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Knockdown of c-Fos suppresses the growth of human colon carcinoma cells in athymic mice

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Abstract

Here we have investigated whether inhibition of c-Fos expression in RKO human colon carcinoma cells (HCCCs) would result in reduced TGFβ1 expression and suppression of tumor growth in athymic mice. We stably transfected RKO cells with c-Fos small interfering RNA (siRNA) or with the corresponding control siRNA. Using these stable cell lines, we demonstrated that siRNA-c-Fos significantly suppressed both AP-1 binding, promoter reporter activity at the proximal AP-1 site in the TGFβ1 promoter, and TGFβ1 production. Further, we established colon cancer xenografts with each of RKO-siRNA-EV, RKO-siRNA-Ctrl, and RKO-siRNA-c-Fos cells. By 24 days, the tumor size of RKO-siRNA-c-Fos xenografts was 40% that of either RKO-EV or RKO-siRNA-Ctrl. Immunohistochemistry (IHC) of tumor xenografts demonstrated that siRNA-c-Fos significantly blocked c-Fos expression, and consequently expression of TGFβ1. However, expression of TGFβ2 and TGFβ3 were unaffected. Overall, our results demonstrate that blockade of TGFβ1 production by siRNA-c-Fos effectively suppressed tumor growth *in vivo*.

Keywords

TGFβ; AP-1; c-Fos; colon carcinoma; xenograft

Introduction

Transforming growth factor β (TGFβ), a natural pleiotropic growth factor, has the capacity to regulate diverse biologic processes for a variety of cell types.^{1, 2} In normal cells, TGFβ suppresses proliferation,³ whereas, in malignant cells, TGFβ displays transforming effects as the tumor cells lose responsiveness to the negative growth control signals of TGFβ.^{4–7} This escape of the cancer cells from TGFβ-mediated growth control is often associated with mutations in the type II TGFβ receptor (RII) gene and/or with alterations in TGFβ signaling pathways.^{8–11} In addition, cancer cell-secreted TGFβ can result in a pro-oncogenic microenvironment by promoting angiogenesis,¹² inhibiting immune surveillance,¹³ and up-regulating extracellular matrix components. Therefore, specifically blocking the production of TGFβ¹⁴ would be expected to inhibit the paracrine, tumor-enhancing effects of TGFβ in cancerous cells that have become refractory to TGFβ-mediated growth inhibition.

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Conflict of interest

None.

Normal colon epithelial cells express three isoforms of TGF β (TGF β s1, 2 and 3).¹⁵ Among these three isoforms, TGF β 1 is the most frequently over-expressed in tumor cells.⁵ Although three TGF β s share more than 70% identity, different promoters¹⁶ control their encoding genes and expression. Several transcriptional factor binding motifs have been identified in the human TGF β 1 promoter, including stimulating protein 1, nuclear protein 1, and activating protein 1 (AP-1).¹⁶ Interestingly, among these gene-regulatory motifs, previous studies implicated the AP-1 binding motif in the human TGF β 1 promoter as the critical site for expression regulation of TGF β 1 expression.^{17, 18} Two AP-1 consensus sequences, a distal site (–418 TGACTCT –412) and a proximal site (–371 TGTCTCA –365), were identified in the human TGF β 1 promoter.^{17, 19} Kim et al demonstrated that TGF β 1 had the ability to activate its own expression and reported that a TGF β 1 promoter region between –453 to +11 displayed the highest responsiveness to TGF β 1 treatment.¹⁷ Additionally, of the two AP-1 binding sites in the human TGF β 1 promoter, the proximal one functioned as a high-affinity one, and this site was required for auto-induction of TGF β 1 expression.^{17–19}

The transcription factor AP-1 consists of a variety of dimers composed of members of the Fos, Jun, and activating transcription factor (ATF) families of proteins.²⁰ We have shown that in untransformed epithelial cells (UECs), the major AP-1 components binding to the proximal site of human TGF β 1 promoter were Fra-2 and JunD.^{18, 21} More interestingly, we reported that in FET6 HCCCs, it is c-Fos, and not Fra-2 and JunD, which bind the proximal AP-1 site of the human TGF β 1 promoter.¹⁴ Moreover, we have shown that c-Fos binding to the TGF β 1 promoter is required for TGF β 1 production by the tumor cells.¹⁴ For example, knockdown of c-Fos suppressed total TGF β 1 production both in poor-differentiated RKO HCCCs, as well as in well-differentiated FET6 HCCCs.¹⁴ Furthermore, decreases in TGF β 1 production by the HCCCs blocked fibroblast mitogenesis and migration in a paracrine fashion.¹⁴ Moreover, addition of a neutralizing anti-TGF β 1 antibody to cultures of FET6 siRNA control cells mimics the effects of c-Fos siRNA. Thus, these *in vitro* effects of c-Fos knock down were likely the result of the reduced TGF β 1 secreted by the FET6 siRNA-c-Fos cells.¹⁴

In contrast to the effects of c-Fos knockdown on these cellular responses *in vitro*, the requirement for c-Fos binding to the proximal TGF β 1 AP-1 site has not been examined *in vivo*. Here we show that, in the poorly differentiated RKO HCCCs, in similarity to well-differentiated HCCCs,¹⁴ c-Fos is the major component of the AP-1 complex at the proximal AP-1 promoter of TGF β 1. Further, our results demonstrate that c-Fos is required for TGF β 1 production by the poorly differentiated RKO HCCCs. More importantly, we show that c-Fos knock down blocks TGF β 1 expression by the tumor cells and tumor progression *in vivo*. IHC revealed that c-Fos blockade not only inhibited TGF β 1 expression in the xenograft tumor tissues, but it also down-regulated TGF β 1 expression in the dermal tissues surrounding the tumors in a paracrine fashion. Taken together, c-Fos plays a critical role in TGF β 1 production and cancer cell growth in human colon carcinoma and may represent as a novel therapeutic target for cancer prevention and treatment.

Materials and Methods

Reagents

Penicillin, streptomycin, McCoy's 5A modified growth medium, and fetal bovine serum was obtained from Invitrogen (Grand Island, NY, USA). Antibodies against c-Jun (sc-45x), JunB (sc-46x), JunD (sc-74x), c-Fos (sc-52x), FosB (sc-48x), Fra-1 (sc-183x), Fra-2 (sc-604x), TGF β 1 (sc-146), TGF β 2 (sc-90), TGF β 3 (sc-82), and normal rabbit serum were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Salmon sperm DNA and agarose beads were purchased from Upstate (Lake placid, NY). pDNA Tin-H1.2 siRNA vector was purchased from GenScript (Piscataway, NJ).

Cell culture

RKO HCCCs and siRNA-c-Fos, siRNA-scrambled control (siRNA-Ctrl), and empty vector (EV) stable transfectants thereof were cultured in McCoy's 5A (Life Technology, Inc.) medium, supplemented with amino acids, pyruvate, and antibiotics (streptomycin, penicillin), and 10% fetal bovine serum as described.¹⁴ The cells were routinely screened for *mycoplasma* using Hoechst 33258 staining.

siRNA plasmid preparations and transfections

The pDNATin-H1.2 siRNA vector (Piscataway, NJ) was used for siRNA-c-Fos preparation. The primers for the siRNA target genes were designed using the siRNA Target Finder and Design Tool from the company. Five sequences for siRNA-c-Fos were designed. The sense strands of the hairpin c-Fos siRNA corresponded to nucleotides 381–402 (5'-AAGGTGGAACAGTTATCTCCA-3'), 422–443 (5'-AATCCGAAGGGAAAGGAATAA-3'), 465–486 (5'-AACCGGAGGAGGGAGCTGACT-3'), 552–573 (5'-AACCTGCTGAAGGAGAAGGAA-3'), and 760–781 (5'-AACCTGTCAAGAGCATCAGCA-3') of the human c-Fos encoding region respectively. The oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and annealed. The double-stranded DNA primers encoding the siRNA sequence of the target genes, or a scrambled sequence supplied by the company as a siRNA control, were cloned into the linearized pDNATin-H1.2 siRNA vector through *EcoRI* and *HindIII* according to the instruction manual from the company. Plasmids of pDNATin-H1.2 siRNA-c-Fos pool, siRNA-scrambled control, and empty vector (EV) were transfected into RKO cells using Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA) transfection reagents to obtain optimal gene silencing according to the manufacturer's protocol.

The siRNA-c-Fos and siRNA-Ctrl RKO stable transfectants were established by selection of RKO cells, which had been transfected with the siRNA-c-Fos plasmid, the siRNA-Ctrl, or EV by incubation in hygromycin B. The transfected cells were treated with hygromycin B at a concentration of 200µg/ml for four weeks. Clones of the siRNA-c-Fos transfected cells and a pool of the siRNA-control transfectants were collected and tested by Western immunoblotting using a specific antibody to c-Fos. The transfectants were routinely cultured in supplemental medium containing 10% FBS and 100µg/ml of hygromycin B. The siRNA stable transfectants of RKO cells were defined as: RKO cells transfected with empty vector (RKO-EV); RKO cells transfected with siRNA-scrambled control (RKO-siRNA-Ctrl); and RKO cells transfected with siRNA-c-Fos (RKO-siRNA-c-Fos).

Real-time quantitative PCR

RNA was isolated from RKO cells and their siRNA stable transfectants using TRIzol Reagent by Invitrogen Corporation (Carlsbad, CA). Total cDNA was prepared by RT-PCR using Random Hexamer Primers purchased from Invitrogen Corporation (Carlsbad, CA) as the primers and the isolated RNA as the template. The Real-Time Quantitative PCR was performed using SYBR Green I agent by QIAGEN Inc. (Valencia, CA). The cDNA prepared above was used as a template. 18S rRNA was used as an internal control.

Chromatin Immunoprecipitation (ChIP) Assays were performed using a ChIP Assay Kit (Upstate, Lake Placid, NY) as described previously.¹⁴ Primers for the TGFβ1 promoter region containing the AP-1 site were 5'-CTTCCGTTCTGGGTCCCCCT-3' and 5'-CGGCCAAGCGCCACCAAAGC-3'. Primers for the human telomerase catalytic subunit (hTERT) promoter containing an AP-1 site (5'-GTGGTGTTTTAAGCCAATGATAG-3' and 5'-AAGATCGCTGATACCCAGAAG-3') were used as a positive control. The products were resolved by a 3% agarose gel and visualized with ethidium bromide.

Electrophoretic Mobility Shift Assays (EMSAs) were conducted as previously described.¹⁴

Western immunoblotting was performed as described previously.²²

Luciferase Reporter Assays

RKO transfectants were plated in 12-well plates at a density of $4 \times 10^4/\text{cm}^2$ and were grown to 50–60% confluence. The cells were transfected with 0.2 μg phTG5-Lux luciferase reporter plasmid containing a 450 bp fragment of the human TGF β 1 gene promoter and 0.1 μg renilla luciferase control reporter (pRL-SV40) plasmid using Fugene 6 reagents according to the instruction manual provided by manufacturer. Dual luciferase reporter assay was performed as described elsewhere.¹⁴

TGF β 1 production assays

RKO cells and RKO stable transfectants were plated in 12-well plates and grown for 72h. Conditioned medium (CM) was collected for quantitative determination of TGF β 1 using an ELISA kit (TGF β 1 quantitative sandwich enzyme immunoassay kit, R&D Systems, Minneapolis, MN) as previously described.¹⁴

Tumorigenicity studies

Four-week-old Balb/c athymic mice were purchased from Charles River Laboratories (Wilmington, MA). EV, siRNA-scrambled control, siRNA-c-Fos clone #4, and siRNA-c-Fos clone #5 cells (5×10^6) were inoculated subcutaneously behind the right anterior forelimb of the mice (eight mice per group) as described earlier.²³ Tumorigenicities were determined as described.²³

IHC staining

Tumor tissues were excised from the mice after euthanasia, immediately fixed in 10% neutral buffered formalin, and embedded in paraffin for sectioning. IHC staining was performed as described before,²⁴ except that all antibodies used in the studies were diluted by 1:100. The bound primary antibodies were stained using the Cell & Tissue Staining Kit (Catalog number: CTS005, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

IHC assessment

Tissue staining was scored using the following system: 0, no staining; 1, weak staining of a minority of the cells; 2, weak staining of the majority of the cells; 3, strong staining of a minority of the cells; 4, strong staining of the majority of the cells.²⁵

Results

c-Fos is the primary component in the AP-1 complex at the proximal AP-1 site of the TGF β 1 promoter in RKO cells

The RKO HCCC line is poorly differentiated, highly aggressive, and lacks responsiveness to the inhibitory effects of TGF β on cell proliferation.^{11, 26, 27} We and others have previously demonstrated that the AP-1 binding motif in the TGF β 1 promoter region –362 to –355 is critical for mediating TGF β 1 expression.^{14, 17, 18} To identify the transcription factors that bind to this TGF β 1 promoter AP-1 site in RKO cells, ChIP assays were conducted using antibodies specific for the AP-1 members. Our current results show that c-Fos, rather than the other AP-1 family members, is the critical factor binding to the AP-1 site of the TGF β 1 promoter (Fig.1 upper panel). To evaluate possible changes in the AP-1 binding activity of Fos family members upon knockdown of c-Fos through siRNA, ChIP

assays were performed in RKO-EV and RKO-siRNA-c-Fos #4 cells. As expected, c-Fos binding was observed at the AP-1 site of the TGF β 1 promoter in RKO-EV cells (middle panel), while binding of c-Fos was inhibited at this AP-1 site in RKO-siRNA-c-fos #4 cells (lower panel). In addition, no binding was observed by other Fos family members when c-Fos was knocked down. When we used mutated AP-1 (mAP-1) primers, as shown in the Fig.1, no bands were observed, confirming the specificity of the bands.

Takakura et al reported that the AP-1 site in the human telomerase catalytic subunit (hTERT) promoter region between -2000 and -378 has the ability to strongly recruit c-Jun, JunD, and JunB as its transcriptional binding proteins.²⁸ We used the hTERT promoter as a positive control. As shown in the bottom of upper panel of Fig. 1, c-Jun, JunD, and JunB showed a similar DNA binding pattern to the results of the previous report.²⁸ These results demonstrate that all of the Jun antibodies used in the ChIP experiments were capable of detectable binding in this assay, but none of these components were detected at the TGF β 1 AP-1 site in RKO HCCCs.

c-Fos-siRNA suppress c-Fos expression in RKO cells

In order to verify the effect of c-Fos-siRNA constructs on c-Fos expression at the mRNA and protein levels, the stable transfectants were cultured and harvested for RNA isolation using Trizol reagent, and quantitative RT-PCR was performed using specific primers for c-Fos (Fig.2A). In addition, Western blot analyses were performed using protein lysates from the indicated transfectant (Fig. 2B). As depicted in Figs. 2A and B, compared with RKO-EV and RKO-siRNA-Ctrl samples, c-Fos mRNA and protein levels were decreased in RKO-c-Fos-siRNA transfectants.

Knock-down of c-Fos inhibits AP-1 binding, TGF β 1 promoter transactivation, and TGF β 1 production

Since TGF β 1 is the isoform most frequently over-expressed in cancer cells⁵ and it has been reported previously that the AP-1 binding motif (-362 to -335) is critical for mediating TGF β 1 expression,^{17, 18} it was of interest to verify the effect of blocking c-Fos expression on the AP-1 binding complex of the TGF β 1 promoter. The results of EMSAs using the c-Fos siRNA transfectants demonstrated that knock-down of c-Fos significantly suppressed AP-1 binding to the proximal AP-1 binding site of the human TGF β 1 promoter (Fig. 3A). The results suggest that c-Fos is not only the major component of the AP-1 complex, but also that it plays an important role in AP-1 binding to the proximal AP-1 binding site of the TGF β 1 promoter in RKO cells.

Since the AP-1 complex plays a critical role in mediating TGF β 1 expression,⁵ to investigate whether knocking-down c-Fos has the ability to suppress TGF β 1 promoter reporter activity, the stable cell lines were transfected with phTG5-Lux¹⁸ and phTG5 TGF β 1 promoter luciferase reporter activity was determined. As shown in Fig.3B, RKO-EV and RKO-siRNA-Ctrl cells displayed constitutive activation of the phTG5 TGF β 1 promoter luciferase reporter. However, in RKO-siRNA-c-Fos#4 and RKO-siRNA-c-Fos#5 cells, phTG5 TGF β 1 promoter luciferase reporter activity was significantly decreased. The results demonstrate that c-Fos is required for TGF β 1 promoter transactivation in RKO cells.

Based on the effects observed in Figs. 3A and B, it was of interest to determine whether blockade of c-Fos expression had the ability to inhibit secreted TGF β 1 in RKO cells. Thus, TGF β 1 protein levels in CM from RKO stable c-Fos siRNA and control transfectants were quantitated with an ELISA kit. The results showed that TGF β 1 production was significantly suppressed in RKO-c-Fos-siRNA#4 and RKO-c-Fos-siRNA#5 cells, relative to either control parental RKO, RKO-EV, or RKO-siRNA-Ctrl cells (Fig. 3C).

Suppression of TGFβ1 production by siRNA-c-Fos inhibits tumor formation in vivo

We have shown that blockade of TGFβ1 production by RKO-siRNA-c-Fos cells suppressed fibroblast mitogenesis and migration in a paracrine fashion *in vitro*.¹⁴ To investigate the role of TGFβ1 production on *in vivo* tumor growth, we inoculated athymic nude mice with RKO-siRNA-c-Fos (#4 and #5), RKO-EV, and RKO-siRNA-Ctrl cells. The time required to form tumors of approximately 5–10 mm in diameter was 4 days for all mice in the four groups. As showed in Fig. 4, RKO-siRNA-c-Fos#4 or RKO-siRNA-c-Fos#5 tumors grew at a slower rate than those from RKO-EV or RKO-siRNA-Ctrl cells. By day 24, the mean tumor size of RKO-siRNA-c-Fos#4 and RKO-siRNA-c-Fos#5 xenograft tumors was less than 40% that of either RKO-EV or RKO-siRNA-Ctrl tumors (mean volumes of 6.6 cm³ for RKO-siRNA-c-Fos#4 and 8.0 cm³ for siRNA-c-Fos#5 compared to 13.9 cm³ for RKO-EV and 13.3 cm³ for RKO-siRNA-ctrl at day 24 after inoculation). The differences were significantly different between for either RKO-siRNA-c-Fos#4 or #5 and RKO-siRNA-ctrl ($p < 0.01$, $n = 8$). Thus, our data suggest that the suppression of colon tumorigenesis in the mice was most likely mediated by c-Fos blockade of TGFβ1 secretion in the RKO cells.

siRNA-c-Fos effectively blocks TGFβ1 production by RKO cells in vivo

To investigate whether siRNA-c-Fos was capable of blocking c-Fos expression and consequent TGFβ1 production in tumor xenografts, IHC staining was performed on the tumor tissues. For each tumor tissue sample, we performed Hematoxylin & Eosin (H&E) staining, as well as IHC of TGFβ1, TGFβ2, TGFβ3, and c-Fos. The primary antibodies were replaced by anti-rabbit serum for negative controls. As shown in the H&E results (Fig. 5A), the poorly differentiated colon carcinomas had dark staining of nuclei. Negative controls showed no positive staining in tumor tissues. c-Fos protein expression was significantly inhibited in both RKO-siRNA-c-Fos#4 and RKO-siRNA-c-Fos#5 groups. Interestingly, in RKO-siRNA-Ctrl tumor tissues, TGFβ1 was strongly stained, but in RKO-siRNA-c-Fos#4 and #5 tumor tissues, the staining was significantly suppressed (Fig. 5A & B). To test whether knock down of c-Fos affected the expression of TGFβ2 or 3, tumor tissues were investigated for expression of TGFβ2 or 3. We found that neither TGFβ2 nor TGFβ3 expression was impaired by siRNA-c-Fos, suggesting that neither TGFβ2 nor 3 were involved in siRNA-c-Fos induced growth inhibition of the tumor cells (Fig. 5A & B).

Knocking down c-Fos expression by siRNA-c-Fos inhibits TGFβ1 expression in the dermal tissues surrounding tumor xenografts

Alterations in the tumor microenvironment play an important role in the tumor progression, invasion, and metastasis. To investigate whether suppression of c-Fos by siRNA had any effects on the microenvironment, the dermal tissues surrounding the tumor xenografts were removed and analyzed for c-Fos and for TGFβ1, 2, or 3 expression. The results in Figs. 6A & B show that the siRNA-c-Fos expressing RKO cells had no capacity to block c-Fos expression in the surrounding mouse dermal tissues *in vivo* (left side of dashed line). However, the expression of TGFβ1 in the surrounding mouse dermal tissues was decreased compared with the results in siRNA-Ctrl mice (left side of dashed line). In contrast, TGFβ2 and TGFβ3 expression were not impaired by the tumors arising from the siRNA-c-Fos transfectants (Fig. 6A & B). Our results indicate that siRNA-c-Fos can block TGFβ1 expression, not only in the tumor tissues, but also in the tissues surrounding the tumor xenografts.

Discussion

A series of genetic events results in the development of hyperplasia, adenoma, carcinoma, and finally metastasis during the pathogenesis of colon cancer.²⁹ Throughout the colon carcinogenesis process, TGFβs play a pivotal role.^{30, 31} TGFβ can act as both a tumor

suppressor and a stimulator of tumor progression, invasion, and metastasis.³² In colon carcinoma, as with most solid tumors, TGFβ1 switches from a growth inhibitor to a stimulator of *in vitro* and *in vivo* growth and invasiveness as the colon tumor cells progress to more highly malignant states.^{30, 31} Although all of the three isoforms of TGFβs are expressed by normal colon epithelium,^{15, 33, 34} out of the three isoforms, TGFβ1 is the most frequently over expressed in cancer.⁵ Consequently, the therapeutic targeting of TGFβ1 expression in late stage colon cancer may be more beneficial than approaches to suppress production of either TGFβ2 or TGFβ3.

Our results herein revealed that in poorly differentiated RKO HCCCs, the AP-1 family member c-Fos is critical for constitutively inducing TGFβ1 gene expression and production. Abrogation of c-Fos protein expression through siRNA-c-Fos was capable of suppressing AP-1 binding to the proximal AP-1 binding site in the human TGFβ1 promoter. Additionally, decreased c-Fos protein expression in RKO cells led to decreased luciferase reporter activity of pHTG5, which contains the relevant TGFβ1 promoter AP-1 site, as well as diminished TGFβ1 secretion by the HCCCs. Moreover, knocking down c-Fos protein expression inhibited RKO cell growth *in vivo* in xenograft studies in athymic mice. IHC from these studies showed that c-Fos-siRNA significantly suppressed TGFβ1 expression in xenograft tumors, as well as in the surrounding dermal tissues, whereas expression of TGFβ2 and TGFβ3 was unaffected.

We have previously reported that TGFβ could induce AP-1 binding to the proximal AP-1 binding site of the TGFβ1 promoter to increase the expression of TGFβ1 through Ras/extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK) pathways in UECs.^{4, 18, 21} JunD and Fra-2 were the major AP-1 components bound to the proximal AP-1 site of the TGFβ1 promoter in response to TGFβ treatment.¹⁸ However, a different AP-1 complex at the TGFβ1 promoter was identified in well-differentiated HCCCs.¹⁴ c-Fos, but not JunD or Fra-2, played a critical role in AP-1 binding to the proximal AP-1 site of the TGFβ1 promoter, as well as in the subsequent production of TGFβ1 by the well-differentiated HCCCs.¹⁴ Here, our results reveal that in the poorly differentiated RKO HCCCs, as was true for the well differentiated HCCCs,¹⁴ c-Fos played a pivotal role in AP-1 binding to the AP-1 site of the human TGFβ1 promoter. Thus, when comparing AP-1 dependent TGFβ1 production between UECs and HCCCs, transcription factor switching had occurred.

Members of the AP-1 protein complex, including JunD or Fra-2, have been reported to inhibit cell transformation *in vitro*.³⁵ Compared with JunD and Fra-2, c-Fos is more frequently considered as a pro-oncogenic protein. Many studies have demonstrated that c-Fos is involved in carcinogenesis for a variety of cancer types.³⁶⁻³⁹ For example, when widely overexpressed in mice, c-Fos causes osteosarcoma formation by transformation of chondroblasts and osteoblasts.^{40, 41} In addition, c-Fos knock out mice have an intrinsic defect that hinders skin tumorigenesis,³⁷ which would be consistent with high levels of c-Fos inducing tumor formation.^{40, 41} Further, it has been reported that knock-down of c-Fos by antisense cDNA was capable of suppressing breast cancer cell growth, which implicated c-Fos as a critical regulator of human breast cancer *in vitro*.³⁹ More interestingly, clinical evaluation of gastric and squamous cell lung carcinomas has revealed that c-Fos expression is predictive of poor prognosis, including significant correlations with reduced survival rates.^{36, 38} More recently, microRNA-101, which is under-expressed in human hepatocellular and gastric carcinomas, has been shown to negatively target c-Fos,^{42, 43} which implicates c-Fos as a regulator of these carcinomas. In contrast to these earlier studies, our results demonstrate a role for c-Fos in the regulation of carcinogenesis and tumor progression *in vivo*, especially in human colon cancer. More importantly, our findings

implicate c-Fos as a potential target protein that can be exploited to abrogate TGF β 1 production and tumor progression.

Interestingly, c-Fos is not only required for TGF β 1 production, as we have shown here, but it also regulates expression of various gene products involved in apoptosis (Bcl-2), transformation (cyclin D1, cdc2), migration (Cox-2, VEGF), and invasion (MMPs and vimentin).^{44–46} For example, c-Fos was required for metalloprotease expression in immortalized cells,⁴⁷ Cox-2 mediated invasion and angiogenesis of gastric cancer epithelial cells,⁴⁸ and expression of inflammatory protein calgranulins in skin carcinogenesis.⁴⁹ Therefore, knockdown of c-Fos may not only affect TGF β 1 production as we have shown here, but it may also regulate these other cellular processes, most of which would reduce cancer progression.^{44, 46}

Our data indicate that blockade of c-Fos inhibits TGF β 1 expression and production both *in vitro* and *in vivo*. Moreover, analysis of the microenvironment showed that TGF β 1 expression was significantly blocked compared with the results from control animals. However, this abrogation of c-Fos had no effect on the expression of TGF β 2 and TGF β 3. This outcome may be explained by the fact that the signaling pathways mediating TGF β 2 and TGF β 3 production are different from those for TGF β 1. In this regard, we have previously reported that TGF β 3 production was mediated by the CRE site located between –61 and –35 in the TGF β 3 promoter and that cAMP responsive element binding protein-1 (CREB-1) and Smad3, as well as JNKs and p38, but not protein kinase A (PKA), ERKs, or Smad4, were the critical activators of TGF β 3 expression.⁵⁰ Overall then, our results suggest that selective targeting of the signaling events upstream from activation of the promoters for the different TGF β isoforms can be exploited to either specifically or simultaneously diminish the effects of the individual isoforms in the tumor microenvironment. Further, blocking c-Fos, as we have done here, may be more selective for the HCCCs, relative to the surrounding normal UECs. Altogether, our results herein provide convincing evidence that blockade of c-Fos may be a viable therapeutic strategy for colon cancer.

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Fig. 1. c-Fos is the major transcription factor recruited to the proximal AP-1 site of the human TGFβ1 promoter in RKO cells

ChIP assays were performed as described in "Materials and Methods." The antibodies indicated were incubated with cross-linked DNA isolated from either RKO or RKO-EV or RKO-siRNA-c-Fos #4 cells. The human TGFβ1 promoter region from -410 to -260 (upper panel) and the hTERT promoter region from -1724 to -1574 (lower panel) were amplified using template ChIP DNA obtained with the indicated antibodies. mAP-1, mutated AP-1 primer.

Fig. 2. siRNA-c-Fos blocks c-Fos expression in RKO cells

(A) si-RNA-c-Fos suppresses c-Fos mRNA expression in RKO cells. siRNA-c-Fos stable transfectant clone 4 (siRNA-c-Fos #4) and clone 5 (siRNA-c-Fos#5), siRNA-scrambled (siRNA-Ctrl), and empty vector (EV) stable transfectants were used to isolate RNA samples. Real-time quantitative RT-PCR was performed and analyzed as described in the “Materials and Methods.” The data plotted are the mean $\pm$ SE of three independent experiments. * $p < 0.01$ compared with the siRNA-Ctrl. (B) siRNA-c-Fos inhibits c-Fos protein expression in RKO cells. Total cell lysates were isolated from indicated stable transfectants for Western blotting using the indicated antibodies. The DIC protein was also detected in the same membrane using an anti-DIC antibody as a loading control.

Fig. 3. siRNA-c-Fos blocks AP-1 binding, TGFβ1 promoter activity, and TGFβ1 secretion in RKO cells

(A) c-Fos-siRNA blocks AP-1 binding to the proximal AP-1 binding site of the human TGFβ1 promoter. Nuclear protein extracts were prepared from the indicated stable transfectants of RKO cells. EMSAs were performed as described in “Materials and Methods.” A 100-fold excess of unlabeled AP-1 oligonucleotide (unlabeled probe) was added to compete for AP-1 binding (lane 1). (B) siRNA-c-Fos blocks phTG5-Lux luciferase reporter activity. Stable transfectants of RKO cells were transfected with phTG5-Lux for luciferase assays. The data plotted are the mean $\pm$ SE of triplicate samples from each of three independent experiments. The asterisks (*) indicate $p < 0.01$ compared with either siRNA-Ctrl or EV. (C) siRNA-c-Fos inhibits TGFβ1 secretion in RKO cells. Quantitative determination of TGFβ1 in CM from RKO cells and their stable transfectants was described in the “Materials and Methods.” Results were normalized to cell number. Secreted TGFβ1 was expressed as pg/10⁶ cells. Data represent the mean $\pm$ SE of triplicate wells. * $p < 0.01$ compared with the siRNA-Ctrl samples. Control: CM from parental RKO cells culture; EV: CM from RKO-EV cells culture; siRNA-Ctrl: CM from RKO-siRNA-Ctrl cells culture; siRNA-c-Fos#4: CM from RKO-siRNA-c-Fos#4 cells culture; siRNA-c-Fos#5: CM from RKO-siRNA-c-Fos#5 cells culture was used.

Fig. 4. siRNA-c-Fos blocks tumor growth of RKO cells *in vivo* in a xenograft model

Growth curves for xenografts were determined by externally measuring tumors in two dimensions using calipers. Data plotted are the mean $\pm$ SE of established tumors in 8 mice from each of the four groups. * $p < 0.01$ compared with the siRNA-Ctrl tumor volume.

5A

5B

Fig. 5. Decreased tumor growth of RKO-siRNA-c-Fos tumor xenografts is associated with suppression of TGFβ1 expression

(A) IHC of resected tumor tissues using the indicated antibodies. Primary antibodies were omitted for the negative controls. (B) Quantitation of the IHC for TGFβ1, TGFβ2, TGFβ3 and c-Fos expression in tumor xenografts. Results represent mean ± SE; * $p < 0.01$ compared with siRNA-Ctrl group.

6A

6B

Fig. 6. TGFβ1, TGFβ2, TGFβ3, and c-Fos expression in the microenvironment surrounding the tumor tissues

(A) IHC staining of the dermal tissues surrounding the tumor xenografts. Representative fields are shown. Surrounding (S) and tumor (T) tissues are marked by the dashed lines. (B) Quantitation of IHC in surrounding dermal tissues. Results represent mean $\pm$ SE. * $p < 0.01$ compared with siRNA-Ctrl group.