Chapter 5

MAP Kinase-Mediated and MLCK-Independent Phosphorylation of MLC20 in Smooth Muscle Cells

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/47732

1. Introduction

Smooth muscle cells constitute the walls of various organs and tubes in the body, including the blood vessels, gastrointestinal tract, respiratory tract, bladder, and reproductive tracts. The primary function of smooth muscle contraction is to generate force, which is utilized to perform many physiological processes such as blood flow and blood pressure maintenance, gastrointestinal motility, bronchial diameter regulation, bladder evacuation, and fetus expulsion. Smooth muscle contraction is caused by the sliding of myosin and actin filaments over each other. Movement of the two types of filaments happens when the globular myosin heads protruding from myosin filaments attach and interact with actin filaments to form crossbridges. The myosin head first attaches to actin together with the products of ATP hydrolysis, performs a power stroke associated with release of hydrolysis products, and detaches from actin upon binding with a new ATP. The myosin interacts with the actin to convert chemical energy, in the form of ATP, to mechanical energy. The coordinated regulation of contraction is a key property of the smooth muscle. When the smooth muscle functions normally, it contributes to general health and wellness. Contractile abnormalities of the smooth muscle are considered to underlie many diseases and disorders, including hypertension, vasospasm, diabetes-associated microvascular abnormalities, bronchial asthma, preterm labor, urinary incontinence, megacolon, and irritable bowel syndrome. Not surprisingly, inadequate contraction and relaxation of smooth muscle may cause the dysfunction of these hollow organs, which is usually associated with morbidity and mortality. Hence, the precise regulation of smooth muscle contraction is much more important in smooth muscles than in striated muscle. The muscle cells can respond to physiological and pathological signals from the environment to adapt to the environmental demands. This

adaptation is accomplished through signal transduction, which activates factors that signal pathways and ultimately lead to muscle contraction or relaxation. The regulation of smooth muscle contraction has two main mechanisms: neuromuscular and myogenic. In the neuromuscular mechanism, the smooth muscle receives principally neural innervation from the autonomic nervous system inside the same tissue, even though the central nervous system may be involved in the regulation of smooth muscle contraction. The myogenic mechanism, on the other hand, plays a more important role in the regulation of smooth muscle contraction. Under this mechanism, smooth muscle cell contraction is regulated principally by the mechanical (stretch) activation of the contractile proteins myosin and the actin in the intact body. A change in membrane potential, brought by the firing of action potentials or by the activation of stretch-dependent ion channels in the plasma membrane, can also trigger contraction. These smooth muscle cells also develop tonic and phasic contractions in response to changes in load or length. In addition, the contractile state of the smooth muscle is controlled by the hormones, autocrine/paracrine agents, and other local chemical signals. Regardless of the stimulus, the smooth muscle cells use crossbridge cycling between the actin and myosin to develop force and calcium ions (Ca^{2+}) that serve to initiate contraction in light of the current view. The deep mechanism for both myogenic and neuromuscular regulations is involved in cell signaling. Because current therapies for disorders and diseases of smooth muscles are costly and primarily palliative and do not target the cause of the disease, attention is being focused on the detailed understanding of the molecular basis of smooth muscle function and regulation and identification of abnormalities (dysfunctional proteins and signaling pathways) that lead to contractile pathologies and the development of strategies to reverse such abnormalities. The muscle contraction regulation mediated by signaling pathways is the key event. In recent years, a variety of signaling pathways has been implicated in the regulation of muscle contraction. A growing body of evidence shows a fine vista for the research of pathway-mediated regulation of the smooth muscle. One of the widely accepted notions is that the increase in Ca^{2+} concentration activates the Ca^{2+}-calmodulin (CaM)–myosin light chain kinase (MLCK) pathway and stimulates the 20-kDa myosin light chain (MLC20) phosphorylation. The MLC20 phosphorylation at Ser^{19} causes a conformational change that increases the angle in the neck domain of the myosin heavy chain which mobilizes the crossbridges and causes the actin thin filament to slide along the myosin thick filament. Through an unknown mechanism, this interaction between the myosin and actin activates the ATPase activity of the myosin head region and leads to the development of a contractile force. Therefore, in theory, the factors that cause the inhibition of the MLCK activity and expression, such as calcium deprivation and calmodulin inactivation, could abolish consequentially MLC20 phosphorylation and smooth muscle cell contraction. However, increasing evidence supports Ca^{2+}-independent contraction of smooth muscle (Deng et al., 2001; Harnett & Biancani, 2003; Ratz et al., 2009). Here, we will describe an MLCK-independent and mitogen-activated protein (MAP) kinase-mediated phosphorylation of MLC20.

2. Expression and function of MLC20 and MLCK in smooth muscle

The myosin II (also called conventional myosin), a hexameric protein complex, is composed of two identical 200-kD heavy chains and two sets of light chains: the 17-kD light chain (MLC17, also known as ELC) and the 20-kD regulatory light chain (MLC20). The exact function of the MLC17 is unclear but it may contribute to the structural stability of the myosin head along with the MLC20. The MLC20 is a small ring around the neck region of heavy chains of myosin and is also known as the regulatory light chain (also called RLC20, RLC2, and LC2). It is believed to participate actively in muscle contraction. The MLC20 is expressed not only in smooth muscle, but also in cardiac, skeletal, and nonmuscle cells. Only the MLC20 in the smooth muscle appears to play a unique role in increasing the actin-activated ATPase activity. The MLC20 is activated by phosphorylation at multiple serine and threonine residues. The phosphorylation of the MLC20 is thought to play a pivotal role in regulating muscle contraction.

Up to now, the MLC20 is the only known physiological substrate of the MLCK. Protein kinase C (PKC), CaM-kinase II, Rho-kinase, p21-activated kinase, and p34cdc2 kinase also phosphorylate three residues on the N-terminus of the MLC20, Ser-1, Ser-2, and Thr-9. In addition, the integrin-linked kinase and ZIP-kinase are reported to phosphorylate the MLC20 in the absence of Ca^{2+}, at Ser19 and Thr18, thus activating the myosin activity (Niiro & Ikebe, 2001). *In vivo* phosphorylation of the MLC20 isoforms is accomplished by raising free intracellular Ca^{2+} and subsequent activation of the Ca^{2+}-calmodulin-dependent MLCK. Ca^{2+}/calmodulin-dependent MLCK is considered the primary regulator of MLC20 phosphorylation among potential regulators of MLC20 phosphorylation. The dephosphorylation of phosphorylated MLC20 is catalyzed by the myosin light chain phosphatase (MLCP), which counters the MLCK that promotes contraction by phosphorylating MLC20. The degree of phosphorylation of the RLC depends on the ratio of the activities of the MLCK and MLCP.

The myosin light chain kinase (MLCK or MYLK) is a Ca^{2+}/CaM-activated kinase found in smooth, cardiac, and skeletal muscles as well as in many mammalian nonmuscle cells. It is a serine/threonine-specific protein kinase that phosphorylates the MLC20 of myosin II. There are three isoforms of the MLCK, i.e., smooth muscle (smMLCK, ~130-kDa), skeletal muscle (skMLCK, ~220-kDa), and cardiac muscle (cMLCK). The short MLCK (smMLCK, ~130kDa) is best known as the conventional smooth muscle MLCK. The smMLCK is encoded by the mylk1 gene, which expresses three transcripts in a cell-specific manner due to the alternate promoters, long MLCK (210- to 220-kDa), short MLCK (130-kDa), and the noncatalytic gene product, called telokin. The short smooth muscle MLCK (130-kDa) is ubiquitous in all adult tissues with the highest amounts in smooth muscle tissues. Since both the short and long MLCK are found in smooth muscles or cultured/embryonic smooth muscle tissues, they are called smooth muscle MLCK (smMLCK). The two smMLCKs have been extensively described, and their function is to regulate the activity of the nonmuscle and smooth muscle myosin II. Here we will deal only with the short smooth muscle MLCK (smMLCK).

The activation of myosin motors by the MLCK modulates a variety of contractile processes, including smooth muscle contraction, cell adhesion, migration, and proliferation. The dysregulation of these processes contribute to a number of diseases. It is widely accepted that the MLCK phosphorylates the MLC20 of smooth and nonmuscle myosin II in the presence of Ca^{2+} and calmodulin. The phosphorylation of the MLC20 facilitates myosin interaction with actin filaments. Once there is an influx of calcium cations into the muscle, either from the sarcoplasmic reticulum or, more important, from the extracellular space, the contraction of smooth muscle fibers may begin. First, the elevation of the Ca^{2+} concentration in smooth muscles causes the Ca^{2+} to bind to the calmodulin (CaM). Then, the complex of the Ca^{2+} and CaM (Ca^{2+}/CaM) activates the MLCK, which would phosphorylate the myosin light chain (MLC20) at serine residue 19 to generate phospho-MLC20. The phosphorylation of the MLC20 enables the myosin crossbridge to bind to the actin filament and allows contraction to begin (through the crossbridge cycle). The Ca^{2+}-CaM activates the MLCK by reversal of an auto-inhibited state. In contrast, reducing intracellular calcium concentration inactivates MLCK but does not stop smooth muscle contraction since the MLC20 has been physically modified through phosphorylation. This regulatory model of myosin phosphorylation is widely accepted as the intracellular path for the induction of smooth muscle contraction.

However, to a certain extent, this viewpoint about the Ca^{2+}-, MLCK- and MLC20-dependent regulatory mechanism is founded on a presumption. Several observations of smooth muscle contraction cannot be explained by the mode of phosphorylation (Kohama K & K., 1995). For example, when the uterine smooth muscle is subjected to prolonged incubation in a Ca^{2+}-free medium, the oxytocin induces the contraction of the muscle without any signs of MLC20 phosphorylation (Oishi et al., 1991). Another example is the MLC20 phosphorylated in rat embryo fibroblasts, which contains no detectable MLCK (Emmert et al., 2004). In addition, these are increasing evidence to support the calcium- and MLCK-independent mechanism of MLC20 phosphorylation (McFawn et al., 2003; El-Toukhy et al., 2006; Cho et al., 2011). According to the current mode, the expression of the MLCK in tissues/cells is necessarily consistent with the MLC20 phosphorylation. However, there has not been any report about the consistence between the MLCK expression and MLC20 phosphorylation. Herein, we first investigated the consistency between the MLCK expression and phosphor MLC20. In the following section we will describe a recent finding, the significant inconsistence between MLCK expression, and MLC20 phosphorylation in multiple smooth muscles in mice.

3. Inconsistence between MLCK expression and MLC20 phosphorylation

According to the current view, the MLCK expression must co-localize with the MLC20 phosphorylation in cells or tissues. However, by a series of experiments, we recently found that phosphorylation (Ser19) of the MLC20 is inconsistent with 130-kDa MLCK expression in mouse aorta, bladder, large/small intestines, stomach, and uterus (Deng et al., 2011) (Figure.1A and B).

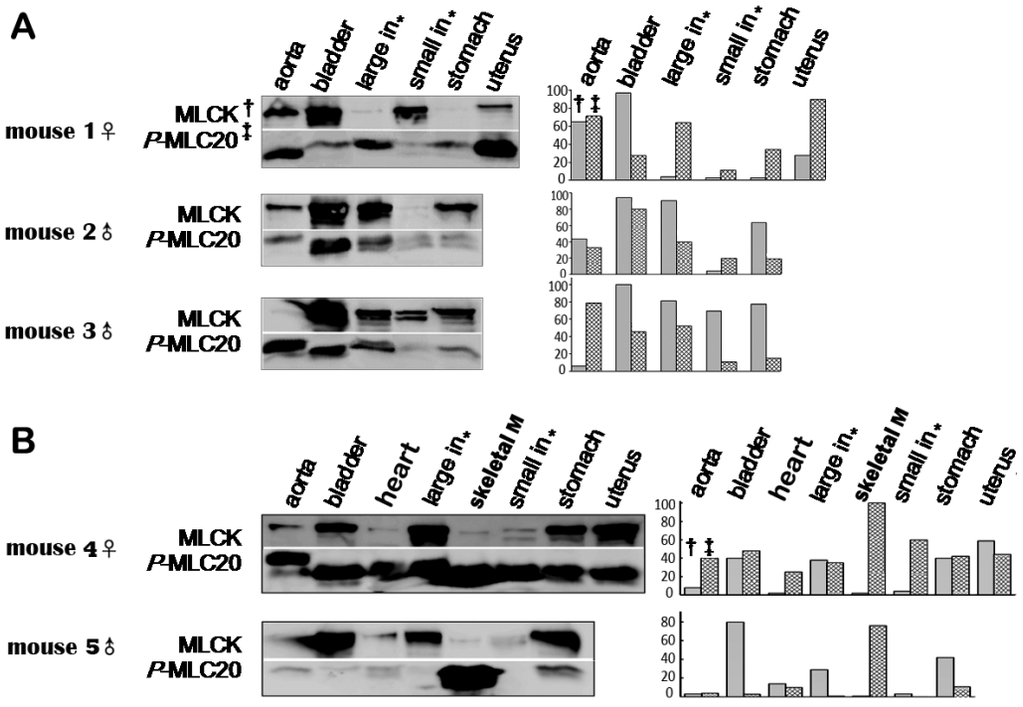

Figure 1. Inconsistence between MLC20 phosphorylation and MLCK expression in smooth muscle tissues. *in, intestine; †, MLCK expression; ‡, phosphor MLC20.

In the experiments, fresh smooth muscle tissues were homogenized on ice with pre-cooled small glass homogenizers within ice-cold lysis buffer (50 mM Tris, 300 mM NaCl, 3 mM EGTA, 0.1 mM sodium orthovanadate, 10% glycerol v/v, 1% NP-40 v/v, 0.3% SDS w/v, protease and phosphatase inhibitor cocktails from Sigma Co., Germany, pH 7.6). Twenty micrograms of protein were loaded onto 10% SDS-PAGE gels that were subjected to electrophoresis in running buffer (BioRad Co., Hercules, CA). The proteins were then transferred to the Immobilon-P Transfer Membrane (Millipore Co., MA), followed by blocking it with 5% fat-free milk in TBST buffer. The MLCK was detected with a specific antibody. After it was washed with 1×PBS for three times, the membranes were re-probed with antibody anti-phospho MLC20. The results show that the levels of MLCK expression and phosphorylated MLC20 in different smooth muscles from the same mouse are markedly different (Figure 1). For instance, the large intestine of mouse 1 contains a high level of phosphor MLC20 but the MLCK expression is low. In contrast, the small intestine has low phosphor MLC20 but the MLCK expression is high. Besides, the MLCK expression and phosphor MLC20 in the same type of smooth muscle tissue are inconsistent among different individuals. For example, the large intestine of mouse 1 contains a low expression of MLCK and high phosphor MLC20. Conversely, mice 2 and 5 express high levels of MLCK in their large intestines but the MLC20 phosphorylation is low. The conspicuous inconsistence between the MLC20 phosphorylation and MLCK expression was subsequently verified by Western blotting in independent experiments. These interesting results cannot be explained by the current regulatory mode. Obviously, they suggest that an alternative regulation system may play an active role in the MLC20 phosphorylation.

4. Unknown substance(s) other than MLCK phosphorylates MLC20

Even though some of the smooth muscle does not show phosphor MLC20 bands (Figure 1), those tissues still contain the MLC20 because we noticed in subsequent experiments that all these frozen lysates had similar levels of phosphor MLC20 after storage in -80°C and several cycles of freezing and thawing. The MLC20 in some tissues was not visualized by any specific antibody against the phosphor MLC20 only because it was not phosphorylated. The MLC20 phosphorylation in MLCK-free tissues suggests that unknown molecule(s) may phosphorylate the MLC20 in phosphor MLC20-containing tissues.

Thus, we proposed a hypothesis that something else other than the MLCK phosphorylates MLC20 in the smooth muscle tissues, which contain no detectable MLCK. Even though we did not know what kind of substance(s) the phosphorylated MLC20 is/are in the phosphor MLC20-containing and MLCK-free tissue, it may mobilize the phosphorylative process of the MLC20. To test our hypothesis, we designed an unconventional experiment, and added a small amount of tissue extract which was freshly prepared from the phosphor MLC20-containing tissues without any detectable MLCK (extract 2), to another extract that contained the unphosphorylated but had no phosphorylated MLC20 and MLCK (extract 1). In other words, we theorized that extract 2 is a catalyst and extract 1 is a substrate of extract 2. By rapid Western blotting assay (with one-hour incubation at room temperature), we chose the tissue extract containing the high phosphor MLC20 but no detectable MLCK as a catalyst (extract 2), and the one containing no phosphor MLC20 and detectable MLCK as the substrate (extract 1). Then, a small amount of exact 2 was added directly into extract 1, as shown in Figure 2A. After the one-hour 37°C incubation in water, as expected, the added extract 2 phosphorylated strongly MLC20 of the extract 1 (lines 2 and 3 in Figure 2A). This suggests that high phosphor MLC20 tissue content may have non-MLCK substance(s) that induce/s MLC20 phosphorylation.

To evaluate the further roles of the MLCK and the possible non-MLCK substance(s) in the MLC20 phosphorylation, we assayed the effects of the isolated MLCK on MLC20 phosphorylation (Figure 2B). Firstly, the MLCK was isolated from the fresh smooth muscle tissue by the anti-MLCK monoclonal antibody from Sigma Co., following conventional immunoprecipitation protocol. Secondly, the isolated MLCK was immediately added to the freshly prepared extract 1, which contains unphosphorylated MLC20 but no detectable MLCK (line 2 in Figure 2B). In the meantime, a small amount of extract 2 that contains a high level phosphor MLC20 but no detectable MLCK was added into the same 'substrate' extract 1 as a control (line 3). The mixtures were incubated in 37°C water for one hour, followed by Western blotting assay. Extracts 1, 2 and isolated MLCK were loaded to lines 1, 5 and 4, respectively, for assays of its own MLCK and phosphor MLC20. No protease and phosphatase inhibitor were added into the reaction system. After the membrane was washed with PBS for three times, the actin and phospho-MLC20 were simultaneously probed by mixture solution of the antibodies against actin and phospho-MLC20 (Membrane was not striped by striping buffer because the sizes of the three targeting proteins were much different). The results show that the immunoprecipitated MLCK failed to

phosphorylate MLC20, while the extract without detectable MLCK phosphorylated MLC20 of extract 1 contains no detectable MLCK (line 3). Several independent experiments were conducted to verify the results. These experiments suggest that MLC20 can be phosphorylated *in vitro* and that non-MLCK substance(s) may trigger the MLC20 phosphorylation.

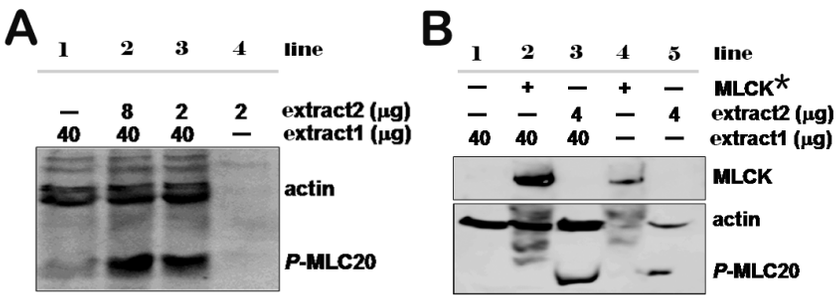

Figure 2. Unknown substance(s) in smooth muscle tissue but not immunoprecipitated MLCK induced MLC20 phosphorylation. (A) Small amount of extract 2 without MLCK induced MLC20 phosphorylation of MLC20 in extract 1 (lines 2 and 3). (B) Precipitated MLCK did not induce MLC20 phosphorylation (line 2). The extract 2 containing no detectable MLCK increased significantly the phosphorylation of MLC 20 in extract 1(line 3). MLCK*, MLCK freshly isolated by co-immunoprecipitation from smooth muscle tissue; *p*-MLC20, phosphor MLC20.

5. Inhibition of MLCK expression and activity do not affect MLC20 phosphorylation

According to the current regulatory model, intracellular calcium ion is the trigger of smooth muscle contraction. The contractile initiation, maintenance, and strength are dependent on the control of intracellular free Ca^{2+} level. The canonical excitation-contraction coupling pathway is triggered by neural, hormonal or myogenic stimulation to elicit the influx of extracellular or intracellular Ca^{2+} (from the sarcoplasmic reticulum) into the cytosol. The Ca^{2+} binds to calmodulin to form the Ca^{2+}/CaM complex. At an increased level of Ca^{2+}, rapid binding of the Ca^{2+} to CaM occurs and triggers MLCK activation. Once activated, the MLCK phosphorylates MLC20 leads to crossbridges cycling and generation of the contractile force. Therefore, several factors may affect the MLC20 phosphorylation, including expression of the MLCK gene, MLCK activity, free Ca^{2+} concentration, and CaM activity. The following experiments were designed to access the effects of these factors on the role of the MLCK in MLC20 phosphorylation: 1) using siRNA to knockdown the MLCK expression in human bladder smooth muscle (hBSM) cells, 2) inactivating the MLCK by inhibitors of calmodulin and MLCK, and 3) depriving the calcium ion of cultured hBSM cells.

We first investigated the effect of alteration in MLCK expression on the MLC20 phosphorylation. To inhibit the MLCK expression, the siRNA against MLCK (from Santa Cruz Biotechnology Inc.) was transfected with Lipofectamine 2000 into cultured hBSM cells at a cell density of 40-50% confluence, which were isolated from the peri-cancer tissue of a bladder cancer patient. Only lower than seventh passage primary cells were used for experiments. Two days after transfection, cells were harvested for Western blotting. MLCK,

pan-actin and phosphor MLC20 were detected by specific antibodies. Western blotting analysis showed that no significant alteration of MLC20 phosphorylation level was observed while the MLCK expression was knocked down (Figure 3A). Together with the data about the expression of MLCK and MLC20 phosphorylation from mice, this experiment provides further proof that MLC20 phosphorylation is independent of MLCK gene expression.

Equally vital to the MLCK gene expression, the MLCK activity or activation may contribute to its functions. Then, the subsequent experiments were designed to study the effects of the MLCK activity on the MLC20 phosphorylation. The MLCK and its upstream molecule, the CaM, were directly inactivated by the corresponding commercial inhibitory peptides (EMD Chemicals Inc., NJ) in both permeabilized or unpermeabilized hBSM cells (Figure 3B, C) and mouse smooth muscle extracts (Figure 3D). The MLC20 phosphorylation was examined by Western blotting. In addition, ethylene glycol tetraacetic acid (EGTA), a Ca^{2+} chelating agent, was added into the medium to prevent the formation of Ca^{2+}/CaM complex in cultured hBSM cells (Figure 3B and C). CaM and MLCK inhibitory peptides were added into mixtures of smooth muscle extracts 1 and 2, in which small amount of extract 2 was able to phosphorylate MLC20 of extract 1 (shown in figure 2). After one-hour incubation at 37°C, *pan*-actin and phosphor MLC20 were assayed by Western blotting. Likewise, none of the inhibitors of the MLCK and CaM, and EGTA inhibit the MLC20 phosphorylation in both cultured hBSM cells and tissue extracts.

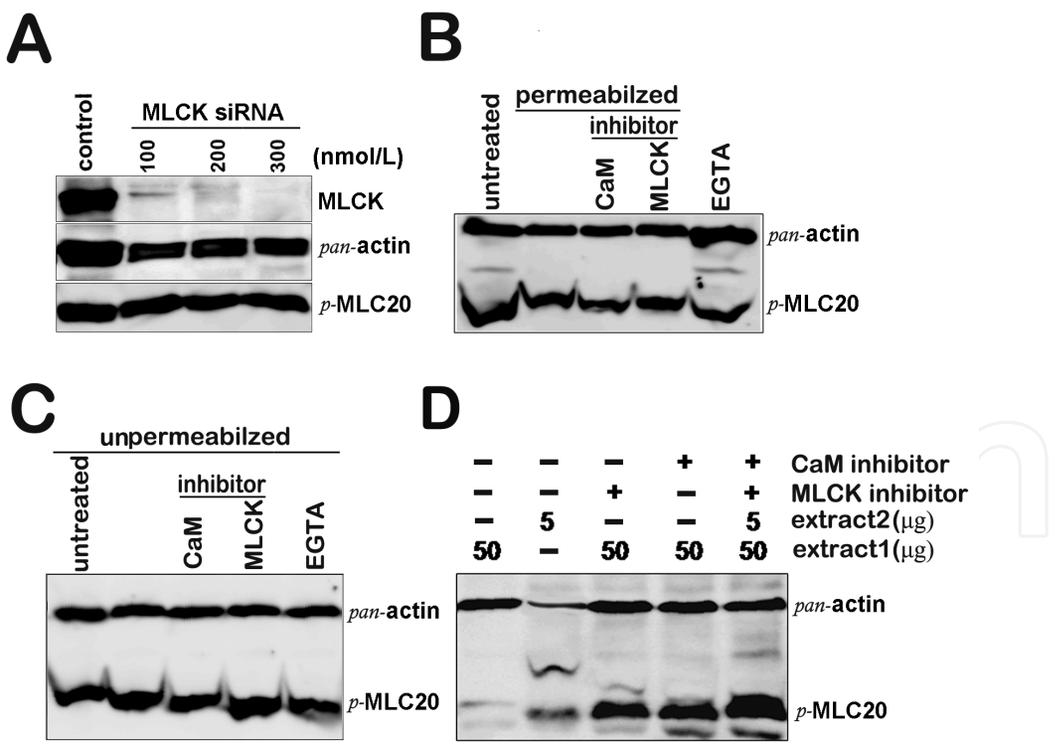

Figure 3. Inhibition of MLCK expression and activity does not block MLC20 phosphorylation. (A) siRNA-mediated MLCK knockdown did not inhibit MLC20 phosphorylation. (B and C) Inhibitors of CaM and MLCK and EGTA did not suppress MLC20 phosphorylation in permeabilized and unpermeabilized cells. (D) Neither CaM nor MLCK inhibitors blocked MLC20 phosphorylation in smooth muscle extracts.

Putting the data together, we firmly believe that an alternative mechanism plays an important part in the regulation of MLC20 phosphorylation. This potential regulatory mechanism is MLCK-independent.

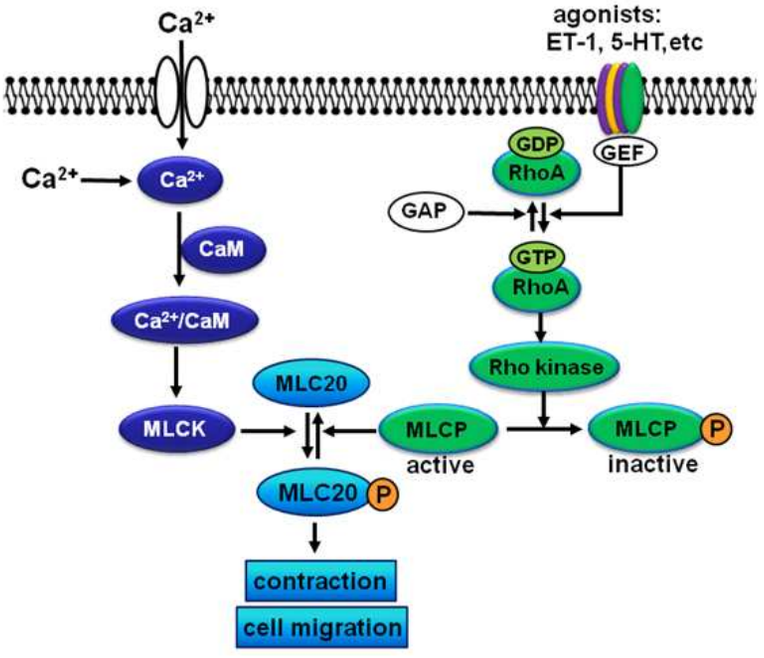

Figure 4. Two main pathways involved in regulation of smooth muscle cell contraction and migration. The contractile response is initiated by a rapid and transient rise in intracellular Ca^{2+}, followed by a Ca^{2+}-calmodulin interaction, the MLCK activation to initiate phosphorylation of the MLC20 and therefore contraction and cell migration (the blue). Agonists (such as ET-1) bind to serpentine receptors on the membrane of the smooth muscle cells to activate the RhoA/Rho kinase signaling pathway, which inactivates the MLC phosphatase (MLCP) by phosphorylation, and resulted in increased unphosphorylated MLCP and sustained smooth muscle contraction (the green). GAP, GTPase activating protein; GEF, GDP/GTP exchange factor.

6. MAP kinase pathways and functions in smooth muscle cells

After we verified the MLCK-independent regulation of MLC phosphorylation, we were interested in searching for the non-MLCK mechanism that may function as the trigger of MLC20 phosphorylation. The cell signaling pathways would be most likely to address this intractable issue. In recent years, a multitude of signaling pathways has been suggested to regulate the smooth muscle contractility. However, these pathways can be broken down into two major pathways (Figure 4): the calcium–calmodulin signalling pathway and Rho/Rho-kinase pathway. The former, the Ca^{2+}/CaM pathway, regulates smooth muscle contraction by the binding of increased intracellular calcium ion with CaM, which activates the MLCK. The MLCK phosphorylates the MLC20 at the neck of the myosin heavy chains. The phosphorylation increases the ATPase activity and thereby produces contraction. In the Rho/Rho-kinase pathway, the Rho A-GTP, activated by multiple stimuli, activates the Rho kinase (also known as Rok or Rock). The activated kinase phosphorylates the myosin phosphatase and therefore modulates the MLC20 phosphorylation.

In the following sections, we will describe a MAP kinase-mediated phosphorylative mechanism of MLC20.

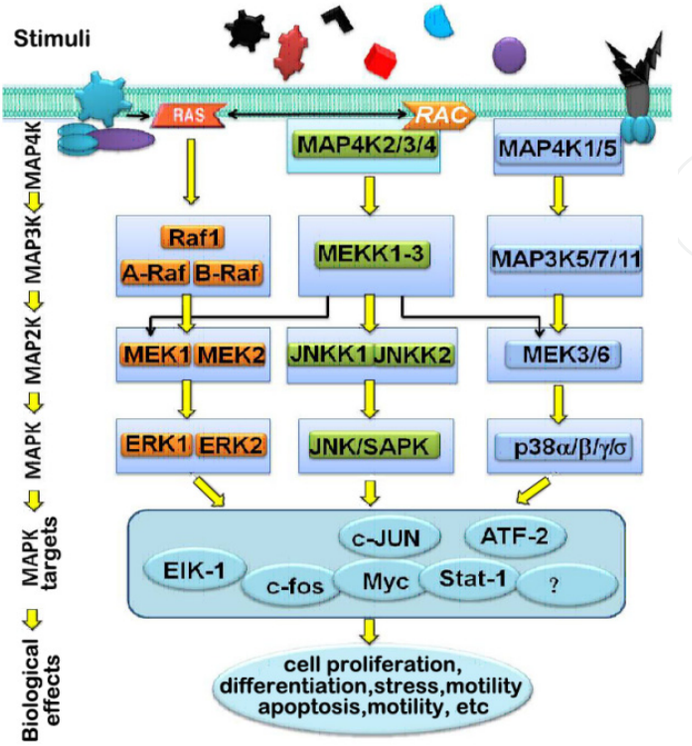

Figure 5. The main components of the three MAP kinase pathways. MAP kinase cascade is typically composed of four kinases that establish a sequential activation pathway comprising a MAP kinase kinase kinase kinase (MAP4K), MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K), and MAP kinase (MAPK). Extracellular/intracellular stimuli activate MAP4K, MAP3K, MAP2K and MAPK sequentially, leading to the multiple biological effects.

MAP kinases (MAPKs) comprise a family of serine threonine kinases, which include three major sets of kinases: extracellular signal-regulated kinases (ERK1 and ERK2), c-Jun amino-terminal kinase/stress-activated protein kinases (JNKs), and p38 MAPKs (Figure 5). These kinases constitute three major discrete cascades and serve as focal points in response to a variety of extracellular stimuli. Members of all three MAPK families, the ERK, JNK, and the p38 MAPKs, are expressed in various types of tissues and cells, including smooth muscles.

6.1. ERK pathway

The best-characterized members of the MAPK family are ERK1 and ERK2, also known as p44 MAPK and p42 MAPK, respectively. The canonical ERK cascade comprises Ras, Rafs, MEK1/2, ERK1/2 and several MAPK-activating protein kinases (MAPKAPK, such as EIk-1, Sap1a and c-Fos). ERK1 and ERK2 are expressed to various extents in all tissues, including various types of smooth muscle. ERK1/2 is distributed throughout quiescent cells, but upon stimulation, a significant population of ERK1/2 accumulates in the nucleus. Immediate upstream kinases that activate ERK cascade are mitogen-activated protein kinase kinase 1/2

(MEK1/2). The ERK is activated by a large variety of diverse extracellular stimuli, including growth factors, cytokines, virus infection, transforming agents, and carcinogens. Activated ERK1/2 phosphorylate numerous substrates, including various membrane proteins (CD120a, Syk, and calnexin), nuclear substrates (SRC-1, Pax6, Elk-1, MEF2, c-Fos, c-Myc, and STAT3), cytoskeletal proteins, and several MAPK-activated protein kinases (MKs), and MAPK-interacting kinases(MNKs). As a consequence of the activation, ERK pathway regulates many distinct and even opposing cellular processing, including proliferation, differentiation, metabolism, morphology, survival and even apoptosis.

ERK pathway may be involved in regulation of contraction, migration and proliferation of smooth muscle cells. PD-098059, a specific inhibitor of MAP kinase kinase (MEK1/2, the upstream of ERK1/2), reduced vascular smooth muscle contraction and increase in blood pressure induced by Ang II and phenylephrine, perhaps by inhibiting ERK1/2 activation (Escano *et al.*, 2008) and airway smooth muscle contraction by isoprenaline (Lelliott *et al.*, 2012). Direct inhibition of ERK1/2 by U-0126 attenuates Ang II- and isoprenaline-induced contraction of bronchial smooth muscle (Sakai *et al.*, 2010; Lelliott *et al.*, 2012). Similar role of ERK1/2 are also observed in other smooth muscle (cells) (Jeong *et al.*, 2011). However, some reports show that the inhibitors of ERK and its upstream do not affect vascular smooth muscle contraction (Do *et al.*, 2009; Bauer *et al.*, 2011; Sathish *et al.*, 2011). Thus, this role of ERK in regulation of smooth muscle contraction is not universal, or even controversial.

The biological process of cell migration is similar to that of muscle cell contraction. The functional role of ERK in the regulation of smooth muscle cell migration is reported by a large number of articles (Aitken & Bagl, 2001; Kavurma & Khachigian, 2003). Many reports about the regulatory model of ERK1/2 in smooth muscle cell migration are consistent, even though it is not entirely clear how ERK activation promotes smooth muscle cell motility. For example, inhibition of ERK by inhibitor and antisense oligonucleotide blocks smooth muscle migration (Graf *et al.*, 1997; Gerthoffer, 2007). Our study confirmed role of ERK1/2 in smooth muscle cell migration by blocking movement by PD-098059(Deng *et al.*, 2011). In addition, Raf-1 kinase, the upstream molecule of MEK1/2 that activates ERK1/2, may be involved in regulation of smooth muscle contraction (Sathishkumar *et al.*, 2010).

6.2. p38 pathway

The p38 cascade is composed of MEKK3, MKK3/4/6, p38 and several MAPKAPKs (EIk-1, CHOP, ATF2 and MEF2A). The p38 family includes four splice variants: p38α, p38β, p38$\gamma$and p38δ. Like the rest of MAP kinases, p38 are ubiquitously expressed in various types of tissues, including various smooth muscle cells. Two main MAPKKs, MEK3 and MEK6, function as the upstream molecules of p38, which are known to activate p38. In addition, MEK4, an upstream kinase of JNK, can aid in the activation of p38α and p38δ in specific cell types. The p38 MAPK cascade is activated by numerous promigratory stimuli, including platelet-derived growth factor (PDGF), Ang II, S1P, and thrombin. This kinase is also responsive to a wide range of environmental stresses, such as ultraviolet irradiation, heat shock, osmotic shock, as well as response to inflammatory cytokines, but less by serum

and growth factors. Substrates of p38 *in vivo* include transcription factors (ATF2, MEF2C), the RNA binding protein tristetraprolin, and several protein kinases, such as MAPKAP kinases 2, 3, and 5. The p38 signaling has been implicated in cellular responses including inflammation, cell cycle, cell death, development, cell differentiation, senescence, and tumorigenesis. Because p38 MAPKs are key regulators of inflammatory cytokine expression, they appear to be involved in many human diseases such as asthma and autoimmunity.

The p38 MAPKs may participate in multiple processes in smooth muscle cells including contraction, oxidative stress signaling, and cytokine synthesis . Contraction induced by some but not all agonists depends on p38 activity. SB203580, the specific inhibitor of p38, blocks spontaneous and agent-induced smooth muscle contraction (Lee *et al.*, 2007; Barona *et al.*, 2011). Besides, by using SB203580 and siRNA, researchers have demonstrated that p38 is involved in regulation of smooth muscle cell migration (Mugabe *et al.*, 2010).

6.3. JNK pathway

The JNK cascade comprises generally MAP-ERK kinase kinase-1 (MEKK1), MKK4/7, JNKs and MAPK-activated C-Jun, JunB and ATF2. JNK family, encoded by three genes, jnk1, jnk2, jnk3, includes three main spliced forms, JNK1, -2, and -3, also known as SAPKγ, SAPKα, and SAPKβ, respectively. JNK1 and JNK2, are believed to be expressed in every cell and tissue type, whereas the JNK3 protein is found primarily in brain (Bode & Dong, 2007). JNK activation is much more complex than that of ERK1/ERK2 owing to inputs by a greater number of MAP4Ks and MAP3Ks. This diversity of upstream messages allows a wide range of stimuli to activate this MAPK pathway, including UV and γ-irradiation, protein synthesis inhibitors (anisomycin), hyperosmolarity, toxins, ischemia/reperfusion injury in heart attacks, heat shock, anticancer drugs (cisplatinum, adriamycin, or etoposide), ceramide, peroxide, and inflammatory cytokines such as TNFα. The activated JNKs translocate to the nucleus where they phosphorylate the effector molecules. A well-known substrate for JNKs is the transcription factor c-Jun. Besides, several other transcription factors have been shown to be phosphorylated by the JNKs, such as ATF-2, NF-ATc1, HSF-1, c-Myc, p53, STAT3, DPC4/ SMAD4/MADH4. Some non-transcription factors can also be regulated by JNKs, such as Bcl-2, paxillin, and Bcl-xL. The dual role of JNK in both apoptotic and survival signaling pathways indicates that the functional role of JNK is complex. JNKs are involved in various physiological and pathological processes, such as apoptosis, neurodegeneration, neural development, cell differentiation and proliferation, inflammation, cytokine production and regulation of responses to stimuli.

Compared with ERK and p38 MAPKs, less is known about the role of JNK MAPKs in contraction and migration of smooth muscle cells. The chemical inhibitors of JNK family members are less selective than MEK (then ERK1/2) and p38 MAPK inhibitors and therefore somewhat less useful for definitive studies of kinase action in cells. However, recent evidences support an important role for JNK in regulation of airway smooth muscle contraction (Lei *et al.*, 2011). SP600125, a JNK inhibitor, attenuates vascular smooth muscle contraction and human prostate smooth muscle induced by norepinephrine and

phenylephrine (Lee *et al.*, 2006; Strittmatter *et al.*, 2012). In addition, overexpression of an inactive mutant of JNK partially inhibits migration induced by PDGF-BB and Ang II (Ohtsu *et al.*, 2005). The mutant may attenuate natural JNK activity by interfering its binding to the up- and down-stream molecules. Besides, SP600125 inhibits smooth muscle migration (Kavurma & Khachigian, 2003).

7. Regulation of MAP kinase pathways on MLC20 phosphorylation

By using the MAPK inhibitors and adenoviral overexpression, we found a MAP kinase pathway-mediated regulatory mechanism of MLC20 phosphorylation.

7.1. Inhibition of MAP kinase pathways increases phosphorylation level of MLC20

Research on the role of the ERK, p38 MAPK, and JNK is facilitated by effective and relatively selective small molecule inhibitors. We first used the p38, JNK, and ERK inhibitors to access the effects of MAP kinase signaling on MLC20 phosphorylation in cultured hBSM cells (Figure 6A).

The SB203580, BIRB 796, SB 202190, and VX-702 have inhibitory effects for the p38 kinase but only the SB 203580, a specific inhibitor of p38α and p38β isoforms, is widely used for the p38 function-related studies. The half maximal inhibitory concentration (IC50) values recommended by the manufacturer are 50 and 500 nM for the p38$\alpha$and p38β, respectively. The concentration ranges used in published reports are 10–50 μM (Kramer et al., 1996; Fatima et al., 2001; Kim et al., 2002). The JNK inhibitors include the SP600125, JNK inhibitor I (a peptide), JNK inhibitor IX, and JNK inhibitor VIII. The SP600125, a JNK inhibitor for three isoforms of JNKs, is often used to inhibit JNKs in most studies. The recommended IC50 values are 40 nM for JNK-1 and JNK-2, and 90 nM for JNK-3. The concentration ranges used by most previous researchers are 10–30 μM (Renlund et al., 2008). The recently used concentration is 20 μM (Takahashi et al., 2011). The ERK inhibitors include the PD98059, ERK Inhibitor II ($C_{18}H_{13}N_7$), U0126, and inhibitory peptide. It is a 13-amino acid peptide that corresponds to the N-terminus of MEK1 and functions as a specific inhibitor of ERK activation. The IC50 recommended by the manufacturer is 2.5 μM. Different concentrations such as 10, 20, 40, and 50 μM were used in previous researches (Mathur et al., 2004; Monick et al., 2008). The concentration of 60 μM was used in our studies. Considering the difficulty to transport a peptide across a cell membrane, we permeabilized cells before the ERK inhibitor treatment, even though the inhibitor peptide is cell-permeable.

In this study, the cultured hBSM cells were exposed to 30 μM of SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), and 60 μM of ERK inhibitory peptide for 40 min. Cells for ERK inhibition were previously permeabilized (Deng *et al.*, 2011). Proteins of cell lysates were separated by SDS-PAGE electrophoresis and then transferred to PVDF membranes. Actin, phosphor MLC20 and MLCK on the same membrane were detected. Inhibition of MAP kinase activity increased MLC20 phosphorylation (Figure 6A). To minimize the effects of the

quantitative difference in protein loading on experimental judgment in Western blotting assay, we always incubated membranes with mixed primary antibodies against *pan*-actin and phosphor MLC20, followed by incubation with corresponding and different second antibody after PBS washing. The actin gene is a housekeeping gene, which is expressed invariably in various cells/tissues usually at different treatments. The *pan*-actin bands can show us how much protein is loaded in the wells of SDS-PAGE gels. Then, the membrane was re-probed with specific antibody against the MLCK. Unexpectedly, all of the three MAP kinase inhibitors appear to increase the phosphor MLC20 level (Figure 6A), especially treatments by the SB 203580 and SP600125 in lines 2–3. The MLCK expression was not altered when the phosphor MLC20 level increased due to the MAP kinase inhibitor treatment.

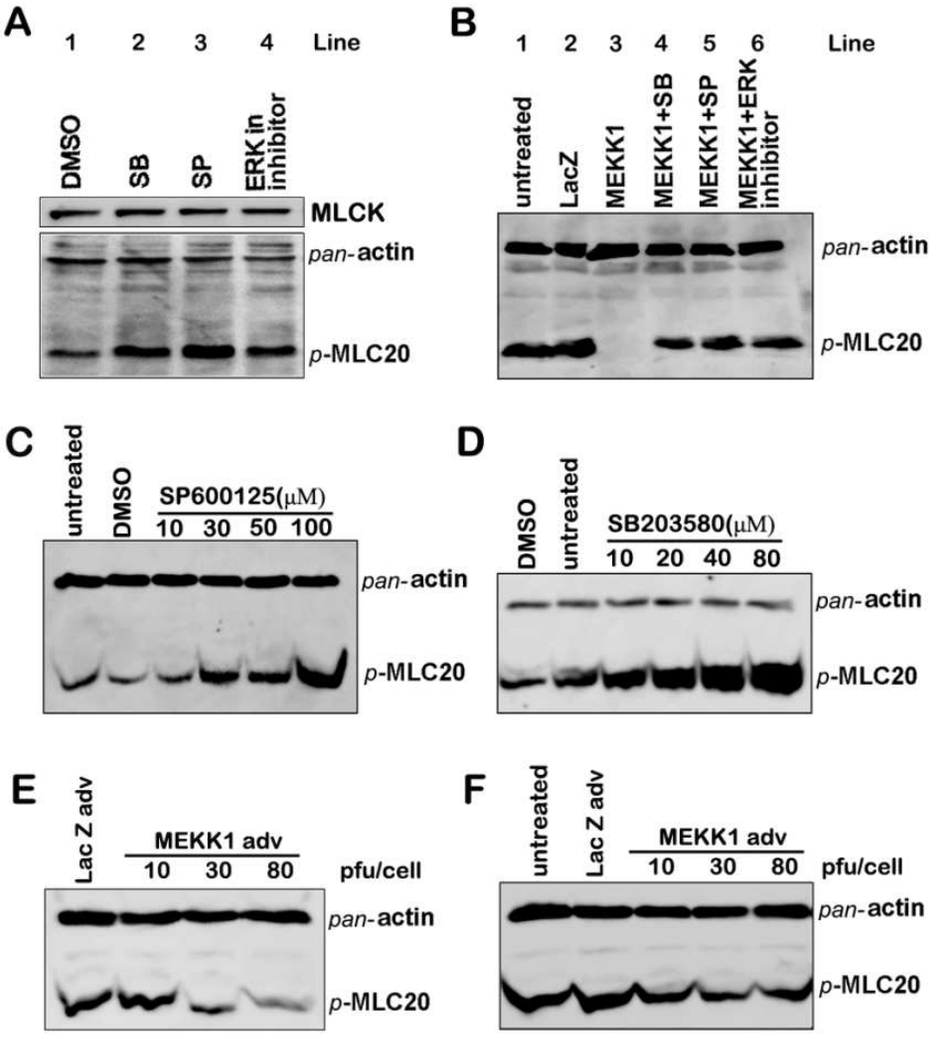

Figure 6. Negative regulation of MAP kinase pathway on MLC20 phosphorylation. (A) All of p38, JNK and ERK inhibitors raised phosphorylation level of MLC20. (B)Activation of MAP kinases by adenovirus-mediated overexpression of MEKK1 inhibited significantly MLC20 phosphorylation (line 3). MAPK inhibitors counteracted inhibition of MEKK1 on MLC20 phosphorylation (line4~6). (C, D) The level of MLC20 phosphorylation increased with JNK and p38 inhibitors in hBSM cells. (E, F) MLC phosphorylation decreased with MEKK1-viral doses. *P*-MLC20, phosphor MLC20; SB, SB203580; SP, SP600125; adv, adenovirus.

7.2. Activation of MAP kinase pathways inhibits MLC20 phosphorylation

Contrary to the MAP kinase-inhibition experiment (Figure 6A), the activity of the MAP kinases was to be increased in this experiment. The activity of the MAP kinase in quiescent cells is relatively low (Graf et al., 1997; Pizon & Baldacci, 2000; Hatton et al., 2003; Kuramochi et al., 2004; Mackeigan et al., 2005), and we observed low activation of MAP kinases in the hBSM cells used in this study. It is difficult to observe the alteration of the MAP kinase activity during a change of MLC20 phosphorylation. Thus, we increased the intracellular levels of the phosphor MAP kinases by adenovirus-mediated overexpression of active human MAP-ERK kinase kinase-1 (MEKK1), the upstream molecule of MAP kinases in hBSM cells.

The generation of MEKK1-containing adenovirus is described in brief as follows. To overexpress MEKK1, an HA-tagged 4.9-kb cDNA for active human wild-type MEKK1 was cloned at the downstream of cytomegalovirus (CMV) promoter of pAdtrack, a shuttle vector that transports target gene MEKK1 to the viral plasmid. To prepare the MEKK1-containing viral DNA, the shuttle vector was recombined with the adenoviral DNA in bacteria. Then, the recombinant adenoviral plasmid was used to produce the adenovirus in packaging cells following conventional protocol for adenovirus preparation. Two control adenoviruses were prepared in the same procedure but human MEKK1 was replaced by the β-galactosidase (LacZ) or green fluorescent protein (GFP). The control virus helped us to distinguish the MEKK1 effects from that of non-MEKK1 virus infection. After the amplification, the viruses were titrated by the agarose overlay plaque assay.

The viral infection efficiency in the cultured hBSM cells was examined by observing the percentage of the GFP-positive living cells under a fluorescence microscope. The viral expressive efficiency of MEKK1 was assayed in an independent experiment by Western blotting using an antibody against the HA-Tag (Deng et al., 2011). The exogenous MEKK1 activity expressed by the adenovirus was estimated by its capacity to activate the downstream molecules, including the JNK, ERK, and p38. These results show that the infection efficiency of the adenoviruses in the cultured hBSM cells reached up to 99% (the cell density was 70% when infected). The MEKK1 expressed by the adenovirus activated strikingly JNK1 and JNK2. In the meantime, it also activated the ERK1 and p38 kinase to a lower extent. Then, the MEKK1 adenovirus was used for the evaluation of the roles of the MAP kinase on MLC20 phosphorylation. When the cell density was between 60–70%, the hBSM cells in 6-well plates were infected with the MEKK1 and the control viruses at different doses (Deng et al., 2011). Three days after infection, the cells were harvested for Western blotting. The experimental results show that the MEKK1 adenovirus infection inhibited the MLC20 phosphorylation significantly (line 3 in Figure 6B). The inhibition of the MAP kinase pathway for the MLC20 phosphorylation is already hinted in Figure 6A but the extent of inhibition is beyond our expectation. Apparently, the inhibitory efficiency depends on viral infection efficiency, expressive capacity of MEKK1 adenovirus, and activating ability of MEKK1 for the downstream molecules.

In addition, we set up other treatments, using MAP kinase inhibitors to counteract the activation of the MEKK1 for the MAP kinases so that we could evaluate more accurately the role of the MEKK1-activated MAP kinase pathway in the regulation of the MLC20 phosphorylation. If it is true that the MEKK1 regulates negatively the MLC20 phosphorylation, the MAP kinase inhibitors would augment the MLC20 phosphorylation through suppression of the activity of the MEKK1 downstream molecules. As expected, the MAP kinase inhibitors neutralized the inhibition of the MEKK1 adenovirus on the MLC20 phosphorylation (lines 4–6 in Figure 6B). Likewise, the actin bands are detected to show a similar amount of loaded proteins. Obviously, the experimental results and even conclusions depend on the balance between the viral doses and inhibitor concentrations. A relatively low-concentration inhibitor may not affect the role of a relatively high-dose infection of MEKK1 adenovirus in MLC20 phosphorylation, and vice versa.

The dose-response relationship is a mathematic relationship between the dose and the organism's reaction to the dose. It describes the change in reaction or effect on an organism caused by differing levels of doses. Herein, we used dose-gradient methods to provide additional evidence that the MLC20 phosphorylation is regulated by the MAP kinase pathways. In the studies, the hBSM cells were exposed to different concentrations of the MAP kinase inhibitors for 40 minutes, followed by Western blotting assays for the phosphor MLC20. The results show that phosphor MLC20 level increases with the doses of inhibitors (Figure 6C and D, ERK inhibitor not shown) (Deng et al., 2011). A similar method was used to examine the effects of the MEKK1 at different infection doses (i.e., pfu/cell) on the MLC20 phosphorylation. The phosphor MLC20 decreased with doses of the MEKK1 adenovirus (Figure 6 E and F).

At this point, our experiments have demonstrated the negative regulation of MAP kinase pathway on the MLC20 phosphorylation.

8. Suggestions for the future investigation

The role of the MLC20 phosphorylation in contraction needs to be reassessed, even though it is accepted widely as a trigger of muscle contraction. A number of experiments have already verified the role of the MAP kinase pathway in the migration of different cell types and smooth muscle contraction. Therefore, according to current views, it is reasonable to assume that the MAP kinases would phosphorylate the MLC20, either via one or more enzymes. However, a series of experiments above showed the negative regulation of the MAP kinase pathways on the MLC20 phosphorylation. Because of conflict between our conclusion and current views about the mechanism of the MLC20 phosphorylation, we subsequently carried out a preliminary experiment to explore the synchrony between the cell migration and MLC20 phosphorylation. The migrating cells were fixed immediately and the phosphor MLC20 inside cells was immunostained with a specific antibody. However, we did not see a difference in the fluorescence intensity of the phosphor MLC20 between the migrating and quiescent cells (Deng et al., 2011).

In addition, when laboratory animals are killed, the muscle is usually relaxed, or rapidly relaxed in seconds (except for some smooth muscles). Because muscle contraction and relaxation are rapid, the MLC20 phosphorylation and dephosphorylation are also very fast, especially in the skeletal muscle. Even for smooth muscles, the muscle contraction and relaxation, such as the bladder emptying by detrusor and relaxation of tracheal/bronchial smooth muscle elicited by agents, may be fast. In one word, the MLC20 is phosphorylated in contracting muscle but unphosphorylated in relaxed muscle, and the transformation between the phosphorylation and nonphosphorylation is rapid. The muscle samples used in a lot of published articles for the MLC20 phosphorylation are almost in non–contracting status. However, those reports show that the phosphorylated MLC20 in various types of muscles are actually in relaxed state. Besides, a lot of data from different laboratories demonstrate that the MLC20 is phosphorylated strongly in cultured cells. As we all know, cells in culture dishes stop moving and are considered quiescent cells when they reach a high density like 90%. This suggests that the MLC20 is heavily phosphorylated in nonmigrating cells. Up to now, there is no direct evidence to support that the MLC20 phosphorylation triggers muscle contraction.

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