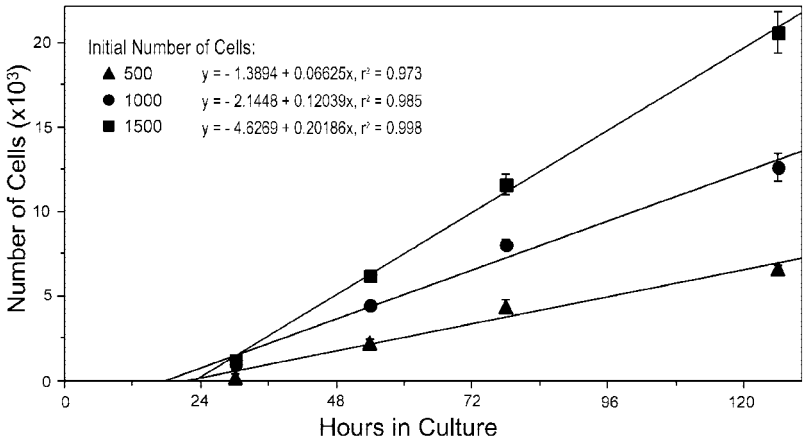

Fig. 3. Growth curve of SM3 cells at various seeding densities. SM3 cells were seeded in 96-well culture plates at 500, 1,000, and 1,500 cells/well. Cells were cultured in DMEM containing 10% FBS at 37°C in 5% CO_2 and measured for cell numbers by crystal violet staining at a series of time points. Results plotted from means ± SD of triplicate experiments show the duration of log-phase growth of SM3 cells under the cultural conditions used in the examination of h2-calponin's effects on the rate of cell proliferation.

with preconfluent monolayer cells were fixed and directly examined by phase-contrast microscopy as described above.

Statistical analysis. The quantitative data of cell proliferation are presented as means ± SD. Regression coefficients were calculated with Microsoft Excel. 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 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 expressed at only low levels in the stomach and bladder muscles of neonatal mice but upregulated during postnatal development to high levels in adult stomach and bladder. In contrast to the postnatal upregulation of h1-calponin, h2-calponin is expressed at high levels in the neonatal mouse stomach and urinary bladder smooth muscles and downregulated during postnatal development. Only a small amount of h2-calponin is present in the adult tissues (Fig. 4). Furthermore, the levels of either calponin isoform differ between the two smooth muscle organs. Although the expression of h1- and h2-calponin appeared 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 previous studies showing 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 (Figs. 1 and 4). Interestingly, the Western blot in Fig. 4*B* detected an additional protein band with a higher molecular weight than that of h2-calponin in both the 1-mo and 6-mo mouse urinary bladder. The amount of this protein seems to be increased in the 6-mo vs. 1-mo bladder. Further studies are under way to determine

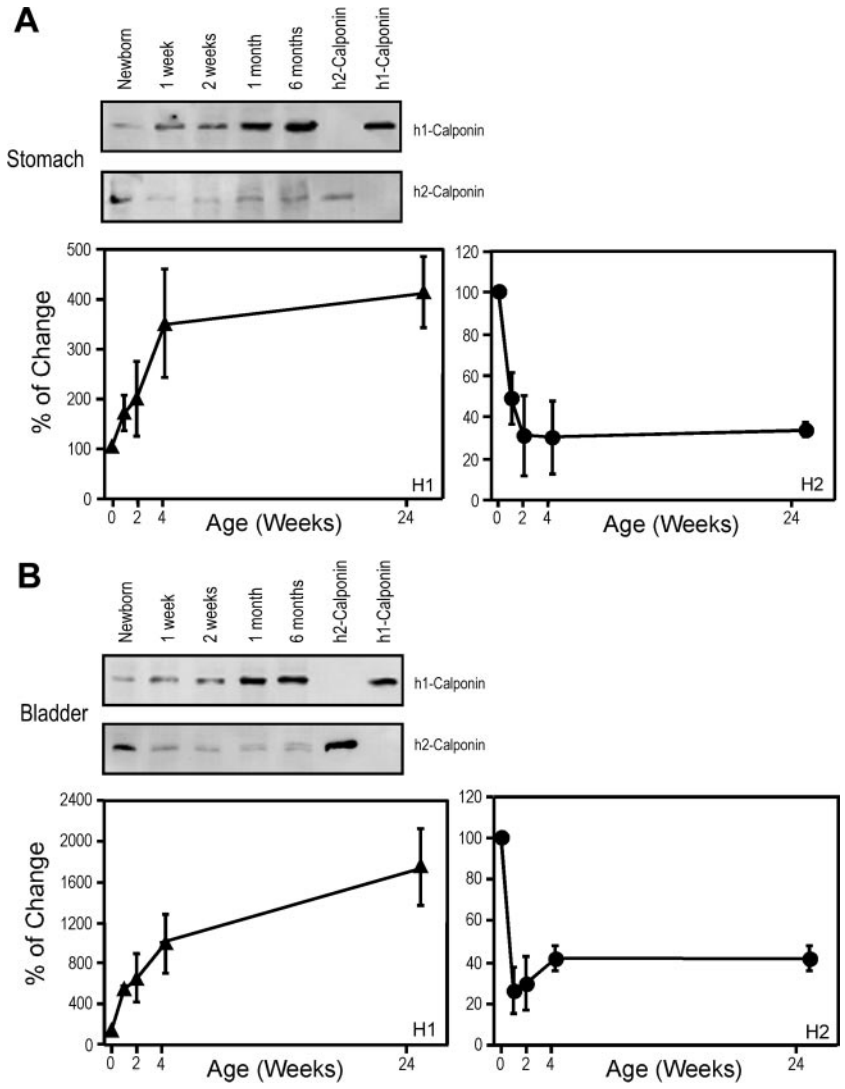

Fig. 4. Developmentally regulated expression of h1- and h2-calponins. Total protein extracts from mouse stomach (*A*) and urinary bladder (*B*) at a series of time points during postnatal development were analyzed by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting with the anti-h1-calponin MAb CP3 and the anti-h2-calponin MAb CP21. Purified mouse h1- and h2-calponins were used as controls. Western blots show a developmental upregulation of h1-calponin in both organs. Expression of h2-calponin is downregulated 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-mo and 6-mo mouse bladder. Curves from densitometry quantification of multiple Western blots (means ± SD; n = 3–4) summarize the changes in calponin isoform expression.

whether this protein is another calponin isoform or a phosphorylation variant of h2-calponin, either of which would 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 with the anti-h1-calponin MAb CP3 and the anti-h2-calponin MAb CP21 showed high-level h1-calponin expression in the nonpregnant and late-term uterus smooth muscle vs. high-level h2-calponin expression in the rapidly growing uterus of midterm pregnancy (Fig. 5). The high-level expression of h1-calponin in prelabor 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 inhibited the rate of cell proliferation. The Western blots in Fig. 6*A* show that although 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 preconfluent, 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. 6*B* show that the h2 sense, but not antisense, cDNA stable-transfected SM3 cells expressed a significant amount of h2-calponin.

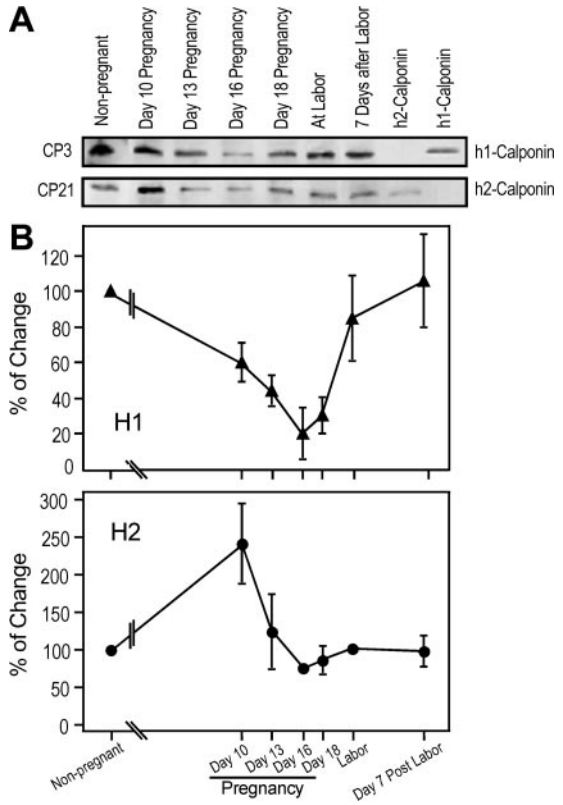

Fig. 5. Regulated expression of h1- and h2-calponins in uterus smooth muscle during pregnancy and involution. Total protein extracts from the smooth muscle layer of mouse uterus were analyzed on SDS-PAGE and transferred to nitrocellulose membrane for Western blotting with the anti-h1-calponin MAb CP3 and the anti-h2-calponin MAb CP21. *A*: blots show high-level expression of h1-calponin in the uterus smooth muscle before labor vs. high-level expression of h2-calponin in the rapidly growing midterm uterus. Purified h2- and h1-calponins were used as controls. *B*: curves from densitometry quantification of multiple Western blots (means ± SD; $n = 3$–4) summarize the changes in calponin isoform expression.

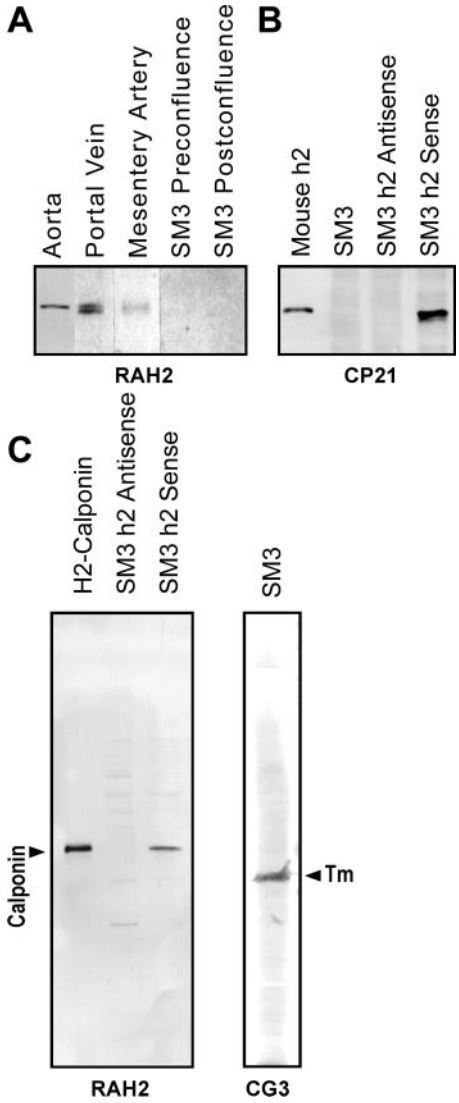

Fig. 6. Transfective expression of h2-calponin in SM3 cells. *A*: total protein extract from rabbit blood vessels and SM3 cells before and after reaching confluence in culture were examined by Western blot with anti-calponin antibodies (see Fig. 1). Results show that although both h1- and h2-calponins 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 antisense, cDNA stable-transfected SM3 cells. *C*: Western blot analysis on total cellular proteins extracted from SM3 cells confirmed the specificity of the anti-calponin RAH2 and the anti-tropomyosin (Tm) CG3 antibodies.

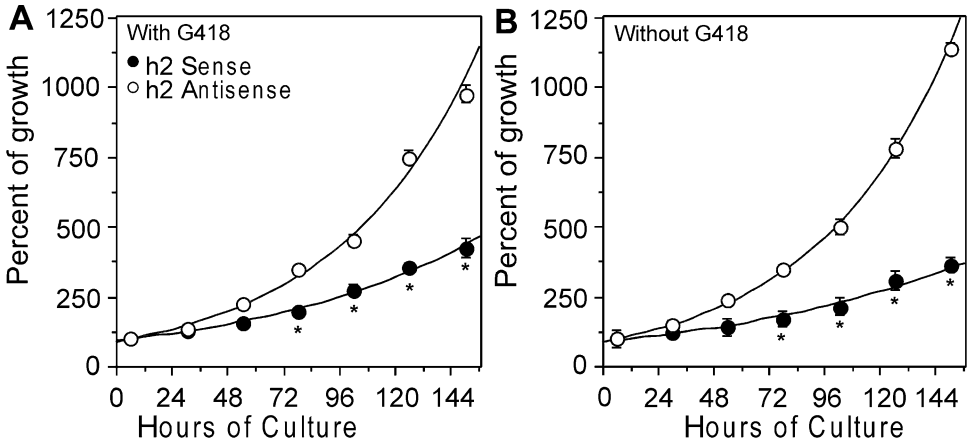

Fig. 7. Inhibition of cell proliferation by stable transfective expression of h2-calponin. SM3 cells stable-transfected with the sense or antisense h2-calponin cDNA were seeded in 96-well culture plates in DMEM containing 10% FBS and cultured at 37°C in 5% CO_2 in the presence (*A*) or absence (*B*) of G418 (500 μg/ml). Cultures were stopped at a series of time points, and the cell numbers were measured by crystal violet staining. Cell growth curves were plotted from means ± SD of 4 experiments. Results demonstrated a decreased proliferation rate in the h2 sense-transfected vs. h2 antisense-transfected cells. *$P < 0.001$.

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

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 vs. h2 antisense cDNA-transfected cells ($P < 0.001$). The Western blot detected a transient expression of h2-calponin in SM3 cells from 24 to 96 h after transfection with the h2 sense cDNA. In contrast to the continuously inhibited proliferation rate in the h2 sense stable-transfected SM3 cells (Fig. 7), the inhibition of cell proliferation was transient in the transiently transfected SM3 cells depending on the expression of h2-calponin (Fig. 8). After the cells ceased h2-calponin expression at 120 h after transfection, their proliferation rate returned to a level similar to that of the h2 antisense cDNA transfected and nontransfected SM3 cell controls (Fig. 8). In the stable transfection experiments, the inhibitory effect of h2-calponin was seen as early as 30 h after replating 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 nonspecific changes in individual stable-transfected cell lines.

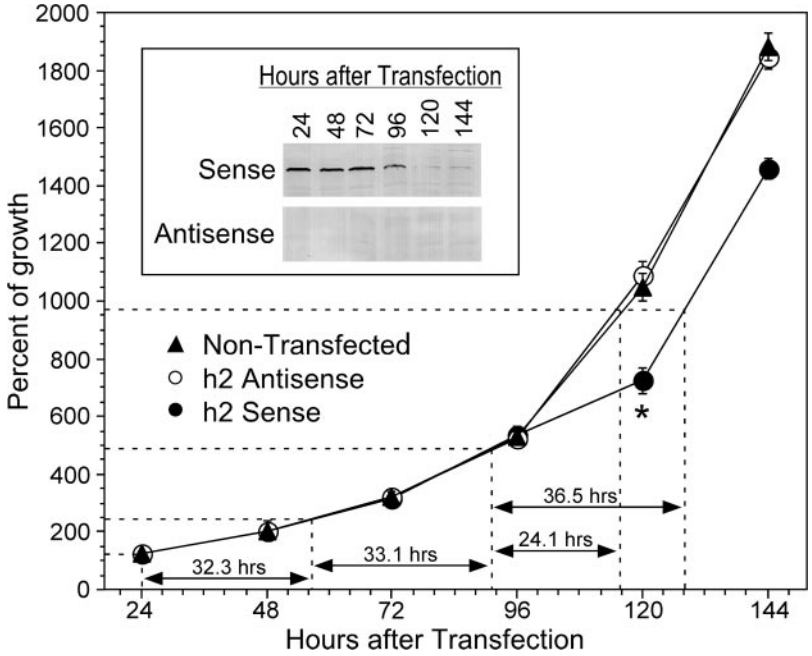

Fig. 8. Inhibition of cell proliferation by transient transfective expression of h2-calponin. SM3 cells were transiently transfected with the h2 sense and h2 antisense expression vectors and cultured in DMEM containing 10% FBS at 37°C in 5% CO_2 for 18 h before being replated in 96-well culture plates at 1,000 cells/well in fresh medium. Cultures were stopped at a series of time points, and the expression of h2-calponin was examined by Western blot with the anti-h2-calponin antibody RAH2. Results in the *inset* show transient expression of h2-calponin in the h2 sense, but not h2 antisense, cDNA-transfected SM3 cells. Cell numbers were measured at these time points by crystal violet staining. Cell growth curves were plotted from the means ± SD of 4 experiments. Dashed lines outline the doubling time of the cultures. Growth curve of h2 antisense cDNA transfected cells is similar to that of the nontransfected 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 nontransfected and h2 antisense cDNA transfected cells was significantly delayed (*$P < 0.001$). Cell proliferating rate of the h2 sense-transfected cells resumed ~24 h after h2-calponin ceased expression.

It is worth noting that the proliferation rates of nontransfected and h2 antisense cDNA-transfected SM3 cells began to accelerate 90 h after replating (cell number doubling time shortened from 32–33 h to 24 h). In h2 sense-transfected SM3 cells, the decrease in cell proliferation rate seen 96 h after transfection (90 h after replating and at least 72 h after the expression of h2-calponin became detectable) had prevented the acceleration of proliferation rate. 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 rate of cell proliferation. The inhibitory effect remained until 120 h after 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/well yielded comparable results in both stably and transiently transfected cultures (only 1,000 cells/well data are shown in Figs. 7 and 8). In all experiments, the proliferation rates of the h2 antisense cDNA-transfected cells and nontransfected cells were almost identical (Fig. 8), indicating that the transfection procedure and the integration of the vector DNA did not have a significant nonspecific effect.

Association of h2-calponin to actin-tropomyosin filaments in transfected SM3 cells. Immunofluorescence microscopy with anti-h2-calponin antibody demonstrates that the force-expressed h2-calponin localizes in the stress fiber structures (Fig. 9). By taking advantage of the fact that the rabbit anti-h2-calponin antiserum and the anti-tropomyosin MAb are recognized by different second antibodies with FITC or TRITC labels that can be distinguished by viewing through different filter sets, double-staining immunofluorescence microscopy clearly showed the colocalization of h2-calponin and tropomyosin in the stress fibers (Fig. 9*C*). Tropomyosin is a 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, because 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. This hypothesis is supported by the fact that 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. 6*C*).

Increased number of binuclear cells in SM3 cultures force-expressing h2-calponin. The number of binuclear cells was significantly increased in the h2 sense cDNA-transfected cells (25.08 ± 0.30%) vs. the h2 antisense

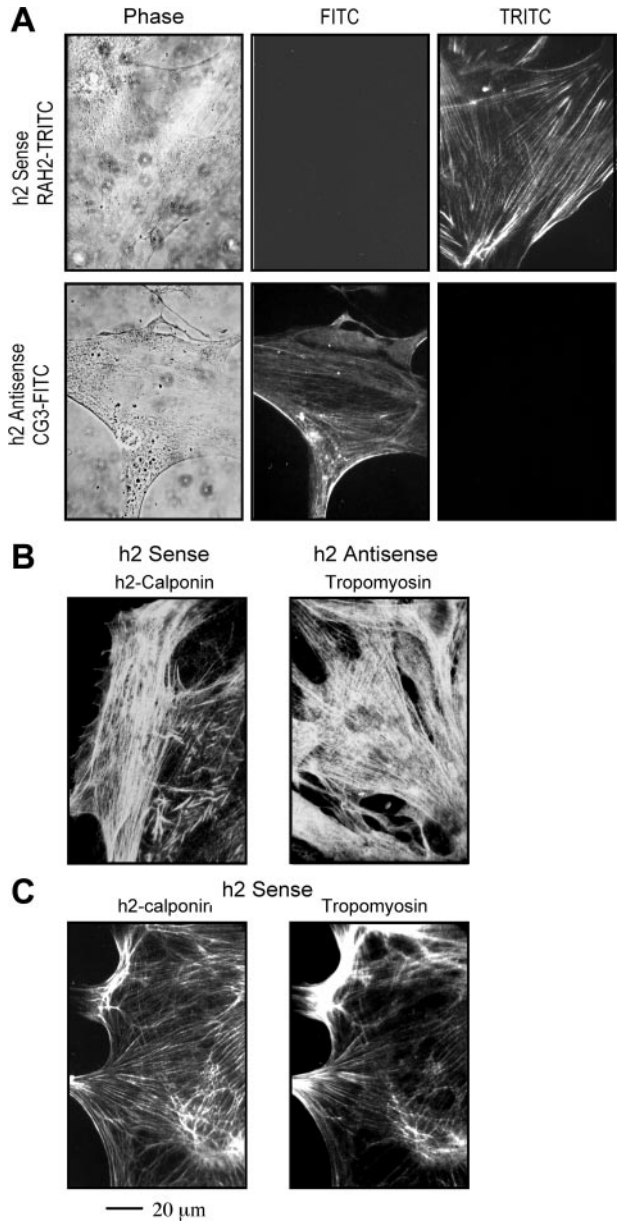

Fig. 9. Association of h2-calponin with the actin-tropomyosin filaments in transfected SM3 cells. SM3 cells stable-transfected with h2 sense or h2 antisense cDNA were cultured on gelatin-coated coverslips. Preconfluent monolayer cell samples were examined by immunofluorescence microscopy with the rabbit 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, were used to selectively detect the localization of h2-calponin and tropomyosin, respectively. Phase-contrast and fluorescence microscopic images were photographed. *A*: 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 colocalization of h2-calponin and tropomyosin.

cDNA-transfected (9.85 ± 0.44%) and nontransfected (9.83 ± 0.30%) SM3 cultures (Fig. 10; $P < 0.001$). The increase in the number of binuclear cells indicates that the forced expression of h2-calponin does not directly reduce the rate of DNA replication to decrease cell

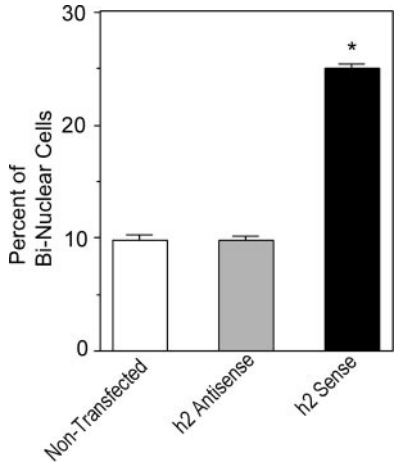

Fig. 10. Increase of binuclear cells in h2-calponin expressing cultures. Stable h2 sense and h2 antisense cDNA-transfected and nontransfected SM3 cells were cultured on gelatin-coated coverslips. Preconfluent monolayer cell samples were collected and fixed with acetone for microscopic examination. Two thousand cells were examined on each coverslip to calculate the rate of binuclear cells. Results (means ± SD) summarized from 3 coverslips in each group demonstrated a significantly increased number of binuclear cells in the h2 sense-transfected cultures (*$P < 0.001$).

proliferation rate but rather inhibits the function of the actin cytoskeleton during cytokinesis, which in turn results in slowed 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 binuclear 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 also play a role in nuclear division. In contrast to the broad stress fiber distribution of tropomyosin (Fig. 11*A*), the enriched association of h2-calponin with the nuclear ring structure (Fig. 11*B*) 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. Upregulated 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 observed increased expression of caldesmon in pregnant uterus smooth muscle, possibly playing a role in suppressing contractility for the maintenance of pregnancy (49).

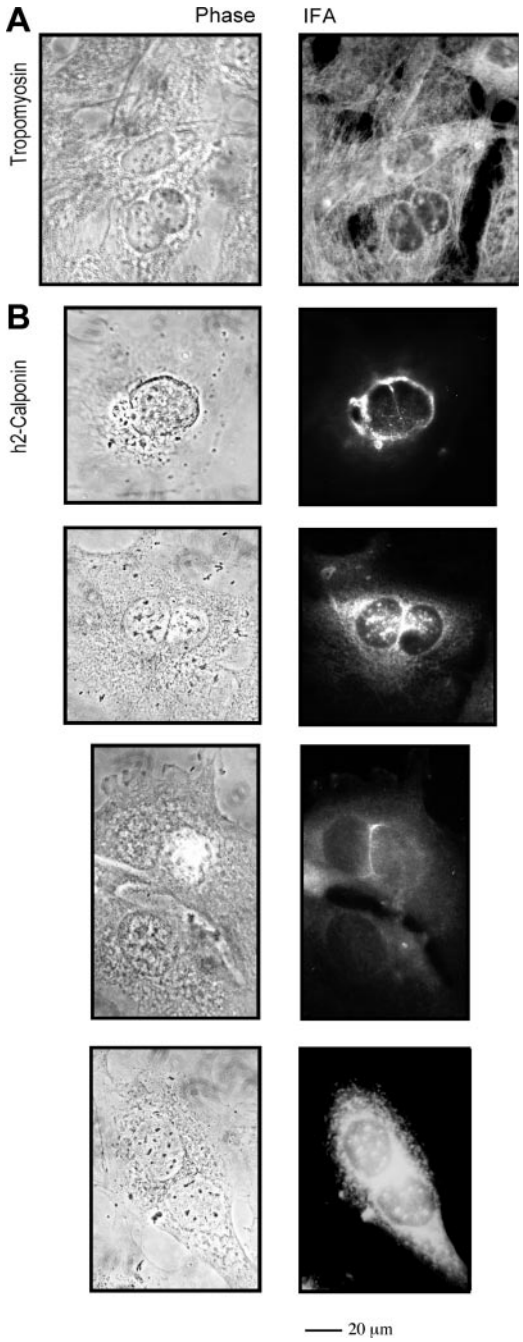

Fig. 11. Nuclear ring structure formed in the transfected SM3 cells by h2-calponin-containing stress fibers. Preconfluent monolayer cultures of h2-sense cDNA-transfected SM3 cells on the coverslips were fixed with acetone and examined by immunofluorescence microscopy with the anti-tropomyosin CG3 MAb or the anti-h2-calponin antibody RAH2 and TRITC-conjugated second antibody. Phase-contrast and immunofluorescence (IFA) 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*).

Thus the decreased expression of h1-calponin in the midterm pregnant uterus (Fig. 5) may also contribute to the suppression of uterus smooth muscle contractility. Western blots with 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 downregulated 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, whereas 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 nonmuscle cells.

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 nonmuscle 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 nonmuscle 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 noncontractile actin cytoskeleton (34, 35) and that 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. Calponin has been detected in the cytoplasm of human osteosarcoma cells, and the survival rate of patients whose tumors exhibit calponin is significantly higher than that of those whose tumors do not express calponin (50). Consistently, the h1-calponin knockout 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).

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 example of this fact (3, 4, 36, 39). The contraction of the contractile ring is most likely generated by the interaction between 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). A calponin homologue in *Xenopus* has been found to regulate cell motility during embryonic development by inhibiting actin-myosin interactions (31). 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 role 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 binuclear cells in h2-calponin-expressing cultures indicates an inhibition of cytokinesis after chromosome replication. h2-Calponin in the binuclear cells was concentrated around the nuclei, specifically in a "nuclear ring" structure that, like the contractile ring, is formed by actin filaments (Fig. 11). 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. Although 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. 11) that is regulated by h2-calponin. We have observed that calponin selectively binds low-molecular-weight nonmuscle 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. Because 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.

We thank Dr. Jim J.-C. Lin for providing the CG3 MAb and Jill O. Jin for proofreading the manuscript.

This work was supported by a grant from the March of Dimes Birth Defect Foundation (J.-P. Jin).

REFERENCES

1. **Applegate D, Feng W, Green RS, and Taubman MB.** Cloning and expression of a novel acidic calponin isoform from rat aortic vascular smooth muscle. *J Biol Chem* 269: 10683–10690, 1994.
2. **Cande WZ.** A permeabilized cell model for studying cytokinesis using mammalian tissue culture cells. *J Cell Biol* 87: 326–335, 1980.
3. **Cao LG and Wang YL.** Mechanism of the formation of contractile ring in dividing cultured animal cells. I. Recruitment of preexisting actin filaments into the cleavage furrow. *J Cell Biol* 110: 1089–1095, 1990.
4. **Cao LG and Wang YL.** Mechanism of the formation of contractile ring in dividing cultured animal cells. II. Cortical movement of microinjected actin filaments. *J Cell Biol* 111: 1905–1911, 1990.
5. **Danninger C and Gimona M.** Live dynamics of GFP-calponin: isoform-specific modulation of the actin cytoskeleton and autoregulation by C-terminal sequences. *J Cell Sci* 113: 3725–3736, 2000.
6. **De Lozanne A and Spudich JA.** Disruption of *Dictyostelium* myosin heavy chain gene by homologous recombination. *Science* 236: 1086–1091, 1987.
7. **Draeger A, Gimona M, Stuckert A, Celis JE, and Small JV.** Calponin: developmental isoforms and low molecular weight variant. *FEBS Lett* 291: 24–28, 1991.
8. **Field C, Li R, and Oegema K.** Cytokinesis in eukaryotes: a mechanistic comparison. *Curr Opin Cell Biol* 11: 68–80, 1999.
9. **Gao J, Hwang JM, and Jin JP.** Complete nucleotide sequence, structural organization and an alternatively spliced exon of mouse *h1*-calponin gene. *Biochem Biophys Res Commun* 218: 292–297, 1996.
10. **Gillies RG, Didier N, and Denton M.** Determination of cell number in monolayer cultures. *Anal Biochem* 159: 109–113, 1986.
11. **Gimona M, Sparrow MP, Strasser P, Herzog M, and Small JV.** Calponin and SM 22 isoforms in avian and mammalian smooth muscle. Absence of phosphorylation in vivo. *Eur J Biochem* 205: 1067–1075, 1992.
12. **Glotzer M.** The mechanism and control of cytokinesis. *Curr Opin Cell Biol* 9: 815–823, 1997.
13. **Glukhova MA, Frid MG, and Koteliansky VE.** Developmental changes in expression of contractile and cytoskeletal proteins in human aortic smooth muscle. *J Biol Chem* 265: 13042–13046, 1990.
14. **Gould KL and Simanis V.** The control of septum formation in fission yeast. *Genes Dev* 11: 2939–2951, 1997.
15. **Han EKH, Guadagno TM, Dalton SL, and Assoian RK.** A cell cycle and mutational analysis of anchorage-dependent growth: Cell adhesion and TGF-beta1 control G1/S transit specificity. *J Cell Biol* 122: 461–471, 1993.
16. **Horiuchi A, Nikaido T, Ito K, Zhai Y, Orii A, Taniguchi S, Toki T, and Fujii S.** Reduced expression of calponin h1 in leiomyosarcoma of the uterus. *Lab Invest* 78: 839–846, 1998.
17. **Horiuchi A, Nikaido T, Taniguchi S, and Fujii S.** Possible role of calponin h1 as a tumor suppressor in human uterine leiomyosarcoma. *J Natl Cancer Inst* 91: 790–796, 1999.
18. **Huang QQ, Chen A, and Jin JP.** Genomic sequence and structural organization of mouse slow skeletal muscle troponin T gene. *Gene* 229: 1–10, 1999.
19. **Jiang Z, Grange RW, Walsh MP, and Kamm KE.** Adenovirus-mediated transfer of the smooth muscle cell calponin gene inhibits proliferation of smooth muscle cells and fibroblasts. *FEBS Lett* 413: 441–445, 1997.
20. **Jin JP.** Cloned rat cardiac titin class I and class II motifs: expression, purification, characterization and interaction with F-actin. *J Biol Chem* 270: 6908–6916, 1995.
21. **Jin JP, Walsh MP, Resek ME, and McMartin GA.** Expression and epitopic conservation of calponin in different smooth muscles and during development. *Biochem Cell Biol* 74: 187–196, 1996.
22. **Knecht DA and Loomis WF.** Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. *Science* 236: 1081–1086, 1987.
23. **Kueng W, Silber E, and Eppenberger U.** Quantification of cells cultured on 96-well plates. *Anal Biochem* 182: 16–19, 1989.
24. **Leinweber B, Parissenti AM, Gallant C, Gangopadhyay S, Kirwan-Rhude A, Leavis PC, and Morgan KG.** Regulation of PKC by the cytoskeletal protein calponin. *J Biol Chem* 275: 40329–40336, 2000.
25. **Lin JJC, Chou CS, and Lin JLC.** Monoclonal antibodies against chicken tropomyosin isoforms: production, characterization, and application. *Hybridoma* 4: 223–242, 1985.
26. **Lin JJC, Warren KS, Wamboldt DD, Wang T, and Lin JLC.** Tropomyosin isoforms in nonmuscle cells. *Int Rev Cytol* 170: 1–38, 1997.
27. **Mabuchi I and Okuno M.** The effect of myosin antibody on the division of starfish blastomeres. *J Cell Biol* 74: 251–263, 1977.
28. **Mabuchi I, Tsukita S, Tsukita S, and Sawai T.** Cleavage furrow isolated from newt eggs: contraction, organization of the actin filaments and protein components of the furrow. *Proc Natl Acad Sci USA* 85: 5966–5970, 1988.
29. **Matthew JD, Khromov AS, McDuffie MJ, Somlyo AV, Somlyo AP, Taniguchi S, and Takahashi K.** Contractile properties and proteins of smooth muscles of a calponin knockout mouse. *J Physiol* 529: 811–824, 2000.
30. **Morgan KG and Gangopadhyay SS.** Cross-bridge regulation by thin filament-associated proteins. *J Appl Physiol* 91: 953–962, 2001.
31. **Morgan R, Hooiveld MH, Pannese M, Dati G, Broders F, Delarue M, Thiery JP, Boncinelli E, and Durston AJ.** Calponin modulates the exclusion of Otx-expressing cells from convergence extension movements. *Nat Cell Biol* 1: 404–408, 1999.
32. **Nigam R, Triggle CR, and Jin JP.** h1- and h2-calponins are not essential for norepinephrine- or sodium fluoride-induced contraction of rat aortic smooth muscle. *J Muscle Res Cell Motil* 19: 695–703, 1998.
33. **Nishida W, Kitami Y, and Hiwada K.** cDNA cloning and mRNA expression of calponin and SM22 in rat aorta smooth muscle cells. Gene 130: 297–302, 1993.
34. **North AJ, Gimona M, Cross RA, and Small JV.** Calponin is localized in both in the contractile apparatus and the cytoskeleton of smooth muscle cells. *J Cell Sci* 107: 437–444, 1994.
35. **Parker CA, Takahashi K, Tang JX, Tao T, and Morgan KG.** Cytoskeletal targeting of calponin in differentiated, contractile smooth muscle cells of the ferret. *J Physiol* 508: 187–198, 1998.
36. **Sanger JM and Sanger JW.** Banding and polarity of actin filaments in interphase and cleaving cells. *J Cell Biol* 86: 568–575, 1980.
37. **Sasaki Y, Uchida T, and Sasaki Y.** A variant derived from rabbit aortic smooth muscle: phenotype modulation and restoration of smooth muscle characteristics in cells in culture. *J Biochem (Tokyo)* 106: 1009–1018, 1989.
38. **Satterwhite LL and Pollard TD.** Cytokinesis. *Curr Opin Cell Biol* 4: 43–52, 1992.
39. **Schroeder TE.** Dynamics of the contractile ring. In: *Molecular and Cell Movement*, edited by Inoue S, and Stephens RE. New York: Raven, 1975, p. 305–334. (Soc Gen Physiologist Ser, Vol 30)
40. **Shirinsky VP, Biryukov KG, Hettach JM, and Sellers JR.** Inhibition of the relative movement of actin and myosin by caldesmon and calponin. *J Biol Chem* 267: 15886–15892, 1992.
41. **Small JV and Gimona M.** The cytoskeleton of the vertebrate smooth muscle cell. *Acta Physiol Scand* 164: 341–348, 1998.

42. **Strasser P, Gimona M, Moessler H, Herzog M, and Small JV.** Mammalian calponin identification and expression of genetic variants. *FEBS Lett* 330: 13–18, 1993.
43. **Takahashi K and Nadal-Ginard B.** Molecular cloning and sequence analysis of smooth muscle calponin. *J Biol Chem* 266: 13284–13288, 1991.
44. **Takahashi K, Yoshimoto R, Fuchibe K, Fujishige A, Mitsui-Saito M, Hori M, Ozaki H, Yamamura H, Awata N, Taniguchi S, Katsuki M, Tsuchiya T, and Karaki H.** Regulation of shortening velocity by calponin in intact contracting smooth muscles. *Biochem Biophys Res Commun* 279: 150–157, 2000.
45. **Trabelsi-Terzidis H, Fattoum A, Represa A, Dessi F, Ben-Ari Y, and Der Terrossian E.** Expression of an acidic isoform of calponin in rat brain: Western blots on one- or two-dimensional gels and immunolocalization in cultured cells. *Biochem J* 306: 211–215, 1995.
46. **Ueki N, Sobue K, Kanda K, Hada T, and Higashino K.** Expression of high and low molecular weight caldesmons during phenotypic modulation of smooth muscle cells. *Proc Natl Acad Sci USA* 84: 9049–9053, 1987.
47. **Wang J and Jin JP.** Conformational modulation of troponin T by configuration of the NH_2-terminal variable region and functional effects. *Biochemistry* 37: 14519–14528, 1998.
48. **Winder SJ, Allen BG, Clement-Chomienne O, and Walsh MP.** Regulation of smooth muscle actin-myosin interaction and force by calponin. *Acta Physiol Scand* 164: 415–426, 1998.
49. **Word RA, Stull JT, Casey ML, and Kamm KE.** Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. *J Clin Invest* 92: 29–37, 1993.
50. **Yamamura H, Yoshikawa H, Tatsuta M, Akedo H, and Takahashi K.** Expression of the smooth muscle calponin gene in human osteosarcoma and its possible association with prognosis. *Int J Cancer* 79: 245–250, 1998.
51. **Yoshikawa H, Taniguchi SI, Yamamura H, Mori S, Sugimoto M, Miyado K, Nakamura K, Nakao K, Katsuki M, Shibata N, and Takahashi K.** Mice lacking smooth muscle calponin display increased bone formation that is associated with enhancement of bone morphogenetic protein responses. *Genes Cells* 3: 685–695, 1998.