Early growth response gene-1 plays a pivotal role in down-regulation of a cohort of genes in uterine leiomyoma

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Abstract

Microarray studies have identified many genes that are down-regulated in uterine leiomyoma compared with myometrium, including early growth response gene-1 (*EGR1*). However, the mechanisms underlying coordinated down-regulation of this gene cohort remain unknown. To address the transcriptional role of *EGR1* in leiomyoma, *EGR1* binding to promoter sequences on target genes was assessed by chromatin immunoprecipitation (ChIP) assay in leiomyoma tissues and myometrium-derived KW cells. Computer analysis demonstrated that 50 out of 135 genes listed as down-regulated in array reports possessed potential binding sites for *EGR1* within 1 kb promoter sequence. ChIP assay was performed for a random selection of 13 genes possessing potential binding sites for *EGR1* (Group A), 3 genes known as *EGR1* targets in other tissues (Group B), and 4 control genes. Decreased *EGR1* bindings were significant for 11 out of 16 genes (Group A+B) in leiomyoma tissues compared with myometrium, and mRNA levels in tissue samples were actually decreased for 7 out of the 11 genes. ChIP analyses performed on KW cells showed induction of *EGR1* binding to the promoter region of all genes except one Group A+B gene, but for none of the control genes. These results indicate that *EGR1* is a key player in coordinated down-regulation of genes in leiomyoma. Application of ChIP-quantitative PCR assay with the aid of computer-assisted analysis of genome databases appears useful for the comprehensive interpretation of array data.

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Introduction

Gene array studies have been conducted to clarify the alteration of gene expression profiles in uterine leiomyoma and have identified numerous genes for which expression is up- or down-regulated compared with levels in normal myometrium (Tsibris *et al.* 2002, Catherino *et al.* 2003, Chegini *et al.* 2003, Skubitz & Skubitz 2003, Wang *et al.* 2003, Weston *et al.* 2003, Hoffman *et al.* 2004, Quade *et al.* 2004). Although the total number of genes analyzed in each array has varied, 5–358 genes per study have been identified as down-regulated in leiomyoma, greatly exceeding the number of up-regulated genes in eight out of nine independent array-based studies (Tsibris *et al.* 2002, Ahn *et al.* 2003, Catherino *et al.* 2003, Chegini *et al.* 2003, Skubitz & Skubitz 2003, Wang *et al.* 2003, Weston *et al.* 2003, Quade *et al.* 2004, Arslan *et al.* 2005). Down-regulated genes would play an important role in leiomyoma phenotype, similar to or more important than up-regulated genes. The next important step in array studies is to interpret these genome-wide results in a comprehensive manner and identify the master regulators of altered expression among the identified genes.

Computer-aided review of these expression array data and the genome database revealed that a substantial proportion of genes reported as being down-regulated in leiomyoma; possess potential binding sites in the promoter regions for early growth response gene-1 (*EGR1*), a pleiotropic transcription factor. We have shown that *EGR1*, a tumor suppressor gene, is consistently down-regulated in leiomyoma compared with surrounding myometrium (Shozu *et al.* 2004). We therefore reasoned that a shortage of *EGR1* in leiomyoma would cause synchronized down-regulation of a cohort of genes sharing potential binding sites for *EGR1*, allowing accelerated proliferation of leiomyoma cells.

EGR1 plays diverse roles in the physiology and pathology of numerous organs and cells, including cell cycle and proliferation, immune responses, memory, arteriosclerosis, pulmonary fibrosis, and tumor suppressor function, through the transcriptional regulation of various target genes (Huang *et al.* 1997, McCaffrey *et al.* 2000, Calogero *et al.* 2001, Lee *et al.* 2004). In the tissues of most cancers other than prostate cancer, *EGR1* expression is reduced and re-expression of *EGR1* leads to retarded tumor cell growth, probably through cell cycle arrest and apoptotic transcriptional activation of target genes such as those for p21, p53, phosphatase and

tensin homolog (PTEN), transforming growth factor- β 1, fibronectin, and growth arrest and DNA damage inducible gene (Gadd)45 (Shin *et al.* 2006). As mentioned earlier, uterine leiomyoma consistently expresses low levels of EGR1. Uterine leiomyoma, although benign, is similar to malignant tumor cells in this regard. We have shown that myometrium-derived KW cells lose virtually all EGR1 expression upon establishment of rapid proliferation and that re-expression of EGR1 in turn retards cellular growth, suggesting that reduced EGR1 in leiomyoma contributes to tumorigenic growth (Shozu *et al.* 2004).

To address the possible impact of EGR1 on transcription of a cohort of down-regulated genes, we examined binding of EGR1 to promoter sequences of potential target genes using collective chromatin immunoprecipitation (ChIP) assay followed by quantitative real-time PCR (qPCR) and demonstrated that down-regulation of EGR1 is a common regulator of down-regulated genes, and probably contributes to leiomyoma phenotypes.

Materials and methods

Tissue acquisition

Uterine tissues were obtained from women, at hysterectomy, for uterine leiomyoma. The institutional review board approved all study protocols and written consent was obtained from all patients. Leiomyoma specimens and corresponding myometrial specimens were obtained from 34 women in the early proliferative phase undergoing hysterectomy.

Tissue preparation and usage, storage of tissue samples, and exclusion criteria have been described elsewhere (Shozu *et al.* 2004). All donors had regular menstrual cycles (mean, 28 days; range, 20–32 days) and had received no medications for ≥ 2 cycles before surgery.

qPCR assay

DNA template for PCR standards was amplified from cDNA or genomic DNA then subcloned into pCR2.1 vector (Invitrogen). Fidelity of amplicons was confirmed by sequencing. Primer sequences are listed in Table 1. For mRNA quantification, amplicons (~ 200 bp) were designed to span ≥ 2 exons and not to include polymorphic regions. For ChIP assay, amplicons (~ 100 bp) were designed to include the most probable EGR1 binding site. For fibroblast growth factor 8 (*FGF8*), v-src sarcoma viral oncogene homolog (*SRC*), and insulin-like growth factor-2 (*IGF2*), primer pairs outside the EGR1 site did not yield the specific product and were eventually set close to, but outside, the site.

Synthesis of cDNA and quantitative PCR were performed as described elsewhere (Kasai *et al.* 2004,

Shozu *et al.* 2004). Primer pairs used for qPCR were the same as used for construction of DNA standards.

Cell culture

Isolation and culture of smooth muscle cells from leiomyoma and surrounding myometrium and phenotypic validation of cells were performed as described previously (Sumitani *et al.* 2000, Shozu *et al.* 2002). KW cells had previously been established from myometrial smooth muscle cells and characterized (Shozu *et al.* 2002).

Establishment of KWtet-off/EGR1 cells

The open reading frame of EGR1 cDNA (ETR103(#1198); obtained from Riken Bioresource Center, Ibaraki, Japan) was amplified and directionally subcloned into pTRE2hyg (Clontech). The insert sequence was identical to the reference sequence from the National Center for Biotechnology Information database, except for one nonsense mutation at position 1242 (T1242C).

A subline of KW cells that stably express EGR1 in the absence of tetracycline (KWtet-off/EGR1 cell) was established by sequential transfection with a pTet-off and pTRE2hyg EGR1 plasmid, using the Tet-off Gene Expression System (Clontech).

ChIP assay

ChIP assay was performed on KWtet-off/EGR1 cell pellets (1.0×10^6 cells) using a ChIP Assay Kit (#17-295; Upstate, Lake Placid, NY, USA) in accordance with the manufacturer's instructions. DNA was sheared into 200–800 bp fragments using a Bioruptor Ultrasonics Sonicator (Cosmo Bio, Tokyo, Japan). Immunoprecipitation was conducted at 4 °C for 16 h using anti-EGR1 antibody (sc-110X; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or nonimmune rabbit IgG (X0903; Dako Japan, Kyoto, Japan). Immunoprecipitated DNA was quantified by real-time PCR using the primers listed in Table 1.

ChIP assay using tissue samples was performed as described above with some modifications. Briefly, totally minced tissue samples were fixed at room temperature for 15 min in the presence of Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) culture medium with 1% formaldehyde and then incubated with 0.125 M glycine for 5 min. After discarding the medium, the fixed sample was homogenized on ice with 10 mM PBS containing 1 mM EDTA, 1 mM EGTA, 10 mM KCl, protease inhibitor cocktail (Roche Diagnostic), and 0.3% NP-40 using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The resulting homogenate was filtered using a 100- μ m Cell Strainer (BD Falcon, Franklin Lakes, NJ, USA) and centrifuged

Table 1 Oligonucleotide sequences used for mRNA quantification and chromatin immunoprecipitation (ChIP) assay

Primer pairs for mRNA quantification name: sequence (5'–3')		Primer pairs for ChIP assay name: sequence (5'–3')
Gene symbol		
EGR1	EGR1-1497F: AAAGTTTGCCAGGAGCGATG EGR1-1678R: CAGGGGATGGGTATGAGGTG	EGR1-771F: CGCACTCCCGGTTCTGCTCT EGR1-467R: CTCCCTCCTCCCTGGTTCCAA
FOS	FOS-249F: TCACCCGCAGACTCCTTCTC FOS-510R: GGCCTCCTGTCATGGTCTTC	FOS-206F: CAGGAAGTCGCAAATGCTCA FOS-53R: CTGTAAACGTCACGGGCTCA
ATF3	ATF3-267F: GCTAACCTGACGCCCTTGT ATF3-505R: AGGCACTCCGTCTTCTCCTTC	ATF3-839F: TACGGTCTACCACTCGCCCTA ATF3-704R: CCGCCGGTTAACACAAAAGC
JUN	JUN-1843F: CCAAGTGCCGAAAAAGGAAG JUN-2022R: GCTGCGTTAGCATGAGTTGG	JUN-998F: CGCGTTATGTTGTGCGTGTGT JUN-874R: CAGGGTCCAGATGGGAACAAGC
CSRP2	CSRP2-73F: GCCTCCAAATGCCCAAGT CSRP2-169R: GCTCGCACTTGAGGCAGAACT	CSRP2-891F: GGGAGAAGGCACTGGAGTGTCA CSRP2-763R: GTCCACCCCAAGCACTTCCA
SERPINE1	PAI1-66F: ACCCCCTCGCTGGAATCTA PAI1-266R: CGAAGACTGTCCACACAGC	PAI1-167F: GACGGACTCCAGAGCCAGTGA PAI1-75R: TGTGGGCCACTGCCTCCTTTA
CYR61	CYR61-235F: CGCCTTAGTCGTCATCTTC CYR61-507R: CAGGGTCTGCCCTCTGACTG	CYR61-520F: GGTCAAGTATCACTACCAAAC CYR61-401R: GGTAGTTGGAGGGTCTGAGG
VEGF	VEGF-141F: TCAGCGCAGCTACTGCCATC VEGF-350R: ATGTGCTGGCCTTGGTGAGG	VEGF-318F: CCTGTCCGCACGTAACCTCAC VEGF-178R: GCAATGAAGGGGAAGCTCGAC
RORA	ROR-f: TGATCGCAGCGATGAAAGC ROR-iso: AACAGTTCTTCTGACGAGGACAGG	RORA-488F: CCTTGACGTATCAGTGGTCTTG RORA-149R: GAGCACTCGGGGCGATAAATG
SRC	SRC-774F: GAGCGGCTCCAGATTGTCAAC SRC-862R: TTGCTGGGGATGTAGCCTGTC	SRC-581F: GCGGGAAAGCTGCGTCCAGAG SRC-441R: GCGTTGAAGGCTCCGAGGGTCT
PNRC1	PNRC1-638F: CCCCCTCAGGAAAGAGCTTTA PNRC1-836R: TGAAACAGAATCCTGCCAAAAG	PNRC1-190F: CTGCAGCAAGCTGGTTGTTGT PNRC1-89R: TTCCTGCGAAAGCCCAATTAGA
HMGA1	HMGA1-27F: TGCGCTCCTCTAATTGGGACT HMGA1-234R: GAGCAGGTGGAAGAGTGATGG	HMGA1-259F: CCAGAAGCTCCTTCGTGACTCC HMGA1-158R: GAGGCTGGGCTCGGAAC
FGF8	FGF8-476F: GAGCAGGTGGAAGAGTGATGG FGF8-545R: CTTGCCTTTGCCGTTGCTCTT	FGF8-718F: CCAGGGCCTCCTCGGGAGAGTG FGF8-637R: CGGACCCCGCTCCCCTGTTTC
PDGFB	PDGFB-1052F: TCTCTGCTGCTACCTGCGTCG PDGFB-1218R: GGGTCATGTTTCAGTCCAACTC	PDGFB-179F: ACTGAAGGGTGTCTCGGCTCT PDGFB-1R: CTTTCAGCTGTTCCGGCCTTT
F3	F3-706F: TCAGGAAAGAAAACAGCCAAAAC F3-929R: ATGATGACAAGGATGATGACCAC	F3-194F: GGGTCCCGGAGTTTCTTACC F3-42R: GCTCTCCCGCGCCTCTGC
PTP4A1	PRL1-172F: AGGCCACAATCTTCAATGAGT PRL1-345R: CTCTTATGGGGCTTCTTGGT	PRL1-216F: CGGCGTTAGCCATTTCATCAAC PRL1-79R: GCAACCCTCCAGCCACCAATC
TGFB3	TGFB3-737F: AAGCGGAATGAGCAGAGGAT TGFB3-959R: CATTGGGCTGAAAGGTGTGA	TGFB3-234F: CAAGGCAAGGCAAGGATTTTGA TGFB3-152R: GGCGATGGGGAGAAAGTGGGT
IGF2	IGF2-669F: CACCCTCCAGTTCGTCTGTGG IGF2-782R: AGGTACACAGCTGCGGAAACAG	IGF2-920F: CTGAATTCTCTAGAACGGGCATTTCAGCA IGF2-862R: GGGGGCAGGGAGCCGCAGAG
CCNG1	CCNG1-316F: TGGCCTCAGAATGACTGCAAG CCNG1-541R: TGCCAATGGGACATTCTTTCT	
CYP19A1	Arom203F: GCCGAATCGAGAGCTGTAAT Arom205R: CTCCTCACTGGCCTTTTCTC	Arom1b-420F: ATGCTGGAATGCTGGACATAC Arom1b-185R: ACAGATTCAGAGGGCTGTTT
18S	18S-535F: GACTCTTTCGAGGCCCTGTA 18S-696R: CGCTCCCAAGATCCAACCTAC	

at 4 °C for 4 min. The cell pellet was washed twice with ice-cold PBS and prepared for the ChIP assay as described above. After revision of cross-links, DNA samples were recovered and purified using a MinElute Reaction Cleanup Kit (Qiagen) and eventually resuspended in 10 µl elution buffer. Amounts of EGR1–DNA complex were normalized to levels of 18S gene (determined by real-time PCR) in input samples.

Statistical analysis

Differences in transcript levels between two groups were evaluated using the Mann–Whitney *U* test for unpaired

data and the Wilcoxon signed rank test for paired data. Values of *P* < 0.05 were considered statistically significant.

Results

Selection of potential target genes of EGR1

Initially, we reviewed three array-based reports (Tsibris *et al.* 2002, Chegini *et al.* 2003, Skubitz & Skubitz 2003) for genes down-regulated in leiomyomas and picked up all genes (135 genes in total) reported in any one of the three reports. Promoter sequences were obtained from the National Center for Biotechnology Information

(NCBI) database. Computer-based analysis using the TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>) (Computational Biology Research Center, Ibaraki, Japan) revealed that 50 out of the 135 genes possessed one or more potential EGR1 binding sites (>80 threshold score) within 1 kb upstream of the predicted transcriptional start site. Experimental analysis was performed for a random selection of 13 out of these 50 genes (Group A), including *EGR1* itself (supplementary table at <http://jme.endocrinology-journals.org/content/vol39/issue5/>). Another three genes (*PDGFB*, *F3*, and *PTP4A1*) that are known to be regulated by EGR1 in tissues other than leiomyoma, but that have never been reported as down-regulated in any array experiments were also included for validation of array results (Group B). A final four genes (*TGFB3*, *IGF2*, *CCNG1*, and *CYP19A1*) were selected as controls from genes that have been reported as up-regulated in leiomyoma compared with myometrium (Group C; Vollenhoven *et al.* 1993, Sumitani *et al.* 2000, Lee & Nowak 2001, Baek *et al.* 2003). *IGF2* possesses a functional EGR1 binding site in the promoter region identified in cells like HepG2 cells (Bae *et al.* 1999). Paradoxically, expression of *IGF2* is up-regulated in leiomyoma tissue in which EGR1 expression is low, suggesting that no EGR1 binds to the ‘functional’ binding sites in leiomyoma cells. *IGF2* was thus selected as a potential negative control for ChIP assay. Similarly *TGFB3* was selected as another example of a control gene possessing an EGR1 binding

site, and paradoxically up-regulated in leiomyoma (Liu *et al.* 1998, De Falco *et al.* 2006). *CYP19A1* (L4 promoter) possesses a GC-rich sequence similar to, but not functioning as, an EGR1 binding site in the core promoter region. Electromobility shift assay clearly demonstrated that it was not EGR1, but rather Sp1 and Sp3 that bound to the GC-rich sequence in myometrial and leiomyoma cells (supplementary figure at <http://jme.endocrinology-journals.org/content/vol39/issue5/>). The *CYP19A1* promoter sequence thus served as a qualified negative control for EGR1 binding. *CCNG1* were selected as an example of a gene independent of EGR1 expression, as computer analysis predicted no potential EGR1 binding sites.

ChIP assay for EGR1 binding in leiomyoma tissue

EGR1 bindings to the promoter in tissue samples obtained from seven patients were quantitated by ChIP analysis, followed by real-time PCR. EGR1 bindings detected in leiomyoma were significantly decreased for 11 genes (8 out of 13 genes in Group A and all 3 genes in Group B) compared with corresponding myometrium (Fig. 1). EGR1 bindings were not different for the other five genes. No gene in leiomyoma displayed EGR1 binding exceeding that in myometrium. A representative result of gel electrophoresis is shown in Fig. 1B.

Figure 1 ChIP assay in leiomyoma tissue. EGR1 binding to promoters was quantified for seven couples of tissue specimens as described in the Materials and methods. (A) Ratio of EGR1 binding in leiomyoma compared with myometrium was calculated for each pair as a fold change. Closed bars represent mean fold decrease of seven pairs and left extended bars (–) mean decreased binding in leiomyoma tissues compared with myometrium. **P* < 0.05 (Wilcoxon signed rank test). *CYP19A1* and *CCNG1* were not analyzed because both have no potential EGR1 binding sequence. (B) Representative gel electrophoresis of one-paired sample was shown for nine gene promoters. *TGFB3* promoter was shown as a control. DNA samples were collected before immunoprecipitation (Input), after immunoprecipitation with anti-EGR1 antibody (EGR1), or after immunoprecipitation with nonimmune rabbit IgG. Number of amplification cycles (37–40 cycles) depended on genes. Decreased EGR1 binding in leiomyoma tissues was consistent in seven pairs.

Expression of potential target genes in leiomyoma tissue

To confirm differential mRNA expression between leiomyoma and myometrium, mRNA levels in tissue samples were quantified by real-time PCR following reverse transcription. Fold changes (mRNA levels in leiomyoma sample/mRNA levels of corresponding myometrium sample) were calculated for each pair.

In 9 out of 13 Group A genes and 2 out of 3 Group B genes, mRNA levels were significantly lower in leiomyoma, whereas in four Group A genes and one Group B gene, mRNA expression was not decreased in leiomyoma (Fig. 2). Among controls (Group C), *IGF2* and *CYP19A1* were up-regulated in leiomyoma as described in previous reports (Tsibris *et al.* 2002, Skubitz & Skubitz 2003, Hoffman *et al.* 2004, Quade *et al.* 2004, Arslan *et al.* 2005), whereas expressions of *TGFB3* and *CCNG1* genes did not differ, contrasting with previous reports (Baek *et al.* 2003).

ChIP assay for EGR1 binding in KWtet-off/EGR1 cells

In the above experiments using EGR-deficient leiomyoma tissues, we demonstrated that lower EGR1

binding correlated with reduced mRNA expression for at least seven genes (Table 2). We next examined whether increased EGR1 binding induces mRNA expression. To this end, we developed an *in vitro* cell assay system by establishing myometrium-derived KWtet-off/EGR1 cells that express EGR1 protein at a low basal level and at 10- to 20-fold higher levels at 6 h or after induction.

We first examined whether induced EGR1 binds to potential binding sites by ChIP–qPCR assay. Induction of EGR1 increased EGR1 binding to potential sites of all genes in Groups A and B, with the exception of one gene (*FGF8*), but to no sites in the three control genes (*TGFB3*, *IGF2*, and *CYP19A1*; Fig. 3A).

Specificity of the ChIP assay for EGR1 binding was assured using two different control experiments. First, nonimmunized rabbit IgG used instead of anti-EGR1 antibody detected no significant binding for any genes. An example of qualitative analysis of PCR products for detection of JUN promoter is shown in Fig. 3B. Secondly, the GC-rich element of *CYP19A1* promoter, similar to the EGR1 binding site, did not yield any significant increase in EGR1 binding, even under

Figure 2 Levels of EGR1 target gene mRNA quantified by real-time PCR in tissue specimens. The mRNA levels for each gene, normalized to 18S level, were determined on tissue samples as described. The ratio of mRNA in leiomyoma to that in corresponding myometrium was calculated for each pair as a fold change. Crossbars represent mean fold change, with number of pairs in parenthesis. Left extended lines (–) show decreased expression in leiomyoma compared with myometrium and right extended lines show increased expression in leiomyoma. **P* < 0.05 (Wilcoxon signed rank test).

Table 2 Subdivision of genes based on the results of four experiments

	Locus link ID	Reason for gene selection ^a	Results on leiomyoma tissues		Results on KW cells		Regrouped based on EGR1 dependency
			EGR1 binding to promoter region ^b	mRNA expression ^c	EGR1 binding in KW cells ^d	Increase in response to EGR1 induction	
Gene symbol							
EGR1	1958	A	↓	↓	+	Not available	Group 1
ATF3	467	A	↓	↓	+	Early	Group 1
FOS	2353	A	↓	↓	+	Early	Group 1
JUN	3725	A	↓	↓	+	Early	Group 1
RORA	6095	A	↓	↓	+	—	Group 1
F3	2152	B	↓	↓	+	—	Group 1
PDGFB	5155	B	↓	↓	+	—	Group 1
HMGA1	3159	A	↓	→	+	—	Group 2
PNRC1	10957	A	↓	→	+	—	Group 2
SRC	1445	A	↓	→	+	—	Group 2
PTP4A1	7803	B	↓	→	+	—	Group 2
CSRP2	1397	A	→	↓	+	Late	Group 3
SERPINE1	5054	A	→	↓	+	Late	Group 3
CYR61	3491	A	→	↓	+	—	Group 3
VEGF	7422	A	→	↓	+	—	Group 3
FGF8	2253	A	→	→	—	—	Group 4
TGFB3	7043	C	→	→	—	—	Group 4
IGF2	3481	C	→	↑	—	—	Group 4
CYP19A1	1588	C	Not examined	↑	—	—	Group 4
CCNG1	900	C	Not examined	→	Not examined	—	Group 4

^aReason for gene selection was specified in the first paragraph of the results section.
^bDetected EGR1 binding to each promoter was lower (↓) or same (→) in leiomyoma tissue compared with myometrium.
^cmRNA expression level was lower (↓), same (→), or higher (↑) in leiomyoma tissue compared with myometrium.
^dChIP assay detected significant (+) or nonsignificant (—) increase of EGR1 binding to each promoter.

Figure 3 ChIP assay in KWtet-off/EGR1 cells. (A) ChIP assay was performed on cells cultured in the presence or absence of tetracycline. Amounts of EGR1–DNA complex were normalized to levels of 18S genes apparent in input samples and fold increases were calculated for each of six independent experiments. Closed bars represent mean fold increase. *CCNG1* was excluded from analysis due to an absence of potential binding sites or similar for PCR amplification. *CYP19A1* was included in the analysis using a GC-rich sequence as the target sequence. **P* < 0.05 (Wilcoxon signed rank test). (B) A representative result of ChIP products on *JUN* promoter. Cross-linked samples were prepared from KWtet-off/EGR1 cells cultured in the absence (–) or presence (+) of tetracycline for 6 h. DNA samples were collected before immunoprecipitation (IN), after immunoprecipitation with anti-EGR1 antibody (IP), or after immunoprecipitation with nonimmune rabbit IgG (IgG). PCR products of 32 cycles were detected by PAGE.

conditions of EGR1 excess. Our ChIP assay was thus specific for EGR1–DNA complexes, and excess amounts of EGR1 did not interfere with assay results.

Regulation of gene expression by EGR1 in KWtet-off/EGR1 cells

To examine the transcriptional roles of extrinsic EGR1 on the selected genes, mRNA levels in KWtet-off/EGR1 cells were determined at 0–60 h after EGR1 induction (Fig. 4). Four genes (*EGR1*, *ATF3*, *FOS*, and *JUN*) showed significant increases at 6 h and two genes (*SERPINE1* and *CSRP2*) showed significant increases at 12–60 h. The remaining 12 genes showed no changes during EGR1 induction.

The early response at 6 h indicates that direct binding of EGR1 to DNA alone was able to elicit initiation of transcription, whereas the late response observed at 12–20 h indicates that binding of EGR1 to promoter alone was insufficient and some secondary event triggered by induced EGR1 was needed for transcriptional initiation. EGR1 mRNA showed an initial profound increase followed by a second enhancement at 60 h. The total increase in EGR1 mRNA would be determined as the sum of transcripts from both extrinsic (EGR1 cDNA plasmids) and intrinsic genes. Given that expression of target genes in the Tet-off Gene Expression System usually reaches a maximum at 6 h or earlier and continues without regulation (Gossen & Bujard 1992), switching on of the intrinsic *EGR1* gene, probably as a secondary event following extrinsic EGR1 expression, may explain the late peak observed at 60 h.

All results are summarized in Table 2, where genes were reordered and divided into four groups based on

Figure 4 Levels of target gene mRNA after induction of EGR1 in KWtet-off/EGR1 cells. The mRNA level in KWtet-off/EGR1 cells, normalized to 18S, was determined before (time 0) and after removal of tetracycline (time 6–60 h). Relative mRNA level was expressed as a fold change compared with mRNA level at time 0. Each time point represents an average of five to six independent experiments. * $P < 0.05$ versus time 0 (Mann–Whitney U test).

the nominal results. In Group 1 genes (*EGR1*, *ATF3*, *FOS*, *JUN*, *RORA*, *F3*, and *PDGFB*), results were compatible with EGR1-dependent expression, with EGR1 binding and mRNA expression simultaneously decreased in EGR1-deficient leiomyoma tissues. EGR1 up-regulated transcription in at least three genes of this group in KWtet-off/EGR1 cells. EGR1 bound to the promoter in Group 2 genes, but this binding did not affect mRNA level. This was supported by experiments conducted on KWtet-off/EGR1 cells. Though EGR1 bound to promoters, it would not be sufficient to initiate transcription in these genes. In Group 3 genes, expression levels of genes were decreased in leiomyomas, whereas no EGR1 bindings to the promoter were detected, suggesting that EGR1 is not responsible for down-regulated expression in those genes. Group 4 genes included all four control genes as in Group C and one gene (*FGF8*) in Group A. No evidence suggested that EGR1 binds to the promoter and up-regulates transcription.

IGF2, a well-known gene in which expression is positively regulated by direct binding of EGR1 in many tissues other than the uterus, was up-regulated in leiomyoma without binding of EGR1. This indicates that over expression of *IGF2* is unrelated to the altered expression of EGR1 in leiomyoma.

Discussion

This study employed a combined method comprising computer-assisted analysis of a genome-wide database and ChIP assay followed by qPCR for comprehensive interpretation of altered gene expression as depicted by array-based experiments. Nearly one-third of down-regulated genes discovered by array experiments possessed potential EGR1 binding sites within 1 kb of promoter sequences. Our results based on ChIP assay using leiomyoma tissues showed that EGR1 bound to those sites in 7 out of 16 genes (44%), including *EGR1* itself, and up-regulated transcription in myometrium. Consequently, we assume that roughly 15% of down-regulated genes (44% of one-third) are attributable to down-regulated expression of EGR1. This is compatible with the notion that low EGR1 expression represents a cause of down-regulated expression for those target genes in leiomyoma.

EGR1 targets identified in this study include genes playing roles in biological responses including cell cycle (*EGR1*, *ATF3*, *FOS*, and *PTP4A1*), apoptosis (*EGR1*, *ATF3*, *SERPINE1*, and *PDGFB*), angiogenesis (*EGR1*, *PDGFB*, *SERPINE1*, *VEGF*, *CYR61*, and *F3*), differentiation of smooth muscle cells (*RORA* and *CSRP2*), degradation and formation of extracellular matrix (*VEGF*, *SERPINE1*, *CYR61*, and *F3*), responses to hypoxia (*PDGFB* and *RORA*), and metabolism of retinoids (*FOS*, *VEGF*,

PDGFB, *SERPINE1*, *HMGA1*, and *F3*; Diaz *et al.* 2000, Wu *et al.* 2004). EGR1 is thus likely to contribute to the leiomyoma phenotype through down-regulation of these target genes. Using a rough estimation that 15% of down-regulated genes are downstream of EGR1, then EGR1 would play an important role in leiomyoma phenotype.

Of the seven genes assigned to Group 1 showing down-regulated gene expression in accordance with decreased EGR1 binding to the promoter, *RORA*, *F3*, and *PDGFB* showed no significant mRNA increases from KWtet-off/EGR1 cells after EGR1 induction. We still consider that these genes may represent targets of EGR1 *in vivo*. Several possible explanations can be offered for this deficient responsiveness in KWtet-off/EGR1 cells. Transcriptional function of EGR1 depends on EGR1 protein modification status, including phosphorylation and dephosphorylation (Cao *et al.* 1992, Huang *et al.* 1998, Srivastava *et al.* 1998). In the cell system employed in this study, EGR1 was gently induced under the minimum stress of tetracycline removal, which elicited no discernible effects on mammalian cells. EGR1 protein induced in this system may therefore lack the protein modifications (dephosphorylation) that are necessary for transcriptional activation and probably proceeds sequentially or simultaneously under physiological stimuli to induce EGR1 in cells *in vivo*. Modification of EGR1 after inducible stimuli is now under investigation in KWtet-off/EGR1 cells.

A second possible explanation lies in the technical limitations of qPCR. According to the instructions provided for the LightCycler system, the discernible minimum difference is generally considered to be a twofold difference in templates at best. Discernible difference also depends on the absolute amount of transcript: the higher the absolute expression level, the smaller the assay variance, and thus the smaller the discernible difference. Actually, *RORA* and *PDGFB* displayed basal expression at two to three orders of magnitude lower than other Group 1 genes.

In Group 2 genes (*HMGA1*, *PNRC1*, *SRC*, and *PTP4A1*), mRNA expression levels did not decrease in leiomyoma, despite significant EGR1 binding to predicted promoter regions. This apparent discrepancy may be explained in various ways. For example, myometrial cells may be lacking other *cis*-regulatory elements collaborating with EGR1 for transcriptional initiation or may contain some repressor factors negating EGR1 binding. Another possibility is again the limitations of real-time PCR as described above, as repression <50% in mRNA level cannot be detected by real-time PCR.

On the other hand, EGR1 binding in leiomyoma tissues was not decreased for Group 3 genes (*CSRP2*, *SERPINE1*, *CYR61*, and *VEGF*), but mRNA levels were still significantly decreased. This is suggestive of EGR1-independent down-regulation of the genes, but does not necessarily exclude the possible contribution of EGR1.

It may regulate transcription through binding to other sites of the same genes (this was not examined in the present study) or regulate indirectly through binding to other gene promoters. Actually, results of the experiment using KWtet-off/EGR1 cells showed positive responses to EGR1 induction in two genes of this group (*CSRP2* and *SERPINE1*), suggesting that EGR1 up-regulates another gene which in turn up-regulates target genes. The limitations of real-time PCR may also explain failures in the detection of positive binding of EGR1 to promoters in tissue specimens.

ChIP assay showed a wide distribution of EGR1 bindings from 3- to more than 30-fold. Small increases as in *RORA* of KWtet-off/EGR1 cells may not necessarily mean reduced binding to the promoter sequence, as association with other transcription factors may mask the epitopes on EGR1, interfering with immunoprecipitation. The topographical relationship of PCR primers to binding sites for EGR1 may also affect amplification efficiency. These factors may also provide other explanations for failure in detection of EGR1 binding.

The present study analyzed only 1 kb upstream of the predicted transcriptional start site (list of nominated down-regulated genes in supplementary table at <http://jme.endocrinology-journals.org/content/vol39/issue5/>). Functional binding sites can exist outside this region, including coding regions. Our study thus identifies just a part of EGR1 target genes. Even with this methodological limitation, we successfully identified 7 EGR1 target genes out of 16 candidates and showed a possible role of EGR1 in synchronized down-regulation in leiomyomas.

In conclusion, we have shown that EGR1 could regulate gene expression in roughly 15% of down-regulated genes and thus contributes to leiomyoma phenotype. Application of ChIP-qPCR assay with the aid of computer-assisted analysis of genome-wide databases may prove useful for comprehensive interpretation and validation of array experiments.

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