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A high concentration of genistein down-regulates activin A, Smad3 and other TGF-β pathway genes in human uterine leiomyoma cells

Xudong Di[1,3], Danica M.K. Andrews[2], Charles J. Tucker[2], Linda Yu[1], Alicia B. Moore[1], Xiaolin Zheng[1], Lysandra Castro[1], Tonia Hermon[1], Hang Xiao[4] and Darlene Dixon[1,5]

[1]Molecular Pathogenesis Group
National Toxicology Program (NTP) Laboratories Branch, NTP
[2]Microarray Group
Laboratory of Molecular Toxicology
National Institute of Environmental Health Sciences
National Institutes of Health (NIH)
Department of Health and Human Services (DHHS)
Research Triangle Park
North Carolina 27709, USA
[3]Bureau of Health of Wujin District
Changzhou 213159, China
[4]Department of Toxicology
School of Public Health
Nanjing Medical University
Nanjing 210029, China
[5]Corresponding author: Tel, 1-919-541-3814;
Fax, 1-919-541-7666; E-mail, dixon@niehs.nih.gov
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Abbreviations: GnRH, gonadotropin-releasing hormone; SERMs, selective estrogen-receptor modulators; SPRMs, selective progesterone-receptor modulators; UtLM cell, uterine leiomyoma cell

Abstract

Previously, we found that high doses of genistein show an inhibitory effect on uterine leiomyoma (UtLM) cell proliferation. In this study, using microarray analysis and Ingenuity Pathways Analysis™, we identified genes (up- or down-regulated, ≥ 1.5 fold, $P \leq 0.001$), functions and signaling pathways that were altered following treatment with an inhibitory concentration of genistein (50 μg/ml) in UtLM cells. Downregulation of TGF-β signaling pathway genes, activin A, activin B, Smad3, TGF-β2 and genes related to cell cycle regulation, with the exception of the upregulation of the CDK inhibitor P15, were identified and validated by real-time RT-PCR studies. Western blot analysis further demonstrated decreased protein expression of activin A and Smad3 in genistein-treated UtLM cells. Moreover, we found that activin A stimulated the growth of UtLM cells, and the inhibitory effect of genistein was partially abrogated in the presence of activin A. Overexpression of activin A and Smad3 were found in tissue samples of leiomyoma compared to matched myometrium, supporting the contribution of activin A and Smad3 in promoting the growth of UtLM cells. Taken together, these results suggest that down-regulation of activin A and Smad3, both members of the TGF-β pathway, may offer a mechanistic explanation for the inhibitory effect of a high-dose of genistein on UtLM cells, and might be potential therapeutic targets for treatment of clinical cases of uterine leiomyomas.

Keywords: activin A; genistein; leiomyoma; myometrium; oligonucleotide array sequence analysis; Smad3 protein; transforming growth factor β

Introduction

Uterine leiomyomas (i.e., fibroids, myomas) are the leading indication for hysterectomy in the United States with the estimated cumulative incidence of tumors by age 50 being > 80% for black women and nearly 70% for white women in the United States (Walker and Stewart, 2005; Viswanathan *et al*., 2007). These tumors represent a tremendous public health burden and economic cost to society. At present, surgical intervention is still the standard and most effective treatment for uterine leiomyomas (Rackow and Arici, 2006; Tulandi, 2007). Although therapeutic agents, such as gonadotropin-releasing hormone (GnRH) agonists are being used clinically (Chegini *et al*., 2002), and selective estrogen-receptor modulators (SERMs) (Liu *et al*., 2007), selective progesterone-receptor modulators (SPRMs) (Chwalisz *et*

Table 1. Functions and genes[a] that were significantly altered in uterine leiomyoma (UtLM) cells following genistein (50 μg/ml) treatment for 24 h

Gene function	Genes	Accession No.	Description	Fold change
Cancer				
	C5ORF13	NM_004772	Chromosome 5 open reading frame 13	-4.45
	CASP3	NM_004346	Caspase 3, apoptosis-related cysteine protease	-1.65
	CAV1	BC006432	Caveolin 1, caveolae protein, 22kDa	-2.84
	CLG	AK026111	Likely ortholog of mouse common-site lymphoma/leukemia GEF	-1.97
	CNN2	NM_004368	Calponin 2	-1.9
	CST	NM_004861	Galactose-3-O-sulfotransferase 1	3.7
	CYR61	Z97068	Cysteine-rich, angiogenic inducer, 61	-2.47
	ENC1	AY049781	Ectodermal-neural cortex (with BTB-like domain)	-2.72
	FGF5	M37825	Fibroblast growth factor 5	-2.14
	FGF13	NM_004114	Fibroblast growth factor 13	-2.06
	FGFR3	NM_000142	Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	1.85
	HAS2	NM_005328	Hyaluronan synthase 2	-4.88
	HMGA2	NM_003483	High mobility group AT-hook 2	-3.06
	HMOX1	NM_002133	Heme oxygenase (decycling) 1	4.99
	ID1	NM_002165	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	1.67
	ID3	NM_002167	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1.54
	ID4	U16153	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	2.68
	IGF2R	NM_000876	Insulin-like growth factor 2 receptor	1.89
	IRS1	NM_005544	Insulin receptor substrate 1	1.6
	IRS2	AF073310	Insulin receptor substrate 2	7.88
	MMP1	NM_002421	Matrix metalloproteinase 1 (interstitial collagenase)	-2.26
	MMP14	NM_004995	Matrix metalloproteinase 14 (membrane-inserted)	1.88
	RARB	NM_000965	Retinoic acid receptor, beta	-1.84
	SHC1	NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	-1.77
	TGFB2	AK021874	Transforming growth factor, beta 2	-3.03
	TGFBR3	NM_003243	Transforming growth factor, beta receptor III (betaglycan, 300kDa)	1.52
	VEGF	AK098750	Vascular endothelial growth factor	-1.6
	VEGFC	NM_005429	Vascular endothelial growth factor C	-2.92
Cell cycle				
	MYBL1	X66087	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	-3.41
	PDGFRA	NM_006206	Platelet-derived growth factor receptor, alpha polypeptide	-2.63
	HGF	X16323	Hepatocyte growth factor (hepapoietin A; scatter factor)	-2.32
	SHC1	NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	-1.77
	RAD21	NM_006265	RAD21 homolog (S. pombe)	-1.69
	CASP3	NM_004346	Caspase 3, apoptosis-related cysteine protease	-1.65
	VEGF	AK098750	Vascular endothelial growth factor	-1.6
	IRS1	NM_005544	Insulin receptor substrate 1	1.6
	PLK3	NM_004073	Polo-like kinase 3 (Drosophila)	2.25
	PCNA	NM_002592	Proliferating cell nuclear antigen	2.36
	IL6R	X12830	Interleukin 6 receptor	3.11
	HMOX1	NM_002133	Heme oxygenase (decycling) 1	4.99
	PLAB	NM_004864	Growth differentiation factor 15	7.57
	IRS2	AF073310	Insulin receptor substrate 2	7.88
Cell death				
	EGR1	NM_001964	Early growth response 1	-6.76
	MYBL1	X66087	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	-3.41
	PDGFRB	NM_002609	Platelet-derived growth factor receptor, beta polypeptide	-3.25
	TGFB2	AK021874	Transforming growth factor, beta 2	-3.03
	VEGFC	NM_005429	Vascular endothelial growth factor C	-2.92
	CDK6	BC027989	Cyclin-dependent kinase 6	-2.91
	SHC3	NM_016848	Src homology 2 domain containing transforming protein C3	-2.35
	MMP1	NM_002421	Matrix metalloproteinase 1 (interstitial collagenase)	-2.26

Table 1. Continued

Gene function	Genes	Accession No.	Description	Fold change
	FGF5	M37825	Fibroblast growth factor 5	-2.14
	CDK5	NM_004935	Cyclin-dependent kinase 5	-1.87
	SHC1	NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	-1.77
	PCNA	NM_002592	Proliferating cell nuclear antigen	2.36
	IRS2	AF073310	Insulin receptor substrate 2	7.88
Cell signaling				
	IRS1	NM_005544	Insulin receptor substrate 1	1.6
	OXTR	NM_000916	Oxytocin receptor	-3.32
	TRPV1	NM_080706	Transient receptor potential cation channel, subfamily V, member 1	2.56
Cell-to-cell signaling and interaction				
	ESM1	NM_007036	Endothelial cell-specific molecule 1	-8.99
	CASP3	NM_004346	Caspase 3, apoptosis-related cysteine protease	-1.65
	VEGF	AK098750	Vascular endothelial growth factor	-1.6
	BSN	NM_003458	Bassoon (presynaptic cytomatrix protein)	5.32
	IRS2	AF073310	Insulin receptor substrate 2	7.88
Cellular growth and proliferation				
	IRS2	AF073310	Insulin receptor substrate 2	7.88
	IL6R	X12830	Interleukin 6 receptor	3.11
	CDKN1C	NM_000076	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	2.36
	PCNA	NM_002592	Proliferating cell nuclear antigen	2.36
	IGF2R	NM_000876	Insulin-like growth factor 2 receptor	1.89
	FGFR3	NM_000142	Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	1.85
	IRS1	NM_005544	Insulin receptor substrate 1	1.6
	VEGF	AK098750	Vascular endothelial growth factor	-1.6
	CASP3	NM_004346	Caspase 3, apoptosis-related cysteine protease	-1.65
	DLG7	NM_014750	Discs, large homolog 7 (Drosophila)	-1.94
	EGR1	NM_001964	Early growth response 1	-6.76
	PHLDA1	BC037430	Pleckstrin homology-like domain, family A, member 1	-13.74

[a]Identified by analysis using Ingenuity Pathway Analysis software.

al., 2005), anti-fibrotic agents and growth factor antagonists (Rackow and Arici, 2006) were assessed experimentally for the treatment of these tumors, there is still a paucity of effective nonsurgical therapies. Therefore, searching for potential novel targets for fibroids treatment is a major goal in this field.

Recently, microarray analysis has provided a novel means of elucidating the mechanisms of fibroid development (Leppert *et al*., 2006; Pan *et al*., 2007; Litovkin *et al*., 2008). For example, gene microarray was used to characterize the molecular environment of leiomyomas and matched myometrium during growth, as well as gene expression profiles of leiomyoma and myometrial-derived smooth muscle cells in response to (TGF-β) and GnRH (Levens *et al*., 2005). Numerous genes have been found to be differentially expressed in leiomyoma and myometrial tissues (Litovkin *et al*., 2008). Moreover, based on the results of microarray studies, much emphasis has been placed on the roles of the extracellular matrix (ECM), TGF-β and collagen composition in fibroid development (Leppert *et al*., 2006).

Genistein, the major isoflavone in soy, has been shown *in vivo* to be beneficial in the prevention of a wide variety of chronic diseases, including cancer (Gupta *et al*., 2010). Furthermore, at high doses ($\geq$ 25 μM) that can be reached in cell culture models, genistein shows multidirectional actions, such as inhibition of tyrosine kinase and DNA topoisomerase activities, synthesis and release of TGF-β and increased apoptosis in the live cell (Polkowski and Mazurek, 2000). In an earlier study, we found that high doses of genistein ($\geq$ 10 μg/ml) had an inhibitory effect on uterine leiomyoma (UtLM) cells (Moore *et al*., 2007). Because increased cell proliferation is believed to be the most significant contributor to the growth of uterine leiomyomas (Dixon *et al*., 2002; Leppert *et al*., 2006), we thought that microarray analysis of the inhibitory effect of a high-dose of genistein (50 μg/ml) on UtLM cells might offer insight on genes and pathways that may be important in arresting the growth of UtLM cells. These data would also provide novel ideas to better

Table 2. Signaling pathways and genes[a] that were significantly altered in uterine leiomyoma (UtLM) cells following genistein (50 μg/ml) treatment for 24 h. Genes, CDKN2B, CCNB2, MADH3, MYBL1, INHBA (activin A), INHBB (activin B) and TGFB2 are also named as P15, Cyclin B2, Smad3, A-myb, Activin A, Activin B and TGF-β2

Signaling pathways	Genes	Accession No.	Description	Fold change
Apoptosis signaling pathway				
	BID	NM_001196	BH3 interacting domain death agonist	-1.86
	CASP3	NM_004346	Caspase 3, apoptosis-related cysteine protease	-1.65
	MAP3K14	NM_003954	Mitogen-activated protein kinase kinase kinase 14	-1.67
	TNFRSF6	NM_000043	Tumor necrosis factor receptor superfamily, member 6	-1.62
Cell cycle G1S checkpoint regulation pathway				
	CDK6	BC027989	Cyclin-dependent kinase 6	-2.91
	CDKN2B	NM_078487	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	2.2
	E2F4	BC033180	E2F transcription factor 4, p107/p130-binding	-1.61
	MADH3	U68019	SMAD, mothers against DPP homolog 3 (Drosophila)	-1.78
	TGFB2	AK021874	Transforming growth factor, beta 2	-3.03
	MYBL1	X66087	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	-3.41
Cell cycle G2M DNA damage checkpoint regulation pathway				
	CCNB2	NM_004701	Cyclin B2	-1.96
	YWHAE	U28936	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	-1.62
Death receptor signaling pathway				
	BID	NM_001196	BH3 interacting domain death agonist	-1.86
	CASP3	NM_004346	Caspase 3, apoptosis-related cysteine protease	-1.65
	MAP3K14	NM_003954	Mitogen-activated protein kinase kinase kinase 14	-1.67
	TNFRSF6	NM_000043	Tumor necrosis factor receptor superfamily, member 6	-1.62
ERK MAPK signaling pathway				
	DUSP6	NM_001946	Dual specificity phosphatase 6	-2.14
	EIF4E	NM_001968	Eukaryotic translation initiation factor 4E	-1.61
	MAPKAPK5	NM_139078	Mitogen-activated protein kinase-activated protein kinase 5	-1.89
	MKNK2	NM_017572	MAP kinase-interacting serine/threonine kinase 2	2.57
	PLA2G4A	NM_024420	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	-1.7
	PPP1R14B	AF318364	Protein phosphatase 1, regulatory (inhibitor) subunit 14B	-1.68
	PPP1R3C	NM_005398	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	-1.69
	PXN	NM_002859	Paxillin	-1.76
	SHC1	NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	-1.77
Fatty acid biosynthesis pathway (Path 2)				
	ACAA2	NM_006111	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	-1.66
	FASN	NM_004104	Fatty acid synthase	1.72
	HADH2	NM_004493	Hydroxyacyl-Coenzyme A dehydrogenase, type II	-1.62
Insulin receptor signaling pathwat				
	ARHQ	NM_012249	Ras homolog gene family, member Q	1.92
	EIF4E	NM_001968	Eukaryotic translation initiation factor 4E	-1.61
	IRS1	NM_005544	Insulin receptor substrate 1	1.6
	IRS2	AF073310	Insulin receptor substrate 2	7.88
	PPP1R14B	AF318364	Protein phosphatase 1, regulatory (inhibitor) subunit 14B	-1.68
	PPP1R3C	NM_005398	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	-1.69
	SHC1	NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	-1.77
Integrin signaling pathway				
	ACTA2	NM_001613	Actin, alpha 2, smooth muscle, aorta	-4.46
	ACTB	NM_001101	Actin, beta	-1.67
	ACTG1	NM_001614	Actin, gamma 1	-1.83
	ACTG2	NM_001615	Actin, gamma 2, smooth muscle, enteric	-4.86
	ARHQ	NM_012249	Ras homolog gene family, member Q	1.92
	ARPC2	NM_152862	Actin related protein 2/3 complex, subunit 2, 34kDa	-1.66
	ARPC3	NM_005719	Actin related protein 2/3 complex, subunit 3, 21kDa	-1.66
	BCAR3	NM_003567	Breast cancer anti-estrogen resistance 3	-2.26

Table 2. Continued

Signaling pathways	Genes	Accession No.	Description	Fold change
	CAV1	BC006432	Caveolin 1, caveolae protein, 22 kDa	-2.84
	COL1A1	NM_000088	Collagen, type I, alpha 1	-3.41
	ILK	NM_004517	Integrin-linked kinase	-1.57
	ITGA2	NM_002203	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	-1.64
	LAMA1	AK091949	Laminin, alpha 1	3.72
	MYLK	NM_053025	Myosin, light polypeptide kinase	-3.24
	PARVA	NM_018222	Parvin, alpha	-1.88
	PPP1R12A	AF086032	Protein phosphatase 1, regulatory (inhibitor) subunit 12A	-2.38
	PPP1R12B	NM_032105	Protein phosphatase 1, regulatory (inhibitor) subunit 12B	-3.98
	PXN	NM_002859	Paxillin	-1.76
	SHC1	NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	-1.77
	VASP	NM_003370	Vasodilator-stimulated phosphoprotein	-2.07
P38 MAPK signaling pathway				
	DDIT3	NM_004083	DNA-damage-inducible transcript 3	2.42
	MAPKAPK5	NM_139078	Mitogen-activated protein kinase-activated protein kinase 5	-1.89
	MKNK2	NM_017572	MAP kinase-interacting serine/threonine kinase 2	2.57
	PLA2G4A	NM_024420	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	-1.7
	TGFB2	AK021874	Transforming growth factor, beta 2	-3.03
	TNFRSF6	NM_000043	Tumor necrosis factor receptor superfamily, member 6	-1.62
Purine metabolism pathway				
	ADARB1	NM_015833	Adenosine deaminase, RNA-specific, B1 (RED1 homolog rat)	1.67
	ADCY7	NM_001114	Adenylate cyclase 7	-2.23
	AK2	NM_172199	Adenylate kinase 2	-2.65
	AK5	BC012467	Adenylate kinase 5	-1.9
	ATIC	NM_004044	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	-1.59
	DGUOK	NM_080916	Deoxyguanosine kinase	-1.56
	ENPP1	NM_006208	Ectonucleotide pyrophosphatase/phosphodiesterase 1	2.13
	HPRT1	NM_000194	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	-1.69
	IFNAR1	NM_000629	Interferon (alpha, beta and omega) receptor 1	-2.07
	IMPDH2	NM_000884	IMP (inosine monophosphate) dehydrogenase 2	1.5
	ITPA	NM_033453	Inosine triphosphatase (nucleoside triphosphate pyrophosphatase)	-1.67
	NT5C	NM_014595	5', 3'-nucleotidase, cytosolic	-1.55
	PAICS	X53793	Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	-1.61
	PAPSS2	AF074331	3'-phosphoadenosine 5'-phosphosulfate synthase 2	-1.72
	PNPT1	NM_033109	Polyribonucleotide nucleotidyltransferase 1	-1.63
	POLI	NM_007195	Polymerase (DNA directed) iota	-1.86
	REV3L	NM_002912	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	-1.82
	RRM2	BC030154	Ribonucleotide reductase M2 polypeptide	-2.9
TGF-β signaling pathway				
	BMP2	NM_001200	Bone morphogenetic protein 2	2.81
	INHBA	AK001903	Inhibin, beta A (activin A, activin AB alpha polypeptide)	-2.06
	INHBB	NM_002193	Inhibin, beta B (activin AB beta polypeptide)	-1.64
	MADH3	U68019	SMAD, mothers against DPP homolog 3 (Drosophila)	-1.78
	SERPINE1	NM_000602	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-2.1
	SMURF1	NM_020429	E3 ubiquitin ligase SMURF1	-1.68
	TGFB2	AK021874	Transforming growth factor, beta 2	-3.03
VEGF signaling pathway				
	ACTA2	NM_001613	Actin, alpha 2, smooth muscle, aorta	-4.46
	ACTB	NM_001101	Actin, beta	-1.67
	ACTG1	NM_001614	Actin, gamma 1	-1.83
	ACTG2	NM_001615	Actin, gamma 2, smooth muscle, enteric	-4.86

Table 2. Continued

Signaling pathways	Genes	Accession No.	Description	Fold change
	EIF2S2	NM_003908	Eukaryotic translation initiation factor 2, subunit 2 beta, 38 kDa	-1.69
	ELAVL1	BC003376	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	-1.6
	PXN	NM_002859	Paxillin	-1.76
	SHC1	NM_003029	SHC (Src homology 2 domain containing) transforming protein 1	-1.77
	VEGF	AK098750	Vascular endothelial growth factor	-1.6
	VEGFC	NM_005429	Vascular endothelial growth factor C	-2.92
	YWHAE	U28936	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	-1.62

[a]Identified by analysis using Ingenuity Pathway Analysis software.

understand the function of how cells and integrated biological mechanisms are involved in UtLM growth inhibition, and possibly provide novel targets for clinical intervention.

Results

Microarray analysis of significantly altered genes

The expression profile of over 41,000 genes was studied in genistein-treated UtLM cells (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vjcjruqaseesmps&acc=GSE19477). Vehicle treated UtLM cells served as the reference for the expression profile. Signature genes were generated by Rosetta Resolver based on the criteria that genes were $\geq$ 1.5-fold and $P \leq 0.001$ throughout all replicates. A total of 541 differentially expressed genes were observed. Of these, 150 genes were up-regulated and 391 genes were down-regulated in genistein-treated compared to vehicle-treated UtLM cells. These genes were considered signature genes and were used for further analysis using IPA.

IPA

To further analyze the biological significance of signature genes, genes were characterized into networks, functions and signaling pathways by using IPA software according to Ingenuity Pathways Knowledge Base (IPKB, http://www.ingenuity.com). These networks, functions and signaling pathways were ranked according to IPA calculated scores, which is based on the significance of involved genes. Based on IPKB, signature genes were classified into multiple functions and pathways by IPA. Among these, six functions and twelve pathways which were identified by IPA with significance values less than or equal to 0.05 (Tables 1 and 2). In the present study, we selected the following genes, INHBA (activin A), INHBB (activin B), MADH3 (Smad3) and TGF-β_2 involved in TGF-β signaling pathway, and genes, CDK6, CDKN2B (P15), MYBL1 (A-myb) and CCNB2 (cyclin B2) in cell cycle regulation for further evaluation. We found that most of all selected genes were down-regulated in the high concentration of genistein treated UtLM cells except the CDK inhibitor, P15, which was up-regulated.

Validation of selected function and signaling pathway genes

Based on the analysis results from IPA software, the eight selected genes were further validated by real-time RT-PCR. As shown in Figure 1, we found that the results of real-time RT-PCR showed similar patterns to that of the microarray, however, the data calculated from the results of real-time RT-PCR was higher compared to microarray data. For example, the expression of P15 was increased in genistein treated UtLM cells about 2-fold by microarray and 11-fold by real-time RT-PCR, while gene expression of cyclin B2, CDK6 and A-myb displayed down-regulation of 1.96, 2.96, 3.41-fold change, respectively, in microarray analysis and showed 5, 3, 3-fold decreased expression, respectively, by using real-time RT-PCR (Figure 1A). For TGF-β signaling pathway genes, we found that gene expression of activin A, activin B, Smad3 and TGF-β_2 were down-regulated by a 2.06, 1.64, 1.78 and 3.03-fold change, respectively, in microarray analysis, and down-regulated by a 2, 4, 2 and 3-fold change, respectively, by real-time RT-PCR (Figure 1B). The differences in the results has been attributed to differences in RT-PCR vs. microarray methodologies.

Decreased protein expression of activin A and Smad3 in genistein-treated UtLM cells

Activin A, is an active TGF-β ligand dimer assembled by two INHBA isoforms (activin A) which initiates cellular responses by binding to its receptors, that

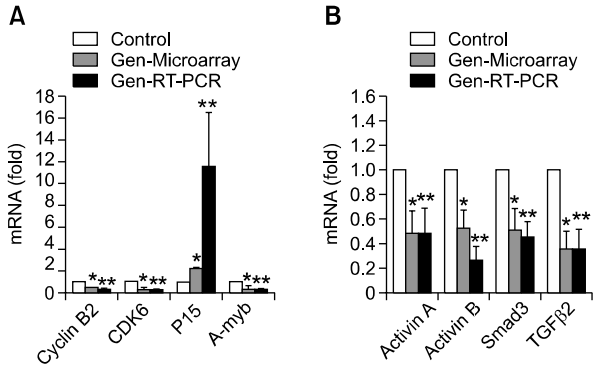

Figure 1. Relative mRNA expression changes of selected pathway genes in uterine leiomyoma (UtLM) cells following genistein (50 μg/ml) treatment for 24 h, as measured by microarray (Gen-Microarray) and real-time RT-PCR (Gen-RT-PCR). Results are calculated as the mRNA fold change by dividing values by the vehicle control (set at 1). Data are presented as mean ± S.E. of three experiments. A statistically significant difference ($P \leq 0.05$) is indicated by asterisk(s) (*: genistein treated versus vehicle control with the method of microarray; **: genistein treated versus vehicle control with the method of real-time RT-PCR). A house keeping gene, GAPDH, was used for normalization. (A) Genes related to cell cycle regulation. (B) Genes in TGF-β signaling pathway.

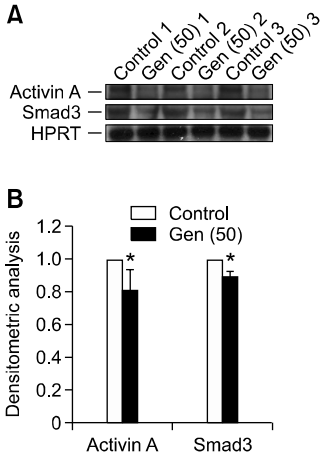

Figure 2. (A) Protein expression of activin A and Smad3 in uterine leiomyoma (UtLM) cells following genistein (50 μg/ml) treatment for 24 h. Hypoxanthine phosphoribosyl-transferase (HPRT) was used as a protein loading control for each experiment. (B) Bar graphs show the mean ± S.E. values of densitometry data of three experiments by dividing values by the vehicle control (set at 1). A statistically significant difference ($P \leq 0.05$) is indicated by an asterisks (*: genistein treated versus vehicle control).

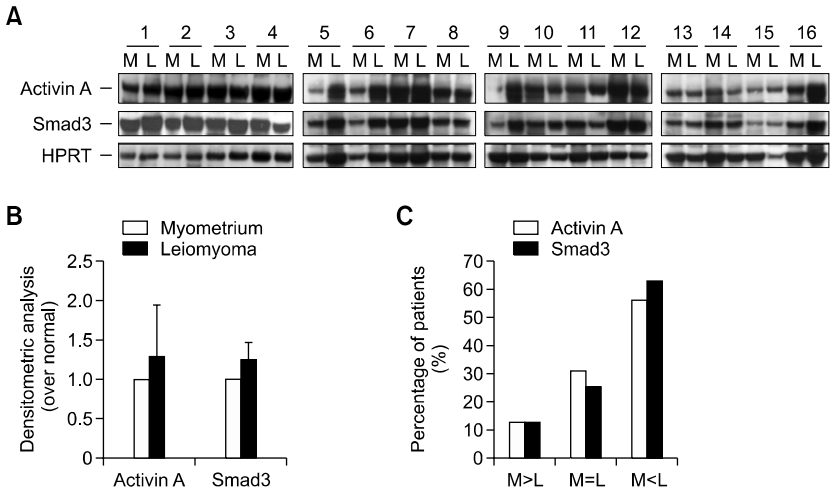

Figure 3. (A) Protein expression of activin A and Smad3 in uterine leiomyomas (L) and patient-matched myometrium (M). Hypoxanthine phosphoribosyl-transferase (HPRT) was used as a protein loading control for each experiment. (B) Bar graphs show the mean ± S.E. values of densitometry data of three experiments by dividing values by patient-matched myometrium (set at 1). (C) Bar graphs shows the comparison of activin A and Smad3 protein expression between leiomyoma and patient-matched myometrial tissue.

have intrinsic serine/threonine kinase activity, and activating downstream Smad proteins (Abe *et al*., 2004). Because gene and mRNA expression of activin A, activin B and Smad3 was down-regulated in genistein-treated UtLM cells, we did further studies to determine if the protein expression of activin A and Smad3 correlated with gene down-regulation in genistein-treated UtLM cells. Using western blotting, we found that the protein expression of both activin A and Smad3 was decreased in genistein-treated UtLM cells compared to vehicle-treated (Figure 2), suggesting that the decreased protein expression of activin A and Smad3 might due to the down-regulation of INHBA and Smad3 genes, possibly leading to the inhibitory effects of genistein on UtLM cells.

Comparison of activin A and Smad3 protein expression between uterine leiomyomas and matched myometrium in tissue samples

We examined the protein expression of activin A and Smad3 in sixteen leiomyomas and patient-matched myometrial tissue samples by western blotting to determine whether both of the protein levels of activin A and Smad3 are overexpressed in uterine leiomyomas compared to the matched myometrial tissue. We found that both protein expression of activin A and Smad3 were detectable in uterine leiomyomas and myometrium (Figure 3A). Although

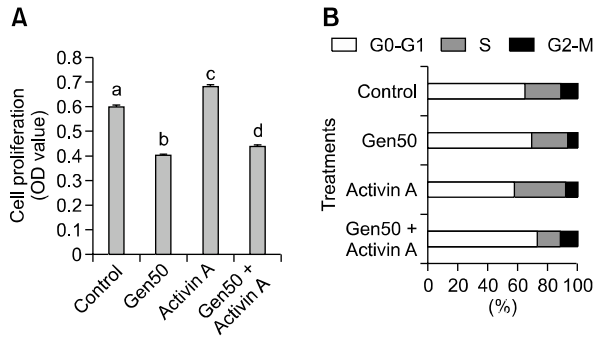

Figure 4. (A) Cell proliferation assay in treated UtLM cells, vehicle (control), 50 mg/ml of genistein (Gen-50), 20 nM activin A or genistein plus 20 nM activin A (Gen-50 + Acitivin A) for 96 h. The bar graph shows the mean ± S.E. values of absorbance of three experiments that have been normalized to the vehicle control (set at 1). a, b, c and d indicate groups which are statistically different ($P \leq 0.05$). (B) Cell cycle analysis in treated UtLM cells, vehicle (control), 50 mg/ml of genistein (Gen-50), 20 nM activin A or genistein plus 20 nM activin A (Gen-50 + Acitivin A) for 48 h. The values represent the number of cells in the different phases of the cell cycle as a percentage of total cells observed.

no statistical significance was found, in the comparison of uterine leiomyomas and matched myometrial tissue (Figure 3B), activin A protein levels were higher in leiomyoma than myometrial tissue in 56% (9/16) of the samples; equal in 31% (5/16) of the samples; and lower in 13% (2/16) of the samples (Figure 3C). The protein expression level of Smad3 was higher in 62% (10/16) of the leiomyoma as compared to their matched myometrial tissue; equal in 25% (4/16) of the samples; and lower in leiomyoma than myometrial tissue in 13% (2/16) of the samples. Moreover, a positive correlation was found between the protein expression of activin A and Smad3 in both leiomyomas and matched myometrial tissue (Figures 3A and 3B), suggesting that signaling of TGF-β pathway, from activin A to Smad3 may have a promotional effect on the growth of leiomyomas.

Activin A stimulates growth of UtLM cells

In order to gain information on the biological effects of activin A on UtLM cells growth, we utilized a recombinant activin A protein and examined its effects by cell proliferation assays. We found that when UtLM cells were treated with activin A (20 nM) for 5 days, the growth of UtLM cells was stimulated and the number of UtLM cells was significantly higher than vehicle-treated ($P < 0.05$) (Figure 4). Furthermore, when UtLM cells were treated with a high concentration of genistein in combination with activin A, we found that the inhibitory effect of genistein was partially antagonized ($P < 0.05$) (Figure 4). Therefore, the results further support the hypothesis that downregulation of activin A expression by genistein may inhibit the growth of UtLM cells.

Activin A increases percentage of UtLM cells in S phase

We conducted cell cycle analysis studies using flow cytometry to support our findings of activin A induced increased proliferation in UtLM cells. We found that activin A increased the percentage of cells in S phase in UtLM cells at 48 h (Figure 4). Additionally, when UtLM cells were treated with a high concentration of genistein in combination with activin A, we found the percentage of cells in G2-M increased. These results support the positive growth effects observed in UtLM cells following activin A treatment and the ability of activin A to rescue UtLM cells from the inhibitory growth effects observed with a high concentration of genistien. These data supports the proliferation data and further strengthens the hypothesis that genestein's down regulation of activin A may contribute to its negative growth effect in UtLM cells.

Discussion

The purpose of the present study was to identify the novel pathways and genes that might be involved in the inhibitory effect of a high dose of genistein on UtLM cells. Using DNA microarray and IPA, we identified genes, functions and pathways that may contribute to the inhibitory effect of genistein on UtLM cells. Most importantly, the possible role of TGF-β pathway genes, activin A and Smad3 in the pathogenesis of human uterine leiomyomas was ascertained.

The growth-inhibitory or apoptosis-inducing effects of genistein at high concentrations are mostly reported in *in vitro* studies (Jamadar-Shroff *et al*., 2009; Pavese *et al*., 2010). By using DNA microarray, specific gene expression patterns have been reported in several different cell types under genistein treatment (Piao *et al*., 2006; Lee *et al*., 2007; Lavigne *et al*., 2008). Consistently, in the present study we found that genes, related to cell cycle progression, such as BID, CASP3, MAP3K14, TNFRSF6, A-myb, CDK6, cyclin B2, Smad3 and E2F4 were significantly downregulated in genistein-treated UtLM cells. We also found that p15, a p53 inducible gene, was induced in UtLM cells. This is consistent with the reported genotoxic/DNA damaging effect of high doses of genistein (Ye *et al*., 2004) and also is consistent with our previous report of decreased apoptosis in UtLM cells treated with a high dose of genistein compared to uterine myometrial

(UtSMC) cells (Moore *et al*., 2007). Herein, our results suggest that regulations of cycle-related genes may be a molecular signature and mechanism of action for high doses of genistein on UtLM cell growth.

In addition to these genes discussed above, we also found that genistein at a high dose down-regulates TGF-β signaling pathway genes in UtLM cells. Smad3 is one of two homologous proteins that involved in signaling from TGF-beta/activin to modulate gene transcription (ten Dijke and Hill, 2004). In leiomyoma, the protein expression of Smad3 as well as several other components of the TGF-β system, such as TGF-β receptors and Smad4, was found elevated compared to matched myometrium, and was lowered after GnRHa therapy (Chegini *et al*., 2003; Levens *et al*., 2005). Increased expression of Smad3 and TGF-β receptors may account for the development of leiomyoma and its matrix components. Therefore, downregulation of Smad3 mRNA expression and decreased protein expression in UtLM cells may contribute to the inhibitory effects of high doses of genistein. Consistent with previous studies, in the present study, we have confirmed that the protein expression of Smad3 is increased in the leiomyomas more than matched myometrial tissue (Chegini *et al*., 2003). Additionally, we also demonstrated that activin A which is assembled by two INHBA isoforms, was lowered in genistein-treated UtLM cells. Activin A, as a member of TGF-β family, controls many physiological processes such as cell proliferation and differentiation, immune responses, wound repair and various endocrine activities (Harrison *et al*., 2005). Herein, we speculate that the inhibition effects of genistein in UtLM cells might be due to the down-regulation of activin A and Smad3, or possibly due to the inhibition of the TGF-β signaling, which transducts from activin A to Smad3. Elevation of TGF-βs has been reported in uterine leiomyomas (Levens *et al*., 2005; Luo *et al*., 2005; Zhao *et al*., 2007); however, the protein expression of activin A has never been investigated. It was reported that in the ovaries of vertebrates, activins are expressed predominantly in the follicular layer of the oocyte where they regulate processes such as folliculogenesis, steroid hormone production, and oocyte maturation (Hogg, *et al*., 2010; McLaughlin, *et al*., 2010). In our present study, the presence of an increased protein expression of activin A in leiomyomas provided further information that activin A may play a role to elevate the growth of uterine leiomyoma. Herein, the results from tissue samples are further supportive of the importance of the TGF-β system as key regulator of leiomyoma growth. Activin A has various effects on diverse biological systems (Abe *et al*., 2004). In contrast to the report that activin A mediates growth inhibition and cell cycle arrest through Smads in human breast cancer cells (Burdette *et al*., 2005), the stimulatory effect on mesenchymal cells such as fibroblasts has emphasized the importance of activin A in keloid pathogenesis (Mukhopadhyay *et al*., 2007). In the present study, we found that the growth of UtLM cells was increased by activin A and the inhibitory effect of a high-dose of genistein was antagonized by co-treatment with activin A. The stimulatory effect of activin A on UtLM cell growth is similar to TGF-β, which has been emphasized as one of the promoters in the formation of the extracellular matrix (Leppert *et al*., 2006). Also, we found that activin A was able to stimulate UtLM cells to progress into the S phase, in addition to promoting the progression of cells into mitosis when administered with a high concentration of genistein. These results further suggest that signaling of TGF-β pathway; from activin A to Smad3 may have a promotional effect on the growth of leiomyomas.

In summary, by using IPA, along with multiple altered functions and signaling pathways, we were able to identify that cell cycle dysregulation and down-regulation of the TGF-β signaling pathway might contribute to the inhibitory effects of a high dose of genistein in UtLM cells. Using additional complimentary experiments, we were able to show that activin A and Smad3, both members of the TGF-β signaling pathway, may offer a mechanistic explanation for the progression of leiomyomas, and that these pathways and genes might be potential therapeutic targets for uterine fibroids.

Methods

Cell culture

Human uterine leiomyoma (UtLM) cells (GM10964) were purchased from Coriell Institute for Medical Research (Camden, NJ), and cultured as previously reported (Swartz *et al*., 2005). Briefly, cells were grown in Eagle's minimum essential medium (Invitrogen [Gibco], Carlsbad, CA) with 19% fetal bovine serum (FBS) (Sigma, St Louis, MO) and supplemented with vitamins, essential and non-essential amino acids and L-glutamine (Invitrogen). All culture maintenance and experiments were performed at 37°C and 5% CO_2. UtLM cells were seeded in T175 flasks (at passages 8-14 at 80-95% confluence), and 24 h prior to treatment, the media was changed to Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12) (Hyclone Laboratories, Logan, UT) phenol red-free with charcoal dextran treated FBS (Sigma). Cells were then treated with either genistein (50 μg/ml) (4', 5, 7-Trihydroxyisoflavone; Sigma Chemical Company, St. Louis, MO) or the vehicle control [0.3% dimethylsulfoxide (DMSO), Sigma] in phenol red-free, stripped-serum DMEM medium for 24 h.

RNA isolation and microarray analysis

Total RNA preparation for microarray studies was performed as previously described (Swartz *et al*., 2005). Briefly, total RNA was isolated with TRIzol agent (Invitrogen) from treated UtLM cells and purified using a RNeasy MidiKit (QIAGEN, Valencia, CA), according to the manufacturers' protocols. Due to the expense of chip printing and the large amount of RNA needed for each of the hybridizations, the samples from the flasks that received the same treatment were pooled, then concentrated to > 9 μg/μl by Microcon 30 columns (Amicon: Millipore, Bedford, MA). Gene expression analysis was conducted using Agilent Whole Human Genome arrays (Agilent Technologies, Palo Alto, CA). Total RNA was amplified by using the Agilent Low RNA Input Fluorescent Linear Amplification Kit protocol. Starting with 500 ng of total RNA, cyanine (Cy) 3- or Cy5-labeled cRNAs were produced according to manufacturer's protocol. For each two-color comparison, 750 ng each of Cy3- and Cy5-labeled cRNAs were mixed and fragmented using the Agilent *In Situ* Hybridization Kit protocol. Hybridizations were performed for 17 h in a rotating oven using the Agilent 60-mer Oligo microarray processing protocol. Slides were later washed as indicated in this protocol, and then scanned with an Agilent Scanner. Data were obtained using the Agilent Feature Extraction software (v7.5), employing defaults for all parameters. Since only those genes that were consistently under- or over-expressed on three of four chip replicates were used, results from these pooled samples represent mean changes between the replicates.

Rosetta resolver

This procedure was performed as previously described (Kim *et al*., 2006). Images and GEML (gene expression markup language) files, including error and *P*-values, were exported from the Agilent Feature Extraction software and deposited into Rosetta Resolver (version 4.0, build 4.0.1.0.7.RSPLIT) (Rosetta Biosoftware, Kirkland, WA). Ratio profiles were combined and intensity plots were generated for each ratio experiment. "Signature genes" were identified, as those having a *P*-value ≤ 0.001 with a fold-change ≥ 1.5 in all three biological replicates.

Ingenuity pathways analysis (IPA)

Signature genes were further analyzed with IPA software (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com), a web-delivered application that makes use of the Ingenuity Pathways Knowledge Base (IPKB) containing large amounts of individually modeled relationships between gene objects (e.g., genes, mRNAs, and proteins). The procedure was performed as previously described (Liu *et al*., 2006). Briefly, we uploaded a tab-delimited text file with gene identifiers and their corresponding expression values such as fold-change and p-value. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. A fold change cutoff of 1.5-fold and $P < 0.001$ was set to identify genes whose expression was differentially regulated. These genes, called "focus genes," were then used as the starting point for generating biological networks. To start building networks, the application queries the Ingenuity Pathways Knowledge Base for interactions between focus genes and all other gene objects stored in the knowledge base, then generates a set of networks with a network size of approximately 35 genes or proteins. Ingenuity Pathways Analysis then computes a score for each network according to the fit of the user's set of significant genes. The score is derived from a *P*-value and indicates the likelihood of the focus genes in a network being found together because of random chance. A score of 2 indicates a 1 in 100 change that the focus genes are together in a network because of random chance. Therefore, scores of 2 or higher have at least a 99% confidence level of not being generated by random chance alone. Biological functions are then calculated and assigned to each network.

Real-time RT-PCR analysis

Real-time RT-PCR analysis was used to quantify the levels of expression of the selected genes (Supplemental Data Table S1). The forward and reverse primers for selected genes were designed using ABI Primer Express software (Applied Biosystems, Foster City, CA). Total RNA was prepared as above and reverse transcribed with MuLV reverse transcriptase and oligo-dT primers (Applied Biosystems), then subjected to an ABI Prism 7900 Sequence Detection System using SYBR green PCR master mix (Applied Biosystems, Cheshire, UK), according to the manufacturer's recommendations (Applied Biosystems). The threshold cycle (CT) values of the real-time PCR were used in the determination of the relative differences in expression between groups. To quantify the gene transcripts, we monitored GAPDH, which served as the internal quantitative control. Each sample was normalized by quantification against its GAPDH content. To determine the normalized value, $2^{-(\Delta\Delta CT)}$ values were compared between control and genistein treated groups, where the changes in crossing threshold (ΔCT) = $Ct_{\text{Target gene}} - Ct_{\text{GAPDH RNA}}$, and $\Delta\Delta CT = \Delta Ct^{\text{control}} - \Delta Ct^{\text{target}}$ as outlined in the Applied Biosystems protocol, and the results are presented as fold change above control.

Western blotting analysis

Total protein was extracted from treated cells and tissue samples as previously described in our lab (Dixon *et al*., 2002; Swartz *et al*., 2005). Then protein was separated on a 4-12% Nu-Page Bis-Tris (Invitrogen) gel, transferred to a 0.45 μM PVDF membrane and probed with a monoclonal anti- human/mouse/rat activin A (1 : 500; MAB3381, R&D Systems Inc., Minneapolis, MN) and a Smad3 (1 : 1000 dilution; Sc-8332, Santa Cruz Biotechnology, Santa Cruz, CA). Recombinant human/mouse/rat activin A (338-AC, R&D Systems Inc.), was used as positive control. Signals were developed by using ECL Western Blotting Analysis System (Amersham pharmacia, Piscataway, NJ) according to the manufacturer's protocol. A densitometer (Fluor ChemTM8900, Alpha Innotech, San Leandro, CA) was used for quantitation of the band intensities.

Cell proliferation assay

UtLM cells were seeded into 96-well plates at an initial density of 4×10^3 per well in 100 μl Eagle's MEM (Invitrogen) supplemented with vitamins, amino acids and 19% FBS, and then incubated with DMEM/F-12, phenol red-free with charcoal dextran treated FBS for 24 h. The UtLM cells were then cultured with vehicle control (0.3% DMSO), genistein (50 μg/ml) alone or genistein in combination with activin A (20 nM) or activin A alone for 96 h. Cell proliferation was measured by spectrophotometry using CellTiter 96^R AQu^{eous} One Solution Cell Proliferation assay (Promega Corporation, Madison, WI) according to the manufacturer's protocol. All conditions were tested in five replicates in triplicate experiments.

Cell cycle analysis

Flow cytometry was used to determine the percentage of cells in various phases of the cell cycle. The DNA content was determined using propidium iodide (PI) and ethanol fixation. Briefly, UtLM cells were treated with 0 μg/ml (DMSO; control), 50 μg/ml genistein (Sigma), 20 nM activin A (R & D Systems), or a combination of 50 μg/ml genistein and 20 nM activin A, and incubated in culture for 48 h. The UtLM cells were pelleted and fixed by the slow addition of cold 70% ethanol to a volume of approximately 2-3 ml with agitation. The volume was adjusted to 5 ml with cold 70% ethanol and the cells were stored at -20°C overnight. Fixed cells were pelleted from the ethanol, washed once in 3 ml of 1X PBS and stained in 1 ml of 20 ng/ml propidium iodide, 1,000 units RNaseOne (Promega) in 1X PBS. Analysis was done using a Becton Dickinson FACSort flow cytometer and CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Individual cells (7,500 per experimental sample) were selected by gating on a PI area versus width dot plot to exclude cell aggregates and debris. Cells were excited using a 488 nm argon laser and emission was detected at 585 nm. Data was analyzed using Modfit software for Mac version 2.0.

Statistical analysis

The results are expressed as means ± S.E. (standard error). The gene expression data of quantitative RT-PCR experiments were analyzed for statistical significance by a Student's *t*-test for two-tailed unpaired comparisons. Two-sided Mann-Whitney test was performed to determine the statistical significance of the results of protein expression and cell proliferation assay between different groups. A probability level of *P*-value ≤ 0.05 was considered significant.

Supplemental data

Supplemental data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-4-04.pdf.

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