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ORIGINAL RESEARCH

Gene expression profile in pelvic organ prolapse†

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ABSTRACT: It was hypothesized that the processes contributing to pelvic organ prolapse (POP) may be identified by transcriptional profiling of pelvic connective tissue in conjunction with light microscopy. In order to test this, we performed a frequency-matched case–control study of women undergoing hysterectomy for POP and controls. Total RNA, extracted from uterosacral and round ligament samples used to generate labeled cRNA, was hybridized to microarrays and analyzed for the expression of 32 878 genes. Significance Analysis of Microarrays (Stanford University, CA, USA) identified differentially expressed genes used for ontoanalysis. Quantitative PCR (qPCR) confirmed results. Light microscopy confirmed the tissue type and assessed inflammatory infiltration. The analysis of 34 arrays revealed 249 differentially expressed genes with fold changes (FC) larger than 1.5 and false discovery rates $\leq$5.2%. Immunity and defense was the most significant biological process differentially expressed in POP. qPCR confirmed the elevated steady-state mRNA levels for four genes: interleukin-6 (FC 9.8), thrombospondin 1 (FC 3.5) and prostaglandin-endoperoxide synthase 2 (FC 2.4) and activating transcription factor 3 (FC 2.6). Light microscopy showed all the samples were composed of fibromuscular connective tissue with no inflammatory infiltrates. In conclusion, genes enriched for 'immunity and defense' contribute to POP independent of inflammatory infiltrates.

Key words: connective tissue / gene expression / ontoanalysis / pelvic organ prolapse / transcription profiling

Introduction

Pelvic organ prolapse (POP), hernias of the female genital tract, and associated urinary incontinence and defecation dysfunction affect 10–25% of women and often requires surgical correction. Thirty percent of operations for this condition are for recurrence of POP implicating an inherent defect in the tissues. Associated risk factors such as race, age, parity, body mass index (BMI) and socioeconomic factors have been reported; however, the natural history and biological mechanisms of POP are unknown (Olsen *et al*., 1997; Brizzolara *et al*., 2002; Hendrix *et al*., 2002; Nygaard *et al*., 2004). Variation in the quality and quantity of the connective tissue's extracellular matrix (ECM) proteins, such as the collagens, has been investigated, but the results are inconsistent and have not engendered a uniform hypothesis to explain the principal mechanisms leading to POP (Kokcu *et al*., 2002; Phillips *et al*., 2006). A prior *in vivo* expression study of POP used the pubococcygeus muscle and a microarray with only 12 626 genes, reporting differential expression of actin, myosin and various ECM protein-related genes (Visco and Yuan, 2003). Whole-genome microarrays were later used to identify genes involved with elastin metabolism in vaginal tissue from women with stress urinary incontinence without clinical POP (Chen *et al*., 2006).

Fibromuscular connective tissue invests and supports the pelvic organs. Surgery for prolapse primarily aims to restore this support. The combined uterine and upper vaginal support is derived from fibers in the paracolpium that are continuations of the cardinal and uterosacral ligaments (USL) (DeLancey, 1992). Although some investigators propose the use of readily available and more distal vaginal biopsies to represent the pelvic connective tissue, rather than the USL (Chen *et al*., 2006), others have shown the vaginal connective tissue may be more subject to the effects of POP than the proximal USL (Kokcu *et al*., 2002; Phillips *et al*., 2006). Likewise, the round ligament (RL), attached high in the pelvis to the fundus of the uterus, is part of the contiguous pelvic fibromuscular connective tissue (Ozdegirmenci *et al*., 2005), thought to provide orientation of the uterus and is potentially less subject to the traumatic effects of vaginal protrusion in POP than the lower genital tract and vagina. We hypothesize these proximal ligaments will demonstrate significant differential regulation of genes associated with the ECM proteins in POP and provide alternative insights into mechanisms underlying the abnormalities of uterine support. The aim of this unique project was to use

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whole-genome microarrays to identify differences in the transcription profile of POP tissue when compared with those from the control samples. In this report, we have combined the RL and the USL, to characterize proximal uterine support defects to further our knowledge of the molecular, cell and biological processes associated with the connective tissue in genital prolapse.

Materials and Methods

Tissue collection

Unconditional institutional review board approval for the study was obtained from Hawaii Pacific Health. Women scheduled to undergo hysterectomy for POP and other benign indications gave informed consent and were enrolled. Inclusion in the POP group required at least one component of prolapse beyond the hymen (Stage 3 or 4) associated with a uterine prolapse at or beyond the hymen (Baden and Walker, 1972). Exclusion criteria were prior pelvic reconstruction surgery, chronic debilitating disease, autoimmune and connective tissue disorders or cancer. Participant characteristics included: age, parity, BMI, menopausal status, vaginal and/or oral estrogen use, smoking, abdominal versus vaginal hysterectomy, and cervicitis in the hysterectomy specimen. Cases were frequency matched to controls based on menopausal status to minimize the confounding differences of age and menopausal physiology between the POP and the control groups.

The uterosacral and RLs were shaved off hysterectomy specimens providing ~1 g of tissue per sample. One set was immediately snap-frozen individually in liquid nitrogen and stored at −80°C and the other set was embedded in paraffin for microscopic evaluation.

Histology

Paraffin-imbedded samples were cut into 10 μm sections and stained with hematoxylin and eosin. A pathologist, blinded to the source of the samples, reviewed them via light microscopy to confirm that all samples were composed of fibromuscular connective tissue and were not portions of the cervix or uterus. The samples were examined for notable differences between the RL and the USL in POP and controls and inspected for the presence of inflammatory infiltrates. To characterize basic differences in composition of the ligaments, they were sorted into two categories that contained more or less than 50% smooth muscle. A power analysis was performed, which was based on the assumption of a difference in smooth muscle composition for the ligaments in POP versus controls. We defined a difference of interest as such: a minimum of 50% smooth muscle content in 90% of the control ligaments and 40% in the POP ligaments. The analysis revealed that 14 samples from each of these groups were necessary to obtain a 90% confidence limit and 80% power. Additionally, two-tailed *T*-test and Fisher's exact two-tailed computations using Epi-6 [www.cdc.gov/epiinfo/Epi6] were performed to compare the clinical characteristics of the two groups. POP and control participants were compared for differences in age, vaginal births, BMI and cervicitis as noted in the final pathology report.

RNA extraction

Total RNA was extracted using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's protocol and consecutively purified using Qiagen's RNeasy Mini Kit with on-column DNAse treatment (Valencia, CA, USA). RNA yield was quantified by spectrophotometry (NanoDrop, Wilmington, DE, USA) and the integrity was assessed by capillary-electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, CA, USA).

Microarray experiments

Arrays

Applied Biosystems (Foster City, CA, USA) Human Genome Survey Micoarrays V2.0 was used to analyze differential gene expression profiles. Each of these arrays contains 32 878 probes interrogating 29 098 genes.

Probe labeling

Digoxigenin-UTP-labeled cRNA was generated and linearly amplified from 5 μg of RNA using Applied Biosystems Chemiluminescence RT-IVT v 1.0 protocol. The labeled cRNA was washed and the quality and quantity were assessed using the NanoDrop (NanoDrop).

Hybridization

Ten micrograms of cRNA were hybridized to the arrays at 55°C for 18 h. Chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence detection kit, 1700 Chemiluminescent Microarray Analyzer and 1700 Chemiluminescent Microarray Analyzer software v1.1 following the manufacturer's protocol. In brief, after hybridization, the arrays were washed and scanned. The resulting images were auto-gridded. The signal was quantified, corrected for background noise and spot- and spatially normalized. The data were exported and the control probes were removed.

Microarray data analysis

The data were analyzed using the Significance Analysis of Microarrays (SAM), Stanford University, CA, program (Tusher *et al*., 2001). This software program assigns a score to each change in gene expression relative to the standard deviation of repeated measurements. Permutations of the repeated measurements are used to estimate the false discovery rate (FDR), which is the percentage of genes identified by chance. In this process, the data were normalized with median centering and a two class unpaired design was performed using a *t*-test statistic with 100 permutations, *k*-nearest neighbor imputer and neighbors equal to 10. We pooled the arrays from the RLs and USL of controls into one group and of POP into the case group.

The resulting list of differentially expressed genes with >1.5-fold changes (FC) and FDRs of 5.18 or less were then analyzed using Applied Biosystems Software: the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System that groups genes by their functions (Thomas *et al*., 2006). In the first step, proteins are classified into functional families and subfamilies, and then categorized more specifically by molecular function and biological process ontology terms. While PANTHER ontology is comparable with other ontology programs, it is significantly simplified to aid high-throughput analyses. Predominant and statistically significant biological processes were identified by PANTHER ontoanalysis. Quantitative PCR (qPCR) was used to verify selected differentially expressed genes involved in the biological processes of interest to us.

Real-time PCR analysis

To confirm the microarray data, four differentially expressed genes were chosen for quantitative RT-PCR: interleukin-6 (IL-6), activating transcription factor 3 (ATF3), thrombospondin 1 (THBS1) and prostaglandin-endoperoxide synthase 2 (PTGS2 or COX2). These genes had been classified as being part of the biological processes of immunity and defense (IL-6, THBS1 and PTGS2) and mRNA transcription regulation/apoptosis/Toll receptor pathway. To correct for loading variations, a control gene, glyceraldehyde-3-phosphate dehydrogenase, was also assayed with each RNA sample. The oligonucleotide primer sets for these five genes were

designed using the PrimerQuest software and obtained from IDT (Integrated DNA Technologies, Coralville, IA, USA) (Table I).

Complementary DNA synthesized from 22 samples used for the microarray analysis was subjected to qPCR. Analysis was performed on the DNA Engine Opticon II (MJ Research, Waltham, MA, USA), with a SYBR Green Master Mix (Invitrogen, Carlsbad CA, USA). Reactions were set up following the recommended protocol using 0.5 μl of each primer (20 pmol/μl) and 1 μl of template per reaction. Reactions were performed in triplicates for each sample for 45 cycles (95°C/15 s denaturing step; 55°C/30 s annealing/extension step, and a final extension step at 72°C/30 s). Negative controls without cDNA were included in the assays. FC were determined based on the average cycle threshold values for all triplicates.

Results

Tissue was obtained from a total of 26 hysterectomy procedures. The majority of cases were abdominal hysterectomies. Two vaginal hysterectomies were performed using sharp dissection in the POP group and one vaginal hysterectomy was performed with a bipolar knife in the control group. Selection was based upon quantity and quality of the extracted RNA, most significant degree of prolapse, and frequency matched for menopausal status and parity. A total of 17 subjects, eight with POP and nine controls, had adequate quality RNA for use on the microarrays. A total of 34 arrays and slides were processed from these 17 patients. The overall grade of prolapse was 3–4 and the degree of uterine prolapse was Grade 3–4 beyond the hymen except for 2 with uterine prolapse to the hymen accompanied by Grade 3 cystoceles. Clinical characteristics of the study population were similar (Table II).

Histology

The pathologist determined that all the samples were composed of fibromuscular connective tissue, and were not samples of cervix or uterus. Characterization of 16 available ligament pairs (one lost cassette) showed the RL with a tendency for more smooth muscle cells (SMC) than the USL and had more than 50% smooth muscle in 13/16 (81.25%) of samples, whereas the USL had more than 50% smooth muscle in only 6/16 (37.5%) of samples (*P*-value 0.03, Yates corrected). We were unable to identify a significant difference between these categories for POP (USL 3/7, RL 5/7) versus control samples (USL 3/9, RL 6/9), though we did not reach power for any individual ligament. Additionally, one sample contained a small area of vaginal mucosa, one sample contained a peripheral burn artifact from electrocaudery, one control USL sample had a focal lymphocytic infiltrate, and none of the samples had any generalized inflammatory infiltrate.

Table I Primers for qPCR

Gene name	Primers
Interleukin-6 (IL-6)	5′CTGGCTGTGGTTGAACAATG3′ 5′TTTTCTGCCAGTGCCTCTTT3′
Thrombospondin 1 (THBS1)	5′TTCCGCCGATTCCAGATGATTCCT3′ 5′ACGAGTTCTTTACCCTGATGGCGT3′
Activating transcription factor 3 (ATF3)	5′TGCCCGCCTTTCATCTGGATTCTA3′ 5′AGGTACAGACACTGCTGCCTGAAT3′
Prostaglandin-endoperoxide synthase 2 (PTGS2)	5′AGGGTTGCTGGTGGTAGGAATGTT3′ 5′ATCTGCCTGCTCTGGTCAATGGAA3′
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5′TCGACAGTCAGCCGCATCTTCTTT3′ 5′ACCAAATCCGTTGACTCCGACCTT3′

Table II Clinical characteristics

	POP (n = 8), mean (range)	Control (n = 9), mean (range)	*P*-value
Age in years	50.6 (28–70)	47.5 (24–65)	NS*
Parity	3.5 (2–6)	2.1 (1–4)	NS*
Cesarean only births	0	2	NS†
Body mass index	25.2	26.8	NS*
Menopause	3	3	NS†
Hormone use	2	1	NS†
Hypertension	2	1	NS†
Diabetes mellitus	1	0	NS†
Asthma	0	1	NS†
Osteoarthritis	1	0	NS†
Alcohol use	0	2	NS†
Smokes <5 cig/day	0	2	NS†
Caucasian	1	1	NS†
Vaginal hysterectomy	2	1	NS†
Chronic cervicitis	6	5	NS†

*Two-tailed *t*-test.
†Fisher's exact two-tailed.
NS, not significant.

Microarrays

The data from the microarray analysis of the RL and USL samples, using more than 1.5 FC in expression, resulted in 1521 up-regulated and 1193 down-regulated genes. Selecting for genes with FDR values ≤5.18, we identified 249 up-regulated gene probes. None of the down-regulated genes met this stringent FDR. The FDR among the down-regulated genes ranged from 32.4 up to 68.0, and thus these genes were not analyzed further. The list of up-regulated genes was analyzed with an ontoanalysis program, PANTHER. Immunity and defense was the most significant biological process for the POP-affected ligaments (Table III) identifying 53 differentially regulated gene probes involved in these processes (Table IV). Additionally, three pathways were identified: inflammation-mediated chemokine and cytokine signaling, Toll receptor signaling and the apoptosis signaling pathway (Table III). From the group of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases, previously reported to be involved in POP, we identified two MMPs: MMP19 (FC 2.57, FDR 5.18) and MMP2 (FC 5.2, FDR 7.8). The data discussed in this publication have been deposited in National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE-12852 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE-12852).

Real-time PCR

A small subset of genes was chosen to confirm the array results. Genes representing various processes were confirmed by qPCR.

Table III Predominant biological processes and pathways based on gene FC of 1.5 or greater and FDR of 5.18 or less

	AB1700 reference genes	Number of differentially expressed genes	Expected number of genes	*P*-value
Biological process				
Immunity and defense	1393	53	13.17	1.13E−16
Cell proliferation and differentiation	955	30	9.03	3.63E−07
mRNA transcription regulation	1350	37	12.77	1.44E−06
Signal transduction	3259	61	30.82	3.53E−06
mRNA transcription	1776	42	16.79	5.91E−06
Ligand-mediated signaling	387	18	3.66	9.04E−06
Granulocyte-mediated immunity	60	7	0.57	3.09E−04
Apoptosis	514	17	4.86	3.23E−04
Intracellular signaling cascade	854	24	8.08	3.72E−04
Nucleoside, nucleotide and nucleic acid metabolism	3210	54	30.35	5.45E−04
Cytokine and chemokine-mediated signaling pathway	237	12	2.24	7.09E−04
Cell cycle control	393	15	3.72	9.69E−04
JAK-STAT cascade	85	7	0.8	3.96E−03
Interferon-mediated immunity	62	6	0.59	4.72E−03
Cell surface receptor mediated signal transduction	1548	31	14.64	1.05E−02
MAPKKK cascade	178	9	1.68	1.19E−02
NF-kappaB cascade	72	6	0.68	1.46E−02
Oncogenesis	408	12	3.86	1.84E−02
Other metabolism	547	14	5.17	2.55E−02
Inhibition of apoptosis	127	7	1.2	3.45E−02
Macrophage-mediated immunity	127	7	1.2	3.45E−02
Cell communication	1154	24	10.91	4.19E−02
Pathways				
Inflammation mediated by chemokine and cytokine signaling pathway	305	14	2.88	2.40E−04
Toll receptor signaling pathway	70	6	0.66	8.86E−03
Apoptosis signaling pathway	146	8	1.38	1.25E−02

IL-6 (qPCR FC 9.8), THBS1 (qPCR FC 3.5) and PTGS2 (qPCR FC 2.4) and ATF3 (qPCR FC 2.6) were selected from the Toll receptor pathway and apoptosis signaling pathway. qPCR confirmed the elevated steady-state mRNA levels for these four genes.

Discussion

POP is common and frequently recurs or continues after surgical correction. Several prior microarray studies have addressed gene expression changes related to POP. For example, two *in vivo* studies, using RNA isolated from pubococcygeus muscle or vaginal tissue, identified actin, myosin, ECM-related genes as well as chemokines and transcription factors (Visco and Yuan, 2003; Chen *et al.*, 2006). Chronic cyclical mechanical stretch applied to cardinal ligament fibroblasts *in vitro* induced mechano-responsive genes such as regulators of actin and ECM remodeling and cell adhesion regulators among others (Ewies *et al.*, 2008). As the authors of the latter study note, ECM responses to stretch are most likely specific to the tissue and to the mechanism of stretch applied. To our knowledge, this is the first microarray study to analyze the transcription profiles of two sites of uterine attachment in the pelvis, identified as fibromuscular connective tissue. More specifically, we examined the RL and uterosacral ligament using 34 microarrays total from eight subjects with POP and nine control subjects.

Consistent with our hypothesis of significant differences in gene expression in POP, we were able to identify 249 up-regulated genes with an FDR of $<5.2\%$. Surprisingly, down-regulated genes within the threshold were not detected. Several reasons could contribute to this finding. The signature of up-regulated genes was significant enough to pass our stringent FDR threshold whereas due to the heterogeneity of the sample population precluded the identification of down-regulated genes. Additionally, choosing a low FDR inadvertently increases the false negative rate or type II error. The women included in this study were neither all pre-menopausal, at a specific phase of the menstrual cycle, nor were they genetically similar in race. This inter-patient variability may have limited our ability to identify specific down-regulated genes or to identify changes specific to individual ligaments. Considering these factors, our sample size was a limitation; however,

Table IV Biological process: immunity and defense

NCBI	Symbol	Gene name	FC	FDR
3569	IL-6	Interleukin-6	16.1	0
7057	THBS1	Thrombospondin 1	3.83	0
4938	OAS1	2′,5′-Oligoadenylate synthetase 1, 40/46 kDa	2.71	0
10135	PBEF1*	Pre-B-cell colony enhancing factor 1	2.67	0
4929	NR4A2	Nuclear receptor subfamily 4, group A, member 2	2.6	0
115908	CTHRC1	Collagen triple helix repeat containing 1	1.94	0
23643	LY96	Lymphocyte antigen 96	1.69	0
5359	PLSCR1	Phospholipid scramblase 1	1.57	0
3383	ICAM1	Intercellular adhesion molecule 1	6.5	2.94
10135	PBEF1*	Pre-B-cell colony enhancing factor 1	3.52	2.94
5743	PTGS2	Prostaglandin-endoperoxide synthase 2	3.02	2.94
6347	CCL2	Chemokine (C–C motif) ligand 2	3.01	2.94
1520	CTSS	Cathepsin S	2.3	2.94
969	CD69	CD69 molecule	2.25	2.94
6648	SOD2	Superoxide dismutase 2, mitochondrial	2.08	2.94
3588	IL-10RB	Interleukin 10 receptor, beta	1.91	2.94
7852	CXCR4	Chemokine (C-X-C motif) receptor 4	1.89	2.94
9516	LITAF	Lipopolysaccharide-induced TNF factor	1.74	2.94
120892	IRRK2	Leucine-rich repeat kinase 2	1.7	2.94
7058	THBS2	Thrombospondin 2	1.62	2.94
8743	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	1.62	2.94
4792	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.61	2.94
84168	ANTXR1*	Anthrax toxin receptor 1	1.56	2.94
1604	DAF	Decay accelerating factor for complement (Cromer blood group)	1.56	2.94
199	AIF1	Allograft inflammatory factor 1	1.54	2.94
3428	IFI16	Interferon, gamma-inducible protein 16	1.51	2.94
1154	CISH	Cytokine-inducible SH2-containing protein	2.56	3.28
3164	NR4A1	Nuclear receptor subfamily 4, group A, member 1	2.36	3.28
8013	NR4A3	Nuclear receptor subfamily 4, group A, member 3	10.5	3.71
5329	PLAUR	Plasminogen activator, urokinase receptor	4.2	3.71
3659	IRF1	Interferon regulatory factor 1	4.07	3.71
2920	CXCL2	Chemokine (C-X-C motif) ligand 2	3.94	3.71
4616	GADD45B	Growth arrest and DNA-damage-inducible, beta	3.22	3.71
25801	GCA	Grancalcin, EF-hand calcium binding protein	1.92	3.71
4783	NFIL3	Nuclear factor, interleukin 3 regulated	1.91	3.71
3119	HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1	1.6	3.71
5889	RAD51C	RAD51 homolog C (*Saccharomyces cerevisiae*)	1.54	3.71
5966	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	2.46	3.83
29015	PRG2; SLC43A3	Solute carrier family 43, member 3	1.51	3.83
3563	IL3RA	Interleukin 3 receptor, alpha (low affinity)	2.43	4.31
7056	THBD	Thrombomodulin	1.97	4.31
3459	IFNGR1	Interferon gamma receptor 1	1.61	4.31
3437	IFIT4 or IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	1.58	4.31
3553	IL-1B	Interleukin 1, beta	5.35	5.18
2919	CXCL1	Chemokine (C-X-C motif) ligand 1	2.71	5.18
4332	MNDA	Myeloid cell nuclear differentiation antigen	2.65	5.18
4688	NCF2	Neutrophil cytosolic factor 2	2.63	5.18

Continued

Table IV *Continued*

NCBI	Symbol	Gene name	FC	FDR
84168	ANTXR1*	Anthrax toxin receptor 1	2.53	5.18
5996	RGS1	Regulator of G-protein signaling 1	2.5	5.18
7127	TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	1.61	5.18
3460	IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)	1.54	5.18
3117	HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1	1.52	5.18

Genes with an FC of 1.5 or above and FDR of 5.18 or less.
*PBEF and ANTRX1 had more than one probe.
NCBI, National Center for Biotechnology Information.

it exceeds the sample size of many prior publications (Chen *et al*., 2006; Haddad *et al*., 2006; Nikolova *et al*., 2007). Since microarrays experiments use a large number of probes (variables) and a relative small number of samples, the *P*-value concept and the standard sample size calculations from the traditional hypothesis testing framework are not suitable (Pawitan *et al*., 2005). An article by one of the most prominent groups working on the statistical aspects of microarray data even suggested that sample size determination based on classical power considerations is not possible (Yang and Speed, 2003). For these reasons, most array studies to date do not perform any power analysis. On the basis of a meta-analysis of 16 published array studies which used progressive re-sampling of existing data, Pavlidis *et al*. (2003) determined that near-maximal levels of power and stability are achieved using 8–15 samples in each experimental group. We believe that the relative large number of arrays, applicable to tissues from divergent sites, and a heterogeneous sample population demonstrate processes common to the whole pelvic milieu affected with POP. These overriding processes, important in demonstrating an outline of the POP condition, provide an orientation for further investigation of more specific gene targets. Furthermore, many of the processes involved in prolapse may be slow responses taking place over time and subtle changes in expression may be the norm, explaining the moderate FC identified.

Ontological analysis of these genes identified enrichment for several biological processes and pathways. For example, we identified immunity and defense, transcriptional regulation, signal transduction, inflammation mediated by chemokines and the Toll receptor signaling pathway as significant contributors to the patho-biology of POP. Immunity and defense is of particular interest, as it represented the biological process with the largest number of differentially expressed genes. The Toll receptor signaling pathway is a system that recognizes pathogen-associated molecular patterns (Kirschning and Schumann, 2002) in addition to endogenous ligands, such as degradation products of the ECM (Scheibner *et al*., 2006). It also mediates the release of neutrophil-specific chemokines ultimately leading to neutrophil infiltration of tissues (Luster, 2002). IL-6 and Suppressor of Cytokine Signaling 3 (SOCS3) (FC 12.87, FDR 3.71) orchestrate the transition of the leukocyte infiltrate from neutrophil to mononuclear-cell infiltrate, a hallmark of acute inflammation (Heinrich *et al*., 2003; Kaplanski *et al*., 2003). Several genes represented chemokines and their receptors, small proteins implicated in homeostasis, development, hematopoiesis, angiogenesis, wound healing as well as those attracting subsets of leukocytes in inflammatory processes (Le *et al*., 2004). Specifically, CC motif, ligand 2 (CCL2), CXC motif, ligand 1 (CXCL1), CXC motif, ligand 2 (CXCL2) and CXC motif, receptor 4 (CXCR4) (FC 1.89, FDR 2.94) were up-regulated. Additionally, intercellular adhesion molecule 1 (ICAM1), strongly induced in POP, is involved in cell–cell adhesion, intra-cellular signaling, can bind to neutrophils, a step required for neutrophil infiltration (Kaplanski *et al*., 2003) and is a ligand for lymphocyte function-associated antigens (Bella *et al*., 1998). Prostaglandin-endoperoxide Synthase 2 (PTGS2 or COX2) catalyzes the initial steps in prostagladin synthesis. PTGS2 induction has been shown to be mediated by Toll receptor signaling pathway via NF-κB and MAP kinase pathways (Mitsunari *et al*., 2006), all of which showed increase expression levels in our study population. Our analysis also identified genes with increased mRNA levels that promote transduction of extracellular signals into cellular responses, such as c-rel proto-oncogene (REL) (FC 2.46, FDR 3.83) and nuclear factor of kappa light chain gene enhancer in B cells inhibitor, alpha (NFKBIA) both also utilizing the NF-κB and MAP kinase pathways. ATF3 (FC 3.1, FDR 3.8), a gene known to negatively regulate Toll receptor inflammatory responses, was also found to be up-regulated (Gilchrist *et al*., 2006).

As mentioned above, we identified a large group of genes conventionally implicated in the inflammatory process. Surprisingly, we did not find any inflammatory cell infiltrate in our histological evaluation of the ligaments. However, this phenomenon has been reported to occur in the normal course of human labor, a process in which an inflammatory gene expression signature was shown in the absence of chorioamnionitis (Haddad *et al*., 2006). Thus, it is reasonable to hypothesize that there are alternative functions of these inflammatory processes in the human.

The ECM component of connective tissue provides structural integrity and a three-dimensional scaffold that is important for cell adhesion and migration. Previous studies, though at times contradictory, have shown connective tissue remodeling in POP. Differences in MMPs have been investigated as evidence of collagen remodeling (Moalli *et al*., 2005; Gabriel *et al*., 2006). Hence, we hypothesized that RL and USL would demonstrate significant differential regulation of ECM genes in POP when compared with controls. Although we did not detect any changes related to any of the collagen genes, we observed elevated expression of other ECM-related genes in our POP samples. Recently, Nikolova *et al*. demonstrated that a single nucleotide polymorphism is associated with POP in a family with six early onset POP-affected probands. This polymorphism in the promoter region of basement membrane protein laminin C1 (LAMC1) affects

the binding site of the transcription factor nuclear factor, IL-3-regulated (NFIL3). NFIL3 (FC 1.91, FDR of 3.71) was significantly over-expressed in our samples possibly indicating a compensatory mechanism to accommodate for the altered binding to LAMC1 (Nikolova *et al*., 2007). Other ECM genes exhibited increase expression: MMP19 and MMP2. Although little is known about the proteinase MMP19, this is the first report on the involvement of MMP19 with POP. MMP2 barely surpassed our stringent FDR; however, our finding confirms a previous report (Moalli *et al*., 2005). A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) (FC 2.07, FDR 2.94) is another extracellular metalloproteinase found to be up-regulated in our prolapse study population. It is known to participate in a variety of biological processes such as inflammation and angiogenesis (Torres-Collado *et al*., 2006). It has been shown to cleave the matrix proteoglycans aggrecan, versican and the tissue factor pathway inhibitor 2 (TFPI2) (FC 4.20, FDR 12.55). However, it is possible that other substrates exist.

Cells are embedded in the surrounding ECM, which plays a crucial role in communication of the mechanical environment and mediates cellular responses to a variety of stimuli. As the uterus descends in POP, the supporting ligaments are subjected to altered mechanics. Numerous studies have shown that mechanical loading affects the cellular structure and function (MacKenna *et al*., 2000), causing changes in cell morphology, alterations in the cell cycle, and DNA and protein syntheses by employing soluble signaling molecules, changing ion influx through activation of mechanosensory ion channels or via collagen fibrils through integrin receptors (Wang *et al*., 2007). Integrins are a family of cell surface receptors that are essential for transducing mechanical forces from the outside to the inside of the cell and vice versa (Shyy and Chien, 1997). Integrins recognize a number of ECM molecules such as collagens or fibronectins. The conformational changes induced by external forces promote the interaction between ECM and integrins, resulting in the reinforcement of the cellular cytoskeleton (Wang *et al*., 1993) and an activation of MAP kinase (Schmidt *et al*., 1998; MacKenna *et al*., 2000) and NF-κB pathways (Xu *et al*., 1998), thus modifying cell behavior and differentiation. Since both of these pathways have to be considered as general, unspecific transducers of mechanical signals, it is possible that the observed increase in the expression of cytokines is a result of the ECM–integrin–MAPK/NF-κB cascade. Indeed, it is well known that CCL2, CXCL1, CXCL2, CXCR4 and IL-6 are all inducible through these two signaling pathways in one system or another, where CCL2 and IL-6 have been shown to mechano-induced through activation of the MAPK pathway for the former (Suda *et al*., 2001) and NF-κB for the latter (Sasamoto *et al*., 2005).

Mechanical stress and resultant changes in cell shape have also been demonstrated to be critical factors in the transcriptional regulation of inflammatory mediators such as COX2 and IL-1B in several different types of cells and tissues (Tsuzaki *et al*., 2003; Kessler-Becker *et al*., 2004; Yang *et al*., 2005; Kjaer *et al*., 2006). One of the most comprehensive studies on the effects of mechano-stimulation of cells demonstrated that human dermal fibroblasts grown in three-dimensional culture were capable of actively participating in the regulation of inflammatory processes (Kessler-Becker *et al*., 2004). A comparison of their microarray expression analysis and our transcriptional profiling of USL and RL in POP revealed remarkable similarities. Genes encoding receptors and cell surface proteins (ICAM1), cytokines/chemokines/growth factors [IL-6, keratinocyte growth factor 7 (FGF7) (FC 1.60, FDR 3.28)] and insulin-like growth factor binding protein 3 (IGFBP3) (FC 2.03, FDR 2.94) as well as stress response genes (PTGS2 and superoxide dismutase 2 (SOD2), mitochondrial, were up-regulated in both studies. Strikingly, however, this pro-inflammatory phenotype of the dermal fibroblast was observed when the cells were grown in relaxed collagen matrices lacking mechanical load. Presently, we are unable to determine if the fibroblasts or SMC residing in the USL and RL are indeed stretched or relaxed: while the ligaments themselves are extended as a tissue, the individual cells may actually reside in a mechanically relaxed environment due to the effects of the altered extracellular environment. Furthermore, discrepancy in these two studies may stem from the different experimental conditions as well as the origin and type of cells.

Although we are unable to conclusively determine if and which of the changes we have identified precede POP, we propose here that the transcriptional profile indicates fundamental phenotypic changes and re-programming of the gene expression in POP. The observed changes in gene expression and mechanical properties of the ECM are a consequence of changes in cell shape affected by the altered environment of the ligaments, ultimately leading to the transcriptional profile of a sterile inflammation.

At this point, it is too early to use this information for clinical treatment of inflammatory processes in managing POP because the components of inflammation identified in this study may indeed relate to unique biological processes such as those recently described in human labor (Haddad *et al*., 2006). Many of the genes we identified deserve further study of the cells producing these changes and their roles in specific immunity pathways leading to an altered ECM, cell shape, mechanical properties and aberrant inflammatory and healing processes. This information may provide potential targets for intervention during earlier stages of POP, mitigating the extremes of this clinical phenotype.

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