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Early growth response-2 expression in uterine leiomyoma cells: regulation and function

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Abstract

Objective—To investigate the regulation of Egr-2 by TGF- β 3 and its functions in cultured human uterine leiomyoma smooth muscle (LSM) cells.

Design—Laboratory research.

Setting—Academic medical center.

Patient(s)—Primary leiomyoma cells from patients with symptomatic leiomyomata.

Intervention(s)—Tissue culture followed by RNA and protein analysis.

Main outcome Measure(s)—Cell proliferation, alteration in ECM component expression.

Result(s)—*In vivo* mRNA levels of Egr-2 were significantly higher in leiomyoma tissues compared with matched myometrial tissues and correlated significantly with TGF- β 3 mRNA levels in leiomyoma tissues. In primary LSM cells, TGF- β 3 significantly induced Egr-2 gene expression in a dose- and time- dependent manner. Small interfering RNA knockdown of Egr-2 markedly increased level of the proliferation marker proliferating cell nuclear antigen and the expression of proto-oncogene c-myc. On the other hand, ablation of Egr-2 stimulated collagen 1A1 and 3A1 transcription and inhibited dermatopontin gene expression. However, the mRNA levels of α -smooth muscle actin and fibronectin were not affected by Egr-2 knockdown.

Conclusion(s)—We demonstrated that TGF- β 3 regulated Egr-2 gene expression and presented evidence that Egr-2 decreases collagen production and stimulates dermatopontin gene expression.

Keywords

TGF- β 3; Egr-2; collagen; dermatopontin; c-myc; PCNA; leiomyomata

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Introduction

Uterine leiomyomata (fibroids) are the most common pelvic tumors in women of reproductive age. Despite their high prevalence in women, up to 70%, and their impact on reproductive and menstrual function in approximately 25% of women with these tumors, the etiology of uterine fibroids remains unknown (1). Accumulating data support the notion that the family of transforming growth factors (TGF- β s), by regulating cell proliferation or extracellular matrix (ECM) production (2–6), plays an important role in tumor growth.

TGF- β 3 is the dominant isoform of the TGF- β family in cells of mesenchymal origin, including smooth muscle cells (7). Previous studies have shown that TGF- β 3 is 3- to 5-fold overexpressed in leiomyoma versus myometrium (2, 3, 8). In uterine leiomyoma smooth muscle (LSM) cells, TGF- β 1 or - β 3 not only stimulates cell proliferation, but also induces collagen or fibronectin production (3–6). In myometrial smooth muscle (MSM) cells, TGF- β 1 or - β 3 demonstrates biphasic regulation of DNA synthesis: they decrease DNA synthesis at higher doses and increase it at lower doses (4). These findings suggest that TGF- β plays several important roles in leiomyoma growth, however, we know little about the underlying mechanisms.

The early growth response (Egr) family of transcription factors includes Egr-1 (Krox-20), Egr-2 (Krox-24), Egr-3, and Egr-4, along with their endogenous inhibitors NGFI-A binding proteins (NAB1 and NAB2) (9, 10). These early immediate gene products are induced by growth factors, cytokines, hypoxia, and mechanical forces associated with injury and cellular stress. Although these transcription factors show a high degree of homology to each other, it is becoming clear that individual members of the Egr gene family serve non-redundant and occasionally opposing biological functions. For example, genetic targeting studies show that while Egr-1 null mice have no spontaneous phenotype, targeting Egr-2 resulted in early lethality due to defective myelination (11, 12). Moreover, Egr-2 appears to play an important role in the negative regulation of T cell function, whereas Egr-1 enhances T cell activity in response to T cell receptor engagement (13, 14). These observations underline the significant functional differences between these two structurally related transcription factors.

Egr-1 is required for TGF- β -induced fibroblast activation, and plays an important role in the regulation of physiologic and pathological fibrogenesis (15). Egr-1 expression was rapidly induced by TGF- β in normal skin and lung fibroblasts and was sufficient to stimulate the transcription of collagen1A2 and other TGF- β inducible genes (15, 16). Mice with targeted deletion of Egr-1 were protected from bleomycin-induced fibrosis (17). However, Egr-1 expression *in vivo* is lower in leiomyoma compared with myometrial tissues (18).

In contrast to Egr-1, very little is known about the expression, regulation and physiological roles of Egr-2 in fibrotic disorders such as uterine leiomyoma. Thus, we sought to investigate the function of Egr-2 in leiomyoma growth and to determine the role of TGF- β 3 in the regulation of Egr-2 expression.

Materials and Methods

Tissue collection and cell culture

Human uterine leiomyoma and matched myometrial tissues were obtained at surgery from 18 premenopausal women (mean age 40 years, range 33–48), following the protocol approved by the Institutional Review Board for Human Research of Northwestern University. The subjects had not received any hormonal treatment during the 6 months prior to surgery. The size of the tumors varied between 3.5 to 10 cm; and their location was

predominantly intramural. The LSM cells were cultured as previously described (19). Cells used in these experiments were passaged one or two times.

RNA preparation and real-time quantitative PCR

Total RNA from LSM cells, leiomyoma tissue, and matched myometrium were extracted using Tri-reagent (Sigma-Aldrich, St. Louis, MO). Complementary DNA was prepared using Superscript™ III first-Strand Synthesis System (Invitrogen, Carlsbad, CA). The gene expression levels of Egr-2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), c-myc, collagen1A1, collagen3A1, dermatopontin, TGF-β3, α-smooth muscle actin (α-SMA), and fibronectin were analyzed by real-time PCR using SYBR Green Reagent (Applied Biosystems, Foster City, CA) on the ABI 7000 or 7900 HT Sequence Detection Systems. All gene expression was normalized to GAPDH. Primers used for gene expression are available upon request.

Small interfering RNA (siRNA)

To knock down the expression of endogenous Egr-2, LSM cells were transfected with Egr-2 siRNA (Dharmacon, Chicago, IL) using Lipofectamine RNAiMAX (Invitrogen). Non-targeting control siRNA (Dharmacon) was transfected as a negative control.

Immunoblotting

Cell lysates were analyzed by immunoblotting as described previously using monoclonal anti-proliferating cell nuclear antigen (PCNA, GenScript Corp., Piscataway, NJ) antibody, or polyclonal anti-Egr-2 antibody (Covance, Princeton, New Jersey) (20). Equal loading was confirmed using anti-β-actin antibody (Sigma-Aldrich). The intensity of bands was quantified using ImageJ software.

Statistical analysis

Differences between groups were analyzed by the student's *t*-test or one-way ANOVA followed by Fisher's protected least significance difference test. Significance was accepted at $P < 0.05$.

Results

In vivo gene expression of Egr-2

To determine Egr-2 expression *in vivo*, we assessed mRNA levels of Egr-2 in human leiomyoma and matched myometrial tissues from 8 subjects using real-time PCR. To allow comparisons of data obtained from samples of different subjects, the mRNA levels of Egr-2 were normalized to GAPDH, and then the mRNA level in leiomyoma tissue was expressed as a multiple of that in the matched myometrium, allowing the detection of relative mRNA difference between the two tissues. Among the specimens from 8 subjects, 7 leiomyoma samples had distinctly higher levels of Egr-2 than those in myometrial tissues. Interestingly, these 7 subjects also expressed significantly higher levels of TGF-β3 in leiomyoma than in myometrial tissues. The mean levels of TGF-β3 and Egr-2 in leiomyoma tissues were 4.85- and 4.7- fold higher than those in myometrial tissues (Fig. 1A). The paired *t*-test performed to analyze Egr-2 and TGF-β3 gene expression in leiomyoma and myometrial tissues from the same subjects demonstrated that the differences are statistically significant ($P < 0.05$). Importantly, subjects with higher levels of TGF-β3 mRNA in leiomyoma also had comparably higher levels of Egr-2 mRNA, and *vice versa*. Regression analysis indicated a highly significant correlation between TGF-β3 mRNA level and Egr-2 mRNA level ($R = 0.805$, $P = 0.016$) in leiomyoma tissue (Fig. 1B). This suggested that expression of Egr-2 is strongly and positively associated with the TGF-β3 status *in vivo* in leiomyoma tissue.

Regulation of Egr-2 mRNA and protein by TGF- β 3 in cultured LSM cells

To analyze the effect of TGF- β 3 on Egr-2 expression in LSM cells, after overnight starvation, various concentrations (0.01, 0.02, 0.05, 0.1, 0.5, 1, 2.5, 5 ng/ml) of TGF- β 3 or vehicle (4 mM HCL containing 1 mg/ml BSA) were added to the medium for a period of 3 h. Real-time PCR was performed to measure Egr-2 mRNA. TGF- β 3 treatment significantly up-regulated Egr-2 mRNA, with the largest inductions observed at 0.5 and 1 ng/ml (Fig. 2A). To explore the temporal response of Egr-2 expression to TGF- β 3 stimulation, LSM cells were treated with 1 ng/ml of TGF- β 3 for 1, 3, 6, and 24 h. TGF- β 3 stimulated Egr-2 mRNA levels as early as 1h. Egr-2 expression reached 3.5- fold at 3 h, after which the magnitude of stimulation decreased gradually and disappeared by 24 h (Fig. 2B). As shown in Figure 2C, TGF- β 3 (1 or 5 ng/ml) also induced Egr-2 protein levels in cultured LSM cells. These representative experiments were reproduced in LSM cells from 3 different subjects. These results indicated that TGF- β 3 up-regulates Egr-2 mRNA and protein levels in a time- and dose-dependent manner in LSM cells.

Knockdown of Egr-2 is associated with increased LSM proliferation

We knocked down endogenous Egr-2 using siRNA to determine its role in LSM cell proliferation. LSM cells from 3 different subjects were cultured in triplicate replicates for statistical analysis. Real-time PCR analysis demonstrated that Egr-2 siRNA consistently and significantly reduced endogenous Egr-2 mRNA levels by approximately 80% in LSM cells (n=3, Fig. 3A). Egr-2 knockdown increased protein levels of PCNA, a cell proliferation marker (Fig. 3B). A representative experiment is shown in top panel; protein levels quantified by densitometry in LSM cells from 3 subjects are shown in the lower panel (Fig. 3B). Since transcriptional repression of c-myc is a key event in TGF- β controlled cell growth, we determined whether the knockdown of Egr-2 affected the expression of the oncogene c-myc (21). As shown in Figure 3C, ablation of Egr-2 significantly increased the mRNA level of c-myc. In total, these results suggest that Egr-2 may inhibit LSM cell proliferation through suppressing the expression of c-myc.

Knockdown of Egr-2 increased collagen gene expression in cultured LSM cells

Leiomyomata are also called fibroids because they are made up of excessive ECM relative to the surrounding uterine smooth muscle. The central structural components in ECM are collagen subtypes. Collagen concentrations in both small and large leiomyomata are elevated relative to normal myometrium (22). Previous studies demonstrated increased mRNA and protein expression of collagen type I with exogenous TGF- β 3 stimulation (5). Therefore, we investigated whether Egr-2 is also involved in collagen synthesis. Knockdown of endogenous Egr-2 significantly increased the mRNA levels of collagen1A1 (Fig. 4A upper panel) and collagen3A1 (Fig. 4A lower panel), suggesting that Egr-2 inhibited collagen synthesis in LSM cells (n=3 subjects).

Knockdown of Egr2 decreased dermatopontin expression in cultured LSM cells

In human uterine leiomyoma, reduction in dermatopontin relative to adjacent myometrium was associated with an increase in TGF- β 3 mRNA levels in leiomyomata, whereas other genes involved in dermatopontin signaling were not differentially expressed (8). Since Egr-2 is a downstream effector of TGF- β 3, we hypothesized that Egr-2 may inhibit dermatopontin expression. We tested this hypothesis by determining the mRNA levels of dermatopontin after Egr-2 knockdown. Opposite to what we expected, knockdown of Egr-2 reduced dermatopontin mRNA levels by approximately 60% (Fig. 4B), suggesting that endogenous Egr-2 positively regulates the gene expression of dermatopontin (n=3 subjects). Moreover, in the presence of Egr-2 siRNA, we found that TGF- β 3 mRNA was down-regulated to

approximately 58% of that in control siRNA-transfected cells (Fig. 2C), suggesting that the interaction between TGF- β 3 and Egr-2 mRNA expression is bidirectional.

To verify that Egr-2 knockdown specifically affected the LSM cell proliferation and gene expression as described above, we determined whether Egr-2 knockdown changed the gene expression of α -SMA and fibronectin, two genes which were previously reported to be regulated by TGF- β signaling pathway (3, 5, 23). As shown in Figure 4C, ablation of Egr-2 didn't alter the mRNA level of either α -SMA (upper panel) or fibronectin (lower panel), suggesting that Egr-2 knockdown specifically regulated collagen1A1, collagen3A1 and dermatopontin gene expression, as well as LSM proliferation.

Discussion

Significantly higher Egr-2 expression *in vivo* in leiomyoma vs myometrium and its induction by TGF- β 3 are the key findings of this study. Previously, TGF- β signaling was linked to leiomyoma growth and ECM formation (3–6). Surprisingly, we found that the ablation of Egr-2 caused LSM cell proliferation and collagen transcription, suggesting an inhibitory function of this gene in leiomyoma growth. Although, only three subject tissues were employed, so the data may not be highly generalizable, our results are consistent with previously published studies, which have found that natural killer cells, which lack Egr-2, demonstrated hyperproliferation (24). Tang et al reported that in MSM cells TGF- β 3 stimulates DNA synthesis at lower doses and inhibits DNA synthesis at higher doses, suggesting that TGF- β 3 may have dual function in regulating leiomyoma cell proliferation (4), which supports the notion that TGF- β signaling system may be a double-edged sword with respect to the regulation of cell proliferation.

The growth-inhibitory effects of TGF- β are primarily based on its ability to inhibit G1-S phase cell cycle transition and defined by two major events, i.e., the repression of the proto-oncogene c-myc, and the subsequent activation of the cyclin-dependent kinase inhibitors p15^{Ink4b} and p21^{Cip1} (25, 26). In the absence of TGF- β , c-myc partners with the zinc finger protein Miz-1 to bind the transcription initiator element of the p15^{Ink4b} promoter, thus, inhibiting the expression of the p15^{Ink4b} cell cycle regulator and promoting cell cycle progression (27). In response to TGF- β , however, c-myc levels decrease rapidly resulting in the relief of p15^{Ink4b} expression and cell cycle arrest (21). Thus, transcriptional repression of c-myc is a key event in TGF- β growth control. Our results indicated that TGF- β inhibits c-myc gene expression, at least in part through up-regulation of Egr-2. The role of Egr-2 in cell survival is complicated and the results from the literature are conflicting, with some studies suggesting that Egr-2 has pro-survival functions (28), while others indicating that it has pro-apoptotic functions (29). Further studies are needed to clearly understand the function of Egr-2 in leiomyoma cell survival.

Uterine leiomyomata are neoplasms composed of cells with expression patterns notable for dysregulation of ECM genes. Specifically, reduction in dermatopontin mRNA was a consistent characteristic of leiomyomata (8). Keloids are overgrown, disordered and inflexible scars resulting from an overproduction of many ECM proteins during healing. When leiomyoma and keloid dermatopontin protein levels are compared with normal tissue, both growths showed a decrease in dermatopontin protein (8). Kuroda and colleagues demonstrated decreased dermatopontin expression in hypertrophic scar tissue and suggested a role for dermatopontin in the pathogenesis of scar formation (30). Dermatopontin binds to decorin (31), which functions as a tissue organizer by orienting and ordering collagen fibrils (32, 33), a phenomenon seen in wound healing. Targeted disruption of dermatopontin in the mouse was associated with abnormal collagen structures (34). Studies have reported disorganization of ECM formation characterized by disordered collagen fibers in uterine

leiomyomata (8). Based on these observations, it is possible that decreased dermatopontin expression contributes to disordered collagen fiber formation and ultimately to the leiomyoma phenotype.

We found that endogenous Egr-2 stimulated dermatopontin gene expression, suggesting that TGF- β 3 may ultimately enhance normal collagen formation in leiomyoma tissue. Our observations are not without precedent. First, wound healing and scarring studies using embryonic transgenic animals demonstrated that deletion (knockout) of TGF- β 3 resulted in delayed wound healing and scar formation compared to wild-type littermates that healed with no scar. Second, TGF- β 3 had scar reduction/prevention effects, which are associated with markedly improved collagen architecture (35).

Uterine leiomyomata are also characterized by overproduction of ECM. TGF- β 3 has been demonstrated to stimulate collagen expression in primary leiomyoma and myometrial cells (5). Egr-2 knockdown increased collagen expression, suggesting that TGF- β 3 has a bimodal effect on ECM production in LSM cells. It is possible that many genes, which affected cell proliferation or ECM production, were regulated by the TGF- β system and that the net effects of all these genes determine the development and growth of leiomyoma.

Collectively, our findings underscore the complexity of TGF- β action in leiomyoma tissues and cells. The general notion is that TGF- β contributes to cell proliferation and increased ECM formation. When we focused on the TGF- β 3/Egr-2/dermatopontin system, however, we found that TGF- β 3 inhibited cell proliferation and enhanced a normal pattern of collagen formation. Our findings highlight the possible dual effects of TGF- β 3 on the growth of uterine fibroids. Therapeutic approaches should aim to inhibit the TGF- β -induced invasive phenotype, but also to retain its growth-inhibitory and apoptosis-inducing effects.

References

1. Walker CL, Stewart EA. Uterine fibroids: the elephant in the room. *Science*. 2005; 308:1589–1592. [PubMed: 15947177]
2. Lee BS, Nowak RA. Human leiomyoma smooth muscle cells show increased expression of transforming growth factor-beta 3 (TGF beta 3) and altered responses to the antiproliferative effects of TGF beta. *J Clin Endocrinol Metab*. 2001; 86:913–920. [PubMed: 11158066]
3. Arici A, Sozen I. Transforming growth factor-beta3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. *Fertil Steril*. 2000; 73:1006–1011. [PubMed: 10785229]
4. Tang XM, Dou Q, Zhao Y, McLean F, Davis J, Chegini N. The expression of transforming growth factor-beta s and TGF-beta receptor mRNA and protein and the effect of TGF-beta s on human myometrial smooth muscle cells in vitro. *Mol Hum Reprod*. 1997; 3:233–240. [PubMed: 9237249]
5. Joseph DS, Malik M, Nurudeen S, Catherino WH. Myometrial cells undergo fibrotic transformation under the influence of transforming growth factor beta-3. *Fertil Steril*. 2009; 93:1500–1508. [PubMed: 19328471]
6. Ding L, Xu J, Luo X, Chegini N. Gonadotropin releasing hormone and transforming growth factor beta activate mitogen-activated protein kinase/extracellularly regulated kinase and differentially regulate fibronectin, type I collagen, and plasminogen activator inhibitor-1 expression in leiomyoma and myometrial smooth muscle cells. *J Clin Endocrinol Metab*. 2004; 89:5549–5557. [PubMed: 15531510]
7. Derynck R, Lindquist PB, Lee A, Wen D, Tamm J, Graycar JL, et al. A new type of transforming growth factor-beta, TGF-beta 3. *EMBO J*. 1988; 7:3737–3743. [PubMed: 3208746]
8. Catherino WH, Leppert PC, Stenmark MH, Payson M, Potlog-Nahari C, Nieman LK, et al. Reduced dermatopontin expression is a molecular link between uterine leiomyomas and keloids. *Genes Chromosomes Cancer*. 2004; 40:204–217. [PubMed: 15139000]

9. O'Donovan KJ, Tourtellotte WG, Millbrandt J, Baraban JM. The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends Neurosci.* 1999; 22:167–173. [PubMed: 10203854]
10. Svaren J, Severson BR, Golda T, Stanton JJ, Swirnoff AH, Milbrandt J. Novel mutants of NAB corepressors enhance activation by Egr transactivators. *EMBO J.* 1998; 17:6010–6019. [PubMed: 9774344]
11. Lee SL, Sadovsky Y, Swirnoff AH, Polish JA, Goda P, Gavrilina G, et al. Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science.* 1996; 273:1219–1221. [PubMed: 8703054]
12. Le N, Nagarajan R, Wang JY, Svaren J, LaPash C, Araki T, et al. Nab proteins are essential for peripheral nervous system myelination. *Nat Neurosci.* 2005; 8:932–940. [PubMed: 16136673]
13. Collins S, Lutz MA, Zarek PE, Anders RA, Kersh GJ, Powell JD. Opposing regulation of T cell function by Egr-1/NAB2 and Egr-2/Egr-3. *Eur J Immunol.* 2008; 38:528–536. [PubMed: 18203138]
14. Basson MA, Wilson TJ, Legname GA, Sarner N, Tomlinson PD, Tybulewicz VL, et al. Early growth response (Egr)-1 gene induction in the thymus in response to TCR ligation during early steps in positive selection is not required for CD8 lineage commitment. *J Immunol.* 2000; 165:2444–2450. [PubMed: 10946269]
15. Bhattacharyya S, Chen SJ, Wu M, Warner-Blankenship M, Ning H, Lakos G, et al. Smad-independent transforming growth factor-beta regulation of early growth response-1 and sustained expression in fibrosis: implications for scleroderma. *Am J Pathol.* 2008; 173:1085–1099. [PubMed: 18772333]
16. Chen SJ, Ning H, Ishida W, Sodin-Semrl S, Takagawa S, Mori Y, et al. The early-immediate gene EGR-1 is induced by transforming growth factor-beta and mediates stimulation of collagen gene expression. *J Biol Chem.* 2006; 281:21183–21197. [PubMed: 16702209]
17. Wu M, Melichian DS, de la Garza M, Gruner K, Bhattacharyya S, Barr L, et al. Essential roles for early growth response transcription factor Egr-1 in tissue fibrosis and wound healing. *Am J Pathol.* 2009; 175:1041–1055. [PubMed: 19679873]
18. Shozu M, Murakami K, Segawa T, Kasai T, Ishikawa H, Shinohara K, et al. Decreased expression of early growth response-1 and its role in uterine leiomyoma growth. *Cancer Res.* 2004; 64:4677–4684. [PubMed: 15231681]
19. Rossi MJ, Chegini N, Masterson BJ. Presence of epidermal growth factor, platelet-derived growth factor, and their receptors in human myometrial tissue and smooth muscle cells: their action in smooth muscle cells in vitro. *Endocrinology.* 1992; 130:1716–1727. [PubMed: 1311246]
20. Deb S, Zhou J, Amin SA, Imir AG, Yilmaz MB, Lin Z, et al. A novel role of sodium butyrate in the regulation of cancer-associated aromatase promoters I.3 and II by disrupting a transcriptional complex in breast adipose fibroblasts. *J Biol Chem.* 2006; 281:2585–2597. [PubMed: 16303757]
21. Warner BJ, Blain SW, Seoane J, Massague J. Myc downregulation by transforming growth factor beta required for activation of the p15(Ink4b) G(1) arrest pathway. *Mol Cell Biol.* 1999; 19:5913–5922. [PubMed: 10454538]
22. Wolanska M, Sobolewski K, Drozdewicz M, Bankowski E. Extracellular matrix components in uterine leiomyoma and their alteration during the tumour growth. *Mol Cell Biochem.* 1998; 189:145–152. [PubMed: 9879665]
23. Bjorkerud S. Effects of transforming growth factor-beta 1 on human arterial smooth muscle cells in vitro. *Arterioscler Thromb.* 1991; 11:892–902. [PubMed: 2065041]
24. Lazarevic V, Zullo AJ, Schweitzer MN, Staton TL, Gallo EM, Crabtree GR, et al. The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nat Immunol.* 2009; 10:306–313. [PubMed: 19169262]
25. Seoane J, Poupponnot C, Staller P, Schader M, Eilers M, Massague J. TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol.* 2001; 3:400–408. [PubMed: 11283614]
26. Massague J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell.* 2000; 103:295–309. [PubMed: 11057902]

27. Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, et al. Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol.* 2001; 3:392–399. [PubMed: 11283613]
28. Bradley EW, Ruan MM, Oursler MJ. Novel pro-survival functions of the Kruppel-like transcription factor Egr2 in promotion of macrophage colony-stimulating factor-mediated osteoclast survival downstream of the MEK/ERK pathway. *J Biol Chem.* 2008; 283:8055–8064. [PubMed: 18198176]
29. Unoki M, Nakamura Y. EGR2 induces apoptosis in various cancer cell lines by direct transactivation of BNIP3L and BAK. *Oncogene.* 2003; 22:2172–2185. [PubMed: 12687019]
30. Kuroda K, Okamoto O, Shinkai H. Dermatotontin expression is decreased in hypertrophic scar and systemic sclerosis skin fibroblasts and is regulated by transforming growth factor-beta1, interleukin-4, and matrix collagen. *J Invest Dermatol.* 1999; 112:706–710. [PubMed: 10233760]
31. MacBeath JR, Shackleton DR, Hulmes DJ. Tyrosine-rich acidic matrix protein (TRAMP) accelerates collagen fibril formation in vitro. *J Biol Chem.* 1993; 268:19826–19832. [PubMed: 8103522]
32. Kokenyesi R, Woessner JF Jr. Effects of hormonal perturbations on the small dermatan sulfate proteoglycan and mechanical properties of the uterine cervix of late pregnant rats. *Connect Tissue Res.* 1991; 26:199–205. [PubMed: 1769238]
33. Iozzo RV. The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth. *Crit Rev Biochem Mol Biol.* 1997; 32:141–174. [PubMed: 9145286]
34. Takeda U, Utani A, Wu J, Adachi E, Koseki H, Taniguchi M, et al. Targeted disruption of dermatopontin causes abnormal collagen fibrillogenesis. *J Invest Dermatol.* 2002; 119:678–683. [PubMed: 12230512]
35. Occleston NL, Lavery HG, O'Kane S, Ferguson MW. Prevention and reduction of scarring in the skin by Transforming Growth Factor beta 3 (TGFbeta3): from laboratory discovery to clinical pharmaceutical. *J Biomater Sci Polym Ed.* 2008; 19:1047–1063. [PubMed: 18644230]

Figure 1.

Correlation between mRNA levels of Egr-2 and TGF-β3 in human leiomyoma and myometrial tissues *in vivo*. (A) Egr-2 and TGF-β3 mRNA levels in human leiomyoma and matched myometrial tissues *in vivo*. To allow comparisons of data obtained from samples from different subjects, mRNA levels in the myometrial tissues were normalized to 1. The data are shown as the mean $\pm$ SEM. (B) Regression analysis indicated Egr-2 mRNA levels correlated significantly and positively with TGF-β3 mRNA levels ($R=0.805$, $P=0.016$).

* $P<0.05$ versus myometrium tissues.

Figure 2.

Effect of TGF-β3 treatment on the expression of Egr-2 in LSM cells. Serum-starved LSM cells were stimulated with (A) variable concentrations of TGF-β3 (ranging from 0.01 to 5 ng/ml) or vehicle (4 mM HCL containing 1 mg/ml BSA) for 3 hours; (B) TGF-β3 (1 ng/ml) or vehicle for 1, 3, 6, 24 hours; or (C) TGF-β3 (0.5, 1, or 5 ng/ml) or vehicle for 72 hours; Messenger RNA levels of Egr-2 at each dose (A) and time point (B) were measured by real-time PCR and normalized by GAPDH. Egr-2 protein levels were measured by immunoblot (C). β-actin was used as a loading control. Results are reported as fold change compared with cells treated with vehicle only and represented as the mean ± SEM of 3 independent experiments. * $P < 0.05$ as compared with vehicle treatment.

Figure 3.

The effects of siRNA knockdown of Egr-2 on LSM cell proliferation. A, Knockdown efficiency and specificity of the Egr-2 gene was examined by real-time PCR. B, Cell proliferation was determined by measuring PCNA protein levels using western blot. Blots were reprobed with a β -actin antibody for loading control. A representative experiment of 3 using LSM cells from 3 different subjects is shown. Western blot densities ($n=3$) were quantified using ImageJ software. C, Effect of Egr-2 knockdown on c-myc mRNA levels. Results are reported as fold change compared with control siRNA transfected cells and represented as the mean $\pm$ SEM of 3 independent experiments. * $P<0.05$ as compared with control siRNA transfected cells.

Figure 4.

The effects of Egr-2 knockdown on the expression of ECM genes in LSM cells. A. Knockdown of Egr-2 increased the mRNA levels of collagen1A1 (upper panel) and collagen3A1 (lower panel) in LSM cells. B. Egr-2 knockdown decreased dermatopontin expression in LSM cells. C. Egr-2 silencing down-regulated TGF-β3 gene expression. D. The mRNA levels of α-SMA (upper panel) and fibronectin (lower panel) were not affected by Egr-2 siRNA in LSM cells. Results are reported as fold change compared with control siRNA transfected cells and represented as the mean ± SEM of 3 independent experiments. * $P < 0.05$ as compared with control siRNA transfected cells.