RESEARCH

A proteomic profile of postpartum cervical repair in mice

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Abstract

A timely and complete uterine cervical tissue repair postpartum is of necessity to prevent obstetrical complications, such as cervicitis, ectropion, hemorrhage, repeated miscarriages or abortions and possibly preterm labor and malignancies. We recently characterized the morphological alterations, as well as changes in angiogenic expression profile in a mice uterine cervix during the immediate postpartum period. Here, we build on this previous study using a proteomic analysis to profile postpartum tissue changes in mice cervix during the same period, the first 48 h of postpartum. The current proteomics data reveal a variable expression of several intermediate filaments, cytoskeletal modulators and proteins with immune and/or wound-healing properties. We conclude that postpartum cervical repair involves a rapid and tightly regulated balance between a host of biological factors, notably between anti- and pro-inflammatory factors, executed by the M1 and M2 macrophage cells, as revealed by proteomics and verified by confocal immunofluorescence. Future studies will assess the suitability of some of the key proteins identified in this study as potential markers for determining the phase of postpartum cervical repair in obstetrical complications, such as cervical lacerations.

Key Words

- cervical repair
- proteomics
- mice

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Introduction

Since the uterine cervix undergoes marked alterations during pregnancy and significant trauma both during normal vaginal delivery (parturition) and immediately after (postpartum) (Timmons *et al.* 2010), a timely and complete tissue repair, postpartum, is of necessity to prevent obstetrical complications. Such (obstetrical) complications include cervicitis, ectropion, hemorrhage, repeated miscarriages or abortions and, possibly, preterm labor and malignancies. Collectively, these physiological, biochemical and biophysical changes are termed cervical remodeling (CR), which is divided into four distinct yet overlapping phases, namely softening, ripening, dilation and postpartum repair (Read *et al.* 2007, Word *et al.* 2007). To date, most of the studies have focused on the first three phases of CR, almost at the exclusion of postpartum repair.

Postpartum repair (sometimes termed reconstruction) consists of a set of complex biological processes that ultimately restores the cervix to its original non-pregnant state, and thus ensures a normal cervical function for subsequent pregnancies (Timmons *et al.* 2010, Mahendroo 2012, Stanley *et al.* 2015). This final phase of CR is generally characterized as an inflammatory and wound-healing response (Timmons & Mahendroo 2007, Bauer *et al.* 2009), as demonstrated by studies that utilized gene microarrays. These studies have shown that a variety of factors, including pro-inflammatory factors, metalloproteases, proteins involved in extracellular matrix (ECM) synthesis and genes governing epithelial differentiation pathways, are all upregulated postpartum (Timmons & Mahendroo 2007, Gonzalez *et al.* 2009).

Immune cells, including neutrophils, eosinophils and both M1 and M2 macrophages, have all been shown to increase in expression postpartum compared to earlier phases of remodeling (Timmons *et al.* 2009, 2010, Mahendroo 2012). We have also recently shown marked and varied changes in the expression of angiogenic factors (Stanley *et al.* 2015). These marked angiogenic and inflammatory responses could serve to promote repair of the cervix after parturition and/or serve a protective role of the birth canal against environmental hazards, such as infection (Gonzalez *et al.* 2009).

Given the facts that postpartum repair is characterized as a pro-inflammatory wound-healing response (Timmons & Mahendroo 2007, Bauer *et al.* 2009, Mahendroo 2012, Stanley *et al.* 2015), in the present study, we sought to identify the signature proteins and their potential roles during the first 48 h of postpartum using the proteomics analysis.

Materials and methods

Animals and postpartum time intervals

Pregnant wild-type mice (C57BL6/129SvEv; Charles River, $n=3$) were used for all the groups in the present study. The time of the first pup was noted as time 0 h and the mothers were killed at time 0, 15 and 48 h after parturition. The forty-eight (48) hour postpartum time point was used as the baseline here, based on findings from our previous studies which showed that the expression of molecules in the rodent cervix restores back to non-pregnancy to early pregnancy levels by 48 h or day 2 postpartum (Mowa *et al.* 2004). All animals were euthanized by a lethal injection of sodium pentobarbital (150 mg/kg body weight, i.p.) and transcardially perfused with 0.9% saline solution. Cervices were carefully harvested under a stereomicroscope to avoid contamination with vaginal and uterine tissues, and the tissues were then processed and analyzed accordingly. All animals were housed under the following conditions: constant room temperature; a 12:12 light and dark cycle, and free access to water and food. All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Appalachian State University and the NIH guidelines (NIH publication number 86-23), with efforts made to minimize both number of animals used and animal suffering.

Proteomics

Whole protein was extracted from cervical tissue at 0, 15 and 48 h postpartum ($n=3$ per time point), as described below.

The supernatant was then immediately stored at -80°C and later transported to the core facility Laboratory of the David Murdock Research Institute (Kannapolis, North Carolina, USA) under dry ice for the proteomics analysis.

Sample preparation: The samples were assayed using the Thermo Scientific Micro BCA Protein Assay kit to determine protein concentration. The samples were diluted to fall within the linear working range of the kit (5–200 $\mu\text{g/mL}$) and the concentrations were calculated based on absorbance values compared to a BSA standard curve. The samples were then filtered using a 3-kDa ultra-centrifugal filter to exchange the dissolution solvent with a mass spectrometry-friendly buffer. 100 μL of the sample was diluted to 500 μL with 50 mM ammonium bicarbonate and filtered. The samples were rinsed with 200 μL ammonium bicarbonate and filtered. The tubes were inverted and centrifuged to collect the sample.

A volume of each sample corresponding to 35 μg of protein (based on the protein quantization results) was used. The sample volumes were made equal by adding 50 mM ammonium bicarbonate (AmBic) to a volume of 29.8 μL . A 1% solution of Rapigest was added to each sample to denature the proteins and the mixture was placed in a shaking heated mixer at 40°C for 10 min. Disulfide bonds were reduced by adding 200 mM dithiothreitol to each sample and heating the tubes to 80°C for 15 min. Free sulfur atoms were alkylated with 400 mM Iodoacetamide by placing the tubes in the dark for 30 min at room temperature. A tryptic digest was performed by adding 0.7 μg Gold-Mass Spectrometry grade trypsin to each tube and incubating overnight at 37°C . Alcohol dehydrogenase (ADH) digest from yeast was added to a final concentration of 50 fmol/ μg protein. The trypsin reaction was stopped and the Rapigest was degraded with the addition of 10% TFA/20% acetonitrile/70% water that was then heated to 60°C for 2 h. The samples were centrifuged and the supernatant pipetted into autosampler vials.

A protein standard (BSA) was carried through the reduction/alkylation and digestion steps and used as a QC of the sample preparation steps and instrument performance. LC solvents for peptide separation included (a) water containing 0.1% formic acid, and (b) acetonitrile containing 0.1% formic acid. The study sequence consisted of the study sample injections bracketed by a pair of QC injections. Data from all study samples were acquired using Data Dependent scans (Nth order double play) on the LTQ Orbitrap XL. Database searches were performed in Elucidator (Rosetta Biosoftware) using MASCOT (Matrix Sciences, London, UK). Analytical results were also viewed in Scaffold (Proteome Software,

Portland, OR, USA). QC and study samples were evaluated to confirm data quality. Liquid chromatography total ion current (TIC) outputs were assessed for signal quality and changes in signal intensity. Results were also monitored for signal trends, such as a consistent increase or decrease in TIC maximum values, and MASCOT search results were used to monitor the quality of the MS data.

Proteomics verification

The proteomics data were verified by confocal immunofluorescence of two proteins and the procedure was performed as described below:

Confocal immunofluorescence was employed to complement Western blot data of the proteins of interest (filamin-A and vimentin). Harvested cervixes (0 and 48 h postpartum) were immersed in a 10% formalin (pH 7.2) aqueous solution. The tissues were fixed in formalin at 4°C for 3–4 days and then transferred into saturated sucrose dissolved in 0.1 M PBS, for at least 2 days prior to sectioning (12 µm) with the cryostat (Leica) at –30°C. After sectioning, the slides were either stained immediately or stored at –20°C until staining. For staining, the sections

were initially incubated with 10% normal goat serum in 0.1 M PBS for 20 min at RT in order to block non-specific protein binding. Next, the sections were washed thrice in 0.1 M PBS and then incubated overnight at 4°C with diluted primary antibodies of interest at 0.5 µg/mL (sc-28284 (filamin-A, rabbit polyclonal IgG), sc-32322 (vimentin, mouse monoclonal); Santa Cruz Biotech). The next day, the sections were washed thrice with 0.1 M PBS buffer followed immediately by incubation in diluted fluoro-tagged secondary antibody at 0.5 µg/mL (filamin-A, mouse anti-rabbit IgG-R (sc-2492); vimentin, goat anti-mouse IgG (sc-2781), Santa Cruz Biotech) for 1 h at RT. Thereafter, the sections were washed thrice with 0.1 M PBS, counterstained with 5 µM Sytox Green, mounted with aqueous Ultracruz Mounting Medium (Santa Cruz Biotech) and then examined with a confocal microscope (Carl Zeiss).

Statistical analysis

Raw MS data files for the study samples, collected by the Thermo Orbitrap XL system, were processed in Elucidator. MS data were grouped in Elucidator based on sample group and alignment (Table 1). Sample groups were used to assist

Table 1 LC and MS instrument parameters used in the study.

System	Parameter	Setting
Autosampler	Temperature	4°C
	Injection	5.0 µL prepared sample
LC Trap Column	Sample load time	5 min
	Trap flow rate	5 µL/min
LC Analytical Column	Column temperature	55°C
	Flow rate	0.4 µL/min
	Gradient	Initial conditions: 98% A t=60 min, 60% A t=65 min, 5% A t=70 min, 5% A t=75 min, 98% A t=95 min, 98% A
		Nth order double play
		5
LTQ Orbitrap XL mass spectrometer	Experiment type	2.0 kV
	# Dependent scans	200°C
	Source voltage	1.0 µA
	Capillary temperature	47.0 V
	Source current	100.0 V
	Capillary voltage	1.0 s
	Tube lens	60,000
	FTMS survey scan time	90 min
	FTMS resolution	ES mode
	Survey time	Positive
	Survey ion mode	375–2000
	Survey polarity	CID
	Survey mass range	35.0
	Activation type	50–2000
	Normalized collision energy	
	ITMS MS/MS mass range	

Analytical samples were analyzed on a Thermo Scientific LTQ Orbitrap XL mass spectrometry system coupled to a Waters nano ACQUITY UPLC system using parameters presented in the table below.

in data alignment and feature identification, and were utilized for QC assessment and group comparisons. Data were processed from retention time, 8–90 min. Thermo Orbitrap data files were searched using the Mascot search engine against the SwissProt mouse database (appended with yeast ADH, March 02, 2010). The aligned mass features were annotated with these database search results using the results from the system Peptide Tellers and a predicted error rate of 1%. MS data were summarized to the feature level, normalized and an error-weighted ANOVA test was performed to compare the expression results between sample groups. Candidate differentially expressed markers were determined based on $P < 0.01$. Features were summarized by protein based on the results of the database search.

Results

Proteomics and confocal immunofluorescence analysis of postpartum cervical tissue

Using the Rosetta Elucidator, several distinct proteins were identified in the cervical tissue of postpartum mice (Fig. 1). Proteomics analysis revealed several groups of proteins that were found to have significant changes

in expression between 0 h and 48 h PP. These proteins were divided into four functional groups, namely: (1) four actin-binding proteins (gelsolin, filamin-A, D isoform of plectin-1 and transgelin) (Fig. 2), (2) four intermediate filaments (keratins types I 8 and II 18, desmin and vimentin) (Fig. 3), (3) six immune-related proteins (glutamine gamma-glutamyltransferase 2, gelsolin, lymphocyte antigen 6D, macrophage migration inhibitory factor (MIF), annexin A1 and phospholipase A2) (Fig. 4) and (4) two hypoxia-induced proteins (transgelin and vimentin) (Fig. 5). Levels of the majority of the functional groups of proteins (three out of four groups, namely actin-binding proteins, intermediate filaments and hypoxia-induced proteins) were most pronounced at 0 h but diminished by 48 h postpartum (Figs 2, 3 and 5, Tables 2 and 3). The proteomics data were verified by confocal immunofluorescence analysis of filamin-A and vimentin (Figs 2 and 5, respectively). In contrast, the expression profiles of proteins involved in immune modulation and/or wound healing were variable, i.e., half of the six proteins (glutamine gamma-glutamyltransferase 2, lymphocyte antigen 6D and gelsolin) were high at 0 h but decreased by 48 h PP, whereas the expressions of the other half (phospholipase A2, annexin A1 and macrophage MIF) were opposite of the first half (Fig. 4 and Table 2).

Figure 1

A distinct proteome-wide expression profile in cervix of postpartum mice in the first 48 h. Cluster analysis reveals differentially expressed proteins of immediate postpartum (0 h) and hours later (15–48 h) in mice cervixes, as determined by proteomics and analyzed by ANOVA analysis. Over 150 proteins were found to be variably expressed, of which about 14 had a statistically significant change between 0 and 48 h postpartum. Red indicates higher protein expression and green indicates lower protein expression compared to baseline control ($n = 3$, $P = 0.05$).

Figure 2

Protein expression profile of actin-binding proteins (ABPs) in postpartum (PP) mice cervix, as revealed by proteomics and verified by confocal immunofluorescence (filamin-A), i.e., gelsolin, plectin-1 and filamin-A showed elevated levels of all the three proteins at 0h PP but decreased at 48h PP in mice cervix. $n=3$. Filamin-A confocal immunofluorescence: A=0h; B=48h. Red indicates higher protein expression, yellow indicates middle and green indicates lower protein expression compared to baseline control.

Discussion

Of the four phases of CR, postpartum repair is the least studied and to date, there is no comprehensive study that has focused exclusively on this fourth and final phase of CR. Here, using proteomics analysis, we identify over 150 proteins that were variably expressed, of which approximately 14 had a statistically significant change between 0- and 48-h postpartum. Through an extensive literature search, these 14 proteins were placed into one or more of the following categories: (a) intermediate filaments, (b) actin-binding proteins, (c) hypoxia-induced proteins and (d) proteins involved in immune modulation and/or wound healing.

Postpartum repair is characterized as a general inflammatory, wound-healing process (Timmons *et al.* 2010, Stanley *et al.* 2015). Specifically, there is a notable influx of tissue monocytes in the cervix shortly before birth as well as increased neutrophils postpartum (Timmons *et al.* 2009). These monocytes are then activated and

differentiated into M1 and M2 macrophages (Timmons *et al.* 2009, 2010). However, the exact mechanisms that underlie the influx and activation of these immune cells during this process are unclear. It is possible that the local VEGF and HIF-1 α surge reported previously, and immediately prior to term (Mowa *et al.* 2004, Stanley *et al.* 2015), could be responsible for both the immune cell influx and the activation for the following reasons: (a) after the birth-induced mechanical trauma (injury), the hypoxia-induced VEGF secretion establishes a gradient that mirrors the hypoxic gradient (Alberts *et al.* 2008), and VEGF receptor-expressing immune cells, including macrophages and neutrophils, respond chemotactically to VEGF, thus migrating to the injury/hypoxic site (Galiano *et al.* 2004, Eming & Krieg 2006, Bao *et al.* 2009); (b) under hypoxic conditions, HIF-1 α stimulates macrophages to secrete M1 pro-inflammatory cytokines (Acosta-Iborra *et al.* 2009) and (c) these recruited immune cells will secrete more VEGF and pro-inflammatory factors, as well as promote wound healing, angiogenesis and VEGF

Figure 3

Protein expression of intermediate filaments proteins in postpartum (PP) mice cervix, as revealed by proteomics, i.e., levels of keratin II 18, keratin I 8, desmin and vimentin were elevated at 0h PP but decreased at 48h PP in mice cervix. $n=3$.

Figure 4

Protein expression analysis of proteins involved in immune modulation and wound healing in postpartum (PP) mice cervix, as revealed by proteomics. Levels of half of the six proteins (glutamine gamma-glutamyltransferase 2, lymphocyte antigen 6D and gelsolin) were high at 0h but low at 48h PP and the expressions of the other half (phospholipase A2, annexin A1 and macrophage migration inhibitory factor) were opposite of the first half. $n=3$.

production in keratinocytes and fibroblasts (Galiano *et al.* 2004, Bao *et al.* 2009), cells that are known to play a critical role in wound healing. Further studies are required to confirm these speculations, as well as to shed more light on the alternate activation of the M2 phenotype.

The four intermediate filaments identified in the present study were keratins (type II 18 and type I 8), desmin and vimentin; overall, their expressions were elevated at 0h PP but dropped at 48h PP. Generally, intermediate filaments are among the three major cytoskeletal elements in eukaryotic cells, which are known to supply cellular strength and integrity by acting as intracellular scaffolds (Fuchs 1994). The two keratins (type II 18 and type I 8) are paired together (Singla *et al.* 2012), and are the major keratins found in epithelium (Tao *et al.* 2009). Desmin is associated with sarcomeres, nucleus and mitochondria of all muscle types (Paulin & Li 2004), and is known to

maintain cell integrity during contractions while helping force transmission and longitudinal load bearing (Paulin & Li 2004, Bonakdar *et al.* 2012). Generally, vimentin is considered as the cytoskeletal element that maintains cell integrity (Goldman *et al.* 1996) and offers resilience to cells undergoing high mechanical strain (Haudenschild *et al.* 2011). However, while the adult phenotypes in mice lacking keratin are obvious and severe, e.g., severe skin fragility (Lloyd *et al.* 1995), it is not so with vimentin. For instance, and of relevance to the current study, vimentin deficiency impairs wound healing both *in vitro* and *in vivo* by diminishing the mobility of fibroblasts and their ability to induce collagen contraction, which is essential in wound healing (Eckes *et al.* 1998, 2000). Generally, wound healing in adulthood is normally characterized by an inflammatory response and by differentiation of the fibroblasts into a specialized contractile cell, the

Figure 5

Protein expression profile of hypoxia-induced proteins in postpartum mice cervix, as revealed by proteomics and verified by confocal immunofluorescence (vimentin), i.e., transgelin and vimentin. Both proteins were elevated at 0h PP and decreased at 48h PP. Vimentin confocal immunofluorescence: A=0h; B=48h. Red indicates higher protein expression, yellow indicates middle and green indicates lower protein expression compared to baseline control. $N=3$. A=0h; B=48h.

Table 2 The key functional proteins significantly expressed in mice cervix the first 48 h postpartum.

Protein functional group	Proteins changing in expression	Overall expression in postpartum
1. Intermediate filaments	<ul style="list-style-type: none">• Keratins type II 18• Keratin type I 8• Desmin• Vimentin	<ul style="list-style-type: none">• All are high at 0 h but drop by 48 h PP
2. Actin-binding proteins	<ul style="list-style-type: none">• Gelsolin• Filamin-A• D isoform of plectin-1• Transgelin	<ul style="list-style-type: none">• All are high at 0 h but drop by 48 h PP
3. Hypoxia-induced proteins	<ul style="list-style-type: none">• Transgelin• Vimentin	<ul style="list-style-type: none">• Both high at 0 h but drop by 48 h PP
4. Immune modulation and/or wound healing	<ul style="list-style-type: none">• Glutamine gamma-glutamyltransferase 2• Gelsolin• Lymphocyte antigen 6D• Macrophage migration inhibitory factor• Annexin A1• Phospholipase 2	<ul style="list-style-type: none">• High at 0 h but drops by 48 h PP• High at 0 h but drops by 48 h PP• High at 15 h but low at 0 h and 48 h PP• Low at 0 h but high by 48 h PP• Low at 0 h but high by 48 h PP• Low at 0 h but high by 48 h PP

myofibroblast (Gabbiani *et al.* 1971, Martin 1997). It is feasible to speculate that the increased vimentin and desmin early in postpartum repair are necessary for the connective-tissue component of cervical repair, while keratin may play a similar role in the epithelial tissue (Eckes *et al.* 2000).

Any cellular process involving movement, such as proliferation, migration and morphological changes, requires continuous and rapid reorganization (assembly, disassembly) of actin filaments (dos Remedios *et al.* 2003). Such processes (actin filament assembly, disassembly and/or organization) are dependent on the presence of actin-binding proteins. Here, we detected four actin-binding proteins, whose expressions were elevated at 0 h postpartum, but decreased by 48 h postpartum. They include cytoplasmic gelsolin, filamin-A, the D isoform of plectin-1 and transgelin.

Gelsolin is a key regulator of actin filament disassembly, in a calcium-dependent manner, but also promotes actin polymerization and reorganization by severing then uncapping actin filaments (Sun *et al.* 1999). Although mice lacking gelsolin are viable and fertile, they have deficiencies in the organization of actin-based domains in osteoclasts and in the migratory capacity of neutrophils and macrophages during inflammation and wound healing (Witke *et al.* 1996). Also, gelsolin has been clinically associated with a variety of impairments in inflammation, cell invasion, immune cell recruitment and wound healing (Witke *et al.* 1996, Sun *et al.* 1999, Spinardi & Witke 2007, Gonclaves *et al.* 2010).

Filamins are responsible for crosslinking actin filaments, anchoring transmembrane proteins and scaffolding proteins (Wang *et al.* 2012), whereas plectin is a very large protein found in nearly all mammalian

cells that serve to link all three major components of the cytoskeleton to cellular junctions. By holding the cytoskeleton together, plectin helps maintain cell integrity and elasticity (Wiche 1998). Collectively, in the context of postpartum cervical repair, these actin-binding proteins could regulate a variety of locomotion-dependent processes, notably wound healing and immune responses. Further studies are needed to define their specific roles in the context of cervical repair.

Hypoxia is known to be involved in various physiological and/or pathological responses, but may also play a role in parturition. For instance, in the high-altitude mountain range of the Andes, expecting mothers induce childbirth at term by climbing to high elevations with a lower oxygen environment or hypoxia. Similarly, it is likely that hypoxia could account for the increased risk in preterm labor associated with smoking. Interestingly, the current proteomics approach revealed two hypoxia-induced proteins, transgelin and vimentin, that were highly expressed at 0 h postpartum. Transgelin is an actin-crosslinking protein known to be expressed in fibroblasts and smooth muscle (Kim *et al.* 2009, 2012), and is strongly expressed in both the uterus and the cervix. Recent studies by Kim *et al.* (2012) demonstrated that transgelin is upregulated by hypoxia, independent of HIF-1 α , and that it (transgelin) also activates the insulin-like growth factor receptor 1 (IGFR1) signaling pathway under hypoxic stress. The IGFR1 pathway has recently been shown to promote cell survival under hypoxic stress (Gariboldi *et al.* 2010, Kim *et al.* 2012) by upregulating *Hif1a* gene transcription (Gariboldi *et al.* 2010) and stabilization of HIF-1 α protein (Pieciewicz *et al.* 2012). This upregulation and stabilization of HIF-1 α by the IGFR1 axis have been shown to induce VEGF expression during embryonic vasculogenesis

Table 3 Differentially expressed proteins.

Primary protein name	Protein description	Overexpressed condition (h)
THIO_MOUSE	MCG2706	15
G3P_MOUSE	Glyceraldehyde-3-phosphate dehydrogenase	48
TBA4A_MOUSE	Tubulin alpha-4A chain	15
ALBU_MOUSE	Putative uncharacterized protein	48
SPR1A_MOUSE	Putative uncharacterized protein	15
ENOA_MOUSE	Enolase	15
TAGL_MOUSE	Transgelin	0
Q3US43_MOUSE	Annexin A1	48
TKT_MOUSE	Transketolase	15
GSTO1_MOUSE	Glutathione S-transferase omega-1	15
ACTA_MOUSE	Putative uncharacterized protein	0, 48
Q3UF30_MOUSE	Protein S100-A10	48
GSTA4_MOUSE	Glutathione S-transferase A4	15
GELS_MOUSE	Isoform cytoplasmic of gelsolin	0, 15
Q9CX22_MOUSE	Cofilin-1	15
Q8BT24_MOUSE	Putative uncharacterized protein (fragment)	15
FLNA_MOUSE	Filamin-A	0
PLEC1_MOUSE	Isoform PLEC-1D of plectin-1	0, 15
Q9Z1R9_MOUSE	Protease, serine, 1	48
FABP5_MOUSE	Fatty acid-binding protein, epidermal	15
SAP_MOUSE	Sulfated glycoprotein 1	48
FETUA_MOUSE	Alpha-2-HS-glycoprotein	0
QSOX1_MOUSE	Isoform 2 of sulfhydryl oxidase 1	15
Q3V2C6_MOUSE	Desmin	0, 15
A8DUV1_MOUSE	Alpha-globin	15
CLCA1_MOUSE	Calcium-activated chloride channel regulator 1	15
HBB2_MOUSE	Hemoglobin subunit beta-2	0
PA21B_MOUSE	Phospholipase A2	48
MIF_MOUSE	Macrophage migration inhibitory factor	48
Q3UCF8_MOUSE	Putative uncharacterized protein	0
Q8K1G6_MOUSE	Muc5b protein	15
Q3TFD9_MOUSE	Vimentin	0
LUM_MOUSE	Lumican	48
K2C8_MOUSE	Keratin, type II cytoskeletal 8	0, 15
14335_MOUSE	Putative uncharacterized protein	15
Q8K0P6_MOUSE	Fcgbp protein (fragment)	15
B1AQ77_MOUSE	Keratin, type I cytoskeletal 18	0, 15
TGM2_MOUSE	Protein-glutamine gamma-glutamyltransferase 2	0, 15
PGRP1_MOUSE	Pglyrp1 protein	15
Q91XG5_MOUSE	Protein S100-G	48
LY6D_MOUSE	Lymphocyte antigen 6D	15

Candidate protein markers based on label-free LC/MS/MS differential expression analysis.

(Pieciewicz *et al.* 2012) and angiogenesis in malignant tumor cells under hypoxic stress (Gariboldi *et al.* 2010). Whether transgelin acts upstream of HIF-1 α and VEGF in postpartum cervical repair is for now speculative. The exact and diverse roles of transgelin during postpartum cervical repair require further inquiry. Like transgelin, vimentin has also been associated with hypoxia. For instance, it has been shown to redistribute among the endothelial cell into stable structures in response to hypoxia (Lui *et al.* 2010), is involved in general wound healing (Rogel *et al.* 2011) and is known to play a role in migrating cells, including fibroblasts, macrophages, endothelial cells and invasive cancer cells (Lui *et al.* 2010, Glaser-Gabay *et al.*

2011, Helfand *et al.* 2011, Rogel *et al.* 2011). Future studies are needed to examine the roles of these hypoxia-induced proteins in the postpartum phase of CR.

Postpartum repair has been previously characterized as a pro-inflammatory and wound-healing process (Timmons & Mahendroo 2007, Bauer *et al.* 2009, Gonzalez *et al.* 2009). Our current proteomics data of postpartum cervical tissue are consistent with the earlier studies. Here, we identified several proteins that have known immune-related functions, notably glutamine gamma-glutamyltransferase 2, a transglutaminase, gelsolin, lymphocyte antigen 6D, macrophage MIF, annexin A1 and phospholipase 2. Transglutaminases are ubiquitous

enzymes that are important in posttranslational modification of proteins (Sabatino *et al.* 2012), which participate in coagulation and wound-healing responses (Upchurch *et al.* 1991, Aeschlimann & Paulsson 1994). Glutamine gamma-glutamyltransferase 2, often referred to as transglutaminase 2, is an enzyme that is important in the remodeling of ECM (Sabatino *et al.* 2012) and was found to be highly expressed at 0h postpartum followed by a decrease in expression at 48h postpartum. It is feasible that it may regulate cervical hemorrhage during parturition in addition to the extensive ECM remodeling that takes place after parturition (Timmons & Mahendroo 2007, 2010, Gonzalez *et al.* 2009). The role of gelsolin in inflammation, immune responses and wound healing has been discussed previously (Witke *et al.* 1996, Sun *et al.* 1999, Spinardi & Witke 2007, Goncalves *et al.* 2010) and is consistent with previous studies that report increases in various immune cell populations just after birth (Timmons *et al.* 2009).

Another protein of interest detected at high levels at 0h postpartum compared to 48h postpartum was lymphocyte antigen 6D (LY6D). LY6D is a marker found on mature B cells and specific populations of dendritic cells (Inlay *et al.* 2009) and is implicated in DNA damage control (Kurosawa *et al.* 2012), implying an increase in either dendritic or B cells postpartum. However, such cell types have not yet been reported postpartum.

Another protein noted in the present study involved in the immune response is macrophage MIF, and is one of the earliest cytokines to be discovered in the 1960s. MIF is a pro-inflammatory cytokine involved in the innate immune response (Calandra 2003, Roger *et al.* 2013), and was first characterized to play a large role in delayed hypersensitivity reactions (Bloom & Bennett 1970). These reactions involve interactions between T-cells and macrophages rather than antibodies, and MIF has broad cytokine actions that modulate T-cell responses (Choi *et al.* 2012), as well as T cell-macrophage interactions

Figure 6

Proposed working model of postpartum repair in the mouse. Cervical postpartum repair involves a combination of pro- and anti-inflammatory actions. At parturition and immediately after postpartum, the cervix is large and pliable without losing cell integrity; this is accomplished by ECM remodeling by transglutaminase 2 (TGM2) and cytoskeletal reorganization by both intermediate filaments (IF) and actin-binding proteins (ABPs). Tissue growth increases distances to nearby blood vessels, creating local hypoxia, which regulates certain IF and ABP while inducing VEGF secretion. Monocytes (M), as well as neutrophils (N), respond chemotactically to VEGF and migrate to the site of hypoxia/injury. Postpartum repair is a balance of pro- and anti-inflammatory processes, and monocytes are activated to M1 or M2 macrophage phenotypes. M1 and N will protect the cervix, secrete pro-inflammatory cytokines and aid in the clearance of damaged ECM molecules. M2 promotes tissue repair and keeps inflammation in check, e.g., annexin A1 inhibits the pro-inflammatory enzyme phospholipase A2. Glucocorticoids may induce the M2 phenotype and annexin A1. Tissue size is decreased, O₂ is restored and VEGF production is reduced.

(Nishihira 2000). MIF is also known to be secreted by the pituitary (Nishihira 2000). With MIF being elevated at 48 h postpartum, we speculate that a delayed hypersensitivity reaction could be taking place in the postpartum cervix, providing further support for a protective role of MIF from potential microbial invasion likely to occur immediately after birth.

One of the key changes occurring during the postpartum phase of CR is the conversion of monocytes to activated macrophages (M1, classical pro-inflammatory, and M2, alternative anti-inflammatory) (Timmons *et al.* 2009, 2010). The pro-inflammatory M1 and neutrophils would serve to aid with the clearance of ECM molecules (Timmons *et al.* 2010) while protecting the birth canal from foreign invaders (Gonzalez *et al.* 2009, Timmons *et al.* 2009), whereas the anti-inflammatory effects could serve to keep the pro-inflammatory effects in check (Timmons *et al.* 2010), which if left unregulated could potentially cause damage to the birth canal, putting subsequent pregnancies in jeopardy. In this study, we have identified a few potential players in the balancing act of pro- and anti-inflammatory levels and effects.

Finally, two proteins associated with immune responses, annexin A1 and phospholipase A2, were elevated at 48 h postpartum compared to 0 h postpartum. These two proteins have contradictory effects. Phospholipase A2 is a calcium-dependent enzyme that hydrolyzes glycerophospholipids into free intracellular arachidonic acid, the precursor for eicosanoids (Dennis 1994), including prostaglandins and leukotrienes, and as such is involved in inflammation and host defense. In contrast, annexin A1, also known as lipocortin, is a calcium-dependent phospholipid-binding protein that will inhibit phospholipase A2 as well as mediate VEGF-induced angiogenesis (Pin *et al.* 2012). Glucocorticoids stimulate production of annexin A1 and activation of M2 anti-inflammatory macrophages (Mantovani *et al.* 2006). It is interesting that both of these are elevated simultaneously, i.e., at 48 h postpartum even though they have apparent contradictory effects. This pattern, however, supports earlier suggestions that there is a check and balance in play between pro- and anti-inflammatory factors in the postpartum repair of the cervix.

In summary, our data support the current thinking in the field of CR that postpartum repair involves a combination of pro- and anti-inflammatory actions. From all the data gathered in the present study, along with what has already been characterized previously with regard to postpartum cervical repair, we propose a working model

(Fig. 6). Future studies will assess the suitability of some of the key proteins identified in this study as potential markers for determining the phase of postpartum cervical repair.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Robert Lee Stanley performed most of the work. Takako Ohashi and Jacob Gordon assisted in the Western blot and confocal immunofluorescence. Analysis and interpretation of data and write up of paper were done by Robert Lee Stanley with assistance from Chishimba Nathan Mowa. Chishimba Nathan Mowa is the principal investigator.

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