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ORIGINAL ARTICLE OPEN ACCESS

p53 Deficiency in Colon Cancer Cells Promotes Tumor Progression Through the Modulation of Meflin in Fibroblasts

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Received: 9 October 2024 | **Revised:** 7 February 2025 | **Accepted:** 12 February 2025

Funding: This study was supported by JSPS KAKENHI (grant JP21K15922) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Nippon Kayaku CO., LTD.

Keywords: colon cancer | fibroblasts | Meflin | p53 | tumor microenvironment

ABSTRACT

Cancer-associated fibroblasts (CAFs), a major component of the tumor microenvironment, play an important role in tumor progression. Colon cancer cells deficient in p53 activate fibroblasts and enhance fibroblast-mediated tumor growth. Meflin is a CAF marker capable of inhibiting tumor growth. In this study, we investigated the role of Meflin in fibroblasts using human cell lines (colon cancer HCT116 and fibroblasts CCD-18Co) and clinical specimens. TP53-suppressed HCT116 (HCT116$^{sh\ p53}$) cells cocultured with CCD-18Co cells showed significantly faster proliferation than HCT116$^{sh\ control}$ cells. In xenograft experiments, the volume of tumors induced by coinoculation with HCT116$^{sh\ p53}$ and CCD-18Co cells was significantly larger than that induced by HCT116$^{sh\ control}$ cells co-inoculated with CCD-18Co cells. HCT116$^{sh\ p53}$ cells increased the levels of CAF-like phenotypic markers in CCD-18Co cells. Moreover, Meflin expression was significantly reduced in CCD-18Co cells cocultured with HCT116$^{sh\ p53}$ cells compared to that in CCD-18Co cells cocultured with HCT116$^{sh\ control}$ cells. si-RNA-mediated inhibition of Meflin activated CCD-18Co cells into tumor-promoting CAF-like cells, which significantly promoted xenograft tumor growth. Overexpression of Meflin in CCD-18Co cells using lentivirus suppressed fibroblast-mediated growth of HCT116$^{sh\ p53}$ tumor xenografts. The expression of Meflin in CCD-18Co cells was suppressed by TGF-β and enhanced by vitamin D. These results indicate that colon cancer cells deficient in p53 suppress Meflin expression in fibroblasts, which affects tumor growth by altering the properties of tumor growth-promoting CAFs. Our results suggest that targeting Meflin in fibroblasts may be a novel therapeutic strategy for colorectal cancer.

1 | Introduction

Cancer-associated fibroblasts (CAFs), surrounding cancer cells, are major components of the tumor microenvironment (TME) and play important roles in tumor progression. CAFs secrete a variety of signaling molecules, including growth factors, cytokines, and chemokines, which are involved in tumor progression, immune function, fibrosis, and regulation of angiogenesis

Abbreviations: BMP, bone morphogenetic protein; CAF, cancer-associated fibroblasts; CTGF, connective tissue growth factor; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6; ISLR, immunoglobulin super family containing leucine-rich repeat; miRNA, microRNA; qRT-PCR, quantitative RT-PCR; TGF-β, transforming growth factor-β; TME, tumor environment; VEGF, vascular endothelial growth factor; WST, water-soluble tetrazolium; α-SMA, alpha-smooth muscle actin.

[1, 2]. CAFs are activated into cells with diverse functions by interacting with cancer cells via extensive signaling. Therefore, phenotypic or functional alterations in CAFs have been investigated as a strategy for improving cancer treatment [3–6]. The role of CAFs in the progression of various types of cancers has been extensively evaluated [7–9]. However, nonspecific targeting or deletion of stromal fibroblasts does not inhibit tumor growth [10–12], indicating that CAFs are functionally heterogeneous and possess both tumorigenic and antitumor properties.

p53 is an important tumor suppressor gene that is mutated in almost all human cancers. Its frequency of mutation is approximately 60% in colorectal cancer [13]. As a transcription factor, p53 regulates the expression of genes associated with cell cycle arrest, apoptosis, and senescence [13–15]. The expression of the *tp53* gene in colon cancer cells occurs in a non-cell autonomous manner and affects the cellular microenvironment [16, 17]. p53-deficient colorectal cancer cells secrete numerous proteins, produce reactive oxygen species, and alter the miRNA profile of exosomes, thereby affecting the TME, including CAFs, and promoting tumor growth [18–22]. However, the mechanism underlying the phenotypic changes in normal fibroblasts induced by humoral factors secreted from p53-deficient colon cancer cells remains unclear.

Immunoglobulin superfamily containing leucine rich repeat (ISLR; also known as Meflin) is a CAF marker capable of inhibiting tumor growth. It is a glycosylphosphatidylinositol-anchored membrane protein identified in mesenchymal stem/stromal cells and fibroblasts in various organs [23]. In pancreatic cancer, the invasion of Meflin-positive fibroblasts correlates with favorable patient outcomes [24]. Furthermore, the enhancement of BMP signaling by adeno-associated virus-mediated delivery of Meflin into hepatocytes inhibited hepatic metastasis of colorectal cancer [25]. Therefore, Meflin-positive fibroblasts in the TME may serve as novel therapeutic targets. However, their relationship with the genetic status of cancer cells in tumor progression has not been fully elucidated. To overcome this knowledge gap, in this study, we determined the relevance of p53 in colon cancer cells and Meflin in fibroblasts, especially with regard to cell–cell interactions between p53 wild/suppressed cancer cells and fibroblasts.

2 | Material and Methods

2.1 | Cell Culture

Human colon cancer cell lines HCT116 and RKO showing wild-type p53 expression, HT-29 and SW480 showing mutant-type p53 expression, Caco-2 showing null-type p53 expression, and CCD-18Co human colon fibroblasts were obtained from ATCC (Manassas, VA, USA). All cell lines were authenticated and checked for contamination and were used within 6 months of purchase (National Institute of Biomedical Innovation, Osaka, Japan). HCT116, HT-29, and SW480 cells were cultured in DMEM (D5796; Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. RKO, Caco-2, and CCD-18Co cells were grown in Eagle's Minimum Essential Medium (30–2003; ATCC) supplemented with 10% or 20% FBS. The fibroblast line was used within nine passages.

2.2 | RNA Interference

Lentiviral GFP-IRES-shRNA vectors against TP53 (RHS4430-101161166, 101,162,286, 101,168,779, and 99,365,289) obtained from Thermo Fisher Scientific (Waltham, MA, USA) were used to generate HCT116$^{sh\ control}$ and HCT116$^{sh\ p53}$ cells, as previously described [21]. After colony selection, the cells were cultured with 2 μg/mL puromycin to maintain stable shRNA expression. CCD-18Co and RKO cells were transfected for 48–72 h at 37°C with siRNA against ISLR or TP53 (Invitrogen, Carlsbad, CA, USA) using Lipofectamine RNAiMAX (Invitrogen, 13,778,150), according to the manufacturer's instructions.

2.3 | Expression of Meflin Using the Lentiviral Expression System

To produce lentivirus, psPAX2 (Addgene), vesicular stomatitis virus G protein (VSV-G) vector (Addgene), and pLV-Puro-CMV>hISLR (VectorBuilder) in OptiMEM (Thermo Fisher Scientific) were mixed with Lipofectamine 2000 (Thermo Fisher Scientific). The mixture was added to HEK293 cells. Three days later, the supernatant was collected, filtered, and used to transduce CCD-18Co cells. Positively transduced cells were selected on 2 μg/mL puromycin for 48 h after transduction.

2.4 | Coculture Experiments

Coculture experiments were performed using Transwell inserts (pore size, 0.4 μm; Corning, NY, USA; 353,181 or 353,092). Fibroblasts were seeded in 6-well plates (2×10^5 cells/well) or 12-well plates (5×10^4 cells/well), and cancer cells were seeded in Transwell inserts at the same concentration as the corresponding fibroblasts. After 24 h, the medium in the companion plates and Transwell inserts was replaced with serum-free EMEM, and the Transwell inserts were combined with the companion plates. Further assays were conducted after coculturing for 48–72 h.

2.5 | Cell Growth and Viability

Cell growth and viability were analyzed by seeding cells in 12-well plates (5×10^4 cells/well). The water-soluble tetrazolium (WST) assay was performed using SF cell counting reagent (Nacalai Tesque, Kyoto, Japan). In coculture, only cancer cells were assessed, without including CCD-18Co cells.

2.6 | Quantitative RT-PCR

Total RNA was extracted from cell lines using the RNeasy kit (QIAGEN, Tokyo, Japan). Complementary DNA was synthesized from total RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). qPCR was performed using THUNDERBIRD qPCR Master Mix (Toyobo) on a QuantStudio 6 Flex (Applied Biosystems; Thermo Fisher Scientific Inc.). Thermocycling conditions were as follows: initial denaturation for 20 s at 95°C, followed by 40–60 cycles of 1 s at 95°C for denaturation and 20 s at 60°C for annealing and extension. The mRNA expression was

quantified using the TaqMan gene expression assay (Applied Biosystems, Foster City, CA, USA). Gene expression was normalized to that of B2M (beta-2-microglobulin). The primers list is shown in Table S1.

2.7 | Western Blot Analysis

Cultured cells were lysed in lysis buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1X protein inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and PBS; pH 7.4]. The lysate was incubated on ice for 15 min and centrifuged at 10,000 × *g* for 15 min at 4°C. The medium was collected by centrifuging at 400 × *g* for 5 min. The medium was concentrated 10-fold using Amicon Ultra 3-kDa centrifugal filters (UFC500396, Millipore). Protein content in the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated using SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Antibodies used for western blotting are listed in Table S2.

2.8 | Elisa

TGF-β levels in cell culture supernatants were measured using a human TGF-β and BMP7 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). HCT116$^{sh\ control}$, HCT116$^{sh\ p53}$, and CCD-18Co cells (2×10^5 cells/well) were seeded in six-well plates and cultured for 2 days. The medium was collected, centrifuged at 400 × *g* for 5 min, and the medium was concentrated 10-fold using Amicon Ultra 3-kDa centrifugal filters (UFC500396, Millipore). ELISA was performed using the supernatant, according to the manufacturer's instructions.

2.9 | Vitamin D Analog

Calcitriol (Selleck, Houston, TX, USA), a vitamin D analog, was dissolved in DMSO, and 1 mM stock solutions were stored at −20°C.

2.10 | Xenograft Model and Immunofluorescence Staining

Xenograft experiments were performed using male BALB/c nude mice (Charles River, Yokohama, Japan), aged 5–6 weeks. HCT116 cells (1 × 10 [6]) were suspended in 200 μL PBS and injected subcutaneously into the left and right flanks of mice with or without CCD-18Co (1 × 10 [6]) cells. Tumor size was measured every 3 days to calculate the tumor volume using the following formula: [tumor volume = tumor length × (tumor width) [2]]/2. Xenograft tumor frozen sections were fixed in paraformaldehyde and stained with antibodies listed in Table S2. The secondary antibody reaction was performed using Alexa Fluor 488 (1:500; cat. No. ab150077; abcam) and Alexa Fluor 594 (1:500; cat. No. ab150077; abcam). The stained sections were imaged using a light microscope (VS200; Olympus Corporation, Tokyo, Japan).

2.11 | Immunohistochemistry

Immunohistochemical staining for p53, Meflin, and α-SMA in colorectal cancers with submucosal invasion was performed using endoscopically resected specimens at the Osaka University Hospital between April 2018 and August 2022. Tumor sections were deparaffinized, rehydrated, and labeled with the antibodies listed in Table S2. The stained sections were imaged using a light microscope (VS200; Olympus Corporation). p53 expression in the invasive lesion was classified based on p53 staining in the nucleus as nonsense/frameshift, wild-type, and missense-type pattern [26]. Three randomly selected fields of view (×100) for each lesion were independently evaluated by three observers in a blinded manner. The evaluation agreed upon by two or more observers was adopted. In the same field of view, the area of positive Meflin and α-SMA staining in the interstitial region was used to calculate the percentage of positive area using ImageJ (NIH), and two groups of p53 wild-type and p53 missense-type were compared.

2.12 | Bioinformatics Analysis

RNA sequencing data was obtained from cBioPortal for Cancer Genomics (https://www.cbioportal.org/), and the data was extracted from the Colon Adenocarcinoma cohort of The Cancer Genome Atlas (TCGA) project. Specific gene expression data were downloaded using the RNA Seq RPKM normalized data from cBioPortal [27, 28]. Data on Meflin expression in colon fibroblasts were retrieved from the NCBI Gene Expression Omnibus (GEO) database (accession no. GSE4682).

2.13 | Statistical Analysis

Data from in vitro experiments were expressed as mean ± standard deviation, and data from in vivo experiments were expressed as mean ± standard error. Comparisons between two groups were performed using Student's *t*-test. A one-way or two-way ANOVA with Tukey's post hoc test was performed to analyze the differences among multiple groups. $p < 0.05$ was considered to indicate a statistically significant difference. The statistical analyses were performed using the JMP Pro17 software (SAS Institute Inc.).

3 | Results

3.1 | TP53 Inactivation in Cancer Cells Suppresses Meflin Expression in Fibroblasts and Promotes Fibroblast-Mediated Tumor Growth

To explore the role of p53 in colon cancer cells and of Meflin in fibroblasts in cell–cell interactions, we established the HCT116$^{sh\ p53}$ cell line using shRNA against p53 and confirmed *tp53* suppression using qRT-PCR and western blot analysis (Figure 1A,B). No difference in the viability of HCT116$^{sh\ control}$ and HCT116$^{sh\ p53}$ cells was observed in vitro. However, HCT116$^{sh\ p53}$ cells cocultured with CCD-18Co cells showed significantly greater viability than HCT116$^{sh\ control}$

cells cocultured with CCD-18Co cells (Figure 1C). The volumes of tumors induced by transplanted HCT116$^{sh\ p53}$ and CCD-18Co cells were significantly greater than those induced by HCT116$^{sh\ control}$ and CCD-18Co cells (Figure 1D). In immunofluorescence staining of frozen xenograft tumor sections, the fluorescence intensity of Meflin and α-SMA staining was

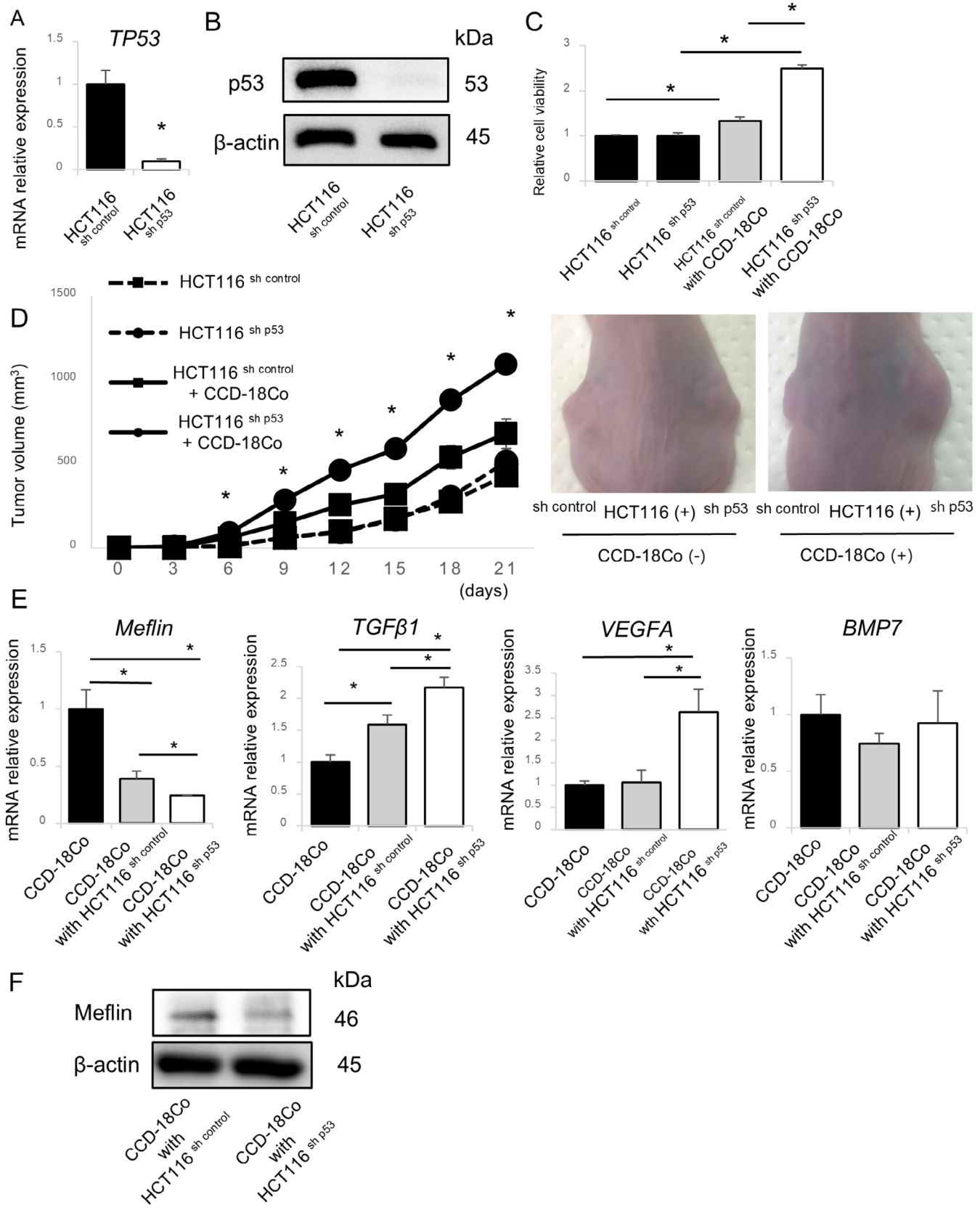

FIGURE 1 | Legend on next page.

FIGURE 1 | TP53 inactivation in cancer cells suppresses Meflin expression in fibroblasts. (A and B) Relative mRNA (A) and protein (B) levels of TP53 in HCT116sh control and HCT116sh p53 cells; $n = 3$; Student's t-test, $^*p < 0.05$. (C) Viability of HCT116sh control or HCT116sh p53 cells cocultured with or without CCD-18Co cells for 72 h, as assessed using the WST assay. Only cancer cells were assessed, without including CCD-18Co cells; $n = 3$; two-way ANOVA, $^*p < 0.05$. (D) Tumor volume in BALB/c nude mice subcutaneously injected with HCT116sh control or HCT116sh p53 cells, with or without CCD-18Co cells; $n = 10$, two-way repeated measures ANOVA; $^*p < 0.05$, compared to the volume in mice injected with HCT116sh control cells together with CCD-18Co cells. (E) Relative mRNA levels of Meflin, TGF-β1, VEGFA, and BMP7 in CCD-18Co cells cocultured with or without HCT116sh control or HCT116sh p53 cells; $n = 3$; one-way ANOVA, $^*p < 0.05$. (F) Western blot analysis of Meflin expression in CCD-18Co cells cocultured with or without HCT116sh control or HCT116sh p53 cells.

higher in tumors induced by HCT116$^{sh\ p53}$ and CCD-18Co cells than in those induced by HCT116$^{sh\ control}$ and CCD-18Co cells (Figure S1). To investigate the effect of different p53 status of cancer cells on the surrounding fibroblasts and the phenotypic changes in fibroblasts, we performed a non-contact coculture of cancer cells and fibroblasts. The relative mRNA expression of *TGF-β1* and *VEGFA* was significantly increased, and the relative mRNA and protein expression of Meflin was significantly decreased in CCD-18Co cells cocultured with HCT116$^{sh\ p53}$ cells compared with that in CCD-18Co cells cocultured with HCT116$^{sh\ control}$ cells. No significant difference in BMP7 expression in fibroblasts was noted, regardless of the p53 status of cancer cells or whether they were cocultured or not (Figure 1E,F). To further explore the effect of p53 status on Meflin expression in fibroblasts, we cocultured other colon cancer cells with CCD-18Co cells. RKO cells were transfected with siRNA to suppress TP53, and inhibition of TP53 was confirmed using qRT-PCR and western blotting (Figure S2). The relative mRNA and protein expression of Meflin was decreased in CCD-18Co cells cocultured with RKO$^{si\ TP53}$ cells compared with that in CCD-18Co cells cocultured with RKO$^{si\ control}$ cells (Figure S3). When HT-29, SW480, and Caco-2 cells were cocultured with fibroblasts, the Meflin mRNA expression in fibroblasts was reduced compared with that in cells cultured alone (Figure S4).

3.2 | Suppression of Meflin Expression in Fibroblasts Promotes Tumor Growth

To elucidate the significance of Meflin in fibroblasts, we knocked down its expression in CCD-18Co cells using siRNA and confirmed the knockdown using qRT-PCR and western blotting (Figure 2A,B). Next, we evaluated the effect of Meflin inhibition on fibroblast tumor growth. Meflin siRNA-transfected CCD-18Co cells showed decreased cell viability compared to the control (Figure 2C). However, HCT116 cells cocultured with Meflin siRNA-transfected CCD-18Co cells showed greater proliferation than those cocultured with control siRNA-transfected CCD-18Co cells (Figure 2D).

Next, we performed xenograft experiments to confirm the effects of Meflin-suppressed fibroblasts on tumor growth using TP53-wild type cancer cells. To eliminate the effects of fibroblast activation by TP53-deficient colon cancer cells and observe the tumor growth effects of Meflin-suppressed fibroblasts, only TP53 wild-type colon cancer cells were used. CCD-18Co cells, with or without Meflin suppression, were subcutaneously injected together with HCT116 cells into nude mice. Tumors derived from Meflin-suppressed CCD-18Co and HCT116 cells grew faster than those derived from CCD-18Co cells without Meflin-suppression and HCT116 cells (Figure 2E). The relative mRNA expression of *TGF-β1*, *VEGFA*, *CTGF*, and *IL-6*, which are phenotypic markers of CAFs that promote tumor growth, was significantly higher in Meflin siRNA-transfected CCD-18Co cells than in control siRNA-transfected CCD-18Co cells (Figure 2F). With regard to the long-term effects of siRNA, we found sustained inhibition of Meflin mRNA expression up to days 6, 9, and 12 of transfection (Figure S5). These results indicate that suppression of Meflin expression in fibroblasts induces a CAF-like phenotype, resulting in tumor growth.

3.3 | Overexpression of Meflin in Fibroblasts Inhibits Fibroblast-Mediated Tumor Growth of p53-Deficient Colon Cancer Cells

To evaluate the effect of exogenous Meflin on tumor progression, we generated Meflin-overexpressing fibroblasts using a lentiviral expression system; qRT-PCR and western blotting confirmed a significant increase in Meflin expression in CCD-18Co cells (Figure 3A). Western blotting also detected Meflin in the culture supernatant of Meflin-overexpressing CCD-18Co cells (Figure 3B). We performed a cell growth and viability assay to examine the effect of exogenous Meflin on fibroblast-mediated cancer cell growth. The viability of Meflin-overexpressing CCD-18Co cells was higher than that of control CCD-18Co cells (Figure 3C). HCT116$^{sh\ control}$ cells cocultured with Meflin-overexpressing CCD-18Co cells showed no significant growth inhibition compared with those cocultured with control CCD-18Co cells. However, HCT116$^{sh\ p53}$ cells cocultured with Meflin-overexpressing CCD-18Co cells showed less proliferation than those cocultured with control CCD-18Co cells (Figure 3D). Furthermore, we inoculated HCT116$^{sh\ control}$ or HCT116$^{sh\ p53}$ cells and control CCD-18Co or Meflin-overexpressing CCD-18Co cells into BALB/c nude mice. Tumor size was reduced when HCT116$^{sh\ p53}$ cells were coinoculated with Meflin-overexpressing CCD-18Co cells compared with that in mice coinoculated with HCT116$^{sh\ p53}$ and control CCD-18Co cells (Figure 3E). These results indicate that Meflin overexpression in fibroblasts inhibited fibroblast-mediated tumor growth in p53-deficient colon cancer cells.

3.4 | TGF-β or Vitamin D Is Related to the Regulation of Meflin Expression in Fibroblasts

To explore factors that regulate Meflin expression in fibroblasts, we performed in vitro experiments using TGF-β1. The addition of recombinant TGF-β1 to CCD-18Co cells decreased

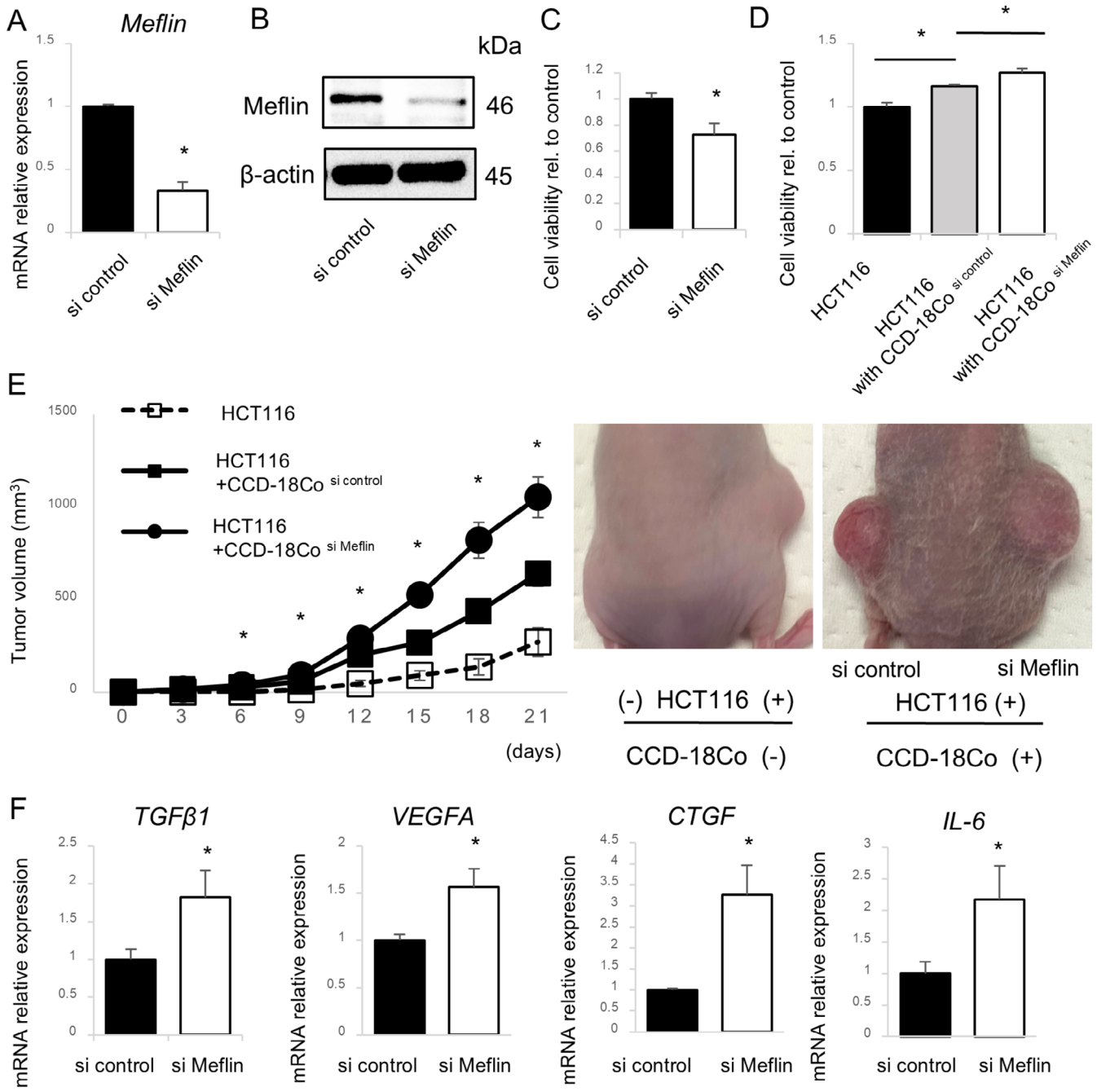

FIGURE 2 | Suppression of Meflin expression in fibroblasts causes phenotypic changes in fibroblasts. (A and B) Relative mRNA (A) and protein (B) levels of Meflin in CCD-18Co$^{si\ control}$ and CCD-18Co$^{si\ Meflin}$ cells; $n = 3$; Student's t-test, $^*p < 0.05$. (C) Viability of CCD-18Co cells transfected with si-control or si-Meflin and cultured for 72 h, as assessed using the WST assay; $n = 3$; Student's t-test, $^*p < 0.05$. (D) Viability of HCT116 cells cocultured for 48 h with or without CCD-18Co$^{si\ control}$ or CCD-18Co$^{si\ Meflin}$ cells. Only cancer cells were assessed, without including CCD-18Co cells; $n = 3$; one-way ANOVA, $^*p < 0.05$. (E) Tumor volume in BALB/c nude mice subcutaneously injected with HCT116 cells together with CCD-18Co$^{si\ control}$ or CCD-18Co$^{si\ Meflin}$ cells; $n = 7$; two-way repeated measures ANOVA $^*p < 0.05$, compared to the volume in mice injected with HCT116 cells together with CCD-18Co$^{si\ control}$ cells. (F) Relative mRNA levels of *TGF-β1*, *VEGFA*, *CTGF*, and *IL-6* in CCD-18Co cells transfected with si-Meflin or control siRNA; $n = 3$; Student's t-test, $^*p < 0.05$.

the mRNA and protein levels of Meflin compared with those in the control and increased the mRNA levels of *TGF-β1* and *VEGFA*, which are markers of CAF that promote tumor growth, compared with those in controls (Figure 4A,B). To identify cell proliferation signals in the interaction between cancer cells and fibroblasts, we analyzed the levels of TGF-β and BMP7 secreted by p53-deficient or p53-wild-type cells using ELISA. No significant differences in TGF-β levels were observed between HCT116$^{sh\ control}$ and HCT116$^{sh\ p53}$ cells. However, the supernatant from the coculture of CCD-18Co and HCT116$^{sh\ p53}$ cells contained more TGF-β than that from the coculture of CCD-18Co and HCT116$^{sh\ control}$. The BMP7 protein concentration in HCT116$^{sh\ p53}$ cell supernatant was lower than that in HCT116$^{sh\ control}$ cell supernatant, but there

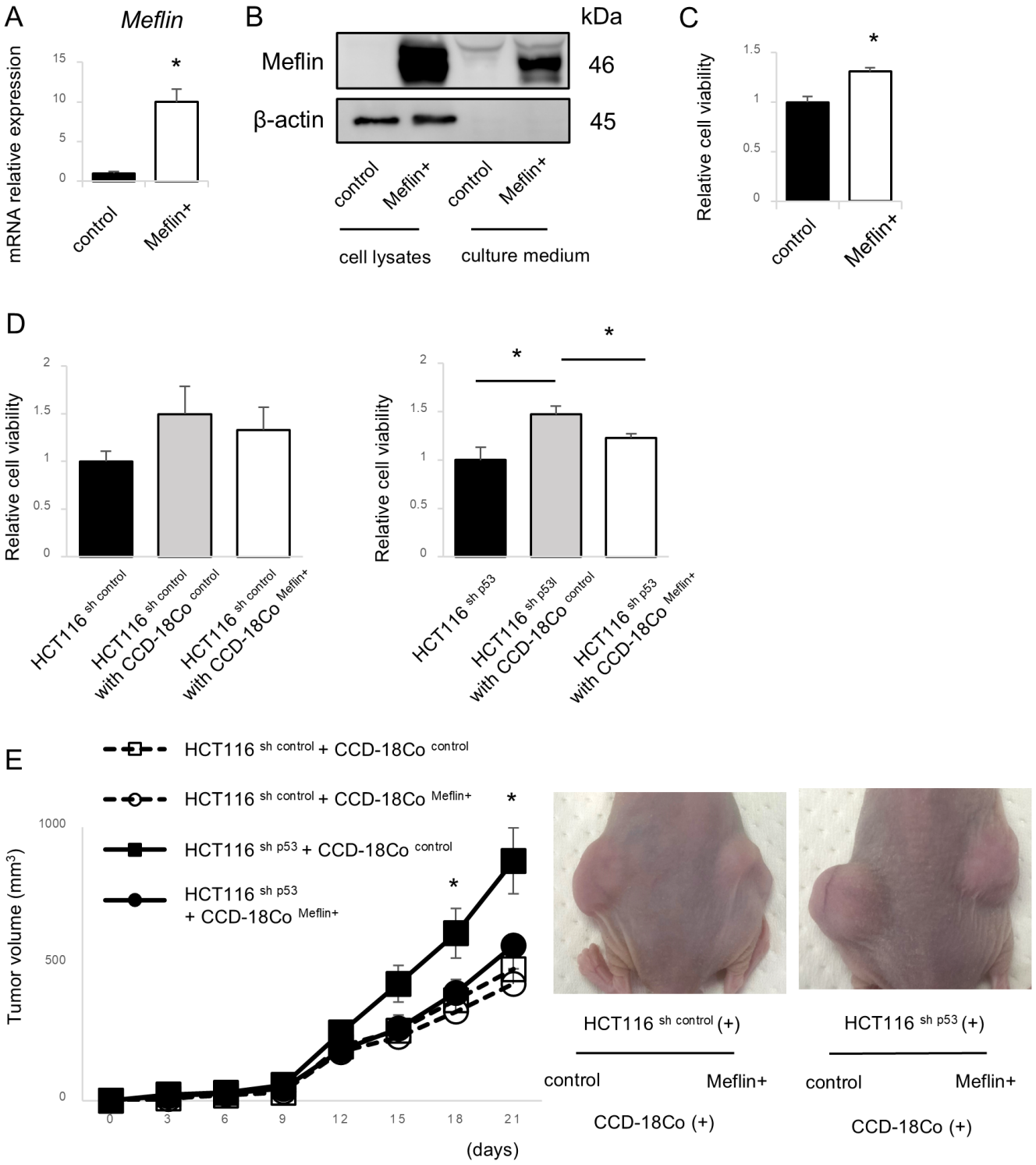

FIGURE 3 | Overexpression of exogenous Meflin in fibroblasts inhibits growth of p53-deficient colon cancer cells. (A and B) Relative mRNA (A) and protein (B) levels of *Meflin* in control CCD-18Co and Meflin-overexpressing CCD-18Co cells; $n = 3$; Student's *t*-test, $^*p < 0.05$. (C) Viability of control CCD-18 and Meflin-overexpressing CCD-18Co cells cultured for 72 h as assessed using the WST assay; $n = 3$; Student's *t*-test, $^*p < 0.05$. (D) Viability of HCT116$^{sh\ control}$ (left) and HCT116$^{sh\ p53}$ (right) cells cocultured for 48 h with or without control CCD-18Co or Meflin-overexpressing CCD-18Co cells, as assessed using the WST assay. Only cancer cells were assessed, without including CCD-18Co cells; $n = 3$; one-way ANOVA, $^*p < 0.05$. (E) Tumor volume in BALB/c nude mice subcutaneously injected with HCT116$^{sh\ control}$ or HCT116$^{sh\ p53}$ cells together with control CCD-18Co or Meflin-overexpressing CCD-18Co cells; $n = 8$–11; two-way repeated measures ANOVA, $^*p < 0.05$, compared to the volume in mice injected with HCT116$^{sh\ p53}$ cells together with control CCD-18Co cells.

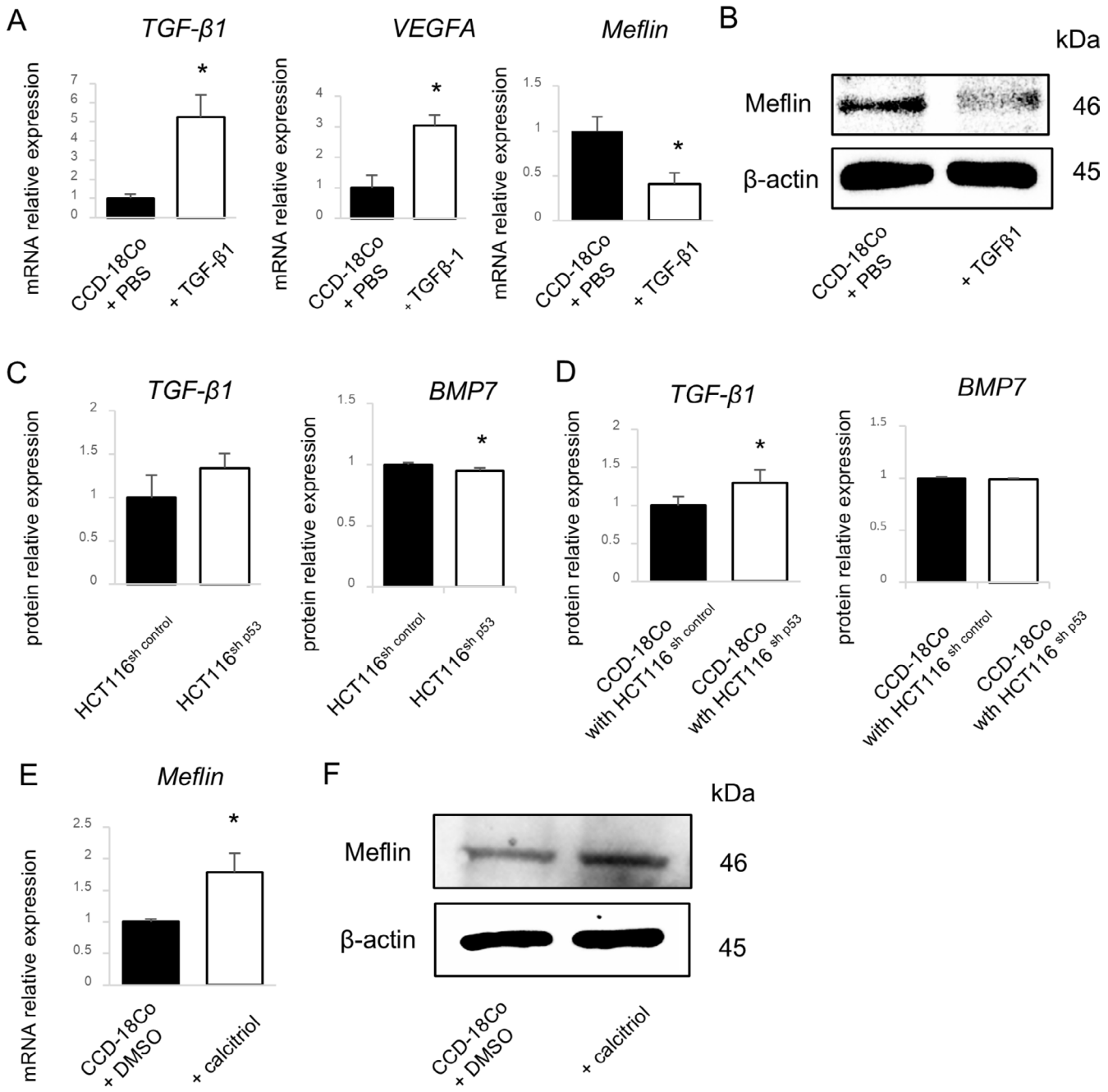

FIGURE 4 | Meflin expression in fibroblasts is regulated by TGF-β and vitamin D. (A) Relative mRNA levels of *TGF-β1*, *VEGFA*, and *Meflin* in CCD-18Co cells treated with or without recombinant TGF-β (10 ng/mL); $n = 3$; Student's *t*-test, $^*p < 0.05$. (B) Western blotting for Meflin in CCD-18Co cells treated with recombinant TGF-β. (C and D) TGF-β and BMP7 levels in the supernatant of HCT116$^{sh\ control}$ and HCT116$^{sh\ p53}$ cells cultured alone (C) or together with CCD-18Co cells (D) were analyzed using ELISA. Equal amounts of HCT116 and CCD-18Co cells were cultured for 48 h in six-well plates; $n = 4$; Student's *t*-test; $^*p < 0.05$. (E and F) Relative mRNA (E) and protein (F) levels of Meflin in CCD-18Co cells treated with or without recombinant calcitriol (100 nM); $n = 3$; Student's *t*-test, $^*p < 0.05$.

was no difference when these cells were cocultured with fibroblasts (Figure 4C,D). These results indicated that fibroblasts cocultured with HCT116$^{sh\ p53}$ secreted higher amounts of TGF-β than those cocultured with the HCT116$^{sh\ control}$.

Next, calcitriol, an active vitamin D preparation that increases Meflin expression in human pancreatic stellate cells [24], was applied to CCD-18Co cells. CCD-18Co cells treated with calcitriol showed increased Meflin mRNA and protein levels compared to the control (Figure 4E,F).

3.5 | Meflin Expression in Fibroblasts Correlates With p53 Staining in Human Colon Cancer

Finally, we assessed the correlation between Meflin and α-SMA expression in fibroblasts and the p53 status of colorectal cancer cells using human clinical specimens. The association between the clinicopathological characteristics of the patients and lesions with p53 status was evaluated (Tables S3 and S4). The Meflin-stained area in cancer stromal fibroblasts was significantly smaller in p53-missense type than in p53-wild type

colorectal cancers. The α-SMA staining was higher in p53-missense type colorectal cancer than in p53 wild-type colorectal cancer, but the difference was not significant (Figure 5). Furthermore, we analyzed the publicly available databases to validate the relationship between p53 status and stromal Meflin expression in human clinical specimens. In the Colon Adenocarcinoma cohort of the TCGA (TCGA-COAD) project obtained via cBioPortal for Cancer Genomics, the mRNA expression of Meflin was significantly lower in samples with missense-type p53 than in those with wild-type p53 (Figure 6). In addition, mRNA expression datasets of fibroblasts extracted from fresh surgical specimens of colorectal carcinoma (CAF group) and normal colonic mucosa (normal colonic fibroblasts, NCF group) were retrieved from the NCBI GEO database (accession no. GSE46824). The datasets revealed that Meflin expression was significantly lower in the CAF group than in the NCF group (Figure S6), and this was the same as in the coculture experiment (Figure S4). These results indicate a relationship between p53-deficient colon cancer cells and Meflin in fibroblasts in invasive colon cancer tissue.

4 | Discussion

Herein, we show that TP53 deficiency in colon cancer cells can suppress Meflin expression in fibroblasts and promote fibroblast-mediated tumor growth. Suppression of Meflin expression activated normal fibroblasts into those exhibiting a CAF-like phenotype that promoted the growth of colon cancer cells. In contrast, Meflin overexpression suppressed fibroblast-mediated effects in p53-deficient colon cancer cells. Meflin expression in fibroblasts was regulated by secreted TGF-β. Vitamin D treatment of normal fibroblasts increased the expression of Meflin. In resected specimens from patients

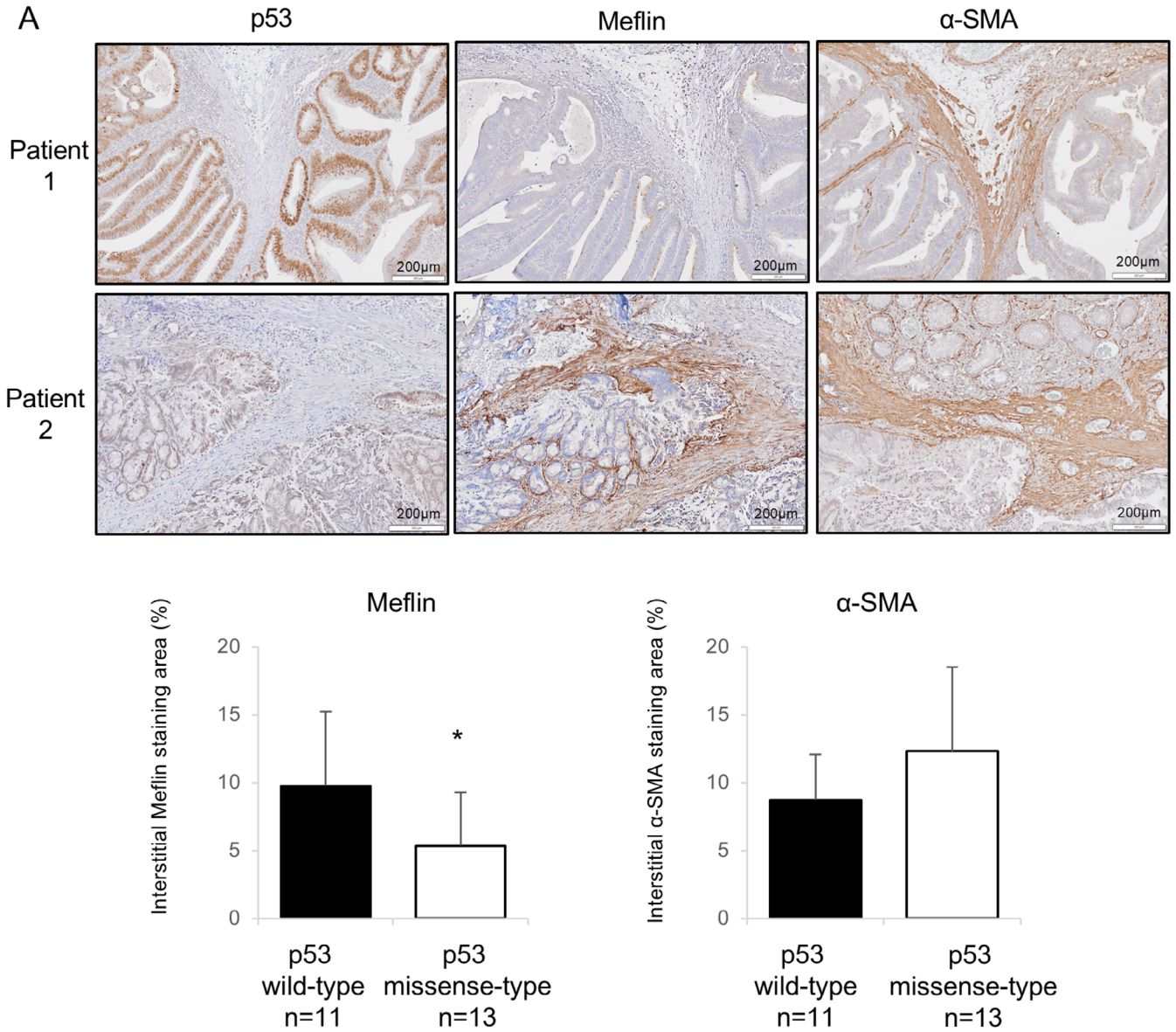

FIGURE 5 | Expression levels of p53 and Meflin in patients with early-stage colorectal cancer were inversely correlated. (A) Representative images of immunostained serial sections from the tumors of two colon cancer patients after labeling for p53, Meflin and α-SMA (scale bar = 200 μm); $n = 24$; Student's t-test, $^*p < 0.05$.

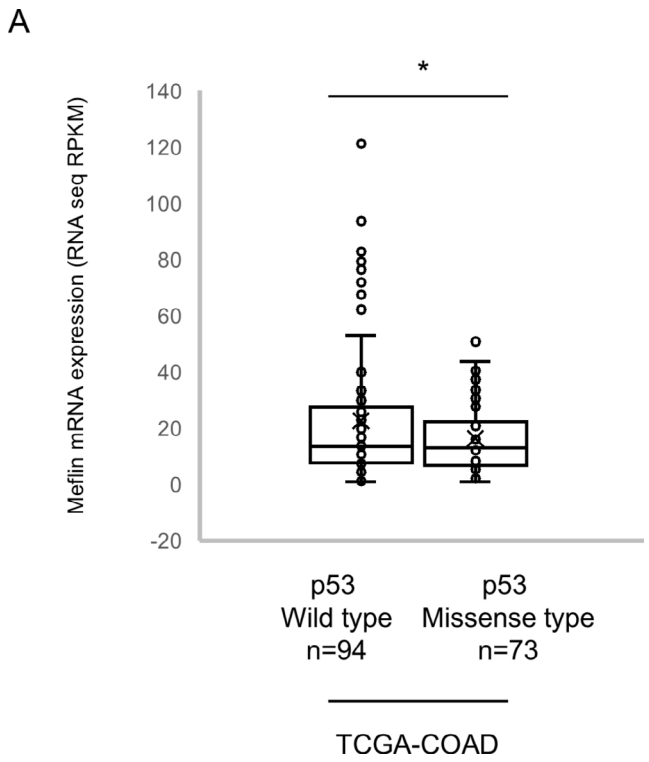

FIGURE 6 | TCGA database analysis of p53 mutations and Meflin expression in human colorectal cancer. (A) Data on mRNA expression of Meflin from p53 wild-type or p53-missense type colon cancer tissues retrieved from the TCGA-COAD database. $n = 167$; Student's t-test, $^*p < 0.05$.

with early-stage colorectal cancer, tumors with a p53 missense mutation in epithelial cells showed lower expression of stromal Meflin.

Meflin inhibits tumor growth in xenograft models generated using pancreatic cancer and stellate cells [23, 24]. Inhibition of tumor growth by Meflin-positive fibroblasts in the TME involves the regulation of BMP signaling or inhibition of collagen cross-linking activity through interaction with lysyl oxidase [25, 29]. In the heart and lungs, Meflin inhibits fibroblast-to-myofibroblast transition by antagonizing TGF-β signaling [30, 31]. Meflin expressed on muscle satellite cells promotes skeletal muscle regeneration by stabilizing Wnt signaling [32]. Meflin secreted by stromal cells promotes intestinal regeneration by suppressing Hippo signaling in epithelial cells [33]. Thus, Meflin-positive fibroblasts affect the surrounding epithelial cells and fibroblasts via multiple mechanisms, creating an environment unfavorable for cancer cell growth. Tumors in the Meflin-knockout pancreatic cancer mouse model were significantly larger and more proliferative than those developed in the wild-type pancreatic cancer mouse model [24]. These findings are consistent with Meflin suppression in fibroblasts promoting cancer cell growth observed in our study.

Normal tissue fibroblasts acquire a CAF-like phenotype in response to the secretion of soluble factors from cancer cells [32]. Phenotypic changes in fibroblasts that support tumor progression are caused by genetic changes in cancer cells [34, 35]. miRNAs in exosomes secreted by cancer cells are key players in fibroblast differentiation [20, 36]. Autocrine TGF-β from CAFs may also be involved in the phenotypic changes involved in their interaction with cancer cells. The conversion of fibroblasts into myofibroblasts with tumor growth characteristics is regulated by cancer cell-derived cytokines such as TGF-β that cause cancer progression via paracrine or autocrine actions [37]. The TGF-β signaling pathway is strongly activated in CAFs but is reduced in epithelial tumor cells [38]. Although the effect of cancer cell-derived humoral factors cannot be completely ruled out, the results of our coculture experiments indicate that fibroblast-derived TGF-β may be involved in CAF activation via Meflin suppression. According to the classification by consensus molecular subtypes, colorectal cancers with a strong stroma-responsive form and TGF-β activity have a poor prognosis. The prognostic potential is derived from genes expressed by stromal cells rather than by epithelial tumor cells [39, 40]. These findings are in agreement with previous reports that Meflin expression in stromal cells in colorectal cancer is regulated by TGF-β and correlates with a favorable prognosis [25].

We also found that overexpression of Meflin in fibroblasts inhibited tumor growth in p53-deficient cancer cells. Thus, drugs targeting Meflin in fibroblasts may benefit patients with p53-mutated colorectal tumors. In other cancers, Meflin-positive fibroblasts in the TME are potential therapeutic targets. Meflin-positive fibroblasts were induced by Am80, a synthetic retinoid, which improved the chemosensitivity of pancreatic cancer by increasing the tumor vascular area and intratumoral drug delivery [29]. Treatment of human pancreatic stellate cells with calcipotriol increased the expression of the Meflin gene [24]. Vitamin D analogs potentiate Meflin expression in human colon fibroblasts. As vitamin D deficiency is associated with a high incidence of colorectal cancer and mortality, vitamin D might protect against this disease. Expression of the vitamin D receptor in stromal fibroblasts predicts a favorable clinical outcome in colorectal cancer [41, 42]. Therefore, Meflin expression in fibroblasts in the colon cancer microenvironment may be modulated by vitamin D and may correlate with a favorable prognosis.

This study had some limitations. Despite these results showing the antitumor effects of Meflin in fibroblasts, it is not clear how Meflin directly acts on cancer cell growth signals. Although the tumor suppressive function of mesenchymal BMP signaling has been reported [43, 44], it was not reduced in fibroblasts activated by p53-deficient colon cancer cells. Despite a slight reduction in BMP7 expression in the supernatant of p53-deficient colon cancer cells, the protein concentration was much lower than that of TGF-β, and its effect appears to be limited. In previous studies, we found that p53-deficient colon cancer cells increase TGF-β expression in fibroblasts [20, 21]. This might involve reactive oxygen species and alterations in the miRNA profile of exosomes, but the mechanism has not been fully elucidated [18–22]. The finding that Meflin-overexpressing fibroblasts only suppress the growth of p53-deficient cancer cells remains mechanistically unclear. Meflin overexpression might antagonize TGF-β secreted by p53-deficient cancer cells or activated fibroblasts, thereby suppressing their differentiation into a CAF-like phenotype [30, 31]. Further research is needed to better understand the

role of fibroblasts in tumor progression and to improve the prognosis and efficacy of chemotherapy in clinical practice by altering the fibroblast phenotype.

This study highlights a novel phenotype of fibroblasts associated with crosstalk between cancer cells in the TME. We propose that Meflin expression is important in fibroblast-mediated tumor suppression, and its targeting may be a novel therapeutic strategy.

Author Contributions

Eiji Kimura: conceptualization, data curation, formal analysis, investigation, visualization, writing – original draft. **Yoshito Hayashi:** conceptualization, formal analysis, supervision, writing – review and editing. **Kentaro Nakagawa:** investigation, writing – review and editing. **Hirotsugu Saiki:** investigation, writing – review and editing. **Minoru Kato:** investigation, writing – review and editing. **Ryotaro Uema:** investigation, writing – review and editing. **Takanori Inoue:** investigation, writing – review and editing. **Takeo Yoshihara:** investigation, writing – review and editing. **Akihiko Sakatani:** investigation, writing – review and editing. **Hiromu Fukuda:** investigation, writing – review and editing. **Ayaka Tajiri:** investigation, writing – review and editing. **Yujiro Adachi:** investigation, writing – review and editing. **Kazuhiro Murai:** investigation, writing – review and editing. **Shunsuke Yoshii:** investigation, writing – review and editing. **Yoshiki Tsujii:** investigation, writing – review and editing. **Shinichiro Shinzaki:** investigation, writing – review and editing. **Hideki Iijima:** investigation, writing – review and editing. **Tetsuo Takehara:** supervision, writing – review and editing.

Acknowledgments

The authors have nothing to report.

Ethics Statement

Approval of the research protocol by an Institutional Reviewer Board. The study followed the principles of the Declaration of Helsinki and was approved by the Institutional Reviewer Board of Osaka University (approval number: 20061, 24,158). Animal studies: All animal protocols were approved by the Animal Care and Use Committee of Osaka University Graduate School of Medicine (No. 25-032-005).

Consent

Written informed consent was obtained from all patients who provided the specimens.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

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