Cell and Tissue Research (2019) 376:443–456
https://doi.org/10.1007/s00441-018-02983-8

REGULAR ARTICLE

Mechanobiology of mice cervix: expression profile of mechano-related molecules during pregnancy

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Received: 16 July 2018 / Accepted: 11 December 2018 / Published online: 22 January 2019

Abstract

There is a known reciprocation between the chronic exertion of force on tissue and both increased tissue density (e.g., bone) and hypertrophy (e.g., heart). This can also be seen in cervical tissue where the excessive gravitational forces associated with multiple fetal pregnancies promote preterm births. While there is a well-known regulation of cervical remodeling (CR) by sex steroid hormones and growth factors, the role of mechanical force is less appreciated. Using proteome-wide technology, we previously provided evidence for the presence of and alteration in mechano-related signaling molecules in the mouse cervix during pregnancy. Here, we profile the expression of select cytoskeletal factors (filamin-A, gelsolin, vimentin, actinin-1, caveolin-1, transgelin, keratin-8, profilin-1) and their associated signaling molecules [focal adhesion kinase (FAK) and the Rho GTPases CDC42, RHOA, and RHOB] in cervices of pregnant mice by real-time PCR and confocal immunofluorescence microscopy. Messenger RNA and protein levels increased for each of these 12 factors, except for 3 (keratin-8, profilin-1, RHOA) that decreased during the course of pregnancy and this corresponded with an increase in gravitational force exerted by the fetus on the cervix. We therefore conclude that size or weight of the growing fetus likely plays a key role in CR through mechanotransduction processes.

Keywords Mechanobiology · Cervical remodeling · Mice · Cytoskeleton · Pregnancy

Introduction

When tissues, such as bone, skeletal muscle and heart are exposed to chronic force, they respond by either increasing their density (e.g., bone) or undergoing hypertrophy (e.g., heart) (Cowin and Hegedus 1976; Choudkroun et al. 1999; Buyandelger et al. 2014; Lyon et al. 2015). Moreover, such changes, in reciprocal fashion, serve to further increase the mechanical forces in these tissues. We suspect a similar situation occurs in the cervix during multiple fetal pregnancies, where the developing fetuses exert an excessive gravitational force that nearly always results in preterm births (Chauhan et al. 2010; Myers et al. 2015; Nott et al. 2016). Cervical remodeling (CR) is a complex biological process involving multiple factors (Timmons et al. 2010; Holt et al. 2011; Mahendroo 2012; Jorge et al. 2014). Most of our previous studies of CR regulation have focused on chemical cues, such as sex steroid hormones and growth factors (Zhao et al. 2013; Donnelly et al. 2013; Stanley et al. 2015, 2018). However, more recently, we used a proteome-wide technology to provide the first evidence of the presence and expression profile of mechano-related signaling molecules in the mouse cervix during pregnancy and cervical repair (Schwabe et al. 2014; Stanley et al. 2018).

CR can be divided into four unique overlapping stages: cervical softening, ripening, dilation and postpartum repair (Read et al. 2007; Timmons et al. 2010; Jorge et al. 2014). In mice, the softening phase of CR occurs between day 10 and day 12 of a 19-day gestation period. Ripening occurs at day 18 (Read et al. 2007). In humans, these processes begin in the first trimester (Timmons et al. 2010; Myers et al. 2010). Together, these biochemical and physical alterations transform the cervix from a sturdy and rigid structure to a dynamic and flexible one that both maintains the fetus in utero while readying the cervix for a timely birth (Timmons et al. 2010; Holt et al. 2011; Mahendroo 2012; Jorge et al. 2014). While preterm birth has long been associated with abnormal acceleration towards ripening and dilation, only recently have there been insights into its molecular mechanisms (Holt et al. 2011), including the role

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of endocrine factors, notably the progesterone and estrogen ratio (Mahendroo 2012). As the influence of both endocrine and mechanical cues on neighboring reproductive tissue (i.e., uterus) has been clearly demonstrated, a similar effect may likely be seen in the cervix as well (Shynlova et al. 2009).

The cervix appears to serve dual functions. Firstly, by remaining closed until full term, it bears the ever-increasing gravitational force exerted on it by the fetus. Secondly, the cervix must simultaneously begin to transform into a state that eventually will be conducive to dilation, thus allowing for timely fetal delivery at parturition (Leppert 1995; Jorge et al. 2014). Clearly, the collagen-rich ECM of the cervix must also undergo significant changes during CR (Aspden 1988; Orr et al. 2006; Read et al. 2007; Word et al. 2007; Myers et al. 2010; Zhang et al. 2012; Leppert et al. 2014; Jorge et al. 2014; Yoshida et al. 2014). This is also true of the uterus where there is a relationship between force and mechanotransduction in normal pregnancy and the pathogenesis of uterine fibroids (Wu et al. 2008; Leppert et al. 2014).

While extensive studies on cervical tissue ECM, morphology and mechanical behavior at the micro- and macro-level have been undertaken, the mechanically associated intracellular changes and underlying molecular mechanism of CR have not been studied. Our recent proteomic and microarray studies were the first to reveal evidence of the presence of and alteration in mechano-related molecules and the most dominantly expressed protein group were cytoskeletal molecules (Mowa et al. 2008; Schwabe et al. 2014; Stanley et al. 2018).

This present study builds on these initial observations by characterizing the intracellular expression profile and cellular localization patterns of these cytoskeletal molecules (cross-linkers, assembly/disassembly) along with mechanosensors and other mechanosignaling molecules, as well as their potential role during CR in normal pregnancy and preterm labor.

Materials and methods

Animal model

Pregnant female C57BL6/129SvEv mice (*Mus musculus*) (days 11, 17 and 19) between 10 and 12 weeks old and non-pregnant mice sourced from Charles Rivers (Wilmington, MA) were used in these studies ($n = 5$), as described below. All animals were housed at room temperature (RT), with a 12-h light:12-h darkness cycle and animals had ad libitum access to water and feed. Ovariectomy was performed on all non-pregnant mice, as previously described (Donnelly et al. 2013), in order to eliminate confounding effects of endogenous ovarian hormones. Non-pregnant and pregnant mice weight was noted as healthy (non-pregnant animal weight range of 30–38 g, pregnant animal weight range of 60–100 g) before animal sacrifice. Prior to tissue harvest, animals were administered intraperitoneally with a lethal dose (30 μL for non-pregnant animals, 60 μL and 100 μL for day 11 and day 17/19 pregnant animals respectively) of euthasol (Euthasol®, Virbac animal health, Fort Worth, TX), perfused intra-cardially followed immediately by harvest of cervical tissues. Tissues were carefully harvested under a stereomicroscope to avoid any tissue contamination from uterine or vaginal tissues and then stored at − 80 °C and or processed appropriately. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH guidelines publication number 86-23 and approved by the local institution at Appalachian State University. Every effort was made to minimize animal suffering and numbers of animals used. Tissues were analyzed using molecular biology techniques (confocal immunofluorescence and real-time PCR) in order to examine the specific mRNA and protein expression profile of select mechano-sensitive molecules and their associated signaling factors in cervical tissues during CR of mice.

Techniques

mRNA expression of select cytoskeletal and Rho GTPase proteins in mice cervix

Gene expression of select cytoskeletal and Rho GTPase of mice cervix of pregnant and non-pregnant mice was examined using real-time PCR, as described previously using three main steps, namely (i) RNA extraction, (ii) cDNA generation and (iii) real-time PCR (Mowa et al. 2008; Donnelly et al. 2013).

Table 1 Details of select primary antibodies used in the present study

Name of target protein	Source of primary antibody	Catalogue number (Santa Cruz)
1. CDC42	Mouse monoclonal	sc-8401
2. RHOA	Mouse monoclonal	sc-418
3. RHOB	Mouse monoclonal	sc-8048
4. Vimentin (VIM)	Mouse monoclonal	sc-32322
5. p-Focal Adhesion Kinase (p-FAK) (Tyr 397)	Rabbit polyclonal	sc-11765-R
6. p-Caveolin-1 (p-CAV1) (Tyr 14)	Goat polyclonal	sc-14037

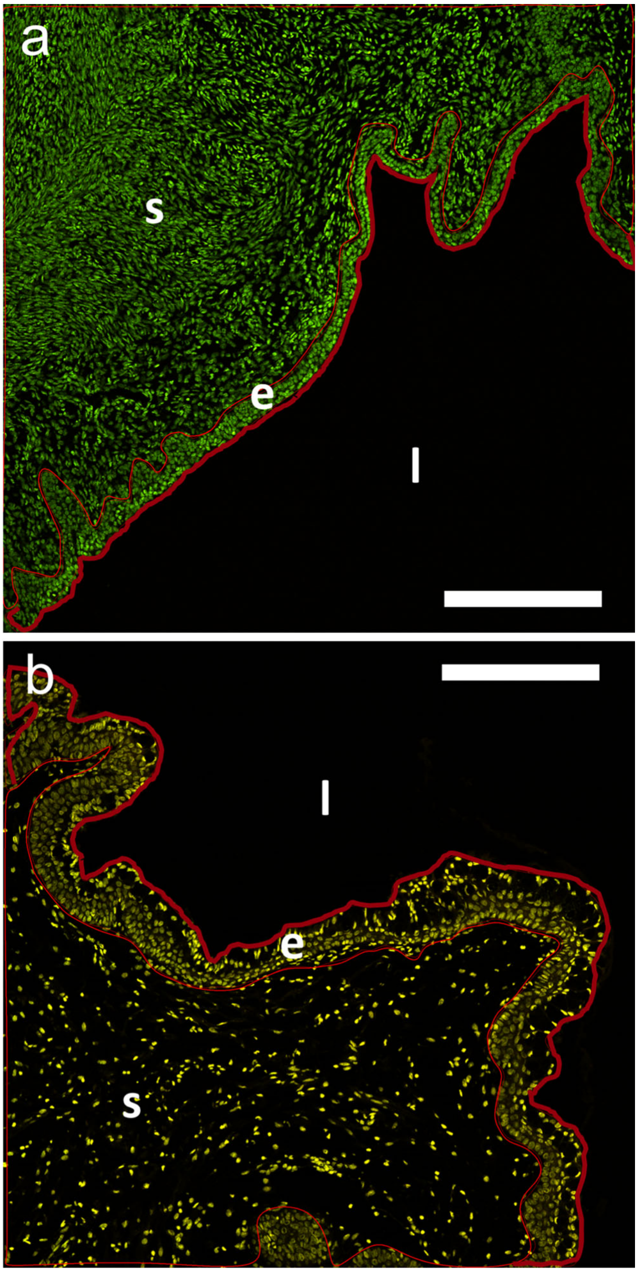

Fig. 1 Quantification of fluorescence intensity in cervical stromal and epithelial cells during pregnancy. The intensity of cell types (stromal versus epithelial) of cervical tissue sections of non-pregnant (**a**) and pregnant mice (day 19 gestation) (**b**) stained with p-FAK primary antibody was measured using the "Histo" tool in the ZEN Black analysis software. The tissue margins of the target cells in field of view were drawn, selected, as shown in red and the image was acquired. The total area within drawn margins was then used to quantify the intensity of fluorescence. Fluorescence intensity studies for CDC42, RHOB, RHOA, VIM and p-CAV were all performed in this manner. s, stromal cells; e, epithelial cells; l, lumen. Scale bar = 200 μm

i. *Total RNA extraction*: Animals were euthanized and transcardially perfused with normal saline at the appropriate pregnancy time points described earlier. The cervices were harvested and immediately stored at − 80 °C. Total RNA was isolated from the cervices of individual animals using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The amount and purity of total RNA for each sample was estimated by spectrophotometric analysis at A260 and A280 using Nanodrop (ThermoScientific, Waltham, MA, USA). Aliquots of total RNA were diluted in RNase-free water and stored at − 80 °C.
ii. *cDNA generation*: Briefly, total RNA from mice cervices were reverse transcribed and amplified by reverse transcriptase enzyme (M-MLV) with reagents (Invitrogen, Carlsbad, CA, USA) using an Eppendorf Master Cycler, according to the manufacturer's instructions, as described previously (Donnelly et al. 2013). No reverse transcriptase was added to negative control groups.
iii. *Real-time PCR*: The generated cDNA was used to evaluate the comparative expression of 12 select genes (see Table 1) in cervices of non-pregnant and pregnant mice. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA), which contain pre-designed and pre-optimized gene-specific probe sets, were used to amplify specific target genes, as previously described (Donnelly et al. 2013). cDNA amplification was performed using the Applied Biosystems 7500 Real-Time PCR system (Foster City, CA, USA). The PCR reactions were set up in 96-well plate in volumes of 25 μL per well. The reaction components were as follows: 5 μL of cDNA, 12.5 μL of TaqMan Universal PCR Mastermix, 1.25 μL of Assays-on-demand™ gene mix (gene-specific) and 6.25 μL of RNase-free water. The program was set as follows: an initial step at 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The comparative amount of mRNA was calculated from the threshold cycles with the instrument's 7500 software v2.0 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The relative expression levels of the target genes were normalized using an endogenous control gene, *Gusβ*. Non-template controls were used as controls for DNA contamination.

Protein expression and cellular localization of select cytoskeletal and Rho GTPase proteins in mice cervix using confocal immunofluorescence microscopy

Confocal immunofluorescence microscopy, equipped with coupled fluorescence intensity analysis, was performed in order to determine changes in the expression of the target proteins, as well as to quantify and localize their cellular expression in the cervix. The proteins of interest that were investigated included mechano-sensitive signaling molecules (CDC42, RHOA, RHOB) and cytoskeletal proteins [vimentin, phosphorylated caveolin-1 (Tyr 14), phosphorylated focal adhesion kinase (Tyr 397)] and tissues were processed as described below.

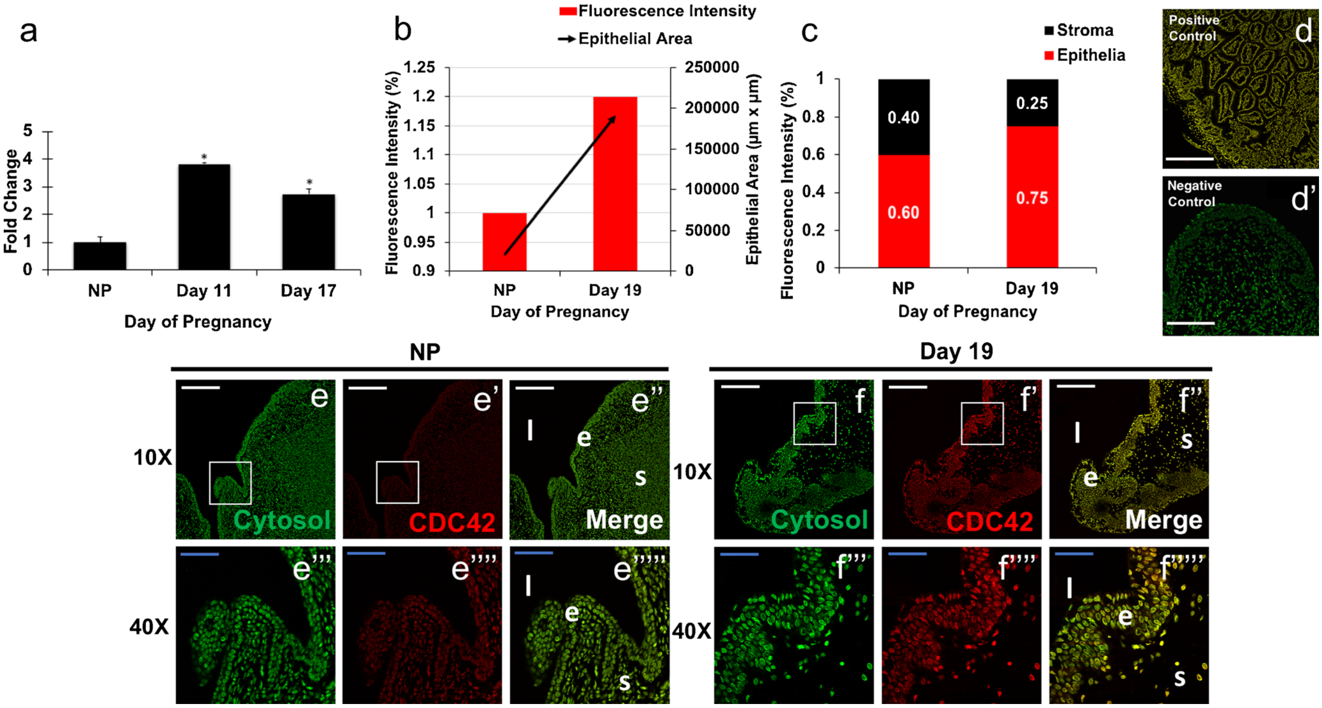

Fig. 2 CDC42 mRNA and protein expression increase over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real-time PCR and confocal immunofluorescence. *Cdc42* mRNA increased overall by 3–4 fold between days 11 and 17 of gestation compared to ovariectomized non-pregnant (NP) in mice cervix (**a**), as revealed by real-time PCR ($n = 5$; $*P < 0.001$ for days 11/17 vs. NP). Consistent with mRNA expression trends, expression of CDC42 protein was more pronounced at day 19 compared to NP (**b**, **e**–**e”””**, **f**–**f”””**). However, only epithelial exhibited an increase in expression of CDC42 protein in day 19 compared to NP (**c**). Images in **e**–**e”””** and **f**–**f”””** are single and merged channels and boxed-in areas in 10× magnification images show expression in selected locations of epithelial and are shown at higher magnification (40×). Tissues were incubated with CDC42 and Texas red antibodies and stained with a nucleic acid-specific marker, i.e., Sytox Green™, appearing in the cytosol as green (**e**, **e”’**, **f**, **f”’**). Any overlap appears as yellow in merged channel images (**e”**, **e”””**, **f”**, **f”””**) with CDC42 appearing red (**e’**, **e””**, **f’**, **f””**). The colon was used as positive control, while the cervix with no primary antibody added was used as a negative control (**d**, **d’**) (scale bar = 100 µm) (l, lumen; s, stromal cells). For all images, except negative control, white scale bar = 200 µm, blue scale bar = 50 µm. $n = 1$

These studies complemented the quantitative expression of mRNA, by real-time PCR, described earlier.

i. *Tissue processing*: Harvested tissues were immediately fixed in 10% formalin for 48 h, dehydrated and embedded in paraffin wax. The embedded tissues were later sectioned at 5 µm and the sections were processed for confocal immunofluorescence, as described previously (Donnelly et al. 2013; Stanley et al. 2015, 2018).
ii. *Confocal immunofluorescence microscopy*: Following deparaffinization and rehydration, tissue sections were initially incubated with either 10% normal goat serum (CDC42, RHOA, RHOB, vimentin) or 10% normal mouse serum (p-caveolin-1, p-FAK) in 0.1 M PBS for 20 min at RT in order to block non-specific protein binding. Next, the sections were washed in 0.1 M PBS and then incubated overnight at 4 °C with primary antibodies [1:100 for p-FAK, 1:50 for the other five proteins of interest (Santa Cruz Biotech., Santa Cruz, CA, USA)] (see Table 1). The following day, the slides were washed thrice in 0.1 M PBS and were immediately incubated in diluted fluoro-tagged secondary antibody at 1:100 dilution (sc-2781, sc-2490 and sc-2492, Santa Cruz Biotech., Santa Cruz, CA, USA) for 45 min at RT. Following this, the slides were washed thrice with 0.1 M PBS and then counterstained with 5 µM Sytox Green™ (Thermo Fisher) according to the manufacture’s guidelines. Slides were mounted with Ultracruz Mounting Medium (Santa Cruz Biotech., Santa Cruz, CA, USA) and examined with a Zeiss 880 laser scanning confocal microscope at 10× and 40× objectives. Images were acquired and processed using ZEN Black imaging software (Carl Zeiss, Peabody, MA, USA). As discussed above, the fluorescence intensity of cervical epithelia versus stroma was quantified between and within each time point (NP and day 19 gestation) at 10× magnification. Specifically, the tissue margins of each cell type were drawn and following capture of the images, their fluorescence intensity was determined using the “Histo” tool of ZEN Black analysis software (see Fig. 1). Images were further analyzed and annotated for figures using Preview, version 9.0 (Apple Inc., Cupertino, CA, USA). Mice colon was

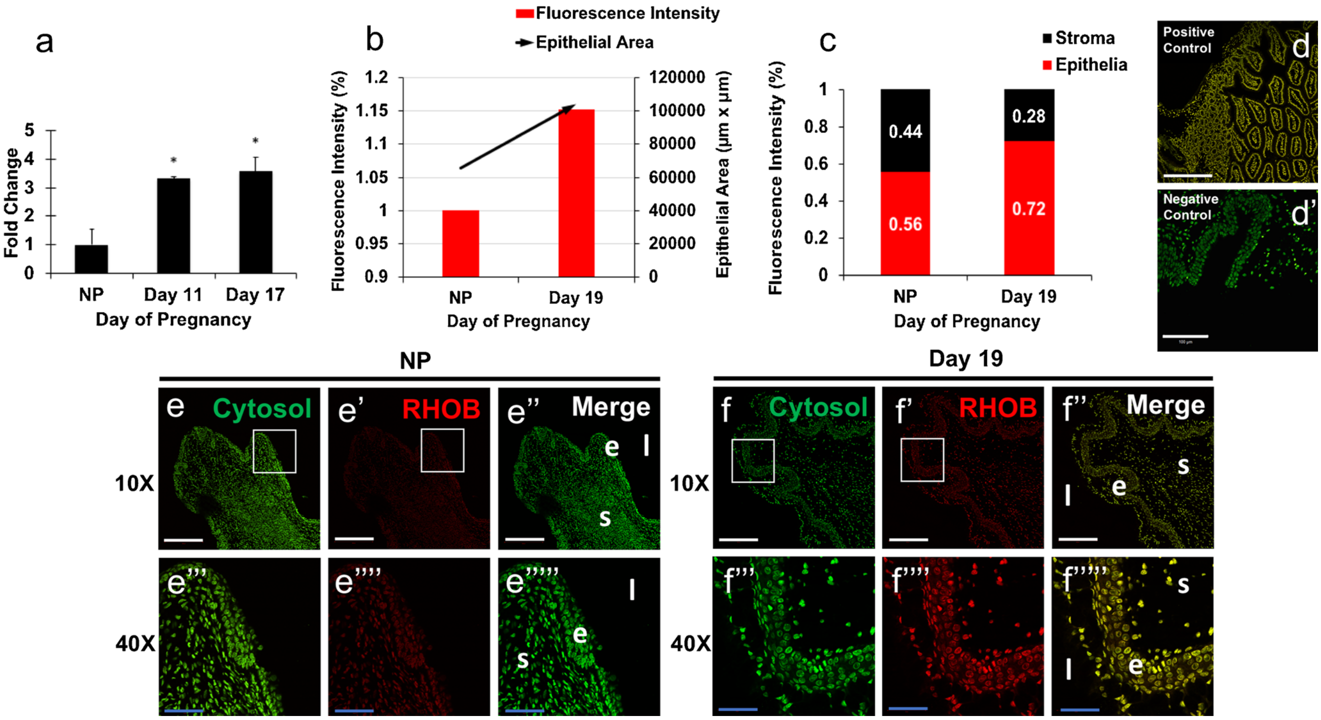

Fig. 3 RhoB mRNA and protein expression increases over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real-time PCR and confocal immunofluorescence. RhoB mRNA increased by 3-fold between days 11 and 17 of gestation compared to ovariectomized non-pregnant (NP) in mice cervix (**a**), as revealed by real-time PCR ($n = 5$; $^*P < 0.001$ for days 11/17 vs. NP). Consistent with mRNA expression trends, expression of RHOB protein was more pronounced at day 19 compared to NP (**b**, **e–e”””’**, **f–f”””’**). However, only epithelial exhibited an increase in expression of RHOB protein in day 19 compared to NP (**c**). Images in **e–e”””’** and **f–f”””’** are single and merged channels and boxed-in areas in 10× magnification images show expression in selected locations of epithelial and are shown at higher magnification (40×). Tissues were incubated with RHOB and Texas red antibodies and stained with a nucleic acid-specific marker, i.e., Sytox Green™, appearing in the cytosol as green (**e**, **e”’**, **f**, **f”’**). Any overlap appears as yellow in merged channel images (**e”**, **e”””’**, **f”**, **f”””’**), with RHOB appearing red (**e’**, **e””**, **f’**, **f””**). The colon was used as positive control, while the cervix with no primary antibody added was used as a negative control (**d**, **d’**) (scale bar = 100 μm) (l, lumen; s, stromal cells). For all images, except negative control, white scale bar = 200 μm, blue scale bar = 50 μm. $n = 1$

used as positive control for CDC42, RHOA and RHOB as it undergoes significant proliferation.

Statistical analysis

Data for real-time PCR analysis were analyzed using ANOVA, followed by the Holm-Sidak test for multiple comparisons. P values of < 0.05 were considered to be statistically significant.

Results

Rho GTPase in the mouse cervix are differentially expressed during gestation

As gestation advanced through day 19, the expression of *Cdc42* and *RhoB* mRNAs, as well as CDC42 and RHOB proteins, increased in mice cervix relative to that of non-pregnant mice, as revealed by real-time PCR and confocal immunofluorescence microscopy, respectively (Figs. 2 and 3). Specifically, the mRNA levels of *Cdc42* increased by 3.7-fold at day 11 and 3.6-fold for *RhoB* at day 17, respectively (Figs. 2 and 3). Both proteins were largely localized in the cervical epithelial cells, with some sparse expression in stromal cells during late pregnancy (day 19). This was in marked contrast to the expression of *RhoA* mRNA and RHOA protein, which decreased drastically with advancing gestation relative to non-pregnant mice (Fig. 4). Indeed, by day 17 of pregnancy, the magnitude of *RhoA* mRNA expression was reduced to less than 5% of control.

Levels of vimentin, FAK and caveolin-1 in the mouse cervix increase during gestation

We next focused on a series of molecules important for cytoskeletal regulation, including vimentin (VIM), FAK and caveolin-1 (CAV1). VIM is an intermediate filament protein common to mesenchymal cells or in epithelial cells following the epithelial to mesenchymal transition (EMT); FAK is an important signaling protein for cell adhesion and migration; and CAV1 classically aids in receptor-mediated endocytosis

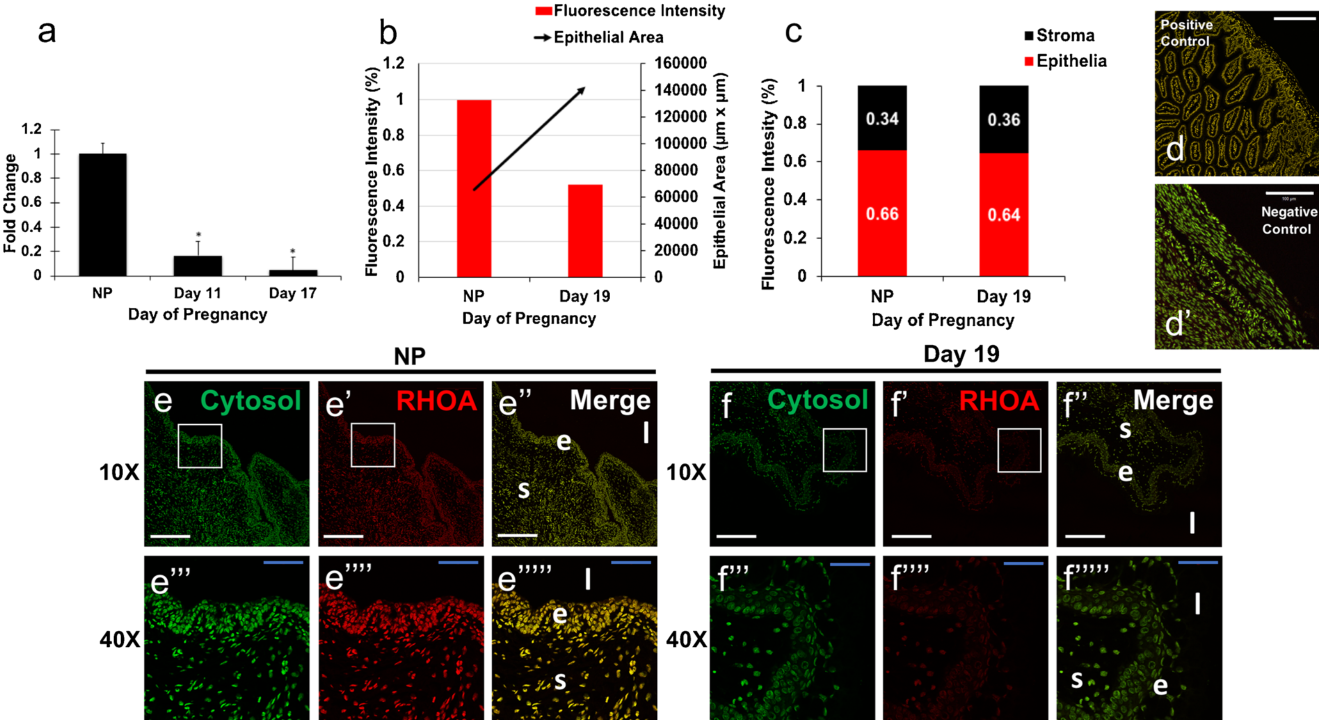

Fig. 4 Expression of RhoA mRNA and protein sharply decrease over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real-time PCR and confocal immunofluorescence. Levels of *RhoA* mRNA in cervix tissue decreases as much as 5-fold between days 11 and 17 of pregnancy compared to non-pregnant mice (**a**) ($n = 5$, $*P < 0.001$ for days 11/17 vs. NP), a trend that was confirmed in confocal immunofluorescence images showing a weaker fluorescence intensity of RHOA protein expression at day 19 compared to NP (**b**, **e**–**e**””’, **f**–**f**””’). Although epithelial (*e*) had almost an intensity that was 2-fold greater in intensity than stromal (*s*), the intensity on both cells between the two time points remained almost unchanged (**c**). Images in **e**–**e**””’ and **f**–**f**””’ are single and merged channels and boxed-in areas in 10× magnification images show expression in selected locations of epithelial and are shown at higher magnification (40×). Tissues were incubated with RHOA and Texas red antibodies and stained with a nucleic acid-specific marker, i.e., Sytox Green™, appearing in the cytosol as green (**e**, **e**’”, **f**, **f**’”). Any overlap appears as yellow in merged channel images (**e**”, **e**””’, **f**”, **f**””’), with RHOA appearing *red* (**e’**, **e**””, **f’**, **f**””). The colon was used as positive control, while the cervix with no primary antibody added was used as a negative control (**d**, **d’**) (scale bar = 100 µm) (l, lumen; s, stromal cells). For all images, except negative control, white scale bar = 200 µm, blue scale bar = 50 µm. $n = 1$

but like FAK can be involved in integrin signaling during adhesion and migration. The mRNA expression of *Vim*, *FAK* and *Cav1* increased in mice cervix over the course of pregnancy by 2.2-fold for *Vim* and *FAK* and a remarkable 14-fold for *Cav1* by day 17 relative to non-pregnant mice (Figs. 5, 6 and 7). The expression pattern for protein of the three molecules (VIM, p-FAK and p-CAV1) was similar to the pattern of their mRNA expression, i.e., they increased with gestation. Moreover, these proteins were largely localized in the epithelial cells of the remodeling cervix (Figs. 5, 6 and 7).

Actin accessory factors in mice cervix are differentially expressed during gestation

The expression pattern of actin accessory molecules, including filamin-1 (*Flna*), gelsolin (*Gsn*), actinin-1 (*Actn1*), transgelin (*Tagln*), profilin-1 (*Pfn1*) and keratin-8 (*Krt8*) were found to be differentially expressed during gestation (Figs. 8 and 9). Specifically, the mRNA expression of most of the molecules increased relative to non-pregnant mice, including *Flna*, *Gsn*, *Actn1* and *Tagln* (Fig. 8). The specific peaks in mRNA expression at various time points were as follows: 7.1-fold for *Flna* at day 11, 6.2-fold change for *Gsn* at day 17, 2.7-fold change for *Actn1* at day 17 and 3.1-fold change for *Tagln* at day 11. In contrast, both *Pfn1* and *Krt8* showed a drastic decrease in expression in the cervix during gestation (Fig. 9). Like *RhoA*, the mRNA levels of these molecules also decreased to less than 5% of the control group (Fig. 9).

Discussion

Cells are influenced by various stimuli in their microenvironment, including biochemical, structural and mechanical signals (Geiger et al. 2001; Dufty et al. 2002). Although we have known the potential influence of fetal weight on CR, most of the work thus far has focused on sex steroid hormones and growth factors. The little we do know on mechanical stimulation is largely focused on tissue response to force at the macroscale and the composition and function of ECM factors (Myers et al. 2008; Yoshida et al. 2014, 2016). Here, we first examined the presence and cellular localization, chronological

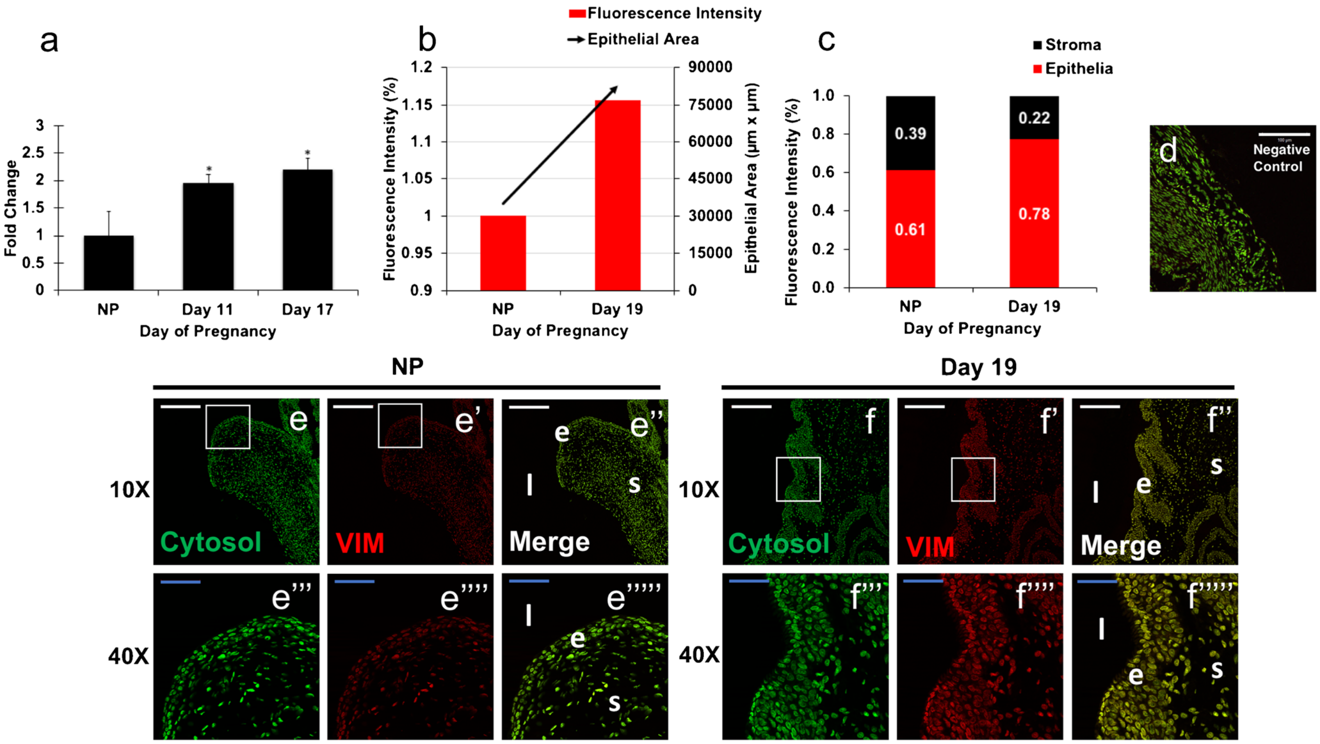

Fig. 5 Vimentin (Vim) mRNA and protein expression increases over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real-time PCR and confocal immunofluorescence. Vim mRNA increased by about 2-fold between days 11 and 17 of gestation compared to ovariectomized non-pregnant (NP) in mice cervix (**a**), as revealed by real-time PCR ($n = 5$; $*P < 0.001$ for days 11/17 vs. NP), consistent with protein expression between day 19 of pregnancy and NP (**b**, **e–e”””**, **f–f”””**). However, only epithelial, but not stroma, exhibited an increase in expression of VIM protein in day 19 compared to NP (**c**). Images in **e–e”””** and **f–f”””** are single and merged channels and boxed-in areas in 10× magnification images show expression in selected locations of epithelial and are shown at higher magnification (40×). Tissues were incubated with VIM and Texas red antibodies and stained with a nucleic acid-specific marker, i.e., Sytox Green™, appearing in the cytosol as green (**e**, **e”’**, **f**, **f”’**). Any overlap appears as yellow in merged channel images (**e”**, **e”””**, **f”**, **f”””**), with VIM appearing red (**e’**, **e””**, **f’**, **f””**). The colon was used as positive control, while the cervix with no primary antibody added was used as a negative control (**d**, **d’**) (scale bar = 100 µm) (l, lumen; s, stromal cells). For all images, except negative control, white scale bar = 200 µm, blue scale bar = 50 µm. $n = 1$

gestational expression pattern and activation of intracellular mechanical machinery in the remodeling cervix of mice. Interestingly, most of the molecules in this study were largely localized to the cervical epithelium, with sparse expression in the stroma, suggesting a significant role for epithelia in mechanical signaling. It is important to note that cervical epithelial cells are known to play a key regulatory role in CR and are located at the interface between the external environment and the body and are thus well-positioned for such a key role. Specifically, some of its (cervical epithelial) roles include providing mechanical and or physical mucus and immune barriers, fluid balance, clearance of particulates, cervical proliferation and repair following vaginal birth. Of relevance to the present study, during pregnancy, cervical epithelial cells are also exposed to increasing mechanical force loads exerted by the growing fetus. This mechanical force has likely profound regulatory effects on the function of epithelial cells and CR. This speculation is supported by our recent proteomic findings revealing that mechano-related proteins are the most abundantly expressed of all proteins in the mouse cervix. This is particularly notable as fetal-induced forces exerted on the cervix increase during pregnancy and their (mechanical-related molecules) levels revert back after births, following removal of fetal force (Schwabe et al. 2014; Stanley et al. 2018). The present findings are consistent with our earlier data and further present key evidence that increasing fetal-induced force on the cervix during pregnancy likely upregulates the expression of cytoskeletal molecules via FAK and Rho-type GTPases. However, because much of the “heavy lifting” of cervical biomechanics is currently attributed to cervical stromal cells, the specific role of cervical epithelial in this process is unclear. The present findings suggest a role for cervical epithelial cells based on localization of mechano-sensitive molecules. The exact role of this cell type for now is unclear but could perhaps be regulatory and upstream of the biomechanical effects of stromal cells.

We investigated the expression pattern of select cytoskeletal-related molecules involved in the modification, assembly/disassembly (*Gsn*, *Tgln*, *Pfn1*) and binding (*Actn1*, *Fln1*) of actin during CR. Of these molecules, four (*Actn1*, *Gsn*, *Tgln*, *Fln1*) increased in expression at the mRNA level, while 1 (*Pfn1*) decreased. This increase in actin

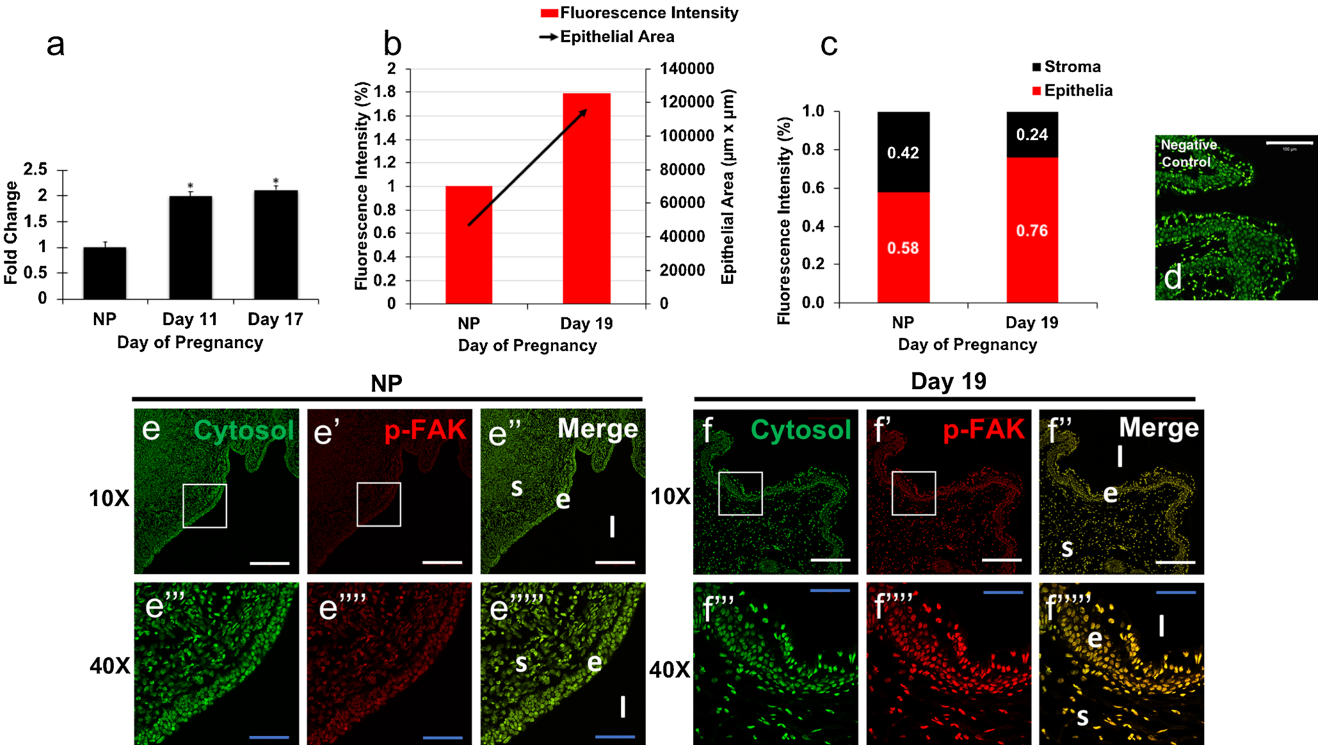

Fig. 6 Levels of focal adhesion kinase (FAK) mRNA and p-FAK protein increase over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real-time PCR and confocal immunofluorescence. FAK mRNA increased by about 2-fold between days 11 and 17 of gestation compared to ovariectomized non-pregnant (NP) in mice cervix (**a**), as revealed by real-time PCR ($n = 5$; *$P < 0.001$ for days 11/17 vs. NP), a pattern consistent with that of phosphorylated protein expression between day 19 and NP (**b**, **e**–**e**””’, **f**–**f**””’). However, only epithelial but not stroma, exhibited an increase in expression of p-FAK protein in day 19 compared to NP (**c**). Images in **e**–**e**””’ and **f**–**f**””’ are single and merged channels and boxed-in areas in 10× magnification images shows expression in selected locations of epithelial and are shown at higher magnification (40×). Tissues were incubated with p-FAK and Texas red antibodies and stained with a nucleic acid-specific marker, i.e., Sytox Green™, appearing in the cytosol as green (**e**, **e**’”, **f**, **f**’”). Any overlap appears as yellow in merged channel images (**e**”, **e**”’”, **f**”, **f**”’”), with p-FAK appearing red (**e**’, **e**””, **f**’, **f**””). The colon was used as positive control, while the cervix with no primary antibody added was used as a negative control (**d**, **d’**) (scale bar = 100 μm) (l, lumen; s, stromal cells). For all images, except negative control, white scale bar = 200 μm, blue scale bar = 50 μm. $n = 1$

levels likely reflects elevated levels of actin assembly that commonly occurs in tissues undergoing remodeling and provides the driving force for changes in cellular shape during proliferation, events that are both likely observed during CR (reviewed by Shekhar et al. 2016). One of the major factors that alter actin organization is the milieu of cues the cell is exposed to in its microenvironment, such as ECM stiffness or compliance, i.e., mechanical force. For instance, when vascular tissues are stretched, the contractile phenotype of their smooth muscle cells stabilizes in a process involving the actin cytoskeleton (Albinsson et al. 2004). Filamin-1 also plays a role in mechanosensing and transmitting force, suggesting that the role of filamin-1 is more than just enforcing stress fibers (reviewed by Zeidan et al. 2003; Nakamura et al. 2015). Could the remodeling cervix constantly bombarded by the ever-increasing weight load of the fetus also stabilize in a similar way? Does the cervix continuously monitor changes in ECM stiffness and force transmission and, thus, in part, adjust and calibrate its responsiveness to the force and thereby hold the fetus in utero? The rise in levels of *Actn1* and *Fln1* and the subsequent bundling and branching of actin into much stronger stress fibers that follow cross-linking appear to suggest so. These resultant structures (stress fibers) effectively transmit force from contractile machinery to ECM structures via focal adhesion (FA) proteins, which in turn play a critical role in inducing mechanically activated signaling pathways (Wozniak et al. 2003; Paszek et al. 2005; Provenzano and Keely 2011). However, although valuable insights are gained from other tissues, there is need to conduct mechanistic and functional studies that are specific to the cervix, in order to evaluate these speculations.

Levels of *Gsn* and *Tgln* mRNA, which code for actin-modifying molecules, also increase, thus likely supporting actin polymerization over the course of pregnancy (Sun et al. 1999; Shekhar et al. 2016). In contrast, there is a decrease in *Pfn1* mRNA levels. This is consistent with previous findings that show that although PFN1 protein is an essential driver of membrane protrusion during cell migration, high concentrations of PFN1 do inhibit cell migration and lead to disappearance of filaments in lamellipodia (Ding et al. 2012;

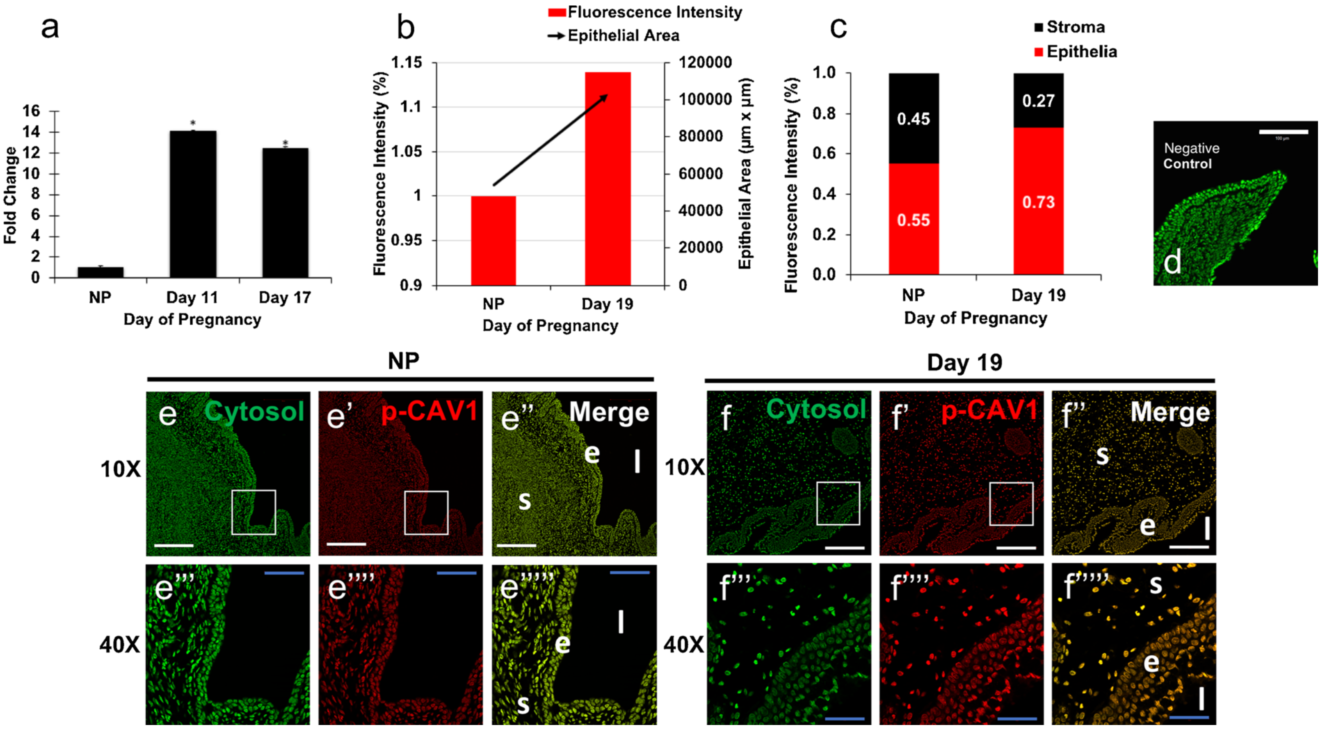

Fig. 7 Expression of caveolin-1 (Cav1) mRNA and p-CAV1 protein increase over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real-time PCR and confocal immunofluorescence. Levels of CAV-1 mRNA increased sharply by as much as 12–14-fold between days 11 and 17 of gestation compared to ovariectomized non-pregnant (NP) in mice cervix (**a**), as revealed by real-time PCR ($n = 5$; $*P < 0.001$ for days 11/17 vs. NP). A similar trend was observed in the intensity of p-CAV1 protein expression between day 19 compared to NP (**b**, **e**–**e**””’, **f**–**f**””’). However, only epithelial but not stroma, exhibited an increase in expression of p-CAV-1 protein in day 19 compared to NP (**c**). Images in **e**–**e**””’ and **f**–**f**””’ are single and merged channels and boxed-in areas in 10× magnification images show expression in selected locations of epithelial and are shown at higher magnification (40×). Tissues were incubated with p-CAV-1 and Texas red antibodies and stained with a nucleic acid-specific marker, i.e., Sytox Green™, appearing in the cytosol as green (**e**, **e**”’, **f**, **f**”’). Any overlap appears as yellow in merged channel images (**e**”, **e**”””, **f**”, **f**”””), with p-CAV-1 appearing red (**e**’, **e**””, **f**’, **f**””). The colon was used as positive control, while the cervix with no primary antibody added was used as a negative control (**d**, **d’**) (scale bar = 100 µm) (l, lumen; s, stromal cells). For all images, except negative control, white scale bar = 200 µm, blue scale bar = 50 µm. $n = 1$

reviewed by Shekhar et al. 2016). It is likely that cell motility events, such as lamellipodia formation and migration, are not as critical during CR as maybe the case with proliferation, except perhaps for immune cell infiltration near term, which is believed to play a role in cervical repair.

We also observed an intriguing characteristic mRNA expression pattern by two other cytoskeletal molecules, *Vim* and *Krt8*, which form intermediate filaments (IFs). IF networks also play a role in mechanotransduction (Goldman et al. 1996 and reviewed by Qin et al. 2010). While both *Vim* mRNA and VIM protein levels increased as expected, to our surprise, mRNA levels of *Krt8*, which ensures integrity of epithelial tissue, decreased in late pregnancy. Previous studies have shown that the loss of keratin-8/18 in simple epithelia decreases the local stiffness of FA (reviewed by Loschke et al. 2015). These unique differences in the expression patterns of IFs and actin in the remodeling cervix is intriguing and future studies should investigate their interaction with ECM and their impact on CR.

Cellular mechanotransduction machinery can be divided into: (i) a mechanosensing module, which contains molecular clutch proteins (e.g., talin, vinculin) that directly link integrin receptors with the actin cytoskeleton; (ii) mechanosignaling proteins (e.g., FAK, paxillin); (iii) cross-linking proteins (e.g., ACTN1, FLNA); and (iv) actin polymerizing factors (e.g., GSN) (Stutchbury et al. 2017). FA complexes are located at cell-ECM attachment sites (Burridge et al. 1988). Different combinations of cytoskeletal molecules can assemble there (FA), depending on whether they are recruited by mechanical or chemical (growth factor) stimuli (Burridge et al. 1988). These molecules (cytoskeleton), in turn, activate specific signaling pathways and control specific biological processes. FAK is a prominent member of the FA complex, is activated early during FA signaling and is critical for mechanosensing (Schaller et al. 1994, Schlaepfer et al. 1998). Since phosphorylation of FAK at tyrosine 397 by SRC is associated with STAT3 involved in growth regulation in other tissues (Schaller et al. 1994; Schlaepfer et al. 1998), the observed increase in levels of phosphorylated FAK at tyrosine 397 in the current study indicates the possible presence and activation of FAK-induced cervical growth. However, the exact biological process mediated by the activated FAK and the

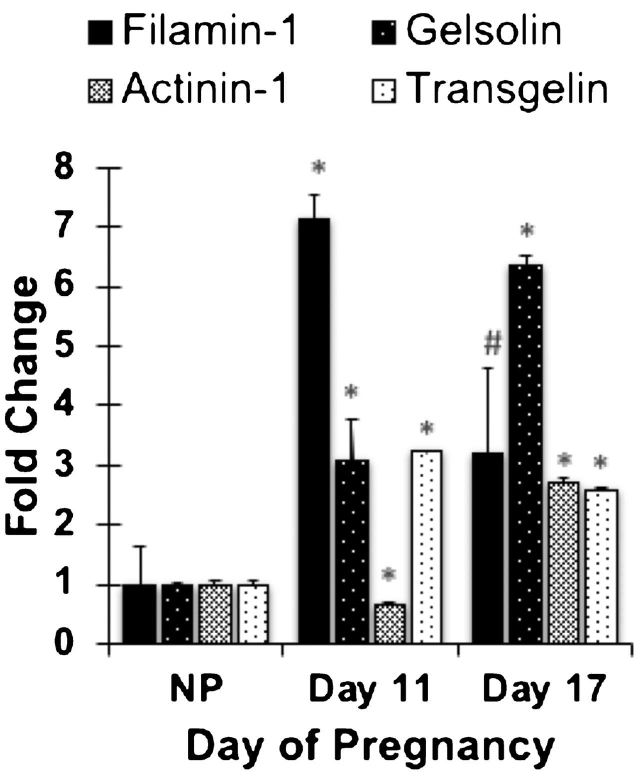

Fig. 8 Expression of cytoskeletal mRNAs in mice cervix increased over pregnancy, as revealed by real-time PCR. Overall, as pregnancy advances the levels of all cytoskeletal mRNA of interest (filamin-1, gelsolin, actinin-1, and transgelin) increased at variable folds between days 11 and 17 of pregnancy compared to NP ($n = 5$; $^{\#}P = < 0.05$, $^{*}P < 0.001$)

specific associated downstream signaling molecules during CR are currently unclear. An increase in Rho-mediated cell contraction in response to stiff microenvironments also drives FAK activation in FA (Pirone et al. 2006). Since FAK is stimulated by various factors, including mechanical ("outside-in" and "inside-out" forces) and chemical (VEGF, EGF, IGF) cues, it is unclear for now which of the two cues are responsible or whether they have a synergistic effect on FAK expression during CR. It is also conceivable that the growing fetus may directly stimulate mechano-sensitive ion channels that are present in the cervix, called piezol, to convert mechanical cues into biochemical signals (Lewis et al. 2017). This may be independent of the collagen-rich ECM whose stiffness in early pregnancy is likely to be a critical microenvironmental stimulus in mechanosensing decreases as pregnancy advances. It is interesting to note that these channels and the cytoskeleton exhibit reciprocal regulation (Lewis et al. 2017). Alternatively, since mechano-sensitive cells are known to have mechanical memory, i.e., in stem cells, the magnitude of response to mechanical cues does not change over time even after the initial degree of stiffness is later altered as is the case during CR, mechanical memory could play a role (Yang et al. 2014). Lastly, VEGF receptors, members of the receptor tyrosine kinase group, are abundantly expressed in the cervix where they help induce cervical growth, among other things (Donnelly et al. 2013) and are also known in other tissues to induce expression of FA molecules (Avraham et al. 2003). Perhaps FA proteins in the cervix cross talk with VEGF receptors during pregnancy. Again, as stated earlier, there is need in the future to conduct functional studies that are specific to the cervix and cell types (epithelial versus stroma), in order to evaluate the current speculations.

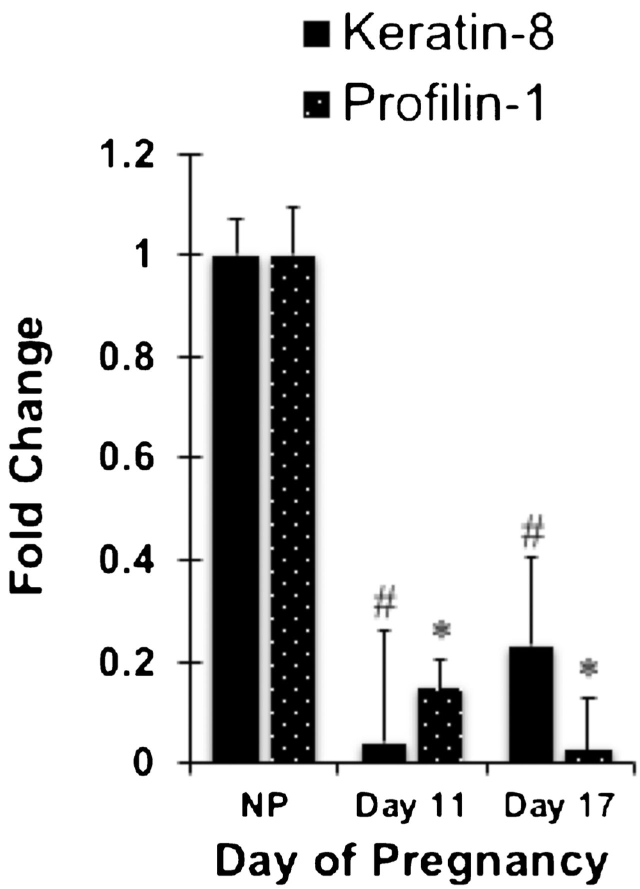

Fig. 9 Expression of keratin-8 and profilin-1 mRNAs sharply decreases in mice cervix during pregnancy, as revealed by real-time PCR. Overall, as pregnancy advances, there was a significant decrease in the mRNA of keratin-8 and profilin-1 compared to NP ($n = 5$; $^{\#}P = < 0.05$, $^{*}P < 0.001$)

We also observed an increase in the expression of *Cav1* mRNA and p-CAV1 protein. Caveolae indentions in the cell membrane of aortic endothelial cells increase in number under laminar or physiological stress and when the tissue is stretched (Yu et al. 2006, reviewed by Shihata et al. 2016). Caveolae and CAV1 are also known to regulate cell cycle and senescence (Quest et al. 2013). CAV1 is in addition believed to interact with other intracellular signaling molecules contained in caveolae. This includes G-coupled receptors and tyrosine kinases and these interactions in turn stimulate downstream signaling, notably the members of the Rho GTPase family (Bender et al. 2002).

The latter half of pregnancy in mice is characterized by pronounced proliferation of the cervical tissue, largely

Table 2 Expression profile of select mechano-related molecules in cervix of pregnant mice

Protein and gene name	Primary function	Trend of protein and/or mRNA expression during pregnancy
1. CDC42, *Cdc42*	A GTPase that regulates signaling in cell division and morphogenesis	Up regulated
2. RHOA, *RhoA*	A GTPase associated with cytoskeletal organization	Down regulated
3. RHOB, *RhoB*	A GTPase associated with cytoskeletal organization	Up regulated
4. Vimentin (VIM), *Vim*	Supports and anchors organelles in cytosol	Up regulated
5. p-Focal Adhesion Kinase (p-FAK), *FAK*	Cellular adhesion and extension of their processes	Up regulated
6. p-Caveolin-1 (p-CAV1), *Cav1*	Links integrin subunits to tyrosine kinase FYN	Up regulated
7. Filamin-1 (FLNA), *Flna*	Aids migration by providing structural crosslink of actin filaments	Up regulated
8. Actinin-1 (ACTIN1), *Actn1*	Actin binding protein in microfilament bundles	Up regulated
9. Transgelin (TAGLN), *Tagln*	Sensitive to change in shape by cross-linking actin/gelling protein	Up regulated
10. Gelsolin (GSN), *Gsn*	Regulator of actin filament assembly	Up regulated
11. Keratin-8 (KRT8), *Krt8*	Cellular structural integrity and signal transduction	Down regulated
12. Profilin-1 (PFN1), *Pfn1*	Regulation of actin polymerization	Down regulated

dominated by epithelial cells (Burger and Sherwood 1998). The Rho family of GTPases plays a key upstream role in physiological and pathological processes of cellular transformation and proliferation using the mechano-sensitive cytoskeleton (Hall 1998; Wennerberg and Der 2004; Karnoub and Der 2013; Thorne et al. 2015; Fujiwara et al. 2016). In the present study, levels of CDC42 and RHOB increased in the remodeling cervix but not RHOA, which was downregulated. Each of these molecules belongs to the Ras superfamily of small GTP-binding proteins (Downward 1990). Rho-type GTPases are known to regulate numerous actin-mediated signaling pathways (Paszek et al. 2005; Provenzano and Keely 2011). Notably, they play a key regulatory role in the response by cells to ECM stiffness and respond to mechanical cues by inducing intracellular contractility via myosin motor proteins (Paszek et al. 2005; Provenzano and Keely 2011), i.e., when motor proteins sense increased stiffness in the ECM, Rho is activated. This, in turn, leads to further induction of intracellular contractility with the goal of generating a force that matches ECM stiffness, a mechanical equilibrium called tensional homeostasis (TH) (Wozniak et al. 2003). The magnitude and tone of TH determines the type of mechano-sensitive signaling pathways activated in FAs and consequently the biological event. In late CR, as collagen is remodeled and the ECM becomes more compliant, cervical stiffness diminishes, as likely does TH. Thus, increasing gravitational force exerted on the remodeling cervix by the weight of the growing fetus and not ECM stiffness, may be directly responsible for inducing the mechanical stimuli and the observed molecular readout in the present study. Also, like FAK, Rho is involved in proliferation (Provenzano and Keely 2011). Notably, Rho is involved in cell cycle progression at several stages and is also associated with various signaling pathways involved in proliferation, including ERK1/2, p38 MAPK and JNK, serum response factor and phosphoinositide 3 kinase-mediated activation of the AKT pathway (Sun et al. 2007; Provenzano and Keely 2011). This pattern of protein expression observed in the present study along with the prominence of proliferation during CR collectively suggests a likely role for mechanotransduction signaling in CR.

The timing in the increase of *Cdc42* mRNA expression in the cervical tissue observed here coincides with the proliferation of cells. CDC42 is normally associated with cytoskeletal reorganization, filopodia formation and motility (Wennerberg and Der 2004). Filopodia are used to probe the extracellular microenvironment. During fetal growth and CR, the ECM becomes very dynamic. The patterns of RHOA and RHOB during CR were opposite, with RHOA decreasing and RHOB increasing as pregnancy advanced. These Rho GTPases, which have as much as 85% homology (Wennerberg and Der 2004), have very similar downstream signaling molecules that mediate the reorganization of cytoskeletal structure and function, notably through the RHO-ROCK pathway (Paszek et al. 2005, Provenzano and Keely 2011). To our surprise, the expression patterns of these two Rho GTPases in the remodeling cervix were different

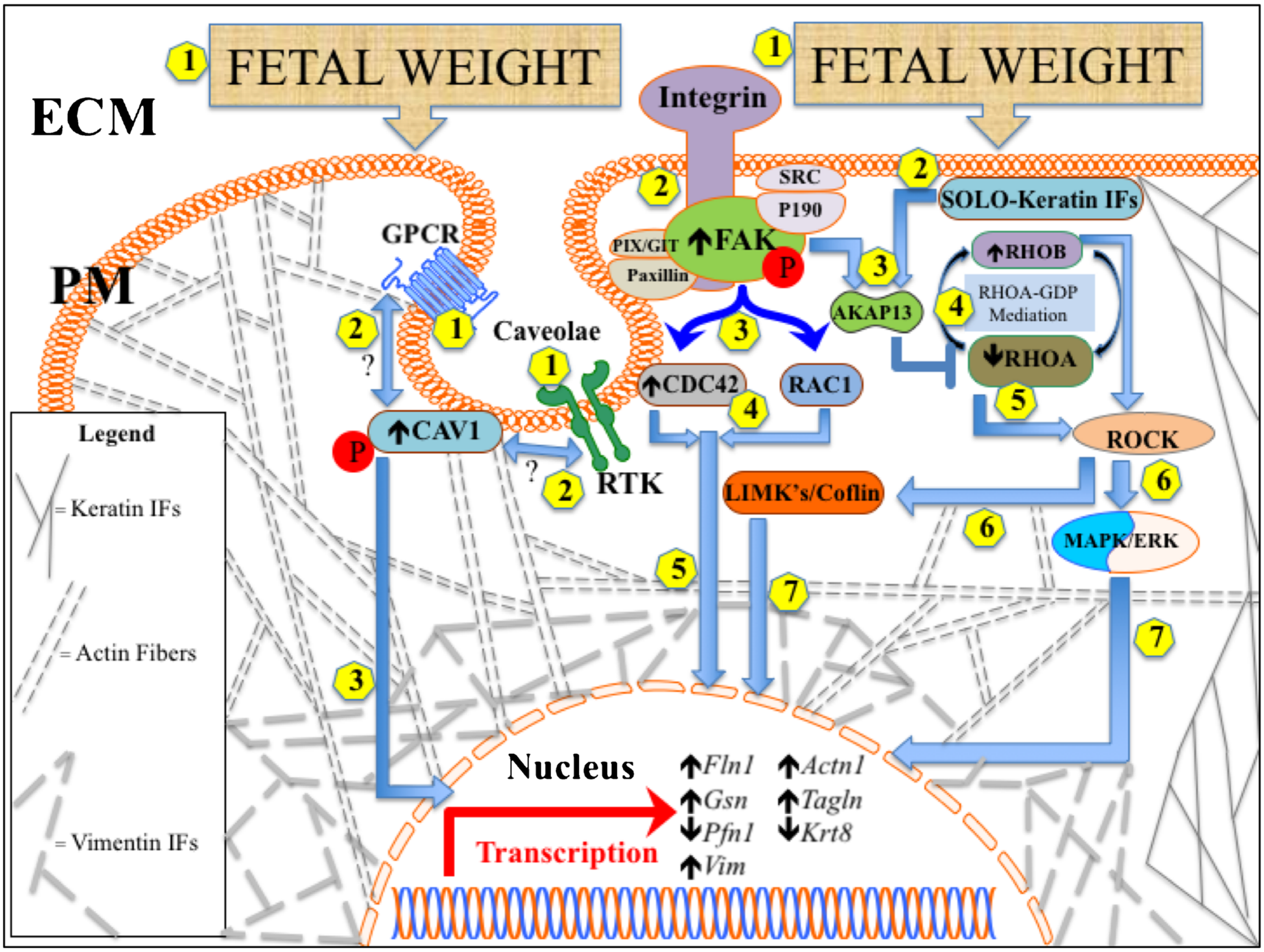

Fig. 10 A proposed model showing likely underlying molecular alterations in expression of mechano-sensitive and cytoskeletal molecules during cervical remodeling in mice. Here, we hypothesize that the mechanical force induced by the growing fetus triggers mechanotransduction. Regulatory factors likely to induce these molecular changes during cervical remodeling may include both mechanical and hormonal factors. Please note that blue arrows signify signaling directionality and the genes/proteins associated with an arrow in the diagram, indicating the pattern of their expression, were investigated in the present study. Further, this model was formulated based on data from the present study as well as existing literature. The numbers in the diagram indicate signaling progression. GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; Ifs, intermediate filaments; PM, plasma membrane; ECM, extracellular matrix. Other literature sources used in the development of this model but not cited in the manuscript include Lappalainen and Drubin (1997), Bamburg et al. (1999), Chang and Goldman (2004), Li et al. (2004), Zhao et al. (2007), Li et al. (2007), Giang Ho et al. (2008), Lawson and Burridge (2014) and Hu et al. (2014)

(decreased RHOA, increased RHOB). This difference in expression pattern is currently unclear. It is, however, important to note that traditionally RHOA contributes to structural integrity of the cell (Wennerberg and Der 2004). Further, a study by Fujiwara and others demonstrated a force-activated RHOA pathway in cultured cells that directly influences actin stress fibers and upregulates keratin networks (specifically keratin 8/18) (Fujiwara et al. 2016). Here, similar to *RhoA* mRNA and RHOA protein expression patterns, we note a decrease in *Krt8* mRNA expression as CR progressed. Our current findings regarding the expression trends of both RHOA and *Krt8* correspond to that of Fujiwara and others (2016). Further, Thorne and others also demonstrated a forced-induced RHOA/ROCK/MAPK/ERK proliferation pathway in myometrial tissue through actin and myosin-induced contractility (Thorne et al. 2015). The difference in our current findings and those observed earlier in myometrial tissue may be based on tissue-specific expression profiles. In the absence of RHOA's influence in the cervix, CDC42 (discussed above) may be the primary modulator of actin organization and mechanically induced proliferation during CR.

In conclusion, the present study demonstrates the existence of dynamic expression patterns in cytoskeletal-related molecules in the remodeling cervix of mice during pregnancy. The prevalence of these cytoskeletal-related molecules, FA proteins and Rho-type GTPases was largely localized in the epithelium, suggesting an important role for epithelial cells in mechanosignaling pathways. Our data provide a snapshot of dynamic cytoskeletal activity during CR and points to the importance of developing and further studying CR-specific signaling pathways that are mechanically stimulated (see trends and proposed model, Table 2 and Fig. 10). Future studies should attempt to delineate the tissue- and cellular-specific roles of the different regulatory cues of CR and whether they synergize with mechanical stimulus. Also, there is need to examine whether an optimal clinically relevant threshold ratio exists between fetal and cervical sizes during pregnancy.

Funding This work was funded by the Appalachian State University Office of Student Research and the College of Arts and Sciences, Department of Biology.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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