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Gene Expression Profiles of Uterine Normal Myometrium and Leiomyoma and Their Estrogen Responsiveness *In Vitro*

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Background : Uterine leiomyomas are common benign smooth muscle tumors among the reproductive aged-women. The research has been aimed to identify the differentially expressed genes between normal myometrium and leiomyoma and to investigate the effects of E_2 on their expression. **Methods :** Gene microarray analysis was performed to identify the differentially expressed genes between normal myomerium and leiomyoma. The data was confirmed at protein level by tissue microarray. **Results :** Gene microarray analysis revealed 792 upregulated genes in leiomyoma. Four genes (tropomyosin 4 [*TPM4*], collagen, type IV, alpha 2 [*COL4α2*], insulin-like growth factor binding protein 5 [*IGFBP5*], tripartite motif-containing 28 [*TRIM28*]) showed the most dramatic upregulation in all leiomyoma samples. Tissue microarray analyses of 262 sample pairs showed significantly elevated expression of TPM4, IGFBP5, estrogen receptor-α, and progesterone receptor (PR) protein in leiomyoma from the patients in their forties, COL4α2 in the forties and fifties age-groups, and TRIM28 in the thirties age-group. PR, insulin-like growth factor 1 (IGF-1), IGF-1 receptor (IGF-1R) and IGFBP5 were induced by E_2 in *in vitro* culture of tissue explants from which cells migrated throughout the plate. Among these, PR, IGF-1, IGFBP5 genes showed higher expression in tissue compared to cells-derived from tissue in leiomyoma and IGF-1R in leiomyoma cell. **Conclusions :** This observation implies the importance of the whole tissue context including the cells-derived from tissue in the research for the understanding of molecular mechanism of leiomyoma. Here, we report higher expression of TRIM28 in leiomyoma for the first time and identify E_2-responsive genes that may have important roles in leiomyoma development.

Key Words : Leiomyoma; 17beta-estradiol; Uterus; Oligonucleotide array sequence analysis; Immunohistochemistry

Uterine leiomyomas, also known as myomas or fibroids, are benign smooth muscle tumors of unicellular origin, composed heavily of extracellular matrix (ECM) like collagen, fibronectin, and proteoglycan. Leiomyomas are associated with dysmenorrhea, recurrent miscarriage, pelvic pain and pressure, and obstetric complications.[1] The reported incidence of leiomyoma in women of reproductive age varies from 5.4% to 77%, depending on the diagnostic method.[2] The American Cancer Society estimated 51,170 new uterine cancer cases including leiomyoma (7.0% of estimated total cancers in females) in the United States during 2008.[3]

Previous studies regarding hormonal, genetic, and growth factors underlying the molecular biology of leiomyoma[1] identified gene expression differences between leiomyoma and normal myometrium.[4] Gene microarray chip (GMA) and tissue microarray (TMA) are powerful tools to compare gene expression levels in healthy and diseased states, because they allow simultaneous interrogation of tens of thousands of genes.[5,6] With these techniques, it is possible to look into the molecular mechanisms of disease development on a genome-wide scale.[5] In array technology, two-color hybridization detection (two probe mixtures that competitively hybridize to a single array) allows the researcher to directly and quantitatively compare the abundance of specific sequences.[5] Similarly, TMA technology can characterize pro-

tein levels for thousands of tissue specimens at a time (depending on the probing method), facilitating rapid translation of molecular discoveries to clinical applications. In addition, TMAs can be used to reveal the cellular localization, prevalence, and by inference the clinical significance of the genes, making it suitable for genomic-based diagnostic and drug target discovery.[6]

Uterine leiomyomas are enriched in ECM with abundant connective tissue elements. Increases in cell proliferation, deposition of ECM, and local growth factors promote leiomyoma growth by paracrine and autocrine signaling mechanisms.[1] Elements of the microenvironment, including ECM, also contribute significantly to the steroid hormone responsiveness that underlies tumor growth.[7] Whole human tissue may therefore be a better model system for human myometrium,[8] although there has not been a thorough experimental examination to this end.

As evidenced by several studies, steroid hormones play a vital role in leiomyoma development and growth. Estrogen in particular has been indirectly implicated by virtue of the high leiomyoma incidence in women of reproductive age[9] and by the ability of GnRH agonists to mediate fibroid regression.[10] In addition, estrogen receptor (ER) modulators, such as ICI 182,780, tamoxifen, and raloxifene, inhibited the proliferation of leiomyoma cells in previous reports.[11] Still, the exact causes of leiomyoma remain largely unknown, and currently there are no reliable treatments aside from myomectomy or full hysterectomy.

Increases in aromatase (mRNA expression and enzymatic activity) mediate local synthesis of E_2 within leiomyoma, again pointing to an E_2 dependency for this tumor type.[12] Interestingly, one report observed substantial differences in aromatase expression when comparing normal myometrium and leiomyoma tissues from women in the follicular phase of the menstrual cycle.[12] The overall objective of the research herein was to identify genes and gene products that were differentially expressed in normal myometrium and leiomyoma using GMA and TMA, and to observe the effects of E_2 upon expression of these genes. The results provide an unprecedented look at differential gene expression within leiomyomas at different points in the menstrual cycle, and the effect of E_2 in differentially expressed genes in normal myometrium and leiomyoma.

MATERIALS AND METHODS

Human tissue collection

Samples of uterine leiomyoma and adjacent myometrium were obtained by hysterectomy upon written informed consent from each patient (Table 1). The specimens used for RNA extraction and cell culture were received from Kyungpook National University Hospital with approval by the standing Research Committee Leuplin-treated samples were obtained from patients who had been subcutaneously administered 3.75 mg GnRH agonist, leuplin (leuprorelin acetate; Takeda Chemical Industries, Ltd., Tokyo, Japan) per month for three months prior to surgery. The stage of the menstrual cycle/endometrial cycle (proliferative or secretory phase) in the women was assumed by patient's menstrual history. Besides, histological examination was performed for all the surgical specimens. During the process (fixation, embedding, slicing, staining. etc.) for final diagnosis of leiomyoma, pathologists determined the endometrial status as well.

Normal myometrial and leiomyoma samples used for the immunohistochemical study were collected during 2005 and 2006 at the Department of Pathology at Yeungnam University Hospital in Korea. Aside from the four leuplin-treated samples described above, all tissue samples were obtained from patients who had not undergone drug or exogenous hormone therapy before surgery.

Table 1. Tissue information with sample ID, age, menstrual cycle, menstrual phase and uterine weight of the patients

Sample ID	Age	Body weight (kg)	Menstrual cycle	Menstrual phase	Uterine weight (g)
3	52	59.8	Irregular	P	434
4	46	74	30	P	347
12	35	56	32	P	343
29	46	62	30	P	402
6	36	52.5	30	P	321
40	35	59	32	P	408
49	41	56	28	P	233
2	42	60	28	L	516
10	49	50	30	L	189
30	44	48	30	L	254
36	44	54	30	L	447
57	44	58	22	L	275
41	39	61	28	L	290
5	54	64	28	*	270
13	45	58	28	*	195
14	51	50	Irregular	*	174
16	39	63	32	*	178

Tissue information. patient number, age, duration of menstrual cycle, and the corresponding phase (P, proliferative phase; L, luteal phase and 4 leuplin treated), body and uterine weight of the patients are listed. Nine samples used for microarray analysis are indicated by underlined sample numbers (4 proliferative and 5 luteal phases). A total of 17 samples (7 proliferative, 6 luteal phases and 4 leuplin treated), including the 9 samples used for microarray analysis, were utilized for real-time reverse transcription polymerase chain reaction analysis. Four samples obtained from the leuplin-treated patients are indicated by asterisk (*) (see Materials and Methods).

RNA extraction and real-time reverse transcription PCR (rtPCR)

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from either frozen tissue or cultured tissue explants and cells, according to the manufacturer's protocol. RNA was reverse-transcribed into cDNA using Superscript-II reverse transcriptase (Invitrogen). Total RNA (1 μg in 20 μL total volume) was primed with oligo $(dT)_{20}$ primers (Bioneer Co., Daejeon, Korea). Subsequently, 2 μL of the 10 × diluted cDNA product and 10 pmoles of each gene-specific primer (Table 2) were used to perform rtPCR (7500 real-time PCR system, Applied Biosystems Inc., Foster City, CA, USA). SYBR Green (Power SYBR® Green PCR Master Mix, Applied Biosystems Inc., Warrington, UK) was used as the fluorescence source. Primers were designed with Primer 3 software (http://frodo.wi.mit.edu) using sequence information listed at the NCBI. rtPCR was carried out under the following conditions: pre-denaturation (95℃, 10 minutes), followed by 40 cycles of denaturation (95℃, 33 seconds), annealing at each gene-specific primer Tm (℃), and extension (72℃, 33 seconds). Proper amplification of the genes of interest was verified by melting point analysis and 1.2% agarose gel electrophoresis.

Tissue explant cultures

Tissue samples were minced into small pieces (3-5 mm) under sterile conditions and rinsed with phosphate buffered saline. ECM of the tissue pieces was digested using 20 mL collagenase type I (Worthington Biochemicals Co., Lakewood, NJ, USA) in Kreb's Ringer bicarbonate in a shaking water bath at 37℃ for 1 hour, accompanied by repeated pipetting to disturb the remaining connective tissue. Digested tissues were then centrifuged at 2,000 rpm for 5 minutes and resuspended in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories Inc., South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Inc.). Digested tissue explants were distributed evenly into 100-mm cell culture dishes (approximately 20 pieces per dish). Culture medium was added to cover half the height of the tissue explants (-5 mL) without disturbing the tissue explants, and the cultures were incubated at 37℃ under 5% CO_2 for about 3-5 days, during which the tissue explants attached to the dish and began emitting smooth muscle cells (SMCs). The volume of the medium was increased to 8 mL per 100-mm cell culture dish after one week as the emitted cells proliferated. Culture continued with daily monitoring of morphology and growth patterns.

Table 2. PCR primers

Gene	NCBI accession gene name	Gene name	Primer	Size (bp)	Tm (℃)
α-actin	J05192	Alpha-actin (ACTA)	F 5′-ggcatccatgaaaccaccta-3′ R 5′-gctggaaggtggacagagag-3′	244	65
β-actin	NM001101	β-actin	F 5′-ggcatcctcaccctgaagta-3′ R 5′-ggggtgttgaaggtctcaaa-3′	203	53
ER-α	X03635	Estrogen receptor-α	F 5′-ggacaggaaccagggaaaat-3′ R 5′-tccagagacttcagggtgct-3′	180	52
PR	M15716	Progesterone receptor	F 5′-gattcagaagccagccagag-3′ R 5′-cgatgcagtcatttcttcca-3′	190	53
IGF-1	X57025	Insulin-like growth factor I	F 5′-cagcagtcttccaacccaat-3′ R 5′-cacgaactgaagagcatcca-3′	213	53
IGF-1R	AY332722	Insulin-like growth factor I receptor	F 5′-gtcgaagaatcgcatcatca-3′ R 5′-gggcttcagcccatgtagta-3′	220	53
ETA-R	D90348	Endothelin-1 receptor	F 5′-tatcctggccattcctgaag-3′ R 5′-ttctcaagctgccattcctt-3′	233	53
TPM4	NM003290	Tropomyosin 4	F 5′-gccatgaaggatgaggagaa-3′ R 5′-cagatgcagcctccagagat-3′	235	53
TRIM28	NM005762	Tripartite motif-containing 28	F 5′-acgtacagaagcgtgtgcaa-3′ R 5′-aagcaaaagggctgtgttgt-3′	233	53
COL4α2	AK025912	Collagen, type IV, alpha 2	F 5′-aggcctgtatggcgagatt-3′ R 5′-tggaaagcctgtttgtcctt-3′	229	54
IGFBP5	NM000599	Insulin-like growth factor binding protein 5	F 5′-gagctgaaggctgaagcagt-3′ R 5′-gaatcctttgcggtcacaat-3′	237	56

Polymerase chain reaction (PCR) primers. The list of genes, Genbank accession numbers, gene names and primer sequences (F, forward; R, reverse), expected PCR product sizes, and annealing temperatures.

Myometrium and leiomyoma tissue explants were maintained in DMEM/10% FBS until the tissue-derived cells reached 50% confluence, at which point the culture medium was changed to phenol-red free medium (DMEM[-]) supplemented with 10% charcoal-dextran-treated FBS for 48 hours. Cultures were then treated with 10 nM E_2 (Sigma-Aldrich Inc., St. Louis, MO, USA) followed by extraction of total RNA as described above and previously.[13]

Gene microarray

RNAs isolated from 9 pairs of frozen tissue (each consisting of normal myometrium and adjacent leiomyoma; 4 tissue pairs were harvested when the patients were in proliferative phase, and 5 were harvested in luteal phase) were used to carry out gene microarray analysis with the Platinum Human Cancer 3.0K oligo microarray containing 3,096 oligonucleiotide (Genocheck, Ansan, Korea), including Operon human oligo subsets (Operon Biotechnologies, Inc., Huntsville, AL, USA), housekeeping genes, and *Arabidopsis* DNA as controls. Equal amounts of the Cy3-(myometrial) and Cy5 (leiomyoma)-labeled cDNA probes were mixed and denatured at 95℃ for 2 minutes before hybridization. Hybridized slides were scanned with an Axon Instruments GenePix 4000B scanner (Axon Instruments Inc., Sunnyvale, CA, USA). Scanned images from tissue samples were analyzed in GenePix Pro 5.1 (Axon Instruments Inc.) and GeneSpring GX 7.3.1 (R package, Agilent Technologies, Santa Clara, CA, USA); images from cell samples were analyzed in GeneSpring 7.2 (Silicogenetics, Foster City, CA, USA). The analyzed data were normalized by Global, Lowess, and print-tip methods (for tissue) and median and quantile normalization (for cell); normalization was also scaled for data reliability. The genes expressed at higher than 200% of control and lower than 50% of control were considered to be upregulated genes and downregulated genes, respectively. The statistical relevance of the data in this study was further confirmed by SAM (Stanford University, Palo Alto, CA, USA). Data were clustered into groups of genes that behaved similarly throughout the time course experiments using GeneSpring GX 7.3.1 (Agilent Technologies). An algorithm designed based on the Pearson correlation was used to separate genes that displayed similar patterns. For SAM analysis, the significance cutoff was set at a median false discovery rate < 5%.

Cytoimmunofluorescence

Myometrial and leiomyoma primary tissue explants were grown on glass chamber slides (Nalge Nunc International, Rochester, NY, USA) until emitted cells reached 70% confluence. The cells were fixed with 4% formaldehyde (Sigma-Aldrich), and nonspecific sites were blocked using Image-IT™, FX signal enhancer (Alexa Fluor® 488 Goat Anti-Rabbit SFX Kit, Molecular Probes, Inc., Eugene, OR, USA) and were incubated with 1 : 100 smooth muscle-specific (SMC) α-actin (Lab Vision Co., Fremont, CA, USA) overnight at 4℃. Alexa Fluor® 488 was used as the secondary antibody at a 1 : 200 dilution. Cells were observed under a microscope (Eclipse TE2000-U, Nikon, Kanagawa, Japan).

Tissue microarray

TMA slides capable of holding a large number of specimens per slide were prepared manually using a dermal punch biopsy needle, as described previously,[14] with slight modifications. In brief, for each tissue sample set, a representative 2 mm-diameter core biopsy was taken from one paraffin-embedded donor tissue block and mounted into the new recipient paraffin blocks using 4% agar. A total of 30 paired myometrial and leiomyoma samples were placed side by side on each slide. For specimens showing variable histological features, the most representative area was selected from the core biopsy. Finally, 3 μm sections were cut from each TMA block. The slides were incubated with each primary antibody. Following is a list of the antibodies and their dilution factors: rabbit polyclonal anti-tropomyosin 4 (TPM4; 1 : 500, CHEMICON Inc., Temecula, CA, USA), mouse monoclonal anti-collagen type IVα2 (COL4α2; 1 : 500, Abcam Ltd., Cambridge, UK), Goat anti-human insulin-like growth factor binding protein 5 (IGFBP5; 1 : 50, R&D systems Inc., Minneapolis, MN, USA), mouse monoclonal anti-TIF1β, (tripartite motif-containing 28 [TRIM28]) (1 : 500, CHEMICON Inc.), mouse monoclonal anti-ER-α (1 : 50, clone 1D5, Zymed Laboratories, San Francisco, CA, USA), mouse anti-progesterone receptor (PR; 1 : 50, clone PR-2C5, Zymed Laboratories). Except for IGFBP5-labeled slides, all slides were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse universal secondary antibody and visualized by EnVision HRP staining (ChemMate™ DAKO EnVision™/HRP, DAKO Inc., Carpentaria, CA, USA). Rabbit anti-goat IgG secondary antibody (DAKO, Glostrup, Denmark) was used for the IGFBP5 slides, which were counterstained with Mayer's hematoxylin, hydrated in an ethanol: xylene gradient, mounted on canadabalsam, and visualized using diaminobenzidine tetrahydrochloride. The intensity of immunoreactivity for each antibody (0, negative; 1+, weak; 2+, moderate; 3+, strong) was evaluated by the standard protocol used in the

Department of Pathology at Yeungnam University Hospital.

Statistical analysis

All values were represented as means ± SEM. Data were analyzed by ANOVA according to the general linear model procedure. The mean values between normal myometrium and leiomyoma were compared by T-test ($p \leq 0.05$) and hormonal effects at different time point (8 hours, 12 hours, 24 hours, and 48 hours) were compared to control (no treatment) by Tukey's Studentized Range (HSD) test (*$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$). All the statistical analyses were performed with the SAS ver. 9.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

GMA revealed four genes that were consistently upregulated in leiomyoma tissue

In order to identify genes that were differentially expressed in normal myometrium and leiomyoma during the proliferative and luteal phases of the menstrual cycle, patient RNA sample pairs were hybridized to a microarray of cancer-related genes (3,096 genes on a human cancer 3.0 K oligo chip). Using this technique, gene expression patterns were compared between nine paired tissue samples from leiomyomas and adjacent normal myometrial tissue. Four of these pairs of tissue samples were harvested while the patient was in proliferative phase and five in the luteal phase, allowing investigation of whether expression of certain genes differed according to the phase of the menstrual cycle. Hierarchical clusters were then constructed from our data to analyze the relationships between the genes expressed in leiomyoma and myometrial tissues (Fig. 1). Samples numbered 2, 10, 30, 36, and 57 were harvested during the luteal phase; among these, sample 2, 10, 30, and 36 showed similar expression patterns, whereas sample 57 revealed a different expression profile on the basis of hierarchical clustering analysis. Among the four proliferative phase samples, numbers 3 and 29 showed similar expression patterns, as did numbers 4 and 12. Overall, 952 proliferative phase genes and 514 luteal phase genes showed higher expression levels in leiomyoma than in myometrium. Of these genes, 42 and 13 genes were elevated by at least two-fold in all of the proliferative and luteal phase samples, respectively. Remarkably, four genes were upregulated in all nine leiomyoma samples compared to adjacent myometrium, regardless of menstrual phase; these genes were *TPM4*, *COL4α2*, *IGFBP5*, and *TRIM28*. In addition to the upregulated genes, 426 proliferative phase genes and 290 luteal phase genes exhibited lower expression in leiomyoma than in myometrium tissue. Of these genes, 49 and 9 genes were more than two-fold lower in the proliferative and luteal phase samples, respectively.

rtPCR confirmed significant upregulation of specific genes in leiomyoma tissue

Based on the results of the array, comparative expression studies were designed to further explore the four upregulated genes in all nine leiomyoma samples (*TPM4, COL4α2*, *IGFBP5*, and *TRIM28*) as well as four additional genes known to be upregulated in leiomyoma: *ER-α*, *PR*, insulin-like growth factor-1 recep-

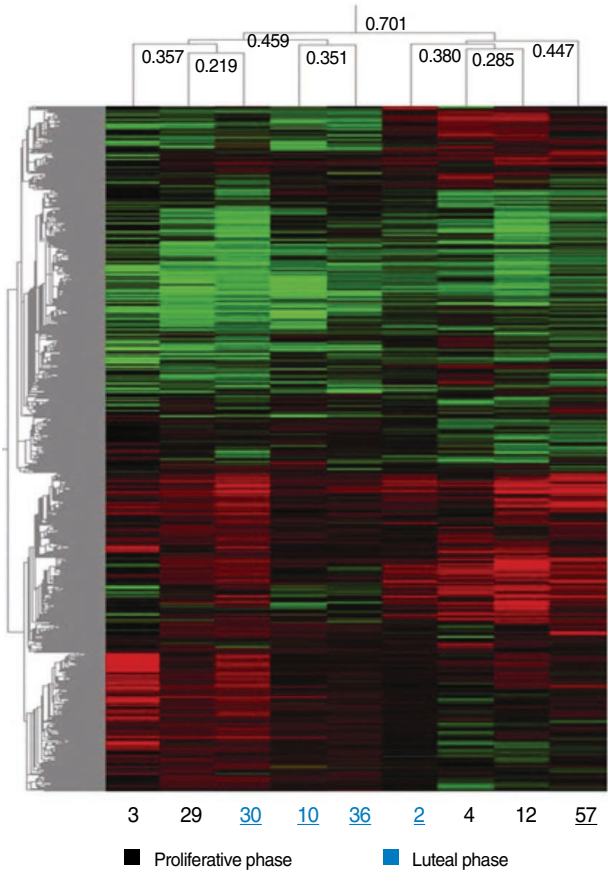

Fig. 1. Gene microarray chip. Hierarchical cluster analysis of nine pairs of samples in which green and red colors indicate genes highly expressed in myometrial and leiomyoma tissues, respectively. RNA extracted from 4 paired tissue samples in proliferative phase and 5 in luteal phase (Table 1) is used for hybridization. The number at the bottom of each column denotes the patient number, either in the proliferative phase (black colored-number without underline) or luteal phase (blue colored-number with underline). The numbers at the top are correlative values.

tor (*IGF-1R*), and endothelin A receptor (*ETA-R*). rtPCR analyses confirmed the gene expression differences of TPM4, COL4-α2, IGFBP5, and TRIM28 observed in the GMA of myometrial and leiomyoma tissue (Fig. 2). These analyses also verified upregulation of *ER*α, *PR, IGF-1R*, and *ETA-R* in the model system, because they were not included in the array analyses. For these experiments, 8 more patient sample pairs (3 from proliferative phase, 1 from luteal phase, and 4 from patients treated with leuplin) were added to the 9 sample pairs used for microarray analysis; thus, a total of 17 samples were analyzed by rtPCR.

All eight genes tested using rtPCR analysis displayed elevated expression in leiomyoma samples compared to corresponding myometrial samples (Fig. 2). On average, compared to the rtPCR analysis, the GMA data yielded slightly higher expression differences for *TPM4*, *IGFBP5*, and *TRIM28* genes and a little lower expression difference for COL4α2 (data not shown). The rtPCR analyses revealed that the differences in mRNA expression level for TRIM28 was higher during proliferative phase and PR was higher in luteal phase compared to leuplin-treated leiomyoma samples.

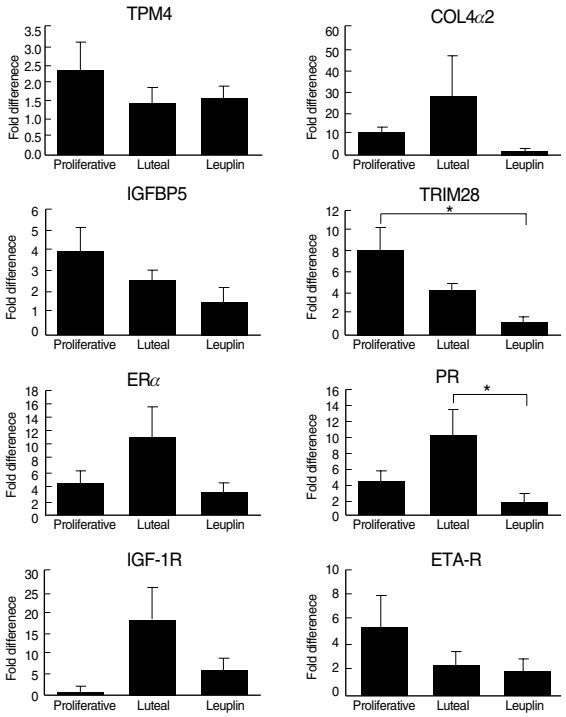

Fig. 2. Real-time reverse transcription PCR (rtPCR). The relative mRNA expression levels in leiomyoma compared to the corresponding myometrium are indicated as fold differences. The average fold differences from 3-4 replicated experiments (with standard deviations) of the data obtained by rtPCR analyses are shown in each picture. A total of 17 paired RNA samples from myometrium and leiomyoma tissues (7 proliferative phase, 6 luteal phase, and 4 leuplin-treated) are used for rtPCR analyses. Among those, RNA from four proliferative phase samples and five luteal phase samples are also used for microarray analysis (*$p \leq 0.05$ by Tukey's test). In addition, statistical difference in gene expression between normal myometrium and leiomyoma is observed by T-test ($p \leq 0.05$).
TPM4, tropomyosin 4; COL4a2 , collagen, type IV, alpha 2; IGFBP5, insulin-like growth factor binding protein 5; TRIM 28, tripartite motif-containing 28; ER, estrogen receptor; PR, progesterone receptor; IGF-1R, insulin-like growth factor-1 receptor; ETA-R, endothelin A receptor.

TMA of upregulated gene products revealed specific subcellular localization and age-dependent expression patterns

To determine whether the gene expression differences agree with protein level expressions between myometrial and leiomyoma tissues, 262 sample tissue pairs (myometirum and leiomyoma) were investigated in an extensive TMA analysis of 6 upregulated genes (*TPM4, COL4*α*2*, *IGFBP5*, *TRIM28*, *ER-*α, and *PR*) (Fig. 3). Cytoplasmic TPM4 staining was observed in myofibrils and blood vessel walls from myometrium and leiomyoma, with no detectable intranuclear staining. COL4α2 expression was also prominent in the vascular walls of both myometrium and leiomyoma, but the staining was stronger in the leiomyoma samples than in the myometrial samples. Interestingly, COL4α2 expression was not observed in the hyalinized lesions (*) of the tumors. IGFBP5 expression was observed not only in the cytoplasm of blood vessel endothelium, but also in the cytoplasm and the nucleus of myofibrils. Weak IGFBP5 immunoreactivity was visible in hyalinized areas (*). TRIM28 localized exclusively to the nucleus in both myometrium and leiomyoma. ER-α displayed diffuse nuclear staining in myometrium and leiomyoma. PR showed nuclear staining in both tissues.

These findings formed the basis of a detailed analysis that categorized protein expression patterns by patient age groups, labeled as follows: "30's" (25 paired samples among 262 total), "40's" (179 samples), "50's" (49 samples) and "60's-70's" (9 samples) (Table 3). Statistical analyses of the TMA data revealed that overall, TPM4, COL4α2, and IGFBP5 levels were significantly different between myometrium and leiomyoma ($p \leq 0.05$), but TRIM28, ER-α, and PR did not show significant expression differences. In contrast, analyses that were narrowed by patient age revealed statistically significant differences in ER-α and PR levels between myometrium and leiomyoma in the samples from women in their forties. Moreover, the 40's age group showed significant differences in IGFBP5, COL4α2 and TPM4 protein

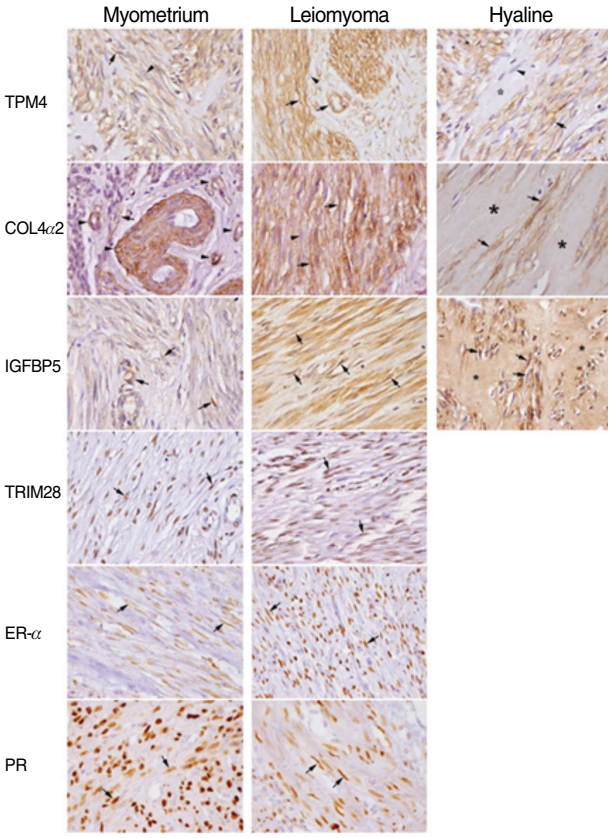

Fig. 3. Tissue microarray. The immobilized paired tissues of myometrium and leiomyoma on each microarray slide are incubated with specific antibodies to localize each protein in the tissue samples. For each antibody staining 262 paired myometrium and leiomyoma samples are used. Each representative image shows typical tissue localization of the target protein for each antibody. Tropomyosin 4 (TPM4) shows positive staining in the cytoplasm of myofibrils and blood vessel walls in myometrium and leiomyoma. Hyalinized foci (*) of the leiomyoma do not show TPM4 expression. Collagen, type IV, alpha 2 (COL4α2) expression is prominent in vascular walls (arrows) of myometrium (weak, +1) and leiomyoma (strong, 3+). Expression is not observed in hyalinized lesions (*) of the tumor. Insulin-like growth factor binding protein 5 (IGFBP5) is detected in the cytoplasm of vascular endothelium in myometrium and in the cytoplasm and nucleus of leiomyoma myofibrils. There is a weak immunoreactivity in hyalinized areas (*) of the tumor. Diffuse nuclear positivity (arrows) is seen in moderate (2+) staining of the normal myometrium and strong (3+) staining of the leiomyomas for estrogen receptor (ER)-α and tripartite motif-containing 28 (TRIM28); the reverse is true for progesterone receptor (PR). Arrows and arrow heads in the picture point the stained and unstained cells, respectively (COL4α2, TRIM28, IGFBP5; ER-α, PR, TPM4).

expression between the two tissue types. TRIM28 and COL4α2 were differentially expressed in the 30's and 50's age groups, respectively. None of the proteins displayed differential expression in the 60's-70's age group.

Table 3. Comparative p-value for different genes between normal myometrium and leiomyoma by paired test (t-test)

	Total (n = 262)	30's (n = 25)	40's (n = 179)	50's (n = 49)	60's-70's (n = 9)
TPM4	0.04	0.38	0.04	0.07	0.72
COL4α2	0.0001	0.26	0.0039	0.0003	0.09
IGFBP5	0.02	0.50	0.03	0.06	1.00
TRIM28	0.94	0.01	0.85	0.18	0.34
ER-α	0.09	1.00	0.04	0.39	0.18
PR	0.37	1.00	0.05	0.68	0.08

Statistical analysis of tissue microarray (TMA) data. 262 pairs of samples (normal myometrium and leiomyoma) were subjected to immunohistochemistry for the six genes (tropomyosin 4 [*TPM4*], collagen, type IV, alpha 2 [*COL4α2*], insulin-like growth factor binding protein 5 [*IGFBP5*], tripartite motif-containing 28 [*TRIM28*], estrogen receptor-α [*ER-α*], and progesterone receptor [*PR*]). The means were compared between normal myometrium and leiomyoma by t-test ($p \leq 0.05$). The numbers in parentheses represent the numbers of samples in each age group (30's, 40's, 50's, and 60's-70's). The data obtained from the immunohistochemical study with TMA were subjected to statistical analysis. Significantly different values are underlined ($p \leq 0.05$).

Several of these genes are upregulated in response to E_2 treatment

These striking differences in relative protein expression levels depending on the patient's age prompted a study to determine whether E_2 administration affected the expression of any of these genes in cells derived from the myometrium and leiomyoma sample pairs. These experiments were performed with tissue explants that had been cultured until the cells that migrated out had achieved 70% confluence (Fig. 4A). Cultures were subjected to E_2 (10 nM) treatment for various time periods (0 hour, 24 hours, or 48 hours), following a 48 hours incubation in steroid-free medium (see Materials and Methods) to eliminate the residual effect of E_2 in the medium prior to experimentation.[15] Following E_2 treatment, the cells were harvested and RNA was extracted for microarray analysis. Samples were hybridized as follows: 0 hour (untreated control) to 24 hours myometrium, 0 hour to 48 hours myometrium, 0 hour to 24 hours leiomyoma, 0 hour to 48 hours leiomyoma (i.e., the arrays compared the effect of a specific E_2 treatment time within each tissue type for each experiment). Surprisingly, E_2 treatment did not mediate significant differences in expression levels for any of the genes (data not shown).

In an attempt to clarify these results, cells emitted from myometrium and leiomyoma were examined to determine whether they were in fact SMCs; if they were not, it might explain why they were unresponsive to E_2. The emitted cells were fixed with formaldehyde and incubated with SMC α-actin. Actin imm-

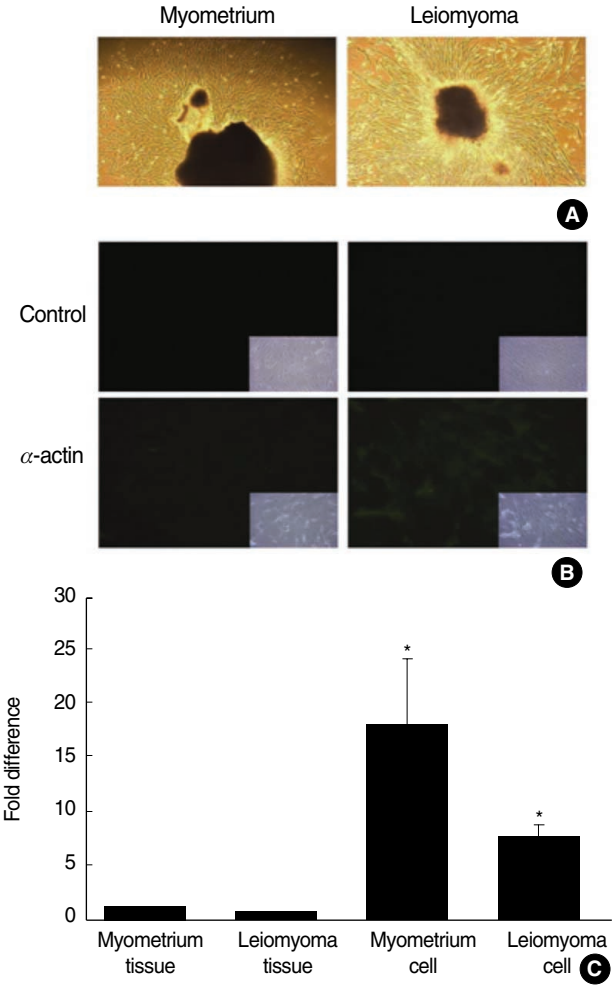

Fig. 4. Cytoimmunofluorescence. Tissue explants from myometrium and leiomyoma are incubated in Dulbecco's modified Eagle's medium/10% fetal bovine serum until the cells derived from each explant reach about 50% confluence. The cell culture images shown are captured on day 12 of culture (A). The cells derived from tissue explants are fixed and incubated with primary antibody to α-actin and secondary Alexa-488-labeled antibodies. Representative cell culture images are shown in the small frames over each α-actin stained picture (B, ×200). The pictures confirm locations of smooth muscle cell α-actin compared to unstained controls. The total RNA extracted from the cells of each tissue explant type and a human α-actin gene-specific polymerase chain reaction (PCR) primer set is used for real-time reverse transcription PCR analysis (C). The relative mRNA expression level in the cells is presented as the fold difference over normal myometrium (C). The average fold differences from 3-4 replicated experiments (with standard deviations) are shown (*$p \leq 0.05$, by Tukey's test).

unoreactivity was apparent in anti-actin-labeled myometrial and leiomyoma samples, but not in negative control samples, indicating that the cells contained actin. rtPCR for SMC α-actin revealed higher expression in cells derived from myometrium and leiomyoma tissue explants than in the digested tissue pieces (Fig. 4), confirming that most of the cells that proliferated out of the tissue samples were SMCs.

Another possible reason for the unresponsiveness to E_2 in the emitted cells could be that the signaling microenvironment within the *in vitro* culture system varied substantially from the microenvironment within the original tissues. To examine this hypothesis, rtPCR was conducted to compare how E_2 administration affected gene expression within the cells that proliferated out from the tissue explants and the whole tissue explants themselves. The 0 hour time point (not subjected to E_2 treatment) for each subject was considered as negative controls. Importantly, the subsequent results revealed that E_2 induced mRNA expression of PR and IGFBP5 in leiomyoma tissue, but not in cells that were cultured from the leiomyoma tissue (Fig. 5). PR was significantly induced at 24 hours and 48 hours E_2-treatment in leiomyoma tissue and 8 hours in myometrial tissue. Similarly, IGFBP5 showed significant induction in leiomyoma tissue at 48 hours E_2-treatment. These results suggested that the *PR* and *IGFBP5* genes displayed E_2 responsiveness only in the intact leiomyoma tissue microenvironment. IGF-1 was induced in response to E_2-treatment in leiomyoma tissue and myometrial cell compared to respective cells and tissue. In contrast to these results, IGF-1R was induced in leiomyoma cell at 12 hours and 24 hours. ERα and *ETA-R* genes were upregulated in myometrial cells where as downregulated in myometrial tissue due to E2 treatment. Moreover, no significant E2-responsiveness was observed for the other genes (*TPM4*, *TRIM28*, and *COL4α2*).

DISCUSSION

Herein, to further study the molecular mechanism of tumorigenesis in benign leiomyoma tumors, gene and TMA studies were used to identify genes potentially responsible for leiomyoma growth. In the initial experiments, mRNA isolated from nine pairs of myometrial and leiomyoma tissue was hybridized to an oligonucleotide microarray containing about 3,000 cancer-related genes. The statistical analyses of the results revealed that among the 792 genes upregulated in leiomyoma relative to myometrium, four genes (*TPM4*, *COL4α2*, *IGFBP5*, and *TRIM28*) showed the highest expression differences in the leiomyoma samples from all nine specimens. rtPCR analysis was subsequently performed to verify the gene microarray data. The rtPCR was used to evaluate the expression of genes known from previous work to be differentially expressed between leiomyoma and myometrium: ER-α, PR, IGF-1R, and ETA-R. Gene repression due to leuplin

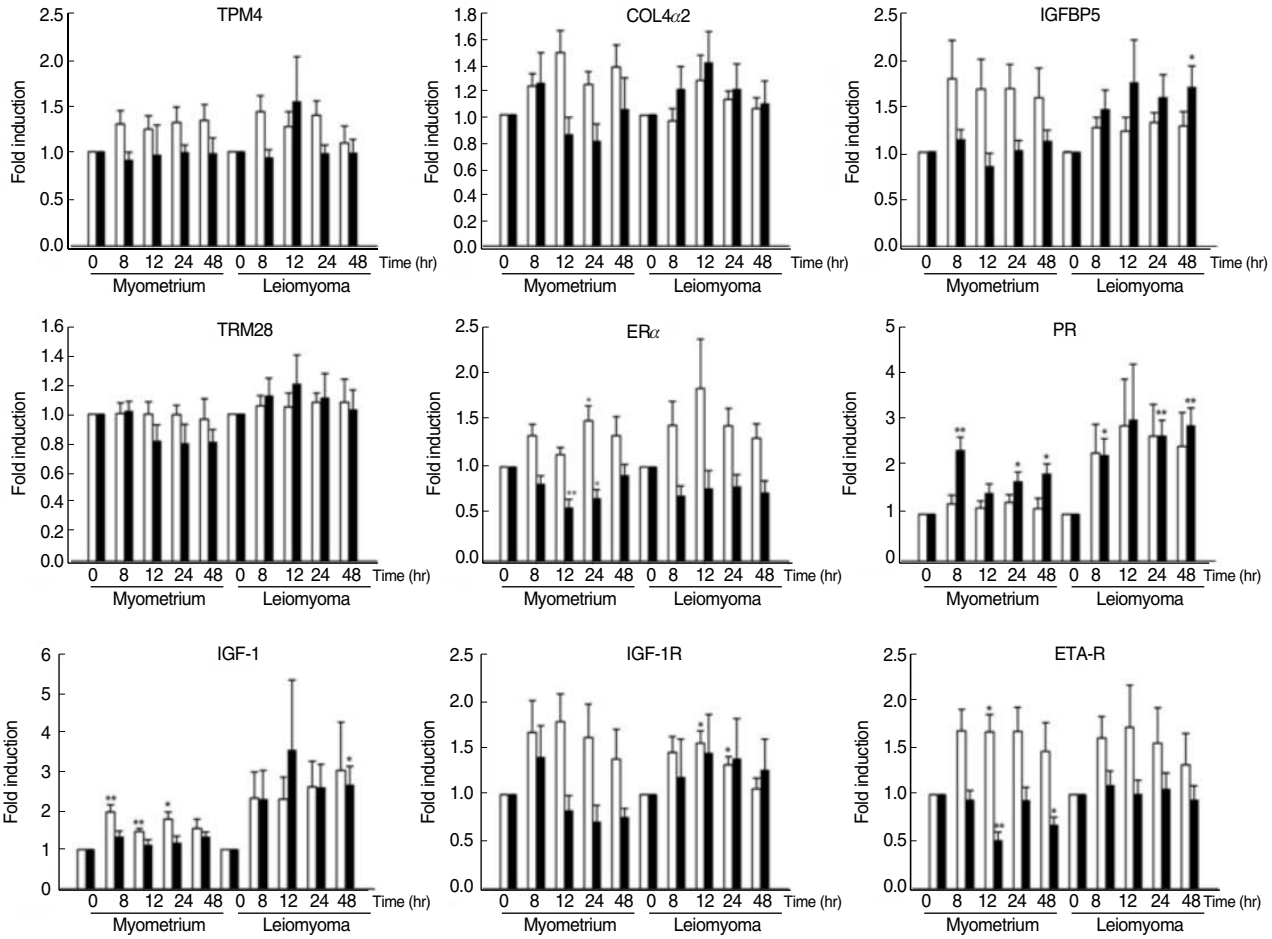

Fig. 5. Effects of E_2-treatment on gene expression in cells and tissue explants of myometrium and leiomyoma. The culture media is changed to Dulbecco's modified Eagle's medium (-)/10% charcoal-dextran-treated fetal bovine serum for 48 hr prior to treatment with E_2 (10 nM) for different time points (0 hr, 8 hr, 12 hr, 24 hr, and 48 hr). Gene expression in tissue explants and cells derived from tissue explants after E_2-treatment is analyzed by real-time reverse transcription polymerase chain reaction. The relative mRNA level in cells and tissues of myometrium and leiomyoma after different periods of hormone treatment is compared to that for 0 hr E_2-treatment (indicated as fold induction in each graph). The white and black bars represent cells derived from tissue explants and tissue explants of myometrium and leiomyoma, respectively. Data shown are normalized to β-actin gene expression (*$p \leq 0.01$, **$p \leq 0.001$ by t- test between 0 hr and each hrs treatment).
TPM4, tropomyosin 4; COL4α2, collagen, type IV, alpha 2; IGFBP5, insulin-like growth factor binding protein 5; TRIM 28, tripartite motif-containing 28; ER, estrogen receptor; PR, progesterone receptor; IGF-1R, insulin-like growth factor-1 receptor; ETA-R, endothelin A receptor.

treatment is not found to be statistically significant except in PR and TRIM28. Leuplin treatment significantly represses the PR and TRIM28 expression compared with luteal phase and proliferative phase respectively.

In agreement with the above data, the TMA study revealed significantly greater corresponding protein expression levels in leiomyoma compared to myometrium. Age-wise grouping of the analyzed data showed that fewer proteins were differentially expressed in leiomyoma from women in the 30's, 50's and 60's-70's age groups than in 40's age-grouped women. Such a difference might be due to lower sample numbers in the other age groups compared to the 40's age group. Sample availability may have been high for the 40's age group because women in their forties have a high relative incidence risk and a tendency to have higher numbers of leiomyomas compared to women in the other age groups.[16]

Most of the previous studies regarding ER-α, PR, IGF-1R, and ETA-R demonstrated significantly higher expression in leiomyoma tissue than in corresponding myometrial tissue,[12] but some recent reports[17-20] showed no difference in ER-α mRNA expression between leiomyomas and myometrium. Vollenhoven *et al.*[17] claimed that the discrepancy between their results and others might be because the genes used for normalization of the data in the other studies were E_2-responsive. Although it is possible that

expression of the β-actin gene used to normalize the data in the present study could have been slightly influenced by E_2 *in vivo*, it is unlikely that the six- to eight-fold ER-α expression difference was completely attributable to biased normalization. In addition, the present analyses yielded similar trends in fold expression differences for every upregulated gene before and after normalization to the β-actin gene (data not shown), suggesting that the data was not greatly affected by the normalization step. Taken together, the data clearly showed that ER-α is upregulated in leiomyoma tissue, regardless of whether they are E_2 responsive. Similarly, the present data showing IGF-1R upregulation in leiomyoma conflict with results from two other studies.[21,22]

This study also identified several upregulated leiomyoma genes; the most notable were *TPM4*, *COL4α2*, *IGFBP5*, and *TRIM28*. The TPM4 protein was mainly localized to the cytoplasm of myofibrils and the blood vessel walls in both myometrial and leiomyoma samples. TPM4 is a member of the tropomyosin family that interacts with actin filaments, and previous studies detected high expression of TPM4 mRNA in rat uterus and fibroblasts.[23] Collagen is one of the major proteins comprising leiomyoma tissue.[12] A study demonstrated slightly higher COL4α2 (type IV collagen) expression in leiomyoma compared to myometrium, which was confirmed by rtPCR, in which 11 of the 18 leiomyoma samples displayed at least 1.5 fold higher mRNA expression.[20] In contrast, the present study measured about 9-fold and 13-fold increases in expression of this gene in leiomyoma, as shown by microarray analysis (data not shown) and rtPCR analysis, respectively. In agreement with the rtPCR analysis, the TMA analysis revealed significantly higher COL4α2 protein expression in leiomyomas, especially from women in their forties ($p \leq 0.0039$) and fifties ($p \leq 0.0003$). Previous studies have shown that several *IGFBP* genes, including IGFBP-2, -3, -4, and -5, were abundantly expressed in leiomyomas.[22] Herein, expression of IGFBP5 mRNA was approximately four and two fold higher in leiomyoma tissues relative to myometrium during the proliferative and leuteal phases, respectively. Significantly higher expression of IGFBP5 protein was observed in leiomyomas from women in their forties, and this protein was localized to the cytoplasm of vascular endothelium and myofibrils. These observations implied that IGFBP5 might play a facilitating role with IGF-1 during the growth stimulation of leiomyomas. The results from microarray and rtPCR analyses revealed higher TRIM28 mRNA expression in leiomyoma than in comparable myometrium, with a more prominent increase in tumors harvested during proliferative phase (8.1 fold) than those taken during luteal phase (4.2 fold). To our knowledge, this report is the first to describe high expression of TRIM28 in leiomyoma. Interestingly, TRIM28 protein was significantly upregulated only in leiomyomas from women in their thirties, with predominant localization in the nuclei of SMCs. The precise role of this protein during leiomyoma tumorigenesis is not apparent from previous functional studies, which putatively implicated TRIM28 in differentiation of the mouse embryonic carcinoma F9 cell[15] but also in DNA repair.[24] Whether either of these TRIM28 functions is involved in leiomyoma tumorigenesis remains to be elucidated in future studies.

Two major culture methods for primary leiomyoma and myometrial cells dominate this research field. In one method, tissue explants are minced into small pieces and grown in culture dishes to promote cellular outgrowth from the tissues.[25] These cells are then passaged and transferred to new culture flasks for experimentation. In the other method, the pieces of tissues are thoroughly digested with enzymes and cells are collected from the digested tissue for culture.[26] Our work suggests that myometrial and leiomyoma cells obtained by the former method produce better results with respect to stability, consistency, and maintenance of morphology during cell culture. Previous reports indicated that the majority of the cells derived from these tissue explants were SMCs that maintained their characteristics in culture (according to immunocytochemical staining with a SMC protein, such as α-actin).[25]

Among the E_2-induced genes *PR, IGF-1*, and *IGFBP5*, except *IGF-1R* showed significant difference in tissue compared to cells-derived from tissue in leiomyoma implying the importance of tissue (microenvironment-extracellular matrix) in at least a few genes for E_2-responsiveness. Especially, PR expression was upregulated due to E_2-treatment only in tissue explants and upregulations were observed in both leiomyoma and normal myometrial tissue explants. Moreover, opposite response toward E_2-treatment by ER-α and ETA-R in tissue (downregulation) and cell (upregulation) in normal myometrium also supports the importance of tissue level study along with cells. This research showed *COL4α2*, *TPM4*, and *TRIM28* to be estrogen independent genes. Additionally, tamoxifen (an antagonist of estrogen; tamoxifen citrate, Tocris Coolson Inc., Ellisville, MO, USA) treatment successfully repressed the E_2-dependent mRNA expression of IGFBP5, PR, and IGF-1 (data not shown). Our study could not show E_2-responsiveness in ETA-R, TPM4, COL4α2, and TRIM28 in leiomyoma. Interestingly, another report revealed significant reductions in the expression of certain genes, including *ER* and *PR*, in cultured leiomyoma SMCs compared to whole tissue.[4] It is speculated that E_2 responsiveness of these genes is only functional in leiomyoma tissue explants, not in normal myometrial

tissue explants or cells derived from either type of tissue, illustrating the important contribution of the unique tissue environment within the leiomyoma to its own maintenance. Our result showed that E_2-responsiveness differs in tissue and cells according to genes. PR, IGF-1, and IGF-1R were upregulated only in tissue explants, in myometrial cells and myoma tissue explants, and only in leiomyoma cells, respectively. Moreover, ER-α and ETA-R showed opposite response in cells and tissue explants. Therefore, elements of the tissue microenvironment (including the ECM) can impact steroid hormone responsiveness.[12]

In conclusion, a DNA microarray system identified four genes (*TPM4*, *COL4α2*, *IGFBP5*, and *TRIM28*) overexpressed in leiomyoma tissue relative to corresponding myometrium. The rtPCR and immunostaining verified the microarray data. TMA analysis demonstrated immunolocalization of TPM4, COL4α2, and IGFBP5 proteins to the blood vessels of both tissue types. Considering that angiogenesis is a key step during tumor growth, these results indicate a possible relationship between upregulation of these genes in leiomyoma and tumor growth. It is worth noting that recent findings revealed antiangiogenic activity for a tropomyosin[27] and for collagen type IV.[28] In addition, it will be interesting to see whether TRIM28 (TIF1β) acts as a corepressor or coactivator in leiomyoma growth. The difference in gene expression levels between the tissue explants and the explant-derived cells, as well as their E_2-responsiveness, suggested that tissue context is critical in gene expression regulation (and presumably tumor growth) within the leiomyomas. In addition, despite a marked decrease of TRIM28 mRNA in leiomyoma in response to GnRH agonist treatment, no E_2-responsiveness for these genes was observed in the leiomyoma tissue explants, implying that even the microenvironment in the tissue explants was not sufficient for the tissue to evoke an E_2 response that recapitulated *in vivo* behavior. These findings cannot rule out the possibility that other factors missing in the tissue explants are required for E_2 responsiveness, or that factor(s) other than E_2 regulate these genes during the GnRH agonist response. In fact, further study might reveal that GnRH and GnRH agonist act directly on leiomyoma tissue, possibly through a GnRH receptor present in leiomyomas themselves.[9]

Although our aim in this research was to observe the effect of estradiol in the differentially expressed genes, three genes (*TPM4*, *COL4α2*, and *TRIM28*) among the four differentially expressed genes did not respond to the estradiol treatment. In addition, we could not show the function of TRIM28 in the uterine leiomyoma, although we identified the gene to be highly expressed in uterine leiomyoma for the first time. Therefore, with respect to the upregulated genes identified herein, further functional analysis of their gene products in the context of leiomyoma will lead to a better understanding of the molecular mechanism underlies their differential expression and roles in leiomyoma growth.

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