5-1997

Myosin Isoform Heterogeneity in Single Smooth Muscle Cells

Thomas J. Eddinger
Marquette University, thomas.eddinger@marquette.edu

Daniel P. Meer
Marquette University

Comparative Biochemistry and Physiology Part B : Biochemistry and Molecular Biology, Vol. 117, No. 1 (May 1997): 29-38. DOI.

Myosin Isoform Heterogeneity in Single Smooth Muscle Cells

Thomas J. Eddinger
Department of Biology, Marquette University, Milwaukee, WI
Daniel P. Meer
Department of Biology, Marquette University, Milwaukee, WI

Abstract

We review the current understanding of the myosin heavy chain (MHC) isoforms and show that the mRNA levels of smooth muscle (SM)1 and SM2 mimic the expressed levels of SM1 and SM2 protein. The reverse transcriptase-polymerase chain reaction technique has been shown to be sufficiently sensitive to examine SM-MHC expression at the single cell level. Most single smooth muscle cells isolated from adult rabbit carotid express both SM1 and SM2. However, expression of these SM-MHC isoforms at the cellular level is nonuniform and highly variable. This work provides a foundation for future investigations as to the possible unique functional characteristics of the SM-MHC isoforms, SM1 and SM2. This methodology may also prove useful when used with mechanical studies to determine the physiological significance of the alternatively spliced myosin isoforms, including the SM-MHC-head and LC_{17} isoforms.

Keywords

Smooth muscle, myosin, heavy chain isoforms, RT-PCR, quantitation, vascular, isozymes

Smooth muscle cells are the major cell type present in many body systems (vascular, digestive, respiratory, reproductive, urinary, etc.) and make up part of every organ and tissue in the body via the vascular system. The importance of the proper functioning of smooth muscle can be appreciated by noting that vascular diseases alone account for most morbidity and mortality in the United States. Unfortunately, our understanding of smooth muscle function is still rather limited despite the significant progress achieved in the past two decades. In this article, we review current literature and work from our laboratory indicating that all smooth muscle cells are not the same. This concept is important for further studies because experimental design and interpretation may need to be reevaluated to assimilate this hypothesis.

Arteries have a unique anatomical structure that is generally divided into three main sections: the tunica intima, tunica media and the tunica adventitia. The tunica intima is the inner lining of the vessel wall and consists of a layer of endothelial cells and a subendothelial layer composed of loose connective tissue and an internal elastic lamina in larger arteries. The tunica media consists of circumferentially arranged smooth muscle cells with small amounts of connective tissue and proteoglycans. The tunica adventitia, or outer layer, is made up of connective tissue and is separated from the media in larger vessels by an external elastic lamina. In healthy vessels, these layers remain discrete.

Smooth muscle (SM) myosin is a hexamer composed of two heavy chains (SM1 and SM2), two regulatory (phosphorylatable) light chains (LC_{20}) and two alkali light chains (LC_{17}) (Fig. 1). Burridge and Bray [7]observed two myosin heavy chains (MHCs) in chicken gizzard. Rovner *et al*. [63]first showed that two different isoforms of MHC were observed in various SM tissues, with the suggestion that they could be important for the unique contractile function present in these cells. Rovner *et al*. [64]and Kawamoto and Adelstein [39]showed that in addition to the two MHCs present in SM cells *in vivo*, there is a third MHC present in these cells in culture. This third form has been identified as a nonmuscle (NM) MHC by gel electrophoresis, Western blotting and peptide mapping 39, 64. These authors also reported that the expression of these three isoforms is regulated by the state of the cells in culture (i.e., confluent, quiescent cells have a high proportion of the SM isoforms, whereas nonconfluent, mitotic cells have a high proportion of the NM isoform). Seidel *et al*. [68]demonstrated that the NM MHC is not required for cytokinesis in these cultured cells and that the expression of SM1 and SM2 are co-regulated. These findings are inconsistent with our hypothesis, suggesting division of labor for these isoforms with cell function (i.e., cytokinesis, synthesis and secretion of various compounds and cell migration by the NM MHCs vs contractile function by the SM MHCs). Grainger *et al*. [29]suggested that the accumulation of NM MHC may be necessary for cytokinesis, which would be consistent with the above stated hypothesis.

Eddinger *et al*. [20]reported that there were, in fact, three MHC isoforms present in smooth muscle cells *in vivo*. Gel electrophoresis and Western blotting verified the third MHC as a nonmuscle isoform with similar electrophoretic and antigenic properties as the NM MHC isolated from platelets. In addition, identification and characterization of the LC_{20} protein from SM cells showed that there are also two isoforms of this protein expressed [26]. In mature swine carotid artery, the major form expressed is the SM LC_{20} and the minor isoform (approximately 16% of the total) is the NM LC_{20}. This was verified by mass spectrometric analysis of tryptic peptides of blots of these proteins. The measured peptide masses correspond to published cDNA sequences proposed to represent SM and NM LC_{20} 45, 74. The amount of the NM LC_{20} isoform (approximately 16%) is similar to the amount of NM MHC (approximately 14%) found in the swine carotid [26]. This agreement in relative content of the NM isoforms may result from limited isoform pairing of the native myosin molecule (i.e., only NM isoforms can combine to form a NM myosin molecule).

A second, and possibly third, NM MHC isoform has also been reported in the literature. Mohammad and Sparrow [52]and Sartore *et al*. [67]observed a third MHC on SDS gels of human pulmonary artery that appears to be a unique NM MHC. Katsuragawa *et al*. [38]and Kawamoto and Adelstein [40]reported two distinct NM MHC mRNAs from chicken that show differential expression. Kawamoto and Adelstein [40]were also able to show two bands on SDS gels that were unique by two-dimensional peptide mapping. Simmons *et al*. [69]reported that the two human NM MHCs are encoded by two genes located on different chromosomes. Saez *et al*. [65]isolated a mammalian (human) NM MHC cDNA clone where alternative polyadenylation is used to generate multiple transcripts from the same protein. Murakami *et al*. [54]produced antisera for NM IIa and NM IIb from peptide sequences from human macrophages and bovine brain. These antisera distinguished between three distinct cellular (NM) MHC isoforms in the tissues surveyed. We resolved two unique NM MHCs in mouse uterus that can be distinguished from the SM MHC by antibody reactivity and distinguished from each other by electrophoresis and peptide mapping [19]. Thus, there are numerous data to support at least two, and possibly three, NM MHCs in a variety of tissues, including smooth muscle.

Differences in LC_{17} have also been reported 9, 32, 33. In a report by Cavaille *et al*. [9], data are provided for the presence of two LC_{17} isoforms that change in ratio with pregnancy. Helper *et al*. [33]have shown that the presence of the two LC_{17} isoforms (LC_{17a} and LC_{17b}) is variable depending on the tissue, with the LC_{17a} form representing as little as 35% of the total in rat aorta to as much as 100% of the total in swine digestive SM. It is interesting to note that in the swine carotid the minor isoform (LC_{17b}) makes up approximately 16% of the total LC_{17} present. This is consistent with the 16% NM LC_{20} and 14% NM MHC reported to be present in this tissue [26]. Thus, it may be that the LC_{17b} is also a “nonmuscle” isoform.

An embryonic LC (LC_{23}) has also been identified in chicken [73], rat [77]and human [12]. This isoform is present transiently during embryonic development and does not appear to be present in postnatal animals.

These results have led to an ongoing controversy about the composition of the native myosin molecule in SM cells. Initial studies identifying the two SM MHC isoforms (SM1 and SM2) [64]reported a 1:1 ratio of these proteins in all tissues examined. Based on their data, the authors suggested that the myosin molecule formed an obligatory heterodimer relative to the heavy chains. This work was supported by the presence of a single myosin isoform on native (nondenaturing) gels where isoforms of skeletal (differing in MHC and/or LC composition) and cardiac (differing in MHC composition) myosin have been resolvable for many years 35, 36, 37. The lack of evidence for isoforms was further substantiated when Persechini *et al*. [60]showed that non-, mono- and diphosphorylated myosin (each differing by a single phosphate) were resolvable on this native gel system. Reports of isoforms of SM myosin on native gels, however, have been plagued with problems, stemming primarily from filamin, another large molecule that can co-migrate with myosin on these native gels [60](Eddinger, unpublished observations). Thus, the failure to observe isoforms can not be used as equivocal evidence that they do not exist.

Numerous studies have since reported ratios other than 1:1 for the SM MHCs 5, 18, 46, 51. In addition, in high resolution gels the NM MHC can be separated from the SM2 isoform. Thus, the ratios of SM1:SM2 reported in the various studies where NM MHC is present but not identified are only accurate as to the amount of NM MHC isoform that is present in those tissues. It is now clear that the ratio of SM1:SM2 is not always 1:1, even when the NM MHC is taken into account [18]. The difference in the ratio of SM1:SM2 brings into question the hypothesis that these two MHC isoforms form an obligatory heterodimer in native myosin.

We recently obtained evidence that the SM MHC from swine (aorta, stomach and uterus) and rabbit (stomach) forms three native smooth muscle isoforms with respect to their MHC composition [75]. In contrast to this, Kelley *et al*. [42]reported that bovine aortic smooth muscle myosin only forms homodimers. Kelley *et al*. used isoform-specific antibodies to immunoprecipitate myosin and for affinity chromatography. Use of the purified

homodimers in *in vitro* motility assays showed no significant differences in their ability to move actin filaments. In addition, phosphorylation of the carboxyl terminal of SM1 was reported to have no effect on filament formation, ability to move actin filaments, actin-activated Mg^{2+} ATPase activity or molecule confirmation [41]. The reason for the difference between their results and ours may be due to the different species used for the experiments.

Further work in this area has revealed that the three MHC isoforms are developmentally regulated 5, 17, 46. The developmental regulation of the MHC helps to explain some of the variability between the reports in the literature in which researchers have found seemingly inconsistent results. It does not, however, explain all the discrepancies, and it greatly increases the complexity of the system. We observed that in a wide variety of SM tissues from swine, rat and guinea pig, all three MHC isoforms are always present 20, 26. Others reported that this is not the case for several other tissues: rabbit aorta 5, 46and swine trachea [52]. In this last study, however, no evidence is provided that the antibody used to detect NM MHC reacts with swine NM MHC. This may be a problem because the monoclonal antibodies we generated proved to be species specific [16].

The developmental regulation of the expression of these MHC isoforms is not as simple as it might appear. Earlier work showed that the NM and SM MHC isoforms quantitatively change in cultured cells with cell cycle 5, 46, 52. We 16, 18and others 5, 46, 52reported a larger percentage of the NM MHC in young animals than in adults. These changes, however, do not fit a simple pattern. In most tissues from a variety of species, NM MHC content is very high in young animals and decreases as the animal develops. The decrease in NM MHC content, however, can be replaced by an increase in either SM1 (rat aorta) or SM2 (swine carotid) [18]. Numerous exceptions and variations of these changes occur. McConnell *et al*. [49]also reported a similar change for the SM MHC isoforms in rabbit bladder with development but did not quantify the NM MHC isoform. Mohammed and Sparrow [52]reported a smaller change in swine trachea for the SM1:SM2 ratio (decrease from 2.1 to 1.0). Their young swine were 30–60 kg or 12–20 weeks. The young animals used by Eddinger and Murphy [17]were approximately 2 kg, 3–5 days. This could explain why their SM1:SM2 ratio in young animals is much lower than what we observed. It is also possible that this difference is due to variability between tissue types. Mohammad and Sparrow [52]reported no NM MHC present in the swine trachea and neither Borrione *et al*. [5]nor Kuro-o *et al*. [46]reported NM MHC in adult rabbit aorta. In addition, Giuriato *et al*. [28]reported that rabbit aorta shows cell-specific developmental changes in the myosin isoforms between the luminal and adventitial sides of the vessel. Thus, although there is substantial evidence for a change in SM MHC isoform content with development, the actual pattern of change can vary greatly.

This variability in myosin subunits allows for the possibility of regulatory control and division of labor within and between SM cells. Quantitatively, the concentration of NM MHC and LC_{20} that is present in smooth muscle cells is 2- to 3-fold greater than that of platelets, Acanthamoeba or adrenal medulla [26]. This large quantity of protein would be expected to have some physiological significance. This may represent the greater capacity of the SM cell for synthesis, secretion, migration and proliferation [79].

Evidence suggests that these myosin isoforms may have discrete localization in SM cells. Small *et al*. [70]described two domains in smooth muscle cells (a contractile domain including myosin and caldesmon and a cytoskeletal domain including filamin and desmin) based on immunohistochemistry. Giuriato *et al*. [28]found unique distributions for the NM myosin isoforms in cultured aorta smooth muscle cells. One population is diffusely distributed throughout the cell, whereas another is localized to the cortical cytoplasm. In addition, DeNofrio *et al*. [14]reported that NM actin is present in membrane ruffles, pseudopods and stress fibers of pericytes, whereas SM actin is localized in stress fibers. Sartore *et al*. [66]stated that SM cells grown *in vitro* show a differential distribution of SM MHC isoforms among cells and a distinct localization of SM1 and SM2 within the same cell. Sparrow and Mitchell [71]found changes in myosin expression, Ca^{2+}-force relationship and pharmacological responsiveness of intact swine airway smooth muscle with development. In addition, cells that

lack most of a specific myosin isoform have been shown to be incapable of cell division 13, 44. Thus, it is not totally unfounded to suggest a physiological significance for these various forms.

In addition to the MHC differences in the tail region, differences have also been identified in the S1 myosin head region. These result from alternate splicing in the 5′ region 1, 30, 43, 78. This alternate splicing at the 25- to 50-kDa junction in the S1 head region generates a seven amino acid insert in SM MHC from visceral tissue that are not present in tonic vascular tissue 1, 30, 43, 78. The presence of the insert in the head region (intestinal myosin) correlates with a higher velocity of movement of actin filaments *in vitro* and higher actin activated Mg^{2+}-ATPase activity than in vascular myosin (aorta) [43]. There are still species differences and other myosin subunit isoforms that confound these results. Increasing the LC_{17a} content in the vascular myosin (no head insert) did not increase its ability to move actin filaments to the rate of the intestinal myosin (having head insert) [43], whereas Hasegawa and Morita [31]reported that a similar exchange in swine aortic myosin increases ATPase activity. The presence or absence of the head insert is associated with the SM1 MHC in rat [78](SM2 was not examined) is only associated with SM2 in the rabbit [1]and is associated with both SM1 and SM2 in turkey [43].

Fig. 2 shows a schematic representation of the possible combinations for the SM MHC. Fig. 2A shows the single SM MHC gene with the optional exons whose alternate splicing is responsible for the four possible mRNAs (Fig. 2B). These four RNAs can be translated to generate four different SM MHC polypeptides (Fig. 2C). Fig. 2D shows the possible SM MHC pairings that can result in four MHC homodimer and six MHC heterodimer molecules. As discussed above, the tail isoforms (3′ end) include homo- and heterodimer molecules. Evidence for or against the head isoform (5′ end) MHC pairings is not available.

Sparrow *et al*. [72]and Hewitt *et al*. [34]reported small positive correlations between SM1 MHC content and V_{max} in rat myometrial tissue. In contrast, others have shown that V_{max} correlates with LC_{17a} content and not the SM1:SM2 MHC ratio in rat uterus [53]. Thus, the possible functional significance of these isoforms continues to be unresolved. Studies using tissues composed of cells showing a mixture of myosin isoforms confound mechanical studies. Using single smooth muscle cells for biochemical and mechanical studies will allow investigators to address these questions without the confounding mixtures of heterogeneous cells.

As mentioned, there are reports of unique function for the presence or absence of the MHC head differences [43], ratio of SM MHC 34, 72and the ratio of LC_{17a} [53]. Other implications for functional diversity within a vessel have also been suggested in a host of studies. Garland and Keatinge 24, 25reported different responses of the inner and outer muscle layers of vessels to various pharmacological agents. Although a significant portion of this variability can be attributed to the variation in innervation and the regional presence of other cell types in the vessel, these results are also consistent with unique SM cell function. Little *et al*. [48]reported unique homo- and heterocellular connections within the cell wall (endothelial–endothelial, SM–SM and endothelial–SM cell junctions). They observed junctions between the endothelial–SM cells showing preferential communication in one direction (endothelial → SM). This may be of significance for *in vivo* function but may also hinder clarifying isoform function in whole tissue experiments.

It has generally been assumed that the smooth muscle cell (SMC) population of the medial layer of the vessel wall is a homogeneous population of cells 8, 10. This does not presume that all SMCs are uniform at any given time, however. SMCs are believed to undergo phenotypic modulation when under specific environmental cues. This shift can vary from a highly differentiated state where the cells' primary function appears to be contraction to a nondifferentiated state where the cells are not contractile but rather active in synthesis, secretion and cell division. This range of phenotypic modulation has been observed in tissue culture where a variety of conditions were shown to shift SMCs back and forth between these extremes.

Numerous reports in the literature indicate distinct SMC populations in the vessel wall [e.g., 23, 80]. These are often based on the presence or absence of the various contractile protein isoforms or morphological changes observed during development, disease or when the cells are cultured. Indeed, these changes may be the result of different populations of SMCs present in the tissue. These changes may also be a single population of SMCs that are moving back and forth along a continuum between a "dedifferentiated" synthetic/mitotic state and a "differentiated" contractile state. The difference here is an important point. In a "normal" vessel, there will be cell turnover with the resultant breakdown of senescent cells and mitosis of other cells for their replacement. Thus, one would expect to find distinct "populations" of SMCs in all vessels. The question is if these are distinct populations of cells or a single population of cells showing different phenotypes along a developmental continuum.

Primary culture SMCs plated at low density rapidly change from an "SMC" into something morphologically and biochemically different than an "SMC." However, if they are left in culture until they reach confluence, they change again (both morphologically and biochemically) to something again resembling "SMCs." Repeated subculture often changes these SMCs into multiple populations of cells that can be distinguished by a number of attributes [58]. It is unclear, however, that the populations of SMCs after multiple platings are not from multiple cell types from the primary culture. In addition, many researchers agree that cells kept in culture for numerous platings are no longer the same as the cells that were put into culture. Thus, these differences may be due to a single population of cells that are in their various roles or multiple populations of cells. Unfortunately, these types of experiments may not resolve this question.

As has been reported by others 23, 28, 80, there is a nonhomogeneous distribution of smooth muscle cells in the vascular wall. Thus, any attempt to assign functional roles to the various MHC must be done at the single cell level. This may explain in part why experiments designed to address this question at the tissue level have resulted in ambiguous results. Measurements made on preparations composed of nonhomogeneous populations of cells can lead to an averaging effect that would mask true correlations. Single cell experiments where protein isoform content and mechanical properties can be measured in the same cell are desirable to test for functional correlations with protein isoforms.

We have tested several methods for determining the relative content of SM1 and SM2 in single cells. Standard protocols for separating and quantitating protein isoforms from tissues were attempted using enhanced sensitivity for detecting the proteins. Using silver staining of gels, we were able to visualize actin and myosin from swine stomach homogenates loaded at 0.1 and 10 ng wet weight tissue, respectively. Using an estimate of 30–50 mg actin and 15 mg myosin/g wet weight tissue [55], it is calculated that this method can detect actin at 3–5 pg and myosin at 150 pg. Unfortunately, this is not enough to be useful at the single cell level.

Using polyclonal antibodies with the enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL), we were able to significantly increase the sensitivity of the assay. This method allows detection of myosin down to 0.1 ng wet weight tissue loaded or 1.5 pg MHC. When single SMCs suspended at a density of 1 cell/μl are loaded onto gels, this method can detect MHC at loadings down to 1 μl, or the equivalent MHC protein from a single cell. Thus, this method may be useful for relative quantitation of protein isoforms from single SMCs. Using colloidal gold (Aurodye, Forte, Amersham) with their silver enhancer was not found to be as sensitive as the ECL.

Although using gel electrophoresis is a viable method for resolving MHC protein isoforms and ECL appears to allow visualization of these isoforms from a single cell, we were interested in a technique that would be applicable over a larger range of proteins with a greater sensitivity. For example, MHC head difference isoforms are not resolvable by standard gel methods. We therefore turned to molecular techniques, which would theoretically allow relative quantitation of the various protein isoforms from single SMCs.

Reverse transcription-polymerase chain reaction (RT-PCR) has been reported to provide the requisite specificity and sensitivity to be useful in analysis of protein isoforms from single cells 22, 47, 62. The known smooth muscle-specific MHC isoforms are all generated from a single gene through alternative splicing mechanisms. The first two smooth muscle-specific MHC isoforms discovered were SM1 and SM2 [63]. The difference between these two isoforms occurs in the 3′ nonhelical domain near their carboxyl termini. Identification of the tail difference region between SM1 and SM2 was obtained by sequencing two cDNAs derived from a rabbit uterine cDNA library that codes for the 3′ ends of SM1 and SM2 56, 57. Using genomic sequencing and RNase protection assays, it was shown that this difference resulted from an alternate splicing mechanism [2]. The difference results from alternative inclusion/exclusion of a 39 nucleotide exon with an inframe stop codon [2]. Inclusion of this exon produces the transcript for the SMC protein that has 9 unique amino acids and is 34 amino acids shorter than SM1 (Fig. 3). Oligonucleotide primers (20 mers) were designed from the known sequence of rabbit smooth muscle myosin [3]to flank the unique exon present in one of the smooth muscle myosin isoforms (SM2). Because the sequence on the mRNA is identical in the region upstream and downstream from this alternate splice site, the same set of primers binds to the 3′ and 5′ end of the alternate splice site in both the SM1 and SM2 mRNA (Fig. 3). These primers are used in a PCR to amplify specific regions of cDNA sequence. Because the primers flank an alternative splice site [2], the DNA fragments generated by PCR (PCR products) should be of two sizes, equal to the distance between the primer sites in the two isoforms (SM1 and SM2). The predicted sizes of the PCR products for the SM1 and SM2 myosin isoforms are 291 and 330 bp, respectively.

Conversion of mRNA to cDNA in RT can be accomplished using either Oligo (dT) primers or random hexamers. The difference between SM1 and SM2 is at the 3′ end, close to the poly A tail. As might be expected, we have found that the oligo (dT) primers give a better yield of cDNA for the sequences of interest. PCR amplifies the sequences of interest logarithmically until the number of product sequences reaches a point when there are not enough primers to pair with all the product. During this plateau, phase amplification continues at something less than a logarithmic rate but does not alter their relative ratio. The number of products produced, their size and their quantity can be determined by running the sample on a gel and visualizing the bands with ethidium bromide followed by densitometric analysis.

To obtain single cells, most isolations most often use a mixture of collagenase and elastase for tissue disruption and cell isolation. We tried these methods and found the cells isolated in this fashion are shorter on average than those obtained using a papain digestion as described by Driska and Porter [15]. The difference is most likely due to variant Ca^{2+} levels in the single cells during these two procedures. Cells from both isolations contract to stimulation with either high K^{+}, histamine or phenylephrine. Using the longer cells provides extra length for attachment to pipettes for mechanical measurements. This is not critical, however, as other laboratories making mechanical measurements on single smooth muscle cells use a collagenase-elastase digestion [6].

To verify that the mRNA levels for SM1 and SM2 can be measured in an accurate and reproducible fashion and that the levels correspond with the protein levels in the cell, numerous controls are required. There are currently no methods that allow quantitation of the SM1 and SM2 protein levels from single cells. Although PCR, in theory, will allow quantitation of these message levels, it needs to be shown that there is some correlation between the mRNA level and the proteins expressed if PCR is to be used to infer relative protein ratios. This was done by using paired tissue samples from five different tissues (i.e., aorta, bladder, carotid, stomach and uterus), all of which express different ratios of SM1:SM2 MHC proteins. By isolating smooth muscle from each of these tissues, we were able to obtain SM2:SM1 protein ratios that varied from approximately 0.1–1.1. These tissues were isolated and cleaned of blood, adipose, loose connective tissue and nonmuscle layers (endothelium, mucosa, endometrium or transitional epithelium). Isolated smooth muscle tissue from each preparation was then homogenized in sample buffer and run on SDS-PAGE to isolate and quantitate the two SM MHC isoforms. Adjacent smooth muscle tissue from each preparation was homogenized in buffer for total RNA extraction. RT-

PCR was then used to generate cDNA and amplify fragments specific for the SM1 and SM2 mRNA. These products could also be run on SDS-PAGE to isolate and quantitate the products amplified.

Because we are using a single set of primers that bind to identical sequences on both SM1 and SM2 cDNA, the binding efficiencies are the same. The SM1/SM2 PCR primers flank intervening sequences in genomic DNA [2]so that only cDNA produces correctly sized PCR products. The amplicons are identical with the exception of the 39 additional nucleotides present in the SM2 cDNA. As has been reported by others [27], there is no difference in amplification of amplicons that use identical primers but differ by a small unique nucleotide sequence. The size difference between the two amplicons allows their separation on SDS-PAGE. The amount of product produced for each sequence is a function of the initial cDNA concentration, which is a function of the levels of mRNA present in the tissue. Thus, quantitation of the two bands (SM1 and SM2 cDNA) from the gel gives the relative ratio of SM1 and SM2 mRNA present in the starting tissue.

Only two bands are observed on the SDS-PAGE gels when the RT-PCR reaction products are run (Fig. 4). The bands were identified by their predicted sizes (291 and 330 bp) by selective enzymatic digestion and by sequencing. Approximately 200 bp of each band has been sequenced, including the difference region, showing a perfect match with their published sequences [3]. Because no other bands are observed, the data are not confused by primer dimerization.

Quantitation of the relative levels of SM2:SM1 mRNA and SM2:SM1 protein from 48 pairs of adjacent tissue regions of five smooth muscle tissues from 17 rabbits gave a correlation coefficient *R* value of 0.92. This large sample size and high *R* values suggest that the expressed protein levels of SM1 and SM2 MHC are regulated primarily by alternative splicing as has been suggested 2, 3. It also suggests that the SM2:SM1 mRNA ratio is a good measure of the SM2:SM1 protein ratio. This is important as there is no method currently available to detect MHC protein levels at the single SMC level, whereas RT-PCR, in theory, is capable of determining the SM2:SM1 mRNA level in single SMCs.

Although there have been reports in the literature of using RT-PCR on single cells 47, 61, we are unaware of anyone having done so with single smooth muscle cells. As mentioned above, SM-MHC provide an ideal system because they allow a single set of primers to be used for their amplification. Theoretically, relative quantitation is possible because all of the criteria required for quantitation of PCR products are met in this system 11, 21, 22, 27, 59, 76. We have done numerous controls to verify that in actuality this is the case for amplification of SM1 and SM2 mRNA from single cells using a single set of primers that span the 3' alternate splice site.

The specificity of the reaction is indicated by the presence of only two amplified products on the gels for all smooth muscle tissue used (vascular, digestive, reproductive, urinary). This is true even when using 60 cycles to amplify low numbers of copies (Fig. 4). Negative controls, which show no product after PCR, also verify the specificity. Leaving RNA out of the RT reaction or leaving cDNA out of the PCR reaction both resulted in no products being amplified.

The accuracy of the reaction was confirmed by control experiments using known quantities of purified SM1 and SM2 cDNA in a variety of ratios for the PCR reaction. SM2 and SM1 DNA were mixed to give ratios of 0, 25, 50, 75 and 100% SM2. PCR was performed on these mixtures and quantitation done on the resulting products. PCR using 35 cycles of amplification on approximately 10^{-9} g starting DNA or 60 cycles of amplification starting with approximately 10^{-18} g DNA both generated products with SM2:SM1 ratios not significantly different than the original ratio. Loading and quantitation of a 16-fold concentration range of PCR product from a gel gave a linear regression for the band intensities of $R > 0.98$. This indicates that the measurements are being made in a quantifiable loading range.

The reproducibility and sensitivity of the RT-PCR method were shown by several more control experiments. Duplicate RT reactions performed using RNA from the same total RNA extraction samples yielded PCR-amplified SM2:SM1 RNA ratios that were not significantly different (n = 6, paired t-test, $P < 0.05$). The control for accuracy described above indicated that the method can accurately amplify a sequence when starting with as little as an estimated 600 copies of cDNA (10^{-18} g) or the number of copies expected to be present in a single cell. Isolated single SMCs can also be used for the RT-PCR. The cells are picked up using a glass micropipette and transferred into a microcentrifuge tube. The RT reaction is performed on the whole cell lysate to prevent possible loss or selective extraction of RNA during standard RNA extraction techniques. Reproducibility and sensitivity of the method can be most clearly observed from a split single cell control. RT reactions were done on isolated single SMCs, which were then split in half so that duplicate PCR reactions could be performed. Two bands (SM2, 330 bp; SM1, 291 bp) were observed for each "split cell" after PCR. The SM2:SM1 ratio for each split cell reaction was not significantly different (n = 11, paired Student's t-test, $P < 0.05$). Thus, RT-PCR works reproducibly on the equivalent of one-half of the SM MHC mRNA from a single smooth muscle cell. [50].

Rabbit carotid tissue samples show a SM2:SM1 MHC protein ratio of approximately 0.5. The SM2:SM1 MHC mRNA ratio for this tissue is also approximately 0.5. Single smooth muscle cells isolated from this tissue also show an average SM2:SM1 mRNA ratio of approximately 0.5. However, the individual cells show a range of SM2:SM1 mRNA ratios from 0.0 to 1.8. This is in contrast to the variability observed at the tissue level for these same tissues that ranged from 0.43 to 0.56. The individual cells show a range of variability that is not observed at the tissue levels, even during perinatal development or in disease states.

The range of SM2:SM1 ratios exhibited by smooth muscle cells isolated from the same carotid is very broad relative to the age-matched interanimal variability of vascular tissue SM2:SM1 ratios. Also, the single cell SM2:SM1 ratio range always encompasses the SM2:SM1 ratio of the tissue from which the cells were isolated. Therefore, by studying the mechanical properties of multicellular smooth muscle preparations, an averaging of the contributions of smooth muscle cells with different SM2/SM1 complements occurs, possibly obscuring unique functional characteristics of these isoforms. These functional differences may also be obscured during *in vitro* motility assays that examine single myosin molecules outside their native filaments. Biochemical studies using purified proteins would not show differences either, unless the amounts of these isoforms were controlled for and their spatial arrangements that exist *in vivo* were duplicated. Ribonuclease protection assays have been used to measure expression of smooth muscle-specific MHC of the mRNA level 2, 4. However, RNase protection assays are not sufficiently sensitive to detect mRNA from a single smooth muscle cell. Thus, to determine whether the smooth muscle-specific MHC isoforms SM1 and SM2 (or any of the other myosin polypeptide isoforms: MHC-head, LC_{20}, LC_{17}) have unique physiological roles, the expression levels of SM2 and SM1 in single cells with known mechanical properties must be determined.

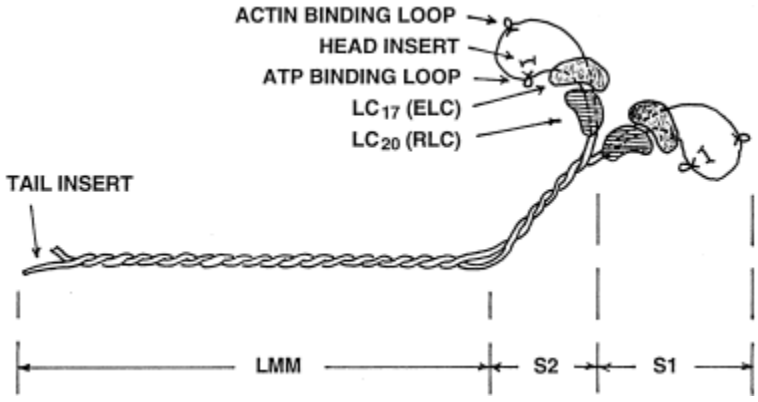

Fig. 1. Schematic representation of the SM myosin molecule. The protein is a hexamer consisting of two MHCs and four LCs. Limited proteolytic digestion with specific enzymes results in MHC fragments as indicated by the

dashed lines (LMM, light meromyosin; S2, subfragment 2; S1, subfragment 1). The unique differences between the two MHCs occur at the carboxyl terminal non-helical tail (tail insert) and at the S1 head region (head insert). One LC_{20} and one LC_{17} associate with each MHC head. There are at least two isoforms of each of these LCs.

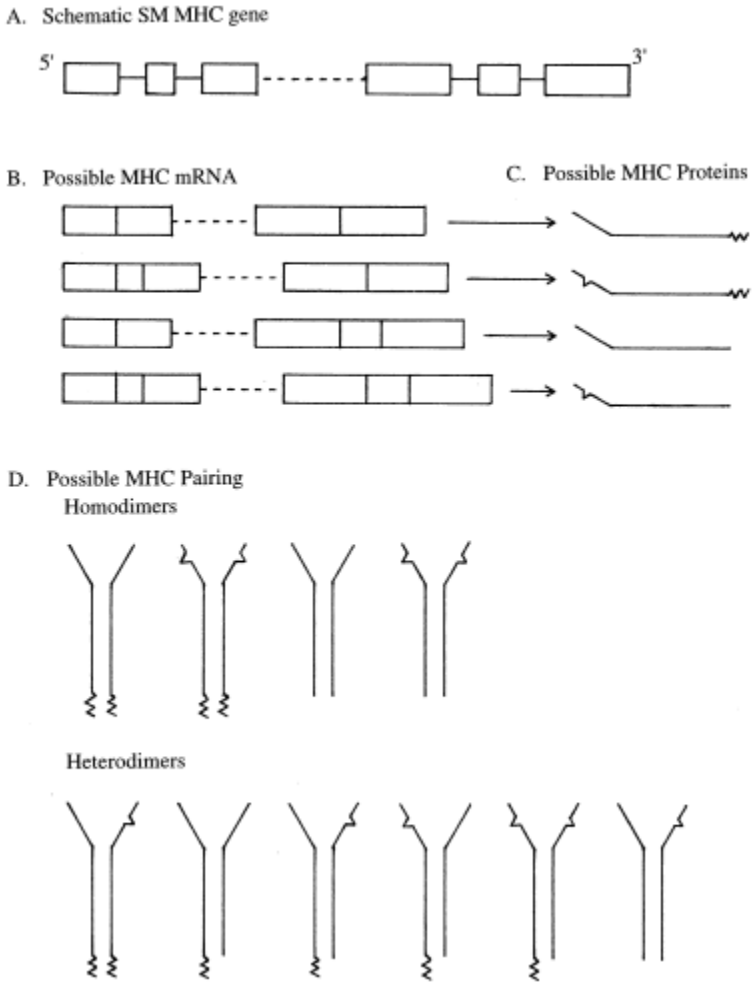

Fig. 2. Schematic flow diagram for possible SM MHC molecule pairing. (A) Single SM MHC gene with 5’ and 3’ exon and introns indicated as boxes and lines. Dashed line indicates sequence not shown. (B) Four possible SM MHC mRNA can result from exclusion or inclusion of the alternatively spliced exons responsible for the head insert (5’ end) and the tail insert (3’ end). (C) Each mRNA may be translated to form a unique SM MHC polypeptide. (D) Possible pairing of the four SM MHCs to form unique myosin molecule isoforms (light chains are not included to minimize confusion). All four SM MHCs may not exist in all SM tissues and, thus, these combinations may not be possible in all tissues.

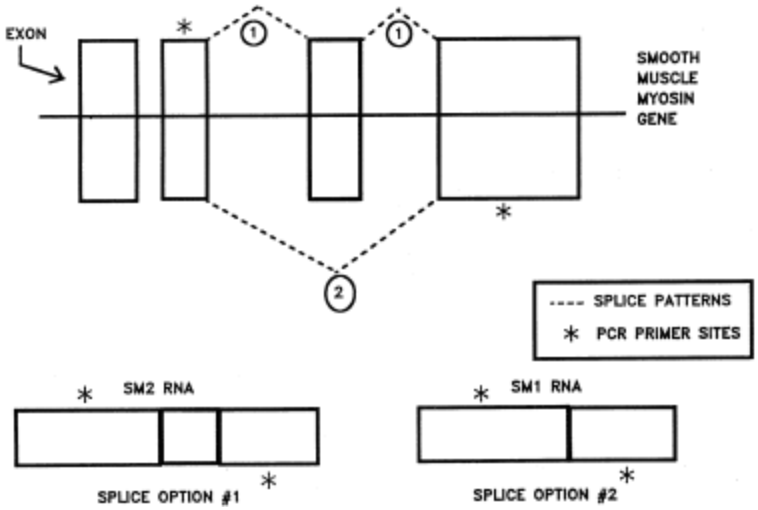

Fig. 3. Schematic drawing of 3' end of SM MHC gene showing alternative splice options. Splice option one includes the alternative exon and the resulting mRNA is shown in lower left (SM2). Splice option two excludes the alternate exon and the resulting mRNA is shown in the lower right (SM1). Although the SM2 mRNA message is longer than the SM1 mRNA message, the SM2 protein is shorter than the SM1 protein due to a stop codon present in the alternative exon. Primer locations are denoted by asterisks. Primer brining sites are in common between both the SM2 and SM1 mRNA and thus generate two different sized products, depending on the presence or absence of the alternative exon.

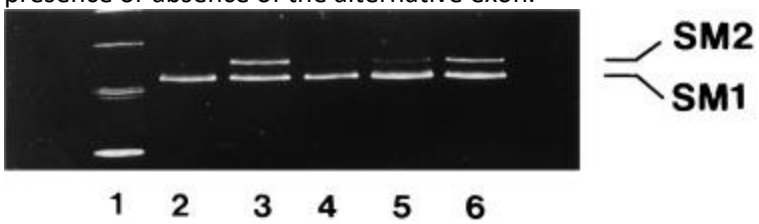

Fig. 4. Eight percent SDS polyacrylamide gel showing RT-PCR products from rabbit carotid tissue (lane 6) and isolated single cells from the same vessel (lanes 2–5). Lane 1 shows 123 bp marker (bands 123–452 shown). For isolated cells and tissues, the upper band (when present) is SM2 mRNA (330 bp) and the lower band is SM1 (291 bp). The tissue sample (lane 6) shows an SM2:SM1 ratio of 0.43 and the four isolated single cells obtained from an adjacent piece of tissue show SM1:SM1 ratio from 0.02 (lane 2) to 0.66 (lane 3).

References

1 P Babij. **Tissue-specific and developmentally regulated alternative splicing of a visceral isoform of smooth muscle myosin heavy chain.** Nucleic Acids Res., 21 (1993), pp. 1467-1471

2 P Babij, M Periasamy. **Myosin heavy chain isoform diversity in smooth muscle is produced by differential RNA processing.** J. Mol. Biol., 210 (1989), pp. 673-679

3 P Babij, C Kelly, M Periasamy. **Characterization of a mammalian smooth muscle myosin heavy-chain gene: Complete nucleotide and protein coding sequence and analysis of the 5' end of the gene.** Proc. Natl. Acad. Sci. USA, 88 (1991), pp. 10676-10680

4 P Babij, S Kawamoto, S White, R Adelstein, M Periasamy. **Differential expression of Sm1 and SM2 myosin isoforms in cultured vascular smooth muscle.** Am. J. Physiol., 262 (1992), pp. C607-C613

5 A.C Borrione, A.M.C Zanellato, G Scannapieco, P Pauletto, S Sartore. **Myosin heavy-chain isoforms in adult and developing rabbit vascular smooth muscle.** Eur. J. Biochem., 183 (1989), pp. 413-417

6 F.V Brozovich, M.P Walsh, K.G Morgan. **Regulation of force in skinned, single cells of ferret aortic smooth muscle.** Pflügers Arch., 416 (1990), pp. 742-749

7 K Burridge, D Bray. **Purification and structural analysis of myosin from brain and other non-muscle tissues.** J. Mol. Biol., 99 (1975), pp. 1-14

8 G.R Campbell, J.H Campbell, J.A Manderson, S Horgan, R.E Rennick. **Arterial smooth muscle: A multifunctional mesenchymal cell.** Arch. Pathol. Lab. Med., 112 (1988), pp. 977-986

9 F Cavaille, C Janmot, S Ropert, A d'Albis. **Isoforms of myosin and actin in human, monkey and rat myometrium.** Eur. J. Biochem., 160 (1986), pp. 507-513

10 J.H Chamley-Campbell, G.R Campbell. **What controls smooth muscle phenotype?** Athersclerosis, 40 (1981), pp. 347-357

11 J Chelly, D Montarras, C Pinset, Y Berwald-Netter, J.-C Kaplan, A Kahn. **Quantitative estimation of minor mRNAs by cDNA-polymerase chain reaction. Application to dystrophin mRNA in cultured myogenic and brain cells.** Eur. J. Biochem., 187 (1990), pp. 691-698

12 P Cummins, K.M Price, W.A Littler. **Foetal myosin light chain in human ventricle.** J. Muscle Res. Cell Mot., 1 (1980), pp. 357-366

13 A DeLozanne, J.A Spudich. **Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination.** Science, 236 (1987), pp. 1086-1091

14 D DeNofriö, R.C Hoock, I.M Herman. **Functional sorting of actin isoforms in microvascular pericytes.** J. Cell Biol., 109 (1989), pp. 191-202

15 S.P Driska, R Porter. **Isolation of smooth muscle cells from swine carotid artery by digestion with papain.** Am. J. Physiol., 251 (1986), pp. C474-C481

16 T.J Eddinger, R.A Murphy. **Two smooth muscle myosin heavy chains differ in their light meromyosin fragment.** Biochemistry, 27 (1988), pp. 3807-3811

17 T.J Eddinger, R.A Murphy. **Changes in smooth and non-muscle myosin heavy chain content with development in smooth muscle.** Biophys. J., 53 (1988), p. 579a

18 T.J Eddinger, R.A Murphy. **Developmental changes in actin and myosin heavy chain isoform expression in smooth muscle.** Arch. Biochem. Biophys., 284 (1991), pp. 232-237

19 T.J Eddinger, J.A Wolf. **Expression of four myosin heavy chain isoforms with development in mouse uterus.** Cell Motil. Cytoskel., 25 (1993), pp. 358-368

20 T.J Eddinger, B.D Gaylinn, P.L Monical, R.A Murphy. **Evidence for non-muscle myosin in smooth muscle cells.** Biophys. J., 53 (1988), p. 579a

21 F Ferre. **Quantitative or semi-quantitative PCR: Reality versus myth.** PCR Methods Appl., 2 (1992), pp. 1-9

22 F Ferre, A Marchese, P Pezzoli, S Griffin, E Buxton, V Boyer. **Quantitative PCR: An overview.** K.B Mullis, F Ferre, R.A Gibbs (Eds.), The polymerase chain reaction, Birkhauser, Boston, MA (1994), pp. 67-88

23 M.G Frid, E.P Moiseeva, K.R Stenmark. **Multiple phenotypically distinct smooth muscle cell populations exist in the adult and developing bovine pulmonary arterial media *in vivo.*** Circ. Res., 75 (1994), pp. 669-681

24 C.J Garland, W.R Keatings. **Constrictor actions of acetylcholine, 5-hydroxytryptamine and histamine on bovine coronary artery inner and outer muscle.** J. Physiol., 327 (1982), pp. 363-376

25 C.J Garland, W.R Keatings. **Action of vasodilator agents on inner and outer muscle of bovine coronary arteries.** Eur. J. Pharmacol., 87 (1983), pp. 327-330

26 B.D Gaylinn, T.J Eddinger, P.A Martino, P.L Monical, D.F Hunt, R.A Murphy. **Expression of nonmuscle myosin heavy and light chains in smooth muscle.** Am. J. Physiol., 257 (1989), pp. C997-C1004

27 G Gilliland, S Perrin, K Blanchard, H.F Bunn. **Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction.** Proc. Natl. Acad. Sci. USA, 87 (1990), pp. 2725-2729

28 L Giuriato, M Scatena, A Chiavegato, M Tonello, G Scannapieco, P Pauletto, S Sartore. **Non-muscle myosin isoforms and cell heterogeneity in developing rabbit vascular smooth muscle.** J. Cell Sci., 101 (1992), pp. 233-246

29 D.J Grainger, T.R Hesketh, J.C Metcalfe, P.L Weissberg. **A large accumulation of non-muscle mysoin occurs at first entry into M phase in rat vascular smooth-muscle cells.** Biochem. J., 277 (1991), pp. 145-151

30 Y Hamada, M Yanagisawa, Y Katsuragawa, J.R Coleman, S Nagata, G Matsuda, T Masaki. **Distinct vascular and intestinal smooth muscle myosin heavy chain mRNAs are encoded by a single-copy gene in the chicken.** Biochem. Biophys. Res. Commun., 170 (1990), pp. 53-58

31 Y Hasegawa, F Morita. **Role of 17-dKa essential light chain isoforms of aorta smooth muscle myosin.** J. Biochem., 111 (1992), pp. 804-809

32 Y Hasegawa, H Veno, K Horie, F Morita. **Two isoforms of 17-kDa essential light chain of aorta media smooth muscle myosin.** J. Biochem., 103 (1988), pp. 15-18

33 D.J Helper, J.A Lash, D.R Hathaway. **Distribution of isoelectric variants of the 17,000-dalton myosin light chain in mammalian smooth muscle.** J. Biol. Chem., 263 (1988), pp. 15748-15753

34 T.E Hewitt, A.F Martin, R.J Paul. **Correlations between myosin heavy chain isoforms and mechanical parameters in rat myometrium.** J. Physiol., 460 (1993), pp. 351-364

35 J.F.Y Hoh. **Neural regulation of mammalian fast and slow muscle myosins: An electrophoretic analysis.** Biochemistry, 14 (1975), pp. 742-747

36 J.F.Y Hoh, G.P.S Yeoh. **Rabbit skeletal myosin isoenzymes from fetal, fast-twitch and slow-twitch muscles.** Nature, 280 (1979), pp. 321-323

37 J.F.Y Hoh, P.A McGrath, P.T Hale. **Electrophoretic analysis of multiple forms of rat cardiac myosin: Effects of hypophysectomy and thyroxine replacement.** J. Mol. Cell Cardiol., 10 (1978), pp. 1053-1076

38 Y Katsuragawa, M Yanagisawa, A Inoue, T Masaki. **Two distinct nonmuscle myosin-heavy-chain mRNAs are differentially expressed in various chicken tissues: Identification of a novel gene family of vertebrate non-sarcomeric myosin heavy chains.** Eur. J. Biochem., 184 (1989), pp. 611-616

39 S Kawamoto, R.S Adelstein. **Characterization of myosin heavy chains in cultured aorta smooth muscle cells.** J. Biol. Chem., 262 (1987), pp. 7282-7288

40 S Kawamoto, R.S Adelstein. **Chicken nonmuscle myosin heavy chains: Differential expression of two mRNAs and evidence for two different polypeptides.** J. Cell Biol., 112 (1991), pp. 915-924

41 C.A Kelly, R.S Adelstein. **Characterization of isoform diversity in smooth muscle myosin heavy chains.** Can. J. Physiol. Pharmacol., 72 (1994), pp. 1351-1360

42 C.A Kelley, J.R Sellers, P.K Goldsmith, R.S Adelstein. **Smooth muscle myosin is composed of homodimeric heavy chains.** J. Biol. Chem., 267 (1992), pp. 2127-2130

43 C.A Kelley, M Takahashi, J.H Yu, R.S Adelstein. **An insert of seven amino acids confers functional differences between smooth muscle myosins from the intestines and vasculature.** J. Biol. Chem., 268 (1993), pp. 12848-12854

44 D.A Knecht, W.F Loomis. **Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*** Science, 236 (1987), pp. 1081-1086

45 C.C Kumar, S.R Mohan, P.J Zhvodny, S.K Nrarula, P.J Leibowitz. **Characterization and differential expression of human vascular smooth muscle myosin light chain 2 isoform in nonmuscle cells.** Biochemistry, 28 (1989), pp. 4027-4035

46 M Kuro-o, R Nagai, H Tsuchimochi, H Katoh, Y Yazaki, A Ohkubo, F Takahu. **Developmentally regulated expression of vascular smooth muscle myosin heavy chain isoforms.** J. Biol. Chem., 264 (1989), pp. 18272-18275

47 H Li, U.B Gyllenstein, X Cui, R.K Saiki, H.A Erlich, N Arnheim. **Amplification and analysis of DNA sequences in single human sperm and diploid cells.** Nature, 335 (1988), pp. 414-417

48 T.L Little, J Xia, B.R Duling. **Dye tracers define differential endothelial and smooth muscle coupling patterns within the arteriolar wall.** Circ. Res., 76 (1995), pp. 498-504

49 J.D McConnell, D.H Ewalt, T.B Boone. **Developmental regulation of myosin heavy chain in the urinary bladder.** J. Cell Biol., 107 (1988), p. 36A

50 D.P Meer, T.J Eddinger. **Heterogeneity of smooth muscle myosin heavy chain expression at the single cell level.** Am. J. Physiol., 270 (1996), pp. C1819-1824

51 M.A Mohammad, M.P Sparrow. **Changes in myosin heavy chain stoichiometry in pig tracheal smooth muscle during development.** FEBS Lett., 228 (1988), pp. 109-112

52 M.A Mohammad, M.P Sparrow. **The distribution of heavy-chain isoforms of myosin in airways smooth muscle from adult and neonate humans.** Biochem. J., 260 (1989), pp. 421-426

53 I Morano, G Erb, B Sogl. **Expression of myosin heavy and light chains changes during pregnancy in the rat uterus.** Pflügers Arch., 423 (1993), pp. 434-441

54 N Murakami, P Mehta, M Elzinga. **Studies on the distribution of cellular myosin with antibodies to isoform-specific synthetic peptides.** FEBS Lett., 278 (1991), pp. 23-25

55 R.A Murphy, S.P Driska, D.M Cohen. **Variations in actin to myosin ratios and cellular force generation in vertebrate smooth muscles.** R Casteels, T Godfraind, J.C Rüegg (Eds.), Excitation-contraction coupling in smooth muscle, Elsevier/North-Holland Biomedical Press, Amsterdam (1977), pp. 417-424

56 R Nagai, D.N.M Larson, M Periasamy. **Characterization of a mammalian smooth muscle myosin heavy chain cDNA clone and its expression in various smooth muscle types.** Proc. Natl. Acad. Sci. USA, 85 (1988), pp. 1047-1051

57 R Nagai, M Kuro-o, P Babij, M Periasamy. **Identification of two types of smooth muscle myosin heavy chain isoforms by cDNA cloning and immunoblot analysis.** J. Biol. Chem., 264 (1989), pp. 9734-9737

58 C.B Neylon, P.V Avdonin, R.J Dilley, M.A Larsen, V.A Tkachuk, A Bobik. **Differential electrical responses to vasoactive agonists in morphologically distinct smooth muscle cell types.** Circ. Res., 75 (1994), pp. 733-741

59 K.E Noonan, C Beck, T.A Holzmayer, J.E Chin, J.S Wunder, I.L Andrulis, A.F Gazdar, C.L Willman, B Griffith, D.D Von Hoff, I.B Roninson. **Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction.** Proc. Natl. Acad. Sci. USA, 87 (1990), pp. 7160-7164

60 A Pereschini, K.E Kamm, J.E Stull. **Different phosphorylated forms of myosin in contracting tracheal smooth muscle.** J. Biol. Chem., 261 (1986), pp. 6293-6299

61 D.A Rappolee, A Wang, D Mark, Z Werb. **Novel method for studying mRNA phenotypes in single or small numbers of cells.** J. Cell. Biochem., 39 (1989), pp. 1-11

62 D.A Rappolee, D Mark, M.J Banda, Z Werb. **Wound macrophages express TFG*α* and other growth factors *in vivo*. Analysis by mRNA phenotyping.** Science, 241 (1988), pp. 708-712

63 A.S Rovner, M.M Thompson, R.A Murphy. **Two different heavy chains are found in smooth muscle myosin.** Am. J. Physiol., 250 (1986), pp. C861-C870

64 A.S Rovner, R.A Murphy, G.K Owens. **Expression of smooth muscle and nonmuscle myosin heavy chains in cultured vascular smooth muscle cells.** J. Biol. Chem., 261 (1986), pp. 14740-14745

65 C.G Saez, J.C Myers, T.B Shows, L.A Leinwang. **Human nonmuscle myosin heavy chain mRNA: Generation of diversity through alternative polyadenylation.** Proc. Natl. Acad. Sci. USA, 87 (1990), pp. 1164-1168

66 S Sartore, A.C Borrione, A.M.C Zanellato, M Tonello, G Scannapieco, P Pauletto, C Dal Palu. **Myosin heavy chain isoforms in bovine smooth muscle and in smooth muscle cells grown *in vitro*. A monoclonal antibody study.** J. Muscle Res. Cell Motil., 10 (1989), pp. 167-168

67 S Sartore, N DeMarzo, A.C Borrione, A.M.C Zanellato, L Saggin, Fabbri, S Schianffino. **Myosin heavy chain isoforms in human smooth muscle.** Eur. J. Biochem., 179 (1989), pp. 79-85

68 C.L Seidel, C.L Wallace, D.K Dennison, J.C Allen. **Vascular myosin expression during cytokinesis, attachment, and hypertrophy.** Am. J. Physiol., 256 (1989), pp. C793-C798

69 M Simmons, M Wang, O.W McBride, S Kawamoto, K Yamakawa, D Gidula, R.S Adelstein, L Weir. **Human nonmuscle myosin heavy chains are encoded by two genes located on different chromosomes.** Circ. Res., 69 (1991), pp. 530-539

70 J.V Small, D.O Furst, J De Mey. **Localization of filamen in smooth muscle.** J. Cell Biol., 102 (1986), pp. 210-220

71 M.P Sparrow, H.W Mitchell. **Contraction of smooth muscle of pig airway tissues from before birth to maturity.** J. Appl. Physiol., 68 (1990), pp. 468-477

72 M.P Sparrow, M.A Mohammad, A Arner, P Hellstrand, J.C Rüegg. **Myosin composition and functional properties of smooth muscle from the uterus of pregnant and non-pregnant rats.** Pflügers Arch., 412 (1988), pp. 624-633

73 H Takano-Ohmuro, T Obinata, M Kawashima, T Masaki, T Tanaka. **Embryonic chicken skeletal, cardiac, and smooth muscles express a common embryo-specific myosin light chain.** J. Cell Biol., 100 (1985), pp. 2025-2030

74 M.B Taubman, J.W Grant, B Nadal-Ginard. **Cloning and characterization of mammalian myosin regulatory light chain (RLC) cDNA: The RLC gene is expressed in smooth, sarcomeric and nonmuscle tissues.** J. Cell Biol., 104 (1987), pp. 1505-1513

75 A.E Tsao, T.J Eddinger. **Smooth muscle myosin heavy chains combine to form three native myosin isoforms.** Am. J. Physiol., 264 (1993), pp. H1653-1662

76 A Wang, M.V Doyle, D.F Mark. **Quantitation of mRNA by the polymerase chain reaction.** Proc. Natl. Acad. Sci. USA, 86 (1989), pp. 9717-9721

77 R.G Whalen, G.S Butler-Browne, F Gros. **Identification of a novel form of myosin light chain present in embryonic muscle tissue and cultured muscle cells.** J. Mol. Biol., 126 (1978), pp. 415-431

78 S White, A.F Martin, M Periasamy. **Identification of a novel smooth muscle myosin heavy chain cDNA: Isoform diversity in the S1 head region.** Am. J. Physiol., 264 (1992), pp. C1252-C1258

79 R.W Wissler. **The arterial medial cell, smooth muscle or multifunctional mesenchyme.** J. Atheroscler. Res., 8 (1968), pp. 201-213

80 A.M.C Zanellato, A.C Borrione, L Giuriato, M Tonello, G Scannapieco, P Pauletto, S Sartore. **Myosin isoforms and cell heterogeneity in vascular smooth muscle. II. Developing and adult bovine aorta.** Dev. Biol., 141 (1990), pp. 431-446