GYNECOLOGY

Uncovering changes in proteomic signature of rat pelvic floor muscles in pregnancy

Lindsey A. Burnett, PhD, MD; Francesca Sesillo Boscolo, PhD; Louise C. Laurent, MD, PhD; Michelle Wong, BS; Marianna Alperin, MD, MS

BACKGROUND: Structural and functional changes of the rat pelvic floor muscles during pregnancy, specifically, sarcomerogenesis, increase in extracellular matrix content, and higher passive tension at larger strains protect the integral muscle components against birth injury. The mechanisms underlying these antepartum alterations are unknown. Quantitative proteomics is an unbiased method of identifying protein expression changes in differentially conditioned samples. Therefore, proteomics analysis provides an opportunity to identify molecular mechanisms underlying antepartum muscle plasticity.

OBJECTIVE: To elucidate putative mechanisms accountable for pregnancy-induced adaptations of the pelvic floor muscles, and to identify other novel antepartum alterations of the pelvic floor muscles.

MATERIALS AND METHODS: Pelvic floor muscles, comprised of coccygeus, iliocaudalis, and pubocaudalis, and nonpelvic limb muscle, tibialis anterior, were harvested from 3-month-old nonpregnant and late-pregnant Sprague-Dawley rats. After tissue homogenization, trypsin-digested peptides were analyzed by ultra-high-performance liquid chromatography coupled with tandem mass spectroscopy using nano-spray ionization. Peptide identification and label free relative quantification analysis were carried out using Peaks Studio 8.5 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Proteomics data were visualized using the Qlucore Omics Explorer (New York, NY). Differentially expressed peptides were identified using the multi-group differential expression function, with q-value cutoff set at <0.05. Proteomic signatures of the pelvic floor muscles were compared to nonpelvic limb muscle and between nonpregnant and pregnant states.

RESULTS: Unsupervised clustering of the data showed clear separation between samples from nonpregnant and pregnant animals along principal component 1 and between pelvic and nonpelvic muscles along principal component 2. Four major gene clusters were identified segregating proteomic signatures of muscles examined in nonpregnant vs pregnant states: (1) proteins increased in the pelvic floor muscles only; (2) proteins increased in the pelvic floor muscles and tibialis anterior; (3) proteins decreased in the pelvic floor muscles and tibialis anterior; and (4) proteins decreased in the pelvic floor muscles alone. Cluster 1 included proteins involved in cell cycle progression and differentiation. Cluster 2 contained proteins that participate in mitochondrial metabolism. Cluster 3 included proteins involved in transcription, signal transduction, and phosphorylation. Cluster 4 comprised proteins involved in calcium-mediated regulation of muscle contraction via the troponin tropomyosin complex.

CONCLUSION: Pelvic floor muscles gain a distinct proteomic signature in pregnancy, which provides a mechanistic foundation for the antepartum physiological alterations acquired by these muscles. Variability in genes encoding these proteins may alter plasticity of the pelvic floor muscles and therefore the extent of the protective pregnancy-induced adaptations. Furthermore, pelvic floor muscles' proteome is divergent from that of the nonpelvic skeletal muscles.

KEY WORDS: pelvic floor muscles, pregnancy adaptations, proteomics, rat

Pelvic floor disorders, including pelvic organ prolapse and urinary and fecal incontinence, are exceedingly common conditions, with a prevalence of 23.7% among community-dwelling women in the United States.[1] These chronic disorders have a tremendous negative impact on quality of life. Pelvic floor muscle (PFM) dysfunction is a major risk factor for the development of pelvic floor disorders, especially pelvic organ prolapse. Although multiple predisposing and promoting factors have been identified, the single most significant event accountable for PFM dysfunction is vaginal delivery. During vaginal birth, PFMs are hypothesized to elongate up to 300% of resting length.[2,3] In the limb skeletal muscles, such strains consistently result in muscle injury, suggesting that all vaginally parous women should sustain PFM injury.[4] Surprisingly, only ~30% of vaginally parous women demonstrate radiologically visible PFM injury.[5] PFMs likely undergo adaptations that change muscle physiological limits to facilitate fetal delivery, while protecting against maternal injury. Given ethical constraints associated with directly probing these muscles in living women, we rely on direct tissue-level studies of PFMs in animal models to help us uncover the reasons for the above. The rat model is used in this study, as it has been previously demonstrated to be an excellent model for tissue changes in pregnancy,[6–8] and the rat pelvic floor muscle anatomy and architecture are similar to those of humans.[9,10]

Our previous investigations have also demonstrated protective pregnancy-induced adaptations of the rat PFMs that favorably alter muscle response to strains associated with delivery.[11] These adaptations, specifically, sarcomerogenesis, increase in intramuscular extracellular matrix content, and higher passive tension at larger strains, appear to be critical to the PFMs' ability to withstand excessive parturition-related strains by attenuating sarcomere

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AJOG at a Glance

Why was this study conducted?

This study elucidates putative mechanisms underlying protective pregnancy-induced adaptations in rat pelvic floor muscles and identifies novel antepartum proteomic alterations of pelvic muscles.

Key findings

- Pregnancy increases pelvic floor muscles' expression of proteins that stimulate cell proliferation and decrease apoptosis.
- Expression of fast skeletal muscle protein isoforms decreases in pelvic floor muscles during pregnancy, suggesting fast to slow fiber type transition.
- Muscle metabolism in pregnancy favors aerobic respiration with increased expression of oxidative enzymes and mitochondrial proteins, consistent with the transition in fiber phenotype.

What does this add to what is known?

We identified candidate modulators of protective pregnancy-induced adaptations of rat pelvic floor muscles, specifically pathways involved in muscle resident stem cell component and muscle metabolism. Understanding antepartum changes in pelvic muscles is essential to harness their endogenous potential to prevent or mitigate maternal birth injury.

hyperelongation and thus preventing muscle injury.[11–13] Consequently, a deeper understanding of the cellular and molecular mechanisms underlying these structural and functional adaptations is needed to be able to harness the endogenous plasticity of PFMs. Building on our previous studies, we sought to obtain a global understanding of the rat PFMs' response to pregnancy, by using an unbiased quantitative proteomic approach.

Quantitative proteomics offers an opportunity to simultaneously identify and quantify thousands of proteins within a tissue. With quantitative mass spectrometry analysis, identification of relatively small changes in protein expression between different conditions can be accomplished. As a result, small but biologically significant protein alterations in multiple signaling pathways can be studied concurrently. Proteomics provides direct assessment of the biologically relevant protein levels, which often only modestly correlate with mRNA expression due to posttranscriptional regulatory mechanisms.[14–16] Here, we used an unbiased quantitative proteomic approach to accomplish the following objectives: (1) to elucidate putative mechanisms accountable for pregnancy-induced adaptations of PFMs, and (2) to identify other novel antepartum alterations of PFMs.

Materials and Methods

Muscle procurement

The University of California San Diego Institutional Animal Care and Use Committee approved all study procedures. Nulligravid nonpregnant (n = 3) and primigravid late pregnant (n = 3) 3-month-old Sprague-Dawley rats were obtained from Envigo (Indianapolis, IN). Nonpregnant animals were in similar parts of the estrous cycle as determined by vaginal smear. Pregnant rats were in day 20–21 of gestation. Animals were sacrificed, and pelvic floor muscles, including coccygeus (C) and the individual components of the levator ani, pubocaudalis (PCa) and iliocaudalis (ICa), were harvested.[17] Tibialis anterior (TA), a hind limb skeletal muscle, served as a nonpelvic control.

Sample preparation for mass spectroscopy

Muscles were snap frozen in liquid nitrogen and sectioned on Leica Cryostat (Buffalo Grove, IL) to mechanically disrupt the tissue. Tissue was suspended in 1% sodium dodecyl sulfate in phosphate-buffered saline solution and incubated overnight at room temperature. Samples were then centrifuged, and pelleted solids were resuspended in 1% sodium dodecyl sulfate in phosphate-buffered saline solution and then disrupted by brief homogenization. Next, tryptic digest and peptide isolation were performed by filter-aided sample preparation, as previously described.[18] In brief, samples in 4% sodium dodecyl sulfate and 10 mM dithithreotide were applied to spin filters, incubated at room temperature for 10 minutes, and boiled at 100°C for 5 minutes. Spin columns were washed with 0.1 M Tris 8M urea. Samples were carboxymethylated with 0.5 mg/mL of iodoacetamide for 20 minutes at 37°C in the dark and then washed with urea solution. Tryptic digest was performed on spin filter with 0.03 mg/mL trypsin in 50 mM ammonium bicarbonate overnight at 37°C. Spin filters were washed with 50 mM ammonium bicarbonate, and peptides were eluted with 0.5 M sodium chloride. Elutant was dried with speed vac and resuspended in 0.5% trifluoracetic acid and 5% acetonitrile. Samples were zip tipped (Millipore, Burlington, MA) per the manufacturer's instructions.

Mass spectroscopy

Trypsin-digested peptides were analyzed by ultra-high-pressure liquid chromatography coupled with tandem mass spectroscopy using nano-spray ionization. The nanospray ionization experiments were performed using a TripleTof 5600 hybrid mass spectrometer (ABSCIEX) interfaced with nano-scale reversed-phase ultra-high-pressure liquid chromatography (Waters Corporation nano ACQUITY, Milford, MA) using a 20 cm–75 μm ID glass capillary packed with 2.5 μm C18 (130) CSHTM beads (Waters Corporation). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of acetonitrile (ACN) at a flow rate of 250 μL/min for 1 hour. The buffers used to create the ACN gradient were as follows: buffer A (98% H_2O, 2% ACN, 0.1% formic acid, and 0.005% trifuoroacetic acid), and buffer B

(100% ACN, 0.1% formic acid, and 0.005% trifuoroacetic acid). MS/MS data were acquired in a data-dependent manner in which the MS1 data were acquired for 250 milliseconds at m/z of 400−1250 Da and the MS/MS data were acquired from m/z of 50−2000 Da. The independent data acquisition parameters were as follows: MS1-TOF acquisition time of 250 milliseconds, followed by 50 MS2 events of 48 milliseconds acquisition time for each event. The threshold to trigger MS2 event was set to 150 counts when the ion had the charge state +2, +3, and +4. The ion exclusion time was set to 4 seconds. Peptide identification and label-free quantification analysis were carried out using Peaks Studio 8.5 software (Bioinformatics Solutions Inc, Waterloo, ON, Canada).

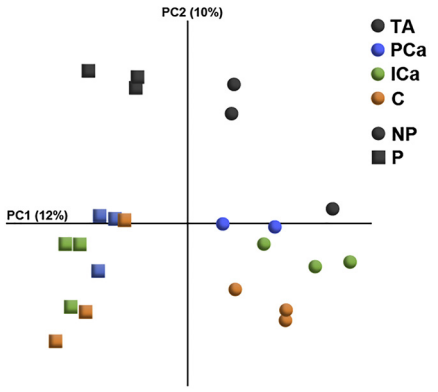
Statistical and functional analysis

For power analysis, the effect size and standard deviation are needed a priori, which is not possible for exploratory studies such as this one, as no prior data are available. We opted for a resource equation approach with repeated-measures analysis of variance, based on which our sample size calculation yielded 3 animals per group. Proteomics data were visualized using the Qlucore Omics Explorer (Qlucore, New York, NY). Differentially expressed peptides were identified using the multigroup differential expression function (equivalent to analysis of variance), with a q-value cutoff set at <0.05 and log2FC set at ≥ 1.5. Proteomic signatures of the pelvic floor muscles were compared to nonpelvic limb muscle, as well as in nonpregnant and pregnant states. Significantly different peptides were imported into the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 to identify biological pathways. *P*-values and Benjamini scores (globally corrected *P* value to control for familywise false discovery rate) were generated by DAVID using previously established methods.[19−23]

Results

We assessed differential peptide expression in PFMs (PCa, ICa, C) and TA, procured from nonpregnant and pregnant rats using principal component analysis. Principal component 1, which accounted for 12% of the variability among samples, drew a clear separation between samples from nonpregnant and pregnant animals (Figure 1). Principal component 2 segregated PFMs from TA, accounting for an additional 10% variability between samples (Figure 1). A total of 63 peptides had significantly different expression between nonpregnant and pregnant states. Of these, 9 proteins were increased and 8 were decreased specifically in PFMs of the pregnant rats, whereas 17 were increased and 13 were decreased in the pregnant group in all muscles examined. This allowed the identification of 4 separate clusters as follows: (1) proteins increased only in PFMs in pregnancy; (2) proteins increased in both PFMs and TA, in pregnancy; (3) proteins decreased in both PFMs and TA in pregnancy; and (4) proteins decreased only in PFMs in pregnancy (Figure 2).

FIGURE 1
Principal component analysis of tryptic peptides from the rat pelvic floor muscles and tibialis anterior



Unsupervised clustering of the data demonstrating a clear separation between samples from nonpregnant (NP) (spheres) and pregnant (P) (squares) rats along principal component 1 (PC1). Clustering separated pelvic floor muscles (pubocaudalis (PCa), blue; iliocaudalis (ICa), green; and coccygeus (C), orange) from tibialis anterior (TA) (black) along principal component 2 (PC2).

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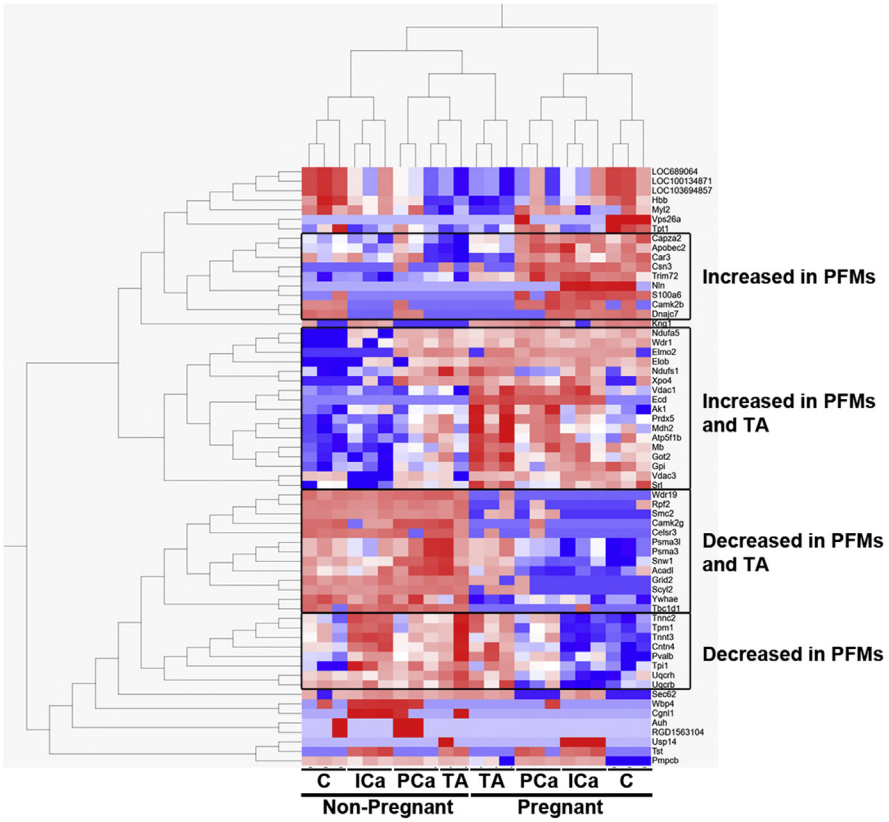
Cluster 1

Pathway analysis of the proteins comprising cluster 1 (Figure 3A), to our surprise, did not identify any pathways containing more than 1 of these proteins. Consequently, we decided to examine the specific expression pattern for each protein. Using this approach, we identified 2 categories of proteins, as demonstrated in Figure 3B: (1) proteins with high expression in nonpregnant rats that undergo minimal changes during pregnancy; and (2) proteins with low expression in nonpregnant animals that undergo large changes during pregnancy.

We focused on the proteins in the second category, as they had the greatest changes in expression during pregnancy. Of the 5 proteins identified, none have been previously well characterized in skeletal muscle. However, two proteins (S100a6 and Camk2b) have known functions in cell proliferation, which was intriguing in the context of sarcomerogenesis that occurs during pregnancy selectively in PFMs.[12,13] S100a6 is a member of the S100 family of proteins, known to regulate cell proliferation and differentiation in a calcium dependent fashion.[24] Camk2b has been shown to prevent apoptosis in hippocampal neurons.[25] Although these proteins seem unrelated, their concurrent change during pregnancy likely contributes to the sarcomerogenesis of PFMs. S100a6, which is known to stimulate cell proliferation, may stimulate resident muscle stem cells, termed

FIGURE 2
Heat map of tryptic digest peptides from rat pelvic floor muscles (PFMs) and tibialis anterior (TA)



Heat map demonstrating 4 major gene clusters (black boxes). Cluster 1: proteins increased in pregnancy only in PFMs. Cluster 2: proteins increased in in pregnancy in both PFMs and TA. Cluster 3: proteins decreased in pregnancy in both PFMs and TA. Cluster 4: proteins decreased in pregnancy only in PFMs. Colors represent relative quantification of protein abundance in $\log^2$ intensity scale, with blue indicating the lowest and red the highest protein expression. Gene names encoding differentially expressed proteins are listed on the right side of the heatmap.

C, coccygeus; *ICa,* iliocaudalis; *PCa,* pubocaudalis.

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satellite cells, that are imperative for sarcomerogenesis, whereas Camk2b may limit apoptosis in the same cellular compartment.

Cluster 2

A total of 17 proteins were significantly increased in all muscles examined during pregnancy (Figure 4A). Using DAVID analysis, we identified 8 pathways that included 9 of these proteins (Figure 4B). Closer examination of these pathways revealed enrichment in metabolic regulation and mitochondrial function. Many pathways seemingly unrelated to skeletal muscles, such as Parkinson, Huntington, and Alzheimer disease pathways, identified components of the mitochondrial electron transport chain, including Atp5f1b (Complex I), and Ndufs1 and Ndufa5 (Complex V). These proteins were also identified as part of the oxidative phosphorylation pathway and metabolic pathway.

The metabolic pathway also included enzymes critical for cellular respiration and energy production, including components of the malate shuttle (Got2, Mdh2) and glycolysis (Gpi, Ak1). Specific expression patterns for each protein were examined and demonstrated 2 patterns. Metabolic enzymes and Complex I components were expressed in high levels in nonpregnant animals with a small increase in pregnancy. On the other hand, components of Complex V had lower expression in nonpregnant animals and greater increase in expression during pregnancy, as illustrated in Figure 4C. Overall, we identified 9 proteins critical for cellular metabolism and regulation of mitochondrial adenosine triphosphate (ATP) generation that were increased during pregnancy, suggesting an upsurge in muscle metabolic activity overall, and mitochondrial respiration in particular.

Cluster 3

A total of 13 proteins were significantly decreased during pregnancy in all muscles examined (Figure 5A). Pathway analysis identified 2 pathways, which included only 4 of the proteins in this cluster (Figure 5B). Thus, we examined protein-specific expression changes to identify proteins with the largest decreases in expression during pregnancy. All members of this cluster had similar baseline expression in nonpregnant animals; however 8 proteins had a dramatic decrease in expression in the pregnant group. Many of these proteins are involved in cell growth (Rpf2, Smc2, Tb1d1) and cell signaling (Celsr3, Scyl2), and 1 protein, Camk2g, has a known function in skeletal muscle: regulation of calcium release from the sarcoplasmic reticulum.[26] Overall, we found that multiple cell growth and signaling proteins were decreased, suggesting restriction of cell growth and muscle fiber size during pregnancy.

Cluster 4

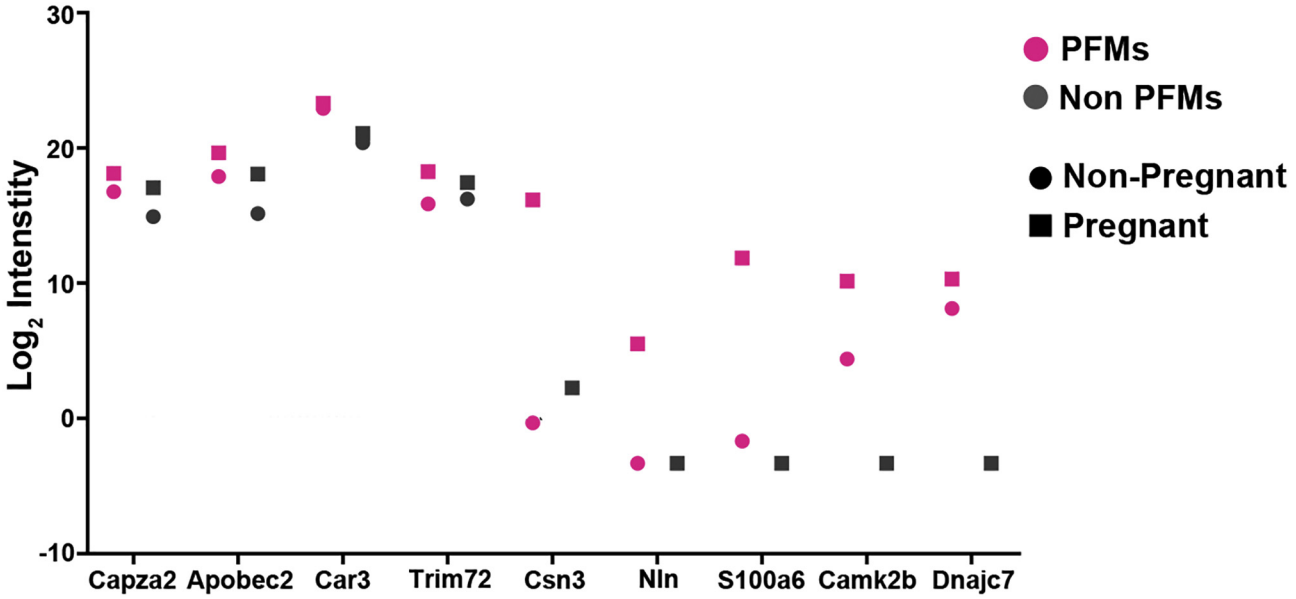
A total of 7 proteins were significantly decreased in pregnancy only in PFMs (Figure 6A). Pathway analysis identified 5 pathways that contained 5 of these proteins (Figure 6B). Interestingly, protein-specific expression patterns showed that all proteins exhibited a similar decrease in expression, as illustrated in Figure 6C. Thus, we focused on the pathways identified, as the pattern of

FIGURE 3
Proteomic analysis of cluster 1 proteins that are increased in pregnancy only in the pelvic floor muscles

A

Gene ID	Protein Name	Gene Name	Coccygeus	Iliocaudalis	Pubocaudalis	Tibialis Anterior	p-value
493810	Capping actin protein of muscle Z-line alpha subunit 2	Capza2	1.25	2.17	0.67	2.14	0.0008
301226	Apolipoprotein B mRNA editing enzyme catalytic subunit 2	Apobec2	1.36	2.32	1.58	2.93	0.0000
54232	Carbonic anhydrase 3	Car3	0.75	0.52	-0.07	0.71	0.0000
29188	Casein kappa	Csn3	18.70	19.38	11.35	0.01	0.0019
365377	Tripartite motif containing 72	Trim72	2.00	3.00	2.15	1.21	0.0001
117041	Neurolysin	Nln	10.70	15.82	0.00	0.00	0.0001
85247	S100 calcium binding protein A6	S100a6	11.46	17.23	11.94	0.00	0.0008
24245	Calcium/Calmodulin Dependent Protein Kinase II Beta	Camk2b	-9.28	17.18	9.38	0.00	0.0004
303536	DnaJ Heat Shock Protein Family (Hsp40) Member C7	Dnajc7	-0.30	17.45	-10.62	0.00	0.0000

B



A, Significantly increased proteins in the pelvic floor muscles during pregnancy with difference in relative quantification of protein expression derived from peptide intensity in coccygeus (C), iliocaudalis (ICa), pubocaudalis (PCa) and nonpelvic limb muscle, tibialis anterior (TA) expressed in $\log^2$. **B,** Graphical representation of protein expression in the pelvic floor muscles (pink) and nonpelvic muscle (black) expressed in $\log^2$. *P* values derived from 2-way analysis of variance, followed by Tukey's range post hoc pairwise comparisons with significance level set to 5%.

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expression did not vary between the proteins in this cluster. Of 5 pathways, 4 included components of mitochondrial Complex III (Uqcrh, Uqcrb). Additional proteins identified in these pathways included members of the troponin complex (Tnnc2 and Tnnt3). The troponin complex, comprising 3 proteins (troponin I, T, and C), is integral for skeletal and cardiac muscle active contraction. Muscle force production in skeletal muscles is controlled primarily by changes in intracellular calcium concentration that alter binding of the troponins. Tnnc2 and Tnnt3 correspond to troponin C and T isoforms found in fast skeletal muscle.[27] Parvalbumin (Pvalb) is also found almost exclusively in fast-contracting muscles; it accelerates the contraction–relaxation cycle of fast twitch muscle by speeding the rate of relaxation via calcium shuttling.[28] Overall, we found that multiple fast skeletal muscle protein isoforms, which are involved in regulation of muscle contraction, are decreased in PFMs in pregnancy, suggesting a transition from fast to slow fiber type.

Comment

This is the first study to examine the molecular mechanisms accountable for pregnancy-induced adaptations in rat pelvic floor muscles. There are 3 primary findings of our study. First, pregnancy fundamentally alters protein expression in all skeletal muscles examined. Second, protein expression in PFMs is different from that of nonpelvic skeletal muscles.

FIGURE 4
Proteomic analysis of cluster 2 proteins that are increased in pregnancy in the pelvic floor muscles and tibialis anterior

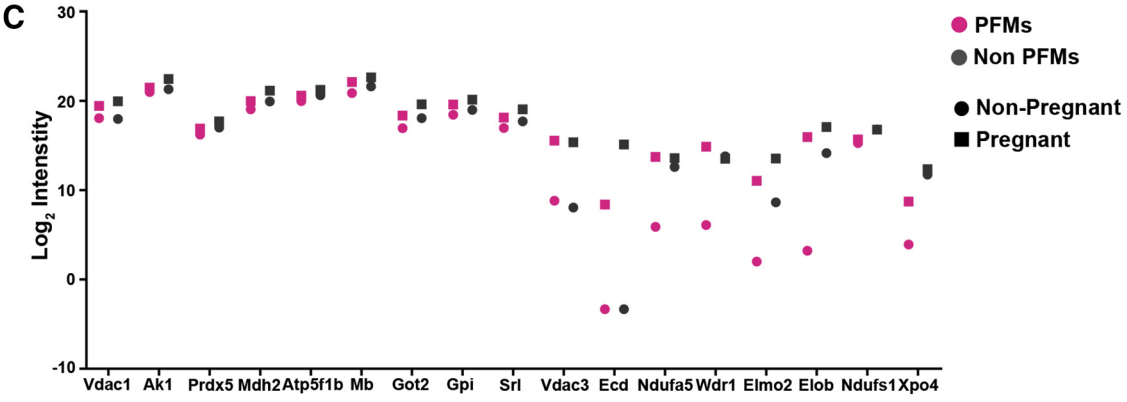
A

Gene ID	Protein Name	Gene Name	Coccygeus	Iliocaudalis	Pubocaudalis	Tibialis anterior	p-value
25488	NADH:Ubiquinone Oxidoreductase Subunit A5	Ndufa5	17.05	6.44	-0.03	-0.97	0.0002
360950	WD Repeat Domain 1	Wdr1	18.25	7.53	0.59	-0.28	0.0001
362271	Engulfment And Cell Motility 2	Elmo2	10.77	16.14	0.19	-4.91	0.0024
81807	Elongin B	Elob	18.64	8.72	10.80	2.94	0.0010
301458	NADH:Ubiquinone Oxidoreductase Core Subunit S1	Ndufs1	1.32	0.94	-1.06	-0.02	0.0008
290280	Exportin 4	Xpo4	5.40	12.43	-3.38	-0.62	0.0018
83529	Voltage dependent anion channel 1	Vdac1	0.63	1.85	1.60	1.99	0.0008
688968	Ecdysoneless cell cycle regualtor	Ecd	0.00	17.68	17.42	18.45	0.0000
24183	Adenylate Kinase 1	Ak1	0.18	0.15	1.16	1.16	0.0011
113898	Peroxiredoxin 5	Prdx5	0.51	0.72	0.81	0.69	0.0003
81829	Malate dehydrogenase 2	Mdh2	1.18	0.85	0.78	1.22	0.0012
171374	ATP synthase mithocondrial F0 comples subunit B1	Atp5f1b	0.71	0.43	0.72	0.62	0.0006
59108	Myoglobin	Mb	1.46	1.75	0.51	1.01	0.0000
25721	Glutamic-oxaloacetic transaminase 2	Got2	1.24	2.44	0.59	1.56	0.0002
292804	Glucose-6-phosphate isolmerase	Gpi	1.72	1.57	0.16	1.15	0.0010
83532	Voltage dependent anion channel 3	Vdac3	0.40	20.45	-0.68	7.30	0.0001
302948	Sarcalumenin	Srl	1.62	2.54	-0.61	1.34	0.0004

B

Term	Count	p-value	Benjamini
Parkinson's disease	5	0.000042	0.0016
Huntington's disease	5	0.00013	0.0025
Biosynthesis of antibiotics	4	0.0031	0.039
Metabolic pathways	7	0.0045	0.042
Carbon metabolism	3	0.012	0.089
Oxidative Phosphorylation	3	0.017	0.1
Alzheimer's disease	3	0.026	0.13
Cysteine and methionine metabolism	2	0.055	0.24

C



A, Significantly increased proteins in the pelvic floor muscles and tibialis anterior during pregnancy with difference in relative quantification of protein expression derived from peptide intensity in coccygeus (C), iliocaudalis (ICa), pubocaudalis (PCa) and nonpelvic limb muscle, tibialis anterior (TA) expressed in $\log^2$. *P* values derived from 2-way analysis of variance, followed by Tukey's range post hoc testing with significance level set to 5%. **B,** Pathways identified by Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of protein significantly increased in PFMs and TA during pregnancy with count of proteins included in each pathway (column 2). *P* values and Benjamini scores (globally corrected *P* value to control for familywise false discovery rate) were generated by DAVID using previously established methods.[19–23] **C,** Graphical representation of protein expression in the pelvic floor muscles (pink) and nonpelvic muscle (black) expressed in $\log^2$.

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Third, individual pelvic floor muscles have unique alterations in protein expression under physiological conditions of pregnancy.

With respect to the first point, we uncovered that proteomic expression changes in all skeletal muscles during pregnancy are mainly related to muscle metabolism. Known characteristics associated with greater oxidative capacity of skeletal muscle include increased mitochondrial density, increased oxidative enzymes, and reduced fiber size.[29,30] We saw increased expression of proteins critical for cellular metabolism and regulation of mitochondrial ATP generation during pregnancy suggesting an increase in metabolic activity, particularly of mitochondrial respiration in response to physiological conditions associated with pregnancy. Consistent with this increase in respiration, we observed a decrease in multiple cell growth and signaling proteins during pregnancy. This decrease is suggestive of restriction of cell growth and muscle fiber size needed to facilitate aerobic respiration in highly oxidative fibers.[31]

In addition to shared protein expression alterations in pregnancy, we also identified differential protein expression in PFMs compared to nonpelvic muscle. These differences were evident in both pregnant and nonpregnant states. Thus, our data indicate that PFMs are inherently different from the limb muscle despite their structural similarity. These findings suggest that PFMs may be uniquely equipped to respond to the stimuli of pregnancy with subsequent specific proteomic alterations resulting in muscle adaptations.

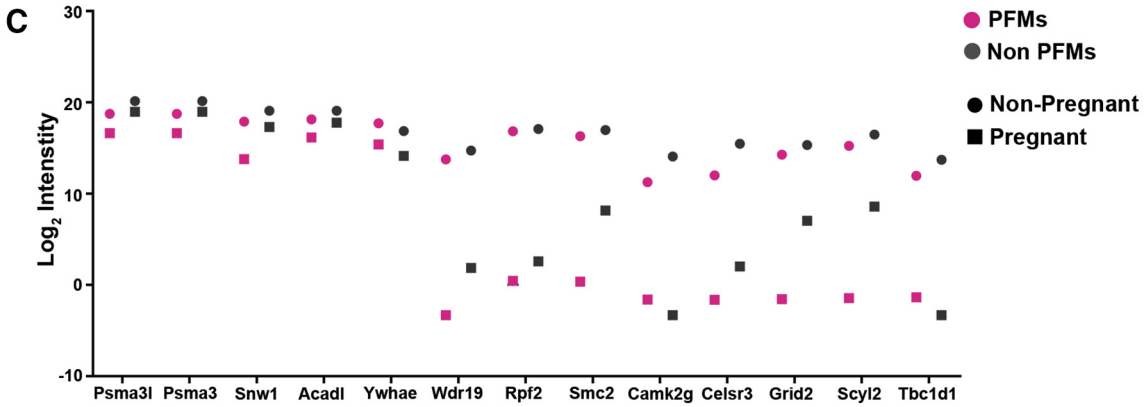
FIGURE 5
Proteomic analysis of cluster 3 proteins that are decreased in pregnancy in the pelvic floor muscles and tibialis anterior

A

Gene ID	Protein Name	Gene Name	Coccygeus	Iliocaudalis	Pubocaudalis	Tibialis anterior	p-value
305349	WD repeat doamin 19	Wdr19	-16.76	-19.92	-17.77	-12.87	0.0000
294436	Ribosome production factor 2 homolog	Rpf2	-14.49	-19.36	-14.77	-14.50	0.0028
362519	Structural maintainance of chromosome 2	Smc2	-19.95	-19.36	-8.49	-8.83	0.0003
171140	Calcium/calmodulin dependent protein kinase II gamma	Camk2g	-16.66	-8.84	-13.09	-17.40	0.0001
83466	Cadherin EGF LAG seven-pass G-type receptor 3	Celsr3	-18.47	-17.81	-4.58	-13.45	0.0027
408248	Proteasome subunit alpha 3-like	Psma3l	-3.12	-1.50	-1.66	-1.17	0.0003
29670	Proteasome subunit alpha 3	Psma3	-3.12	-1.50	-1.66	-1.17	0.0003
500695	SNW domain containing 1	Snw1	-4.99	-2.51	-4.79	-1.78	0.0001
25287	Acyl-CoA dehydrogenase long chain	Acadl	-1.11	-2.03	-2.83	-1.30	0.0001
79220	Glutamate ionotropic receptor delta type subunit 2	Grid2	-17.33	-17.84	-12.31	-8.31	0.0003
314717	SCY1 like pseudokinase 2	Scyl2	-18.16	-18.14	-13.75	-7.88	0.0004
29753	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon	Ywhae	-2.98	-2.04	-1.92	-2.73	0.0001
360937	TBC1 domain family member 1	Tbc1d1	-11.34	-11.36	-17.21	-17.03	0.0009

B

Term	Count	p-value	Benjamini
Proteasome	2	0.042	0.82
Oocyte meiosis	2	0.096	0.87



A, Significantly decreased proteins in the pelvic floor muscles and tibialis anterior during pregnancy with difference in relative quantification of protein expression derived from peptide intensity in coccygeus (C), iliocaudalis (ICa), pubocaudalis (PCa), and nonpelvic limb muscle, tibialis anterior (TA) expressed in $\log^2$. *P* values derived from 2-way analysis of variance, followed by Tukey's range post hoc testing with significance level set to 5%. **B,** Pathways identified by Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of protein significantly decreased in PFMs and TA during pregnancy with count of proteins included in each pathway (column 2). *P* values and Benjamini scores (globally corrected *P* value to control for familywise false discovery rate) were generated by DAVID using previously established methods.[19–23] **C,** Graphical representation of protein expression in pelvic floor muscles (pink) and nonpelvic muscle (black) expressed in $\log^2$.

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Sarcomerogenesis, or addition of sarcomeres in series, that increases resting fiber length is the major structural pregnancy-induced adaptation identified in rat PFMs thus far.[11,13] Sarcomerogenesis is a highly regulated process of sarcomere assembly and addition within muscle fibers associated with increase in myonuclei number.[32–34] During these processes, quiescent resident muscle stem cells (satellite cells) become activated and progress through the myogenic lineage.[35–37] Here we show a higher expression of proteins known to increase cell proliferation and to decrease apoptosis in PFMs during pregnancy. Such changes appear favorable for promoting satellite cell expansion, in turn facilitating sarcomerogenesis of PFMs that occurs under the physiological conditions of pregnancy.

We also discovered a decrease in multiple fast skeletal muscle protein isoforms in PFMs in pregnancy, supporting a transition from fast (glycolytic) to slow (oxidative) fiber phenotype. This fiber phenotype transition is unique to PFMs but complementary to the overall changes observed in all skeletal muscles examined, as slow fiber type is associated with increased aerobic respiration. These novel discoveries set the stage for the future studies aimed at comparing PFMs' metabolic function under nonpregnant and pregnant conditions.

Inherent limitations of our study include the use of an animal model to simulate human conditions. Consequently it is unknown whether these specific protein expression changes occur in women during pregnancy. However, because of the inability to

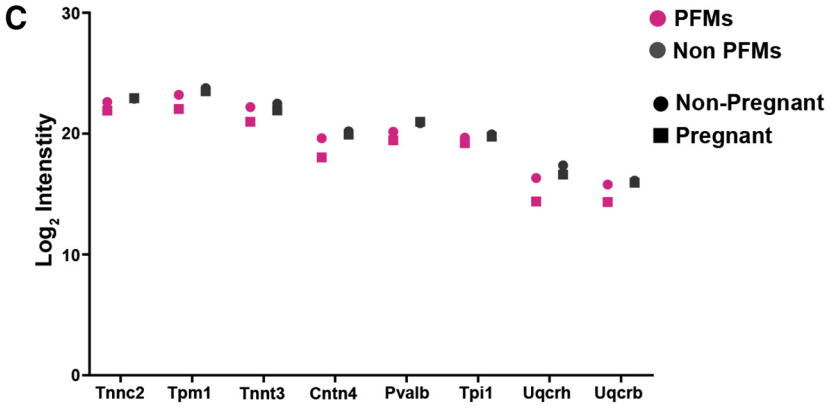
FIGURE 6
Proteomic analysis of cluster 4 proteins that are decreased in pregnancy in the pelvic floor muscles

A

Gene ID	Protein Name	Gene Name	Coccygeus	Iliocaudalis	Pubocaudalis	Tibialis anterior	p-value
296369	Troponin C2	Tnnc2	-0.54	-1.69	0.09	0.05	0.0003
24851	Tropomyosin 1	Tpm1	-0.98	-2.34	-0.17	-0.25	0.0003
24838	Troponin T3	Tnnt3	-1.00	-2.45	-0.23	-0.55	0.0001
116658	Contractin 4	Cntn4	-1.60	-2.67	-0.49	-0.27	0.0001
25269	Parvalbumin	Pvalb	-1.31	-0.90	0.07	0.12	0.0008
24849	Triosephosphate isomerase 1	Tpi1	0.09	-1.13	-0.35	-0.19	0.0024
366448	Ubiquitinol-cytochrome c reductase hinge protein	Uqcrh	-1.44	-3.52	-0.87	-0.75	0.0021
362897	Ubiquitinol-cytochrome c reductase binding protein	Uqcrb	-1.06	-2.26	-1.00	0.17	0.0003

B

Term	Count	p-value	Benjamini
Cardiac muscle contraction	3	0.00059	0.011
Oxidative phosphorylation	2	0.072	0.49
Parkinson's disease	2	0.076	0.38
Non-alcoholic fatty liver disease (NAFLD)	2	0.081	0.32
Alzheimer's disease	2	0.091	0.29



A, Significantly decreased proteins in the pelvic floor muscles during pregnancy, with difference in relative quantification of protein expression derived from peptide intensity in coccygeus (C), iliocaudalis (ICa), pubocaudalis (PCa) and nonpelvic limb muscle, tibialis anterior (TA) expressed in $\log^2$. *P* values derived from 2-way analysis of variance, followed by Tukey's range post hoc testing with significance level set to 5%. **B,** Pathways identified by Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of protein significantly decreased in PFMs during pregnancy with count of proteins included in each pathway (column 2). *P* values and Benjamini scores (globally corrected *P* value to control for familywise false discovery rate) were generated by DAVID using previously established methods.19—23. **C,** Graphical representation of protein expression in the pelvic floor muscles (pink) and nonpelvic muscle (black) expressed in $\log^2$.

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directly sample PFMs in pregnant women, animal models are critical to our understanding of the molecular biology of PFMs. The rat in particular has been identified as a representative model for studies of the pelvic floor during pregnancy and delivery.[7,8,12,38] Despite more favorable maternal pelvic-to-fetal size ratio in the rat compared to humans, rat PFMs demonstrate substantial phenotypic and functional adaptations. The above suggests that the human PFMs also undergo significant adaptations. The current study serves as a basis for identification of possible candidate modulators of pregnancy-induced adaptations. Whether these modulators directly alter pelvic organ prolapse risk is currently unknown. However, our future goals include perturbing these signaling pathways to directly examine their protective role against the untoward effects of birth injury.

The diversity and extent of hormonal and physiological alterations associated with pregnancy that alter functionality of virtually every organ system represent one of the most striking nonpathological transformations observed in nature.[39—41] Our data support the dramatic impact of pregnancy on skeletal muscles, as the pregnant state accounts for the highest degree of variability in muscle protein expression. Future studies are needed to validate and localize the proteomic changes observed in this study, and to identify the specific stimuli during pregnancy that direct protein expression to achieve protective adaptations of the pelvic floor muscles. ■

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