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Focal Adhesion Signaling is Required for Myometrial ERK Activation and Contractile Phenotype Switch Before Labor

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Abstract In late pregnancy rapidly increasing fetal growth dramatically increases uterine wall tension. This process has been implicated in the activation of the myometrium for labor, but the mechanisms involved are unclear. Here, we tested, using a rat model, the hypothesis that gestation-dependent stretch, via activation of focal adhesion signaling, contributes to the published activation of myometrial ERK at the end of pregnancy. Consistent with this hypothesis, we show here that ERK is targeted to adhesion plaques during late pregnancy. Furthermore, myometrial stretch triggers a dramatic increase in myometrial contractility and ERK and caldesmon phosphorylation, confirming the presence of stretch sensitive myometrial signaling element. Screening by anti-phosphotyrosine immunoblotting for focal adhesion signaling in response to stretch reveals a significant increase in the tyrosine phosphorylated bands identified as focal adhesion kinase (FAK), A-Raf, paxillin, and Src. Pretreatment with PP2, a Src inhibitor, significantly suppresses the stretch-induced increases in FAK, paxillin, Src, ERK and caldesmon phosphorylation and myometrial contractility. Thus, focal adhesion-Src signaling contributes to ERK activation and promotes contraction in late pregnancy. These results point to focal adhesion signaling molecules as potential targets in the modulation of the myometrial contractility and the onset of labor. J. Cell. Biochem. 100: 129–140, 2007.

Key words: focal adhesion signaling; myometrium; labor; ERK activation; ERK targeting and Src

The initiation of labor is a complex process, resulting from multiple maternal-fetal interactions. While endocrine, paracrine, and autocrine mechanisms of parturition play important and well-known roles in the activation of myometrium and initiation of labor, the effect of stretch on myometrial activation for labor is relatively little studied. The end of a successful pregnancy marks the time of most rapid fetal growth. The fetal growth rate after 34 weeks of pregnancy is 7× of the rate at 15 weeks. [F. Gary Cunningham et al., 2005]. This rapid growth is generally assumed to lead to a dramatic increase in uterine tension, which is thought to promote the onset of labor [Challis et al., 2000]. It is also clear that multiple gestation pregnancies and polyhydramnios, conditions associated with increased tension/stretch on the uterine wall, cause an increased incidence of premature labor [Slattery and Morrison, 2002]. Thus, there is a strong clinical impression that mechanotransduction contributes to the initiation of the labor and suggests that myocytes are able to sense mechanical tension from the growth of the fetus and adaptively generate the biochemical responses to the mechanical signals.

Focal adhesions (FA) make direct contact with the extracellular matrix (ECM), providing a structural link between the actin cytoskeleton and the ECM. Thus, FA have been postulated as

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mechano-sensors and sites for protein tyrosine kinase mediated signaling pathways [Burridge and Chrzanowska-Wodnicka, 1996]. However, few studies have been focused on this topic in the setting of pregnancy and labor [Macphee and Lye, 2000; Williams et al., 2005]. Studies from the Lye laboratory have suggested that endocrine pathways may not be sufficient alone to bring about myometrial activation for labor, and that additional pathways involving mechanical tension on the myometrium may also be involved [Macphee and Lye, 2000], but the signaling pathways linking mechanical forces to myometrial activation have not been delineated.

We have previously reported that labor in the rat is associated with a significant increase in the phosphorylation of extracellular signal-regulated kinase (ERK) and caldesmon (CaD) [Li et al., 2003], and that an ERK inhibitor delays preterm labor in a rat model [Li et al., 2004]. CaD is an actin-binding, myosin inhibitory protein [Morgan and Gangopadhyay, 2001]. ERK-mediated phosphorylation of CaD is known to reverse CaD's inhibitory effects on actomyosin interaction [Huang et al., 2003] and we have suggested that this mechanism facilitates contraction of the uterus during labor [Li et al., 2003]. However, the upstream events responsible for ERK activation during labor remain elusive. Evidence for ERK involvement in focal adhesion mediated signaling in cultured non-muscle cell types has been reported [Renshaw et al., 1997; Fincham et al., 2000]. However, how focal adhesion stimulation at the cell periphery is spatially transmitted to activate ERK that is presumed to be located in the cytosol or nucleus of the cell is unknown. Furthermore, nothing is known about the actual spatial distribution of ERK in differentiated myometrial cells during pregnancy.

The present study tested the hypothesis that gestation-dependent stretch, via activation of FA signaling could be responsible for subsequent activation of contractility and ERK during late pregnancy. In vitro stretch of myometrial strips was used to study the effect of mechanical stretch on FA signaling, in the absence of circulating hormones and neuronal inputs. We report here that: (1) The subcellular location of ERK is gestation-dependent, with ERK being targeted to FA plaques at late term of pregnancy when stretch is greatest; (2) Myometrial stretch in vitro leads to the phosphorylation of FA signaling proteins, ERK and CaD, and an increase in contractility; (3) A-Raf is an intermediate kinase that transduces FA signaling to ERK/CaD pathway activation; and (4) Stretch-dependent increases in contractility as well as phosphorylation of FA proteins, ERK and CaD are blocked by a Src inhibitor, consistent with this hypothesis.

MATERIALS AND METHODS

Animals and Tissue Collection

All procedures were approved by the BBRI Institutional Animal Care and Use Committee. Sprague–Dawley primigravid pregnant rats were used for the experiments (Taconic, Germantown, NY). For pregnant rats, day 1 = sperm positive, day 0 was arbitrarily taken as midnight after mating. Timed pregnant rats were euthanized by carbon dioxide inhalation, followed by cervical dislocation. Excised uteri were immersed immediately in an oxygenated (95% O_2, 5% CO_2) PSS solution at room temperature. The composition of the PSS was (mM): 120 NaCl, 5.9 KCl, 11.5 Dextrose, 25 $NaHCO_3$, 1.2 $NaH_2PO_4{\cdot}H_2O$, 1.2 $MgCl_2{\cdot}6H_2O$, and 2.5 $CaCl_2$.

Tissue Preparation and Force Recording

The whole-thickness uterine strips were microdissected to strips approximately $8 \times 2 \times 2$ mm, and oriented parallel to the longitudinal axis of muscle bundles. Four myometrial strips were dissected from each rat for in vitro stretch experiments. Isometric force was recorded at 37°C as previously described [Li et al., 2003]. Preparations were allowed to equilibrate at slack length for 3 h before initiation of experiments. For in vitro stretch experiment, the strips were stretched to 50 mN (millinewton) passive tension. The tension was applied gradually over 20–30 s and maintained at 50 mN for the duration of experiment. The contractile activity was digitalized with MacLab/8e, Chart v3.5.4 (AD Instrument, Castle Hill, Australia).

Single Cell Isolation

The single uterine smooth muscle cells were enzymatically isolated from fresh myometrial samples by a modified method developed for vascular smooth muscle [Gallant et al., 2005]. Briefly, for about 50 mg (wet weight) of freshly isolated myometrium (longitudinal smooth muscle strips, cut in size about $1 \times 1 \times 3$ mm),

the digestion medium consisted of 5.1 mg type II collagenase (Worthington, 252 U/mg), 2.1 mg elastase (Sigma, St. Louis, MO Grade II, 4 U/mg) and 5,000 U soybean trypsin inhibitor (type II-S, Sigma) in 7.5 ml of Ca^{2+}-Mg^{2+}-free HBSS, plus 0.2% BSA, GIBCO). The tissue was digested at 34°C for 30 min with oxygenation and gentle shaking. The digestion was repeated in an identical digestion medium with the exception of lowering collagenase to 2.5 mg. Dissociated single cells were poured over glass coverslips and plated on ice for 60 min. The cells were allowed to sediment by gravity onto the coverslips. Isolated cells from one coverslip of each digestion were tested to confirm shortening in response to 51 mM KCl-PSS at room temperature.

Immunofluorescence Staining and Digital Confocal Microscopy

Freshly isolated the cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum and incubated with the appropriate primary antibody overnight at 4°C. Repeated washes removed any cells not strongly adherent. Images were acquired using a Kr/Ar laser (Bio-Rad Radiance 2000) scanning confocal microscope equipped with a Nikon 60X (NA1.4) oil immersion objective in 0.4 μm step size.

Center optical sections were determined by visual inspection of the nuclear location. Surface optical sections were obtained as the highest optical section that could be clearly focused. To quantify translocations, computer-generated line scans (three samples per cell) were used to measure the fluorescence intensity at the cell edge and at the center of the cell in center optical sections.

All cells on each coverslip that met standard lab criteria of length and intensity of staining were included in quantitative analysis. All cells studied had lengths above 120 μM for nonpregnant and 180 μM for pregnant myometrial cells and signal intensities more than 10-fold greater than background.

For co-localization experiments, the lack of cross talk between the fluorophores was confirmed by demonstrating that single-labeled red cells gave no detectable signals on the green channel and single-labeled green cells gave no signal on the red channel at the same settings used to collect data from co-labeled cells.

Immunoblotting

Strips were quick-frozen at 2, 5, and 10 min after a mechanical stretch of 50 mN in dry ice/trichloroacetic acid/acetone slurry containing 10 mM DTT. Also, the lengths of the strips were measured after stretch as to compare to the slack length. The frozen samples were homogenized as previously described [Li et al., 2004]. Protein-matched samples were boiled, separated on an SDS–PAGE, transferred to a PVDF membrane and analyzed by western blotting with appropriate antibodies. The intensity of signals was quantitated by using an Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE).

Immunoprecipitation

The myometrial smooth muscle strips were frozen in liquid nitrogen and pulverized with a mortar and pestle. The tissue powder was dissolved in a buffer containing 50 mM Tris (pH 7.4), 4% glycerol, 1 mM EDTA, 150 mM NaCl, 1% NP40, 1% Na deoxycholate, 20KIU aprotinin, 5 μM leupeptin, 5 μM pepstatin A, 2 mM Na_3VO_4, and 1 mM NaF and centrifuged at 12,000 rpm at 4°C for 15 min. The supernatant was precleared with protein A agarose and then incubated with anti-phospho tyrosine conjugated agarose beads overnight at 4°C with gentle rotation. The immune complex beads were washed, resuspended in sample buffer (25 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 5% β-mercaptoethanol, 1 mM EDTA, and 0.001% bromophenol blue), and boiled at 100°C for 5 min. The proteins of interest were detected by western blotting with specific antibodies.

Materials and Antibodies

Pyrazolopyrimidine 2 (PP-2) was purchased from Calbiochem (La Jolla, CA). Protein A agarose and Vinculin monoclonal antibody (1:10,000) were products of Sigma. The phospho-p44/42 MAP kinase antibody (1:2,000) was purchased from Cell Signaling (Beverly, MA). The phospho-CaD antibody (1:600), phosphotyrosine antibody (1:1,500), MAP kinase1/2 antibody (1:250) were products of Upstate (Lake Placid, NY). C-Src antibody (1:1,000), c-Raf (1:500), A-Raf (1:1,500) and FAK antibodies (1:200) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The paxillin antibody (1:1,500) was a product of BD Transduction Laboratories (San Diego, CA).

Secondary antibodies conjugated with Oregon green 488 or Alexa 568 (1:250) for imaging were purchased from Molecular Probes (Eugene, OR).

Statistical Analysis

Data were analyzed and expressed as means ± SE unless it is specified otherwise. Data were compared by using an unpaired, two-tailed *t*-test. P values <0.05 were denoted as * or $^{\#}$ and $P < 0.01$ were denoted as ** or $^{++}$.

RESULTS

ERK is Targeted to the Cell Surface During the Late Pregnancy

The subcellular localization of ERK in myometrial smooth muscle and whether that localization displays a gestation-dependence is unknown. In the present study, we determined ERK localization by quantitative confocal microscopy of freshly dissociated, fully contractile rat uterine smooth muscle cells. As is shown in Figure 1, in myometrial cells isolated from nonpregnant rats, using an antibody that recognizes only ERK bands in an immunoblot of whole cell homogenate (Fig. 1A), ERK is homogeneously distributed in the cytosol (Fig. 1B). During the hypertrophy phase (at early pregnancy, day 9), ERK remains in the cytosol. Pregnancy in the rat has a duration of 23 ± 0.24 SD days [Li et al., 2004]. Interestingly, in late pregnancy (day 20 to day 21), as is shown in Figure 1B and C, ERK translocates to the cell surface. We quantified the ERK distribution by line scan analysis [Gallant et al., 2005], measuring confocal fluorescence intensity of a

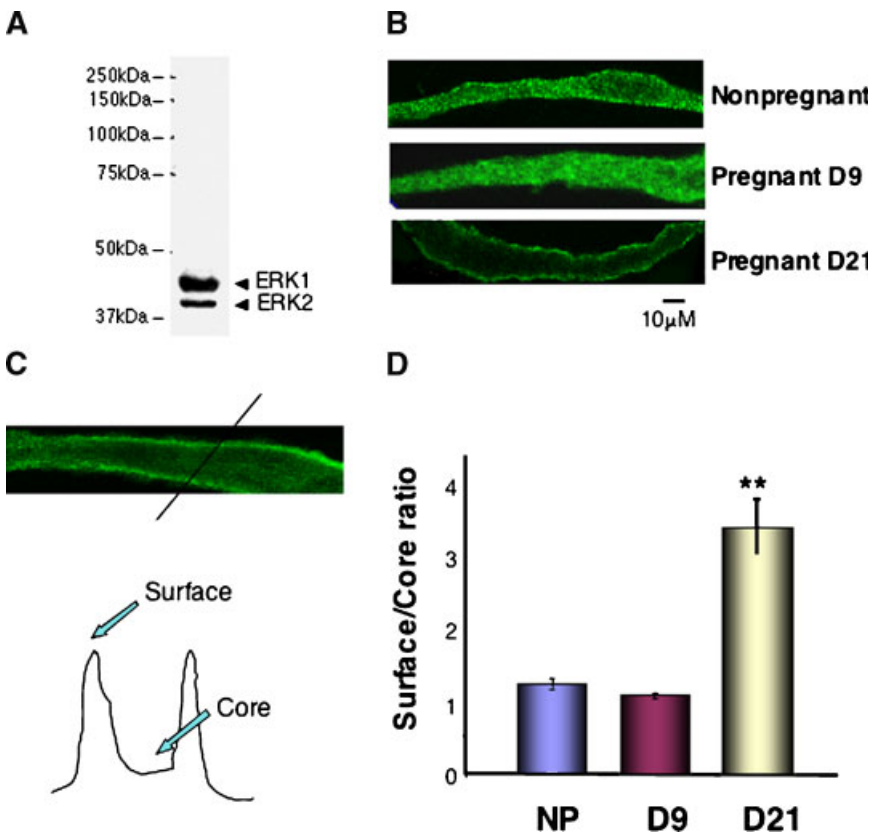

Fig. 1. ERK targeting is gestation-dependent. **A**: ERK immunoblot of myometrial homogenate from a 20-day pregnant rat demonstrating specificity of the antibody used for imaging. **B**: Confocal center optical sections of single, freshly isolated myometrial cells immunostained for ERK. The single myometrial cells are isolated from different rats and immunostained with total ERK antibody in different experiments. Thus, the absolute intensity of ERK signals should not be directly compared between different images. Rather, the images are shown to illustrate typical patterns of distribution of ERK. Bar, 10 μM. **C**: Method for quantification of ERK distribution by measurement of a ratio of the digital fluorescence at the cell surface to that at cell core in a day 21 of pregnancy cell. **D**: Average surface/core ratios. $^{**}P < 0.01$ compared to NP (nonpregnant) and D9 (pregnant day 9) groups. There is no significant difference in the ERK distribution between NP and D9 groups ($P = 0.2$). Data were collected from myometrium isolated from 3 rats and 18–24 cells studied in each group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

center optical section at the edge of the cell and the center of the cell, expressing the data as surface/core ratios (Fig. 1C and D). In myometrial cells from nonpregnant and day 9 pregnant rats, the ratios are 1.26 ± 0.06 and 1.1 ± 0.04, respectively. In late pregnancy, the ratio increases significantly to 3.47 ± 0.39 ($P < 0.0001$ compared to early pregnancy).

Plasmalemmal ERK Co-Localizes With Vinculin in Myometrial Focal Adhesion Plaques

To test the hypothesis that ERK targeting to the cell cortex is related to FA signaling, we determined whether plasmalemmal ERK co-localizes with vinculin as a marker of focal adhesion plaques. Freshly isolated myometrial cells from pregnant day 21 rats were co-stained for ERK1/2 and Vinculin. As is shown in Figure 2, ERK and vinculin co-localize, not completely, but to a high degree (see yellow in composite), both in center optical sections (Fig. 2A), and, in more definitive optical surface sections (Fig. 2B see arrows in insets). Vinculin staining shows the characteristic pattern of adhesion plaque staining in differentiated smooth muscle cells [Moore et al., 2004; Gallant et al., 2005; Marganski et al., 2005]. The antibody used recognizes only vinculin isoforms in a myometrial homogenate (Fig. 2C).

Myometrial Stretch In Vitro Increases Myometrial ERK2 and Caldesmon Phosphorylation

To further test the hypothesis that gestation-dependent stretch is, at least in part, responsible for the activation of ERK and phosphorylation of CaD during the late pregnancy, the effect of in vitro stretch on ERK and CaD phosphorylation was investigated. Myometrial strips from 20-day pregnant rats were equilibrated without stretch for 3 h. Then the strips were stretched to 50 mN (millinewton) passive tension and maintained at 50 mN for the duration of experiment. The final length of strips after stretch is applied is comparable of that the length of uterine segment measured in vivo with the fetus inside (17.75 ± 1 mm vs. 17.43 ± 0.6 mm, $P = 0.29$). Strips at slack length without stretch in vitro (6.5 ± 0.3 mm) were used as a control group.

As is shown in Figure 2D, the levels of phosphorylation of ERK increase within 2 min after the application of stretch and the increase is maintained through 10 min ($P < 0.05$); interestingly, the levels of phospho-CaD increase with stretch but the increase is not significant until 10 min after the application of stretch ($P < 0.001$, Fig. 2E). A significant lag, between ERK phosphorylation and ERK-mediated CaD phosphorylation has also been observed in vascular smooth muscle after agonist activation [Je et al., 2004].

Myometrial Stretch Induces Significant Tyrosine Phosphorylation and Activates Focal Adhesion Signaling

The above data implicate FA signaling in myometrial ERK activation. FA signaling is known to involve tyrosine phosphorylation of many proteins, whereas most other previously reported signaling pathways regulating contractility of differentiated smooth muscle involve serine/threonine phosphorylation. Thus we screened myometrial homogenates by anti-phosphotyrosine immunoblotting. Myometrium was harvested from 20-day pregnant rats. After a 3 h-equilibration in serum-free organ culture, myometrial strips were stretched and maintained at a passive tension of 50 mN for 10 min. As is shown in Figure 3A, myometrial stretch induces a significant increase in the tyrosine phosphorylation of several proteins, the most prominent of which are 125, ~68, and 60 kDa proteins. In a manner similar to what is seen with ERK activation, tyrosine phosphorylation of these proteins is, on average, maximally increased within 2 min after the application of the stretch stimulus (Fig. 3B). The molecular weights of these bands are typical of the adhesion plaque proteins focal adhesion kinase (FAK), paxillin and Src.

To definitively identify these tyrosine-phosphorylated proteins, the protein extracts were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody, and the immune complexes were analyzed by immunoblotting with protein-specific antibodies. As shown in Figure 3C, mechanical stretch significantly increases the amount of FAK (p125), paxillin (p68), and Src (p60) in the phospho-tyrosine immunoprecipitates, compared to resting, unstretched control strips.

A-Raf is an Intermediate Kinase That Links FA Signaling to ERK/CaD Phosphorylation

The availability of two-color infrared Odyssey immunoblot detection enabled us to detect two proteins of interest in the same immunoblot simultaneously, and thus we confirmed that

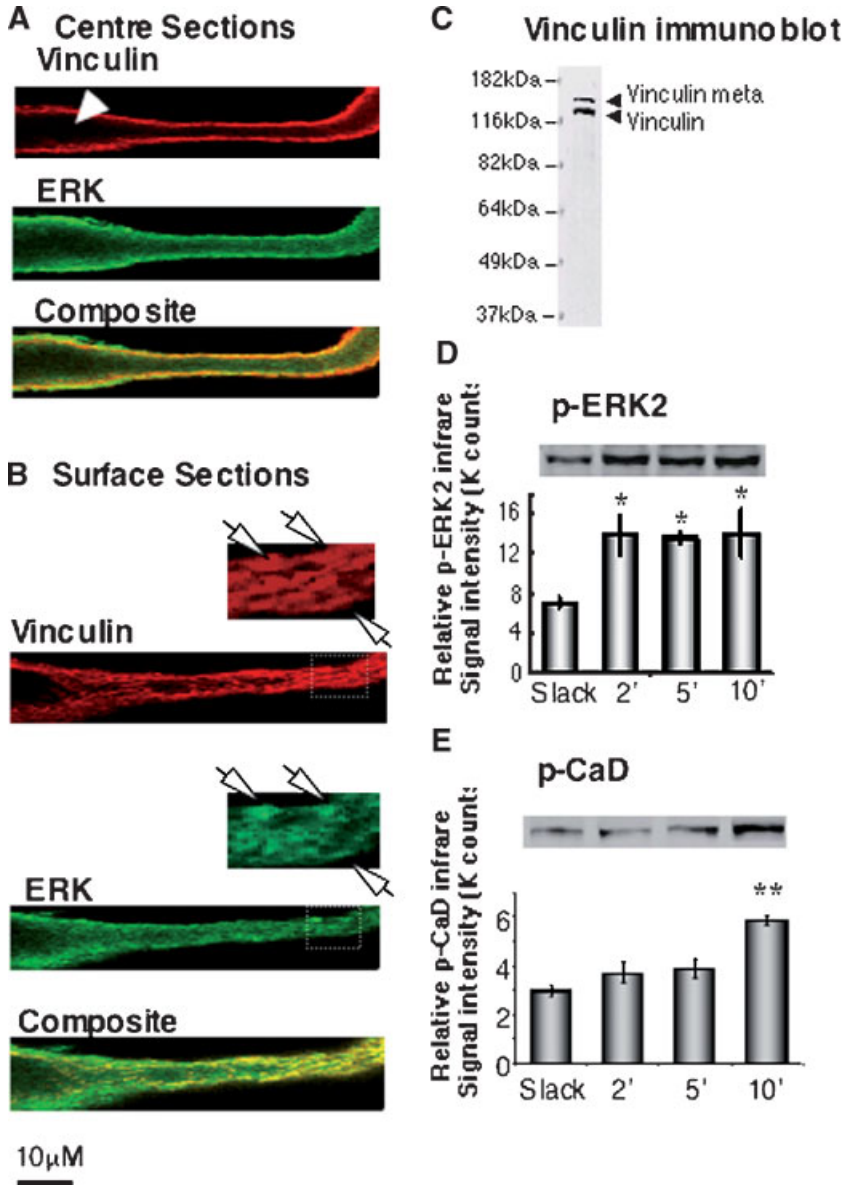

Fig. 2. ERK co-localizes with vinculin in adhesion plaques in late pregnancy and mechanical stretch leads to phosphorylation of ERK and CaD. **A**: Center optical sections of a freshly dissociated myometrial cell, co-stained for vinculin and ERK. Arrowhead indicates the position of the nucleus. **B**: Surface sections from the same myometrial cell co-stained for ERK and vinculin. Composite images show overlap (yellow) of ERK and vinculin in discrete plaque-like structures. Insets show expanded views of Vinculin and ERK staining. Bar, 10 μM. **C**: A vinculin immunoblot of myometrial homogenate from a 20-day pregnant rat demonstrating specificity of the antibody used for imaging. **D**: Densitometry and typical immunoblot with a phospho-ERK antibody of myometrial samples stretched to 50 mN for indicated time in vitro. Signals are normalized by actin protein levels. Data were collected from myometrium isolated from 3 rats and 3 strips were used for each data point. **E**: Densitometry and typical immunoblot with a phospho-CaD antibody. Signals are normalized by actin protein levels. Data were collected from the myometrium isolated from 3 rats and 3 strips for each data point. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared to resting samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tyrosine phosphorylated 125 and 60 kDa bands precisely overlap with signals from FAK and Src specific antibodies respectively (Fig. 3D, in yellow), again, confirming the activation of FA signaling in myometrium in the response to the stretch stimulation. To our surprise, the signal from paxillin does not exactly overlap with the predominant 68 kDa phosphotyrosine band (Fig. 3D), despite the fact that immunoprecipitation confirms that paxillin is a participant in the FA signaling in response to mechanical signals.

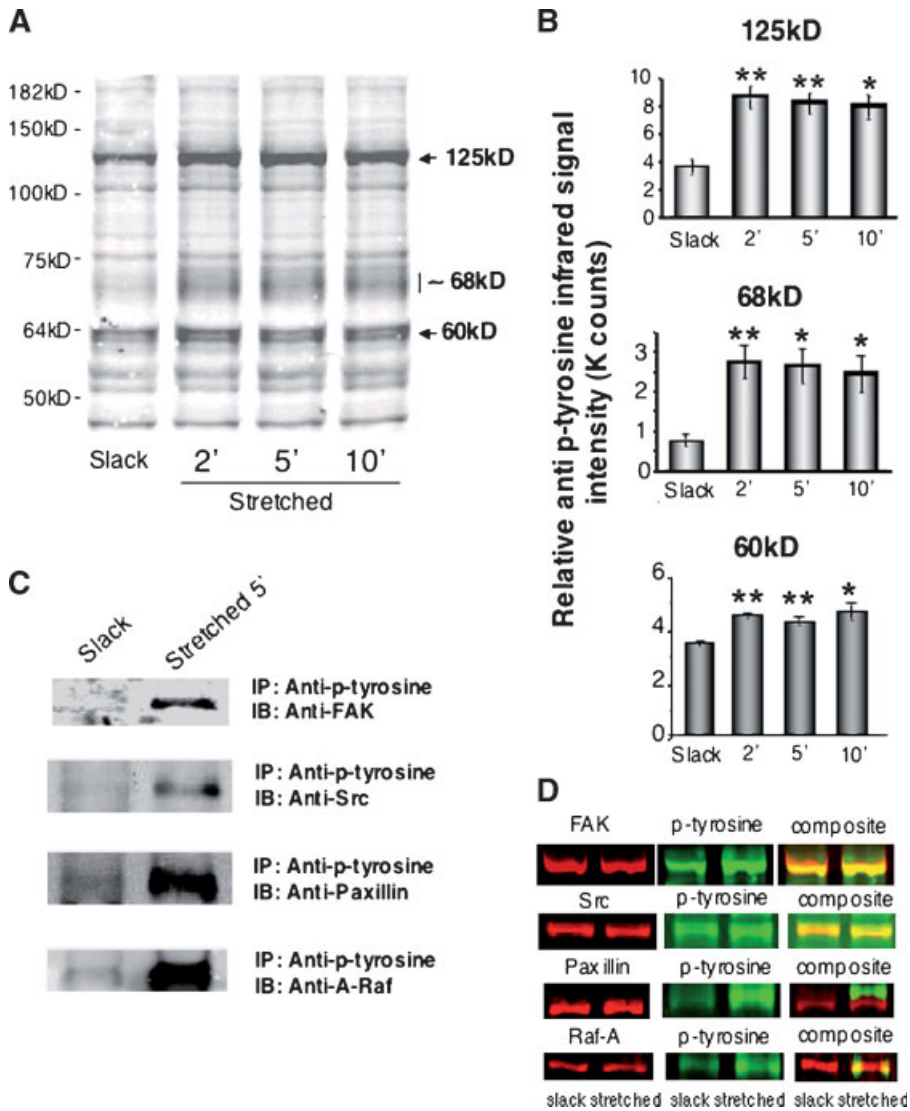

Fig. 3. Identification of proteins tyrosine phosphorylated in response to myometrial stretch. **A**: Phosphotyrosine immunoblot of myometrial smooth muscle strips stretched to 50 mN for the indicated time. Note prominent stretch-induced increases in a band at 125 kDa, a diffuse staining at ~68 kDa and a band at 60 kDa. **B**: Average densitometry of 125, 68, and 60 kDa phosphotyrosine bands. Data were collected from the myometrium isolated from 3 rats and 3 strips for each data point. $^{*}P < 0.05$, $^{**}P < 0.001$ compared to resting group. **C**: Myometrial lysates of unstretched and stretched samples immunoprecipitated with phospho-tyrosine antibody and then analyzed by SDS–PAGE, immunoblotted with FAK, Src, paxillin, and A-Raf antibodies respectively (three rats, three myometrial strips in each group). **D**: Two-color western blotting confirmed the co-localization of phospho-tyrosine 125, 60, and ~68 kDa bands with FAK, Src, and A-Raf proteins, respectively (in yellow), with the exception of paxillin. Similar results were obtained from 4 to 16 samples and 2 to 8 rats for each group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Other phospho-tyrosine containing proteins in the molecular weight range of 68 kDa include mitogen activated protein kinase kinases (MAPKKs) and MAPK kinase kinases (MAPKKKs). Possible candidates are c-Raf (74 kDa, Tyr-341), A-Raf (68 kDa, Tyr-302) and PKC delta (78 kDa, Tyr-311). However, no signals are detected in the phospho-tyrosine immunoprecipitates by using c-Raf and PKC delta-specific antibodies, despite of the presence of each protein in the myometrium (data not shown). Also, the phospho-tyrosine bands in this molecular weight do not overlap on two-color immunoblot with those for c-Raf or PKC delta (data not shown). In contrast, as shown in Figure 3C, A-Raf is significantly increased in the p-tyrosine immunoprecipitates after application of in vitro stretch. Accordingly, when phosphotyrosine signals (in green) and A-Raf (in red) are detected simultaneously in lysates

of slack and stretched myometrium, they are found to overlap (yellow) (Fig. 3D). The above data indicate that A-Raf participates in stretch sensitive myometrial FA signaling.

A Src Inhibitor Prevents Tyrosine Phosphorylation of Focal Adhesion Signaling Proteins and ERK2 Phosphorylation

Src family members have been implicated in the activation and phosphorylation of FAK [Salazar and Rozengurt, 1999, 2001]. To test whether there is a cause and effect relationship between stretch-induced Src phosphorylation and ERK phosphorylation as well as FAK and Raf-A tyrosine phosphorylation, we used PP-2, a potent and relatively selective inhibitor of the Src family of protein tyrosine kinases. After a 3 h-equilibration in serum-free organ culture, myometrial strips were pre-treated with 3 and 10 μM of PP2 for 30 min, then stretched and maintained at 50 mN for 10 min. Pre-treatment with PP2 prior to in vitro stretch causes a significant and dose-dependent inhibition of stretch-induced FA signaling (tyrosine phosphorylation of p125, ~p68 and p60), as well as phosphorylation of ERK2 and CaD compared to the vehicle-control samples (Fig. 4).

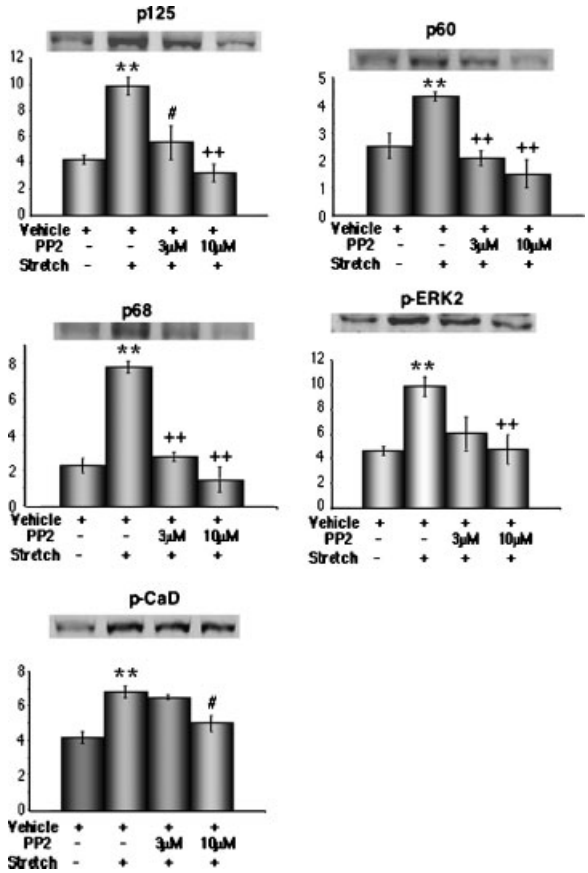

Fig. 4. A Src inhibitor, PP2, significantly inhibits stretch-induced phosphorylation. Typical immunoblots are shown above average densitometry for each protein in the presence or absence of stretch and the presence or absence to two concentrations of PP2. In the slack and stretch groups, the same amount of vehicle (ethanol) was used for control. Data were collected from the myometrium isolated from three rats and three strips for each data point. $^{**}P < 0.001$ compared to unstretched samples. $^{\#}P < 0.05$ $^{++}P < 0.001$ compared to stretched samples without PP2 treatment.

Mechanical Stretch Increases Myometrial Contractility in a Src-Dependent Manner

The above data demonstrate the existence of stretch-sensitive elements in the pregnant myometrium that result in ERK activation. Our past work suggests that ERK activation should lead to corresponding changes in contractility [Li et al., 2003]. As shown in Figure 5A, induced stretch quickly transforms the relatively quiescent status typical of pregnancy to a highly active contractile state, characterized by a significant increase in the frequency of large contractions. The complex changes in rhythmic contractions were quantified by measuring the area under the active tension curve (AUC). The AUC increases in a highly significant manner in response to mechanical stretch (Fig. 5C). Pretreatment with PP2, dramatically and in a dose-dependent manner, supresses the increase in contractility in response to stretch (Fig. 5B and C).

DISCUSSION

The main findings of the present study are (1) ERK targeting to focal adhesion plaques in myometrial smooth muscle is gestation-dependent; (2) Mechanical stretch of myometrium in vitro activates focal adhesion signaling and results in increased ERK and CaD phosphorylation; (3) Inhibition of Src tyrosine kinase activity significantly supresses stretch-dependent myometrial contractility, together with focal adhesion signaling, Raf-A, ERK, and CaD phosphorylation. Together, these findings suggest that focal adhesion signaling proteins may provide novel targets for the development of potential therapeutics in the setting of preterm labor.

The activation of myometrial ERK as a result of stretch-induced focal adhesion signaling is a novel concept. We have shown here that focal

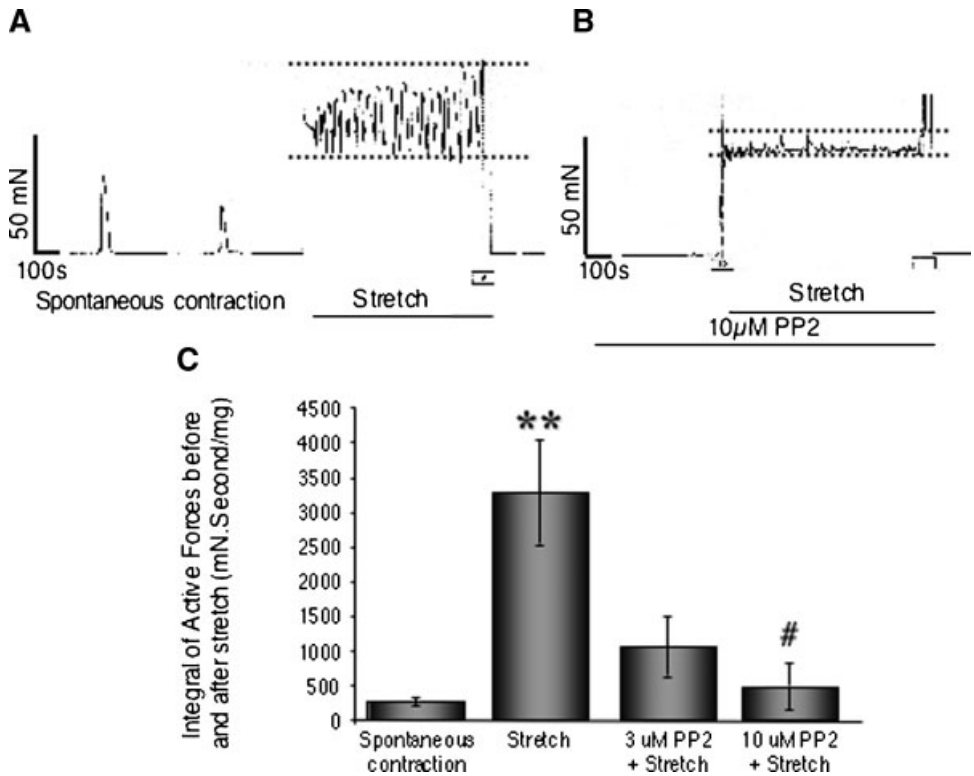

Fig. 5. In vitro stretch induces Src-dependent increases in myometrial contractility. **A**: Representative myograph recording of contractile force from uterine smooth muscle strips from 20-day pregnant rats in the response to in vitro stretch. A 50 mN tension was maintained for 10 min during the stretch. **B**: Representative myograph recording during identical stretch after pretreatment with 10 μM PP2 for 30 min. Note that stretch-induced *active* force (the force between the dotted lines in panel A and B) is essentially abolished by PP2. **C**: Quantitation by measurement of the Area under curve (AUC). AUC is the integral of the *active* force trace over a 10 min time period before and after stretch. $^{**}P < 0.001$ compared to spontaneous contraction. $^{\#}P < 0.05$ compared to stretched strips without PP2 treatment. Data were collected from the 14 myometrial strips and 7 rats for spontaneous contraction and stretch groups; Data were collected from 3 rats and 6 strips in PP2 treatment groups.

adhesion-dependent phosphorylation of ERK can occur independently of uterotonics. Past studies have linked myometrial ERK activation to signal transduction through G protein coupled receptors (GPCR), such as those activated by oxytocin and $PGF_{2\alpha}$ [Nohara et al., 1996; Ohmichi et al., 1997]. However, in vascular smooth muscle, ERK has been shown to be activated by stretch-dependent mechanisms [Franklin et al., 1997]. Similarly, integrin engagement and v-Src involvement have been demonstrated in non-muscle cells such as rat embryo fibroblasts to promote cell motility [Fincham et al., 2000]. Integrin engagement in non-muscle cells is known to lead to the activation of a variety of intracellular signaling events via recruitment of signaling molecules to focal adhesion sites [Schlaepfer and Mitra, 2004].

Little is known regarding focal adhesion signaling in myometrium. But, expression of both integrins and syndecans is known to be elevated in myometrium during late pregnancy and labor [Cluff et al., 2005; Williams et al., 2005], suggesting a possible role of focal adhesion signaling in the regulation of myometrial contractility. In the present study, we have linked gestation-dependent myometrial ERK targeting with focal adhesion signaling. Likely, the effects of focal adhesion signaling and GPCR signaling on ERK activation are additive in the in vivo setting of labor as illustrated in Figure 6. In this model, consistent with the results presented here, stretch-induced integrin clustering can lead to FAK phosphorylation [Schlaepfer and Mitra, 2004], creating docking sites for Src and paxillin. Furthermore, activated Src facilitates the maximal FAK activation through further phosphorylation of FAK [Schlaepfer and Mitra, 2004]. Tyrosine phosphorylation of A-Raf, presumably by Src [Marais et al., 1997], triggers a signaling pathway leading to MEK,

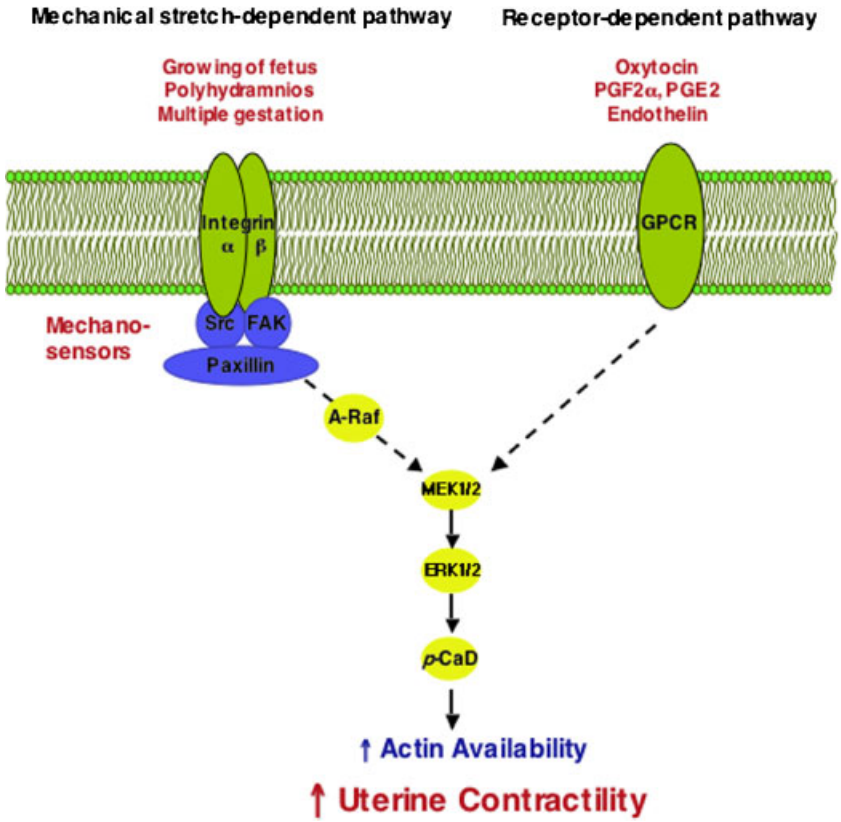

Fig. 6. Model of signaling involved myometrial ERK activation. In addition to classical GPCR-mediated pathways described by others, data presented here suggest that the activation of ERK by focal adhesion signaling also promotes myometrial contraction and contributes to the initiation of labor. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ERK, and CaD phosphorylation and activation of contractility.

We have previously reported that myometrium from day 16 and day 20 pregnant rats displays much lower frequency of spontaneous contractions compared with that from non-pregnant rats [Li et al., 2003], consistent with the clinical impression of a "quiescent" status of uterine smooth muscle during pregnancy in the human. At the end of pregnancy, the rapidly growing fetus presents an increasing stretch stimulus to the uterine wall. As shown here, myometrial stretch alone can activate FA signaling, increase ERK and CaD phosphorylation and lead to a more contractile state of the myometrial smooth muscle (Fig. 5A). The data support the concept of stretch-sensitive elements in the pregnant myometrium that generate biochemical responses leading to increases in the potential capacity to generate forceful contractions and provide a mechanism, synergistic with mechanisms activated by uterotonics, whereby the switch from a quiescent state to highly contractile state can occur.

Stretch-induced phosphorylation of ERK2 and CaD peaks at 2 and 10 min, respectively. The question arises as to the mechanism of the significant delay between the phosphorylation of ERK2 and CaD. It has previously shown that in certain types of vascular smooth muscle, upon stimulation with an α-adrenergic stimulation, ERK initially translocates to the cell cortex, docks there transiently and, while at the cortex, becomes activated [Khalil et al., 1995]. After activation, ERK undergoes a second redistribution to the contractile filament to meet up with its substrate, CaD [Khalil et al., 1995]. ERK-mediated phosphorylation of CaD is known to dis-inhibit actomyosin interactions in vascular muscle [Dessy et al., 1998] and in vitro [Foster et al., 2004]. In the present study we found that a similar translocation of ERK from the cytosol to the surface membrane occurs in late pregnancy in myometrial cells (Fig. 1B) and precedes the previously reported phosphorylation of ERK that occurs during labor [Li et al., 2003]. Thus, the translocation of unphosphorylated ERK to the membrane appears

to precede activation that, in the case of myometrial cells, may "prime" the myometrium for the subsequent initiation of labor in response to hormonal and uterotonic agonist stimuli.

In the present study, our data suggest that A-Raf is an effector recruited by stretch-dependent FA stimulation as part of the activation of myometrial MAP kinase signaling. While A-Raf, B-Raf, and c-Raf (Raf-1) are similar in sequence and function, differential activation and regulation have been observed [Marais et al., 1997; Stokoe and McCormick, 1997]. For example, Src tyrosine kinases have been shown to be synergistic with Ras in the activation of c-Raf as measured by phosphorylation at Tyr-341 [Fabian et al., 1993]. B-Raf, in contrast, does not contain an analogous Src tyrosine phosphorylation site and is activated by Ras alone. A-Raf contains an analogous Src phosphorylation site at Tyr-302 and is more strongly activated by Src than Ras [Marais et al., 1997]. Thus, since Src is activated by myometrial stretch, it is not surprising to see a differential activation of A-Raf in this system.

In conclusion, we have observed that ERK is targeted to adhesion plaques in the cell cortex at late pregnancy, implicating ERK in "priming" myometrium for labor. We have also shown that myometrial stretch in vitro activates focal adhesion signaling, then increases in phosphorylation of A-Raf, ERK and CaD. Inhibition of tyrosine phosphorylation of focal adhesion signaling suppresses myometrial contractility, ERK and CaD phosphorylation in response to mechanical stretch. Our data suggest that the activation of ERK by focal adhesion signaling, in addition to classical GPCR-mediated pathways, facilitates myometrial contraction and plays a distinct role in the switch from the quiescent phase of pregnancy to a more contractile phenotype at the end of pregnancy.

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REFERENCES

Burridge K, Chrzanowska-Wodnicka M. 1996. Focal adhesions, contractility, and signaling. Annu Rev Cell Dev Biol 12:463–518.

Challis JRG, Matthews SG, Gibb W, Lye SJ. 2000. Endocrine and paracrine regulation of birth at term and preterm. Endocr Rev 21(5):514–550.

Cluff AH, Malmstrom A, Tingaker B, David G, Ekman-Ordeberg G. 2005. Normal labor associated with changes in uterine heparan sulfate proteoglycan expression and localization. Acta Obstet Gynecol Scand 84:217–224.

Dessy C, Kim I, Sougnez CL, Laporte R, Morgan KG. 1998. A role for MAP kinase in differentiated smooth muscle contraction evoked by α-adrenoceptor stimulation. Am J Physiol Cell Physiol 275:C1081–C1086.

Cunningham FG, Hauth JCH, Leveno KJ, Gilstrap L III, Bloom SL, Wenstrom KD. editors. 2005. Williams Obstetrics. Fetal Growth Disorders. 22nd edition.. Chapter 38: New York: McGraw-Hill. p 893.

Fabian JR, Daar IO, Morrison DK. 1993. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol Cell Biol 13(11):7170–7179.

Fincham VJ, James M, Frame MC, Winder SJ. 2000. Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. Embo J 19(12):2911–2923.

Foster DB, Huang R, Hatch V, Craig R, Graceffa P, Lehman W, Wang CL. 2004. Modes of caldesmon binding to actin: Sites of caldesmon contact and modulation of interactions by phosphorylation. J Biol Chem 279(51):53387–53394.

Franklin MT, Wang C-LA, Adam LP. 1997. Stretch-dependent activation and desensitization of mitogen-activated protein kinase in carotid arteries. Am J Cell Physiol 273:C1819–C1827.

Gallant C, You JY, Sasaki Y, Grabarek Z, Morgan KG. 2005. MARCKS is a major PKC-dependent regulator of calmodulin targeting in smooth muscle. J Cell Sci 118 (Pt 16):3595–3605.

Huang R, Li L, Guo H, Wang CL. 2003. Caldesmon binding to actin is regulated by calmodulin and phosphorylation via different mechanisms. Biochemistry 42(9):2513–2523.

Je HD, Gallant C, Leavis PC, Morgan KG. 2004. Caveolin-1 regulates contractility in differentiated vascular smooth muscle. Am J Physiol Heart Circ Physiol 286(1):H91–H98.

Khalil RA, Menice CB, Wang C-LA, Morgan KG. 1995. Phosphotyrosine-dependent targeting of mitogen-activated protein kinase in differentiated contractile vascular cells. Circ Res 76:1101–1108.

Li Y, Je HD, Malek S, Morgan KG. 2003. ERK1/2-mediated phosphorylation of myometrial caldesmon during pregnancy and labor. Am J Physiol Regul Integr Comp Physiol 284(1):R192–R199.

Li Y, Je HD, Malek S, Morgan KG. 2004. Role of ERK1/2 in uterine contractility and preterm labor in rats. Am J Physiol Regul Integr Comp Physiol 287(2):R328–R335.

Macphee DJ, Lye SJ. 2000. Focal adhesion signaling in the rat myometrium is abruptly terminated with the onset of labor. Endocrinology 141(1):274–283.

Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. 1997. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. J Biol Chem 272 (7):4378–4383.

Marganski WA, Gangopadhyay SS, Je HD, Gallant C, Morgan KG. 2005. Targeting of a novel Ca+2/calmodulin-dependent protein kinase II is essential for extracellular signal-regulated kinase-mediated signaling in differentiated smooth muscle cells. Circ Res 97(6):541–549.

Moore ED, Voigt T, Kobayashi YM, Isenberg G, Fay FS, Gallitelli MF, Franzini-Armstrong C. 2004. Organization of Ca2+ release units in excitable smooth muscle of the guinea-pig urinary bladder. Biophys J 87(3):1836–1847.

Morgan KG, Gangopadhyay SS. 2001. Invited review: Cross-bridge regulation by thin filament-associated proteins. J Appl Physiol 91:953–962.

Nohara A, Ohmichi M, Koike K, Masumoto N, Kobayashi M, Akahane M, Ikegami H, Hirota K, Miyake A, Murata Y. 1996. The role of mitogen-activated protein kinase in oxytocin-induced contraction of uterine smooth muscle in pregnant rat. Biochem Biophys Res Commun 229(3): 938–944.

Ohmichi M, Koike K, Kimura A, Masuhara K, Ikegami H, Ikebuchi Y, Kanzaki T, Touhara K, Sakaue M, Kobayashi Y, Akabane M, Miyake A, Murata Y. 1997. Role of mitogen-activated protein kinase pathway in prostaglandin F2alpha-induced rat puerperal uterine contraction. Endocrinology 138(8):3103–3111.

Renshaw MW, Ren XD, Schwartz MA. 1997. Growth factor activation of MAP kinase requires cell adhesion. Embo J 16(18):5592–5599.

Salazar EP, Rozengurt E. 1999. Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. J Biol Chem 274(40):28371–28378.

Salazar EP, Rozengurt E. 2001. Src family kinases are required for integrin-mediated but not for G protein-coupled receptor stimulation of focal adhesion kinase autophosphorylation at Tyr-397. J Biol Chem 276(21): 17788–17795.

Schlaepfer DD, Mitra SK. 2004. Multiple connections link FAK to cell motility and invasion. Curr Opin Genet Dev 14(1):92–101.

Slattery MM, Morrison JJ. 2002. Preterm delivery. Lancet 360(9344):1489–1497.

Stokoe D, McCormick F. 1997. Activation of c-Raf-1 by Ras and Src through different mechanisms: Activation in vivo and in vitro. Embo J 16(9):2384–2396.

Williams SJ, White BG, MacPhee DJ. 2005. Expression of alpha5 integrin (Itga5) is elevated in the rat myometrium during late pregnancy and labor: Implications for development of a mechanical syncytium. Biol Reprod 72(5): 1114–1124.