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The BRAF^{V600E} inhibitor, PLX4032, increases type I collagen synthesis in melanoma cells

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Abstract

Vertical growth phase (VGP) melanoma is frequently metastatic, a process mediated by changes in gene expression, which are directed by signal transduction pathways in the tumor cells. A prominent signaling pathway is the Ras-Raf-Mek-Erk MAPK pathway, which increases expression of genes that promote melanoma progression. Many melanomas harbor a mutation in this pathway, BRAF^{V600E}, which constitutively activates MAPK signaling and expression of downstream target genes that facilitate tumor progression. In BRAF^{V600E} melanoma, the small molecule inhibitor, vemurafenib (PLX4032), has revolutionized therapy for melanoma by inducing rapid tumor regression. This compound down-regulates the expression of many genes. However, in this study, we document that blocking the Ras-Raf-Mek-Erk MAPK pathway, either with an ERK (PLX4032) or a MEK (U1026) signaling inhibitor, in BRAF^{V600E} human and murine melanoma cell lines increases collagen synthesis *in vitro* and collagen deposition *in vivo*. Since TGFβ signaling is a major mediator of collagen synthesis, we examined whether blocking TGFβ signaling with a small molecule inhibitor would block this increase in collagen. However, there was minimal reduction in collagen synthesis in response to blocking TGFβ signaling, suggesting additional mechanism(s), which may include activation of the p38 MAPK pathway. Presently, it is unclear whether this increased collagen synthesis and deposition in melanomas represent a therapeutic benefit or an unwanted “off target” effect of inhibiting the Ras-Raf-Erk-Mek pathway.

Keywords

Vemurafenib; TGFβ; pERK; IL-8; MMP-1; Human melanoma; Murine melanoma

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Introduction

Melanoma is the most aggressive and invasive form of skin cancer, with rates steadily increasing to a current lifetime incidence of 1 in 50 individuals [1,2]. Early stage melanoma, classified as radial growth phase (RGP), is confined to the epidermis and is readily curable by surgical excision. In contrast, later stage vertical growth phase (VGP) melanoma is frequently metastatic, with median patient survival rates of only 6 to 9 months. The transition from non-invasive, localized RGP melanoma to invasive and metastatic VGP melanoma is mediated by changes in gene expression, which are directed by signal transduction pathways in the tumor cells. Among the most prominent signaling pathways is the MAPK pathway, Ras-Raf-Mek-Erk, which increases expression of genes that promote melanoma progression, *i.e.* secreted growth factors and chemokines [3,4]. Importantly, many melanomas harbor a mutation in this pathway, $BRAF^{V600E}$, which causes constitutive activation of MAPK signaling and increased expression of downstream target genes [3–11], thereby exacerbating tumor progression. However, in $BRAF^{V600E}$ melanoma, the small molecule inhibitor, vemurafenib (PLX4032), induces rapid tumor regression [5,12–14] and has revolutionized therapy for melanoma. PLX4032 binds to the ATP-binding site of mutated BRAF, inhibiting ERK/MAPK signaling and downstream gene expression in $BRAF^{V600E}$ cells.

The tumor microenvironment (TME) plays a crucial role in the invasive behavior of VGP melanoma. Notably, there is an increase in proteolytic enzymes, particularly the interstitial collagenase, matrix metalloproteinase-1 (MMP-1) [15–18] in VGP melanoma compared to RGP melanoma. Indeed, elevated MMP-1 is linked to the onset of VGP melanoma and to a poor prognosis [16,19]. MMP-1 is also a downstream target of MAPK signaling [11], one mechanism that may explain the high levels of MMP-1 expression seen in $BRAF^{V600E}$ melanomas [8,11]. Likewise, the collagenase MMP-13 is also downstream of MAPK signaling in melanoma cells [20], and has been shown to regulate human and murine melanoma growth [20,21]. The collagenase secreted by human melanoma cells degrades collagens, types I and III, leading to destruction of the extracellular matrix (ECM), which is an essential step in melanoma invasion and metastasis [22].

The triple helical interstitial collagens, types I, II and III, are the most abundant proteins in the body, and type I collagen is the most abundant collagen. It is a heterotrimeric protein, composed of two alpha 1 chains (Col1A1) and one alpha 2 chain (Col1A2) [23]. Since collagens are the major component of the ECM and the main scaffolding protein of the TME, remodeling of the ECM by degradation or synthesis of collagen influences tumor infiltration, angiogenesis, invasion, and migration of tumor cells [24]. Preventing collagen degradation has been shown to prevent melanoma metastasis, suggesting that degradation of collagen is necessary for metastasis [8]. Conversely, blocking collagen synthesis in melanomas by pharmacologic intervention blocks angiogenesis and metastasis [25–27], suggesting that collagen deposition fosters tumor progression. The contradictory nature of these studies suggests that collagen may be a ‘double-edged sword’ [24], in that it may both inhibit and promote the progression of melanomas.

In a recent study, we examined heterogeneity among clones of melanoma cells derived from a single tumor. Our studies revealed that C4 tumors excised from nude mice contained substantial amounts of collagen, as seen with Masson Trichrome stain [28]. Here, we report the unexpected finding that blocking the Ras-Raf-Mek-Erk MAPK pathway, either with an ERK (PLX4032) or a MEK (U1026) inhibitor, in $BRAF^{V600E}$ human and murine melanoma cell lines increases collagen synthesis *in vitro* and collagen deposition *in vivo*. We find that these inhibitors decrease expression of two genes known to be inhibited when this pathway is blocked: the pro-angiogenic cytokine, Interleukin-8 (IL-8) and MMP-1 [11,29]. Since TGFβ signaling through its receptor, TGFβ RI/II, is a major mediator of collagen synthesis, we examined whether blocking TGFβ signaling with a small molecule inhibitor would block this increase in collagen. We found only a minimal reduction in collagen synthesis in response to blocking TGFβ signaling, suggesting that additional mechanisms are involved. Although it is not clear how blocking the Ras-Raf-Mek-Erk pathway increases collagen expression, our data suggest that activation of another MAPK pathway, p38, may be involved. We found that p-p38 increased when melanoma cells were co-treated with PLX4032 and exogenous TGFβ, suggesting cooperativity between MAPK and TGFβ signaling in these cells [30].

Results

Inhibition of the MAPK pathway increases collagen synthesis in human and murine $BRAF^{V600E}$ cell lines

In a recent paper comparing two clones (C4 and C9 cells) derived from a human $BRAF^{V600E}$ cell line, we found higher deposits of collagen in the C4 tumor tissue as well as increased levels of TGFβ mRNA [28], an inducer of matrix synthesis in melanoma [31]. Given that blocking MAPK signaling halts tumor progression in $BRAF^{V600E}$ melanoma, we investigated the effect of MAPK inhibitors on the expression of type I collagen by melanoma cells. We treated several melanoma cell lines with both the $BRAF^{V600E}$ inhibitor, PLX4032, and the MEK inhibitor, U0126, and measured Col1A2 (collagen, type I, alpha 2) expression as a marker of collagen synthesis [32]. Fig. 1A shows the effect of these inhibitors on C4 melanoma cells. Interestingly, a significant increase in collagen mRNA (8–10 folds, $P < 0.0001$) was seen in cells treated with either PLX4032 or U0126 compared to cells treated with the DMSO control. We confirmed that these compounds blocked pERK in cell lysates from parallel experiments (Fig. 1B). Furthermore, immunoblots of C4 cell lysates revealed an increase in pre-pro-collagen in cells treated with PLX4032 or U0126 for 48 h, but only the pro-collagen in control cells treated with DMSO (Fig. 1C (100–150 kD) lower band and upper band, respectively). The presence of pre-pro-collagen may reflect an increase in the synthesis of collagen protein in the process of being prepared for secretion. We conclude that the increase in collagen mRNA is paralleled by an increase in collagen protein.

It has previously been shown that blocking MAPK signaling with either PLX4032 [29] or U0126 [11], decreases expression ofMMP-1 in human $BRAF^{V600E}$ melanoma cell lines. Likewise, we found that MMP-1 mRNA is reduced in C4 cells treated with either inhibitor for 48 h (Fig. 1D). Importantly, we extended our studies to include two additional human

BRAF^{V600E} melanoma cells lines, VMM12 (Fig. 1E–G) and C9 (Supplementary Fig. 1A and B). We found that PLX4032 and U0126 treatment increased collagen and decreased MMP-1 mRNA and protein in both cell lines. In addition, two murine cell lines, D4M.7A and D4M.3A cells [33], derived from a mouse model of BRaf^{V600E}, also showed increases in collagen mRNA with PLX4032 treatment (Supplemental Fig. 1C and data not shown). To test the inhibition of collagenase expression in murine BRaf^{V600E} cell lines, we quantified MMP-13 mRNA in D4M cells, the principal secreted collagenase in the murine system, which is analogous to human MMP-1 [21,22]. Similar to the human cell lines, MMP-13 expression significantly decreased in D4M cells with PLX4032 treatment for 48 h (Supplementary Fig. 1D). Taken together, these data demonstrate that the increase in collagen in response to MAPK inhibitors is not restricted to one particular melanoma cell line or species.

IL-8 expression decreases with MAPK inhibition

It has been reported that IL-8 may suppress collagen expression and regulate its turnover in human fibroblasts [32]. Therefore, to investigate a potential mechanism for the increase in collagen synthesis seen with the treatment of MAPK inhibitors, we measured expression of IL-8 in the presence of these inhibitors. We reasoned that if PLX4032 or U0126 decreased IL-8 expression in cells with increased collagen, then IL-8 might be regulating collagen expression. In fact, a previous study has reported that PLX4032 treatment decreases IL-8 expression in human BRAF^{V600E} mutant cell lines [29], suggesting that IL-8 may constitutively suppress collagen expression and that this suppression is relieved when its expression is decreased. We found that treating three BRAF^{V600E} cell lines with either PLX4032 or U0126 significantly decreased IL-8 expression (Fig. 2A, and data not shown). Therefore, we added exogenous IL-8 to cells treated with PLX4032 to ask if exogenous IL-8 could decrease collagen expression in these cells. However, we found no significant difference in Col1A2 mRNA levels in cells treated with exogenous IL-8 compared to DMSO control cells ($P > 0.05$, Fig. 2B) nor was there a difference between cells treated with PLX4032 alone or with PLX4032 and IL-8 ($P > 0.05$, Fig. 2B). These results suggest that IL-8 is not regulating collagen mRNA.

TGFβ signaling partially affects collagen gene expression

Since the effects of BRAF and MEK inhibitors were nearly identical in BRAF^{V600E} cell lines, in keeping with previous studies [7], we focused on the effects of PLX4032 on collagen synthesis in the C4 melanoma cells. TGFβ signaling is a major mechanism for increasing collagen synthesis [30,34–36], and therefore we examined TGFβ expression in cells treated with PLX4032. First we showed that treating C4 cells with PLX4032 increased expression of TGFβ (Fig. 3A).

Next, we tested the possibility that signaling through TGFβ receptors (TGFβ R1 and RII) might increase collagen synthesis when MAPK signaling was blocked by PLX4032. To this end, we added exogenous TGFβ to the C4 cells and measured collagen mRNA. We found that exogenous TGFβ (2 ng/ml) significantly increased collagen expression by about 2.5-fold (Fig. 3B). PLX4032 treatment increased collagen expression even more than TGFβ alone (4-fold compared to control/DMSO treatment) and the combination of TGFβ and

PLX4032 had an additive effect on Col1A2 mRNA, increasing expression 10-fold higher than control/DMSO treatment (Fig. 3B).

We then blocked TGFβ receptor signaling in the C4 cells with the small molecule inhibitor, SB431542 (10 μM) [37] and measured collagen mRNA (Fig. 3B). Treatment with SB431542 alone, or in combination with exogenous TGFβ, reduced collagen mRNA by 25% compared to DMSO control (Fig. 3B), suggesting that TGFβ signaling plays a role in mediating expression of collagen. In addition, compared to PLX4032 treatment alone, SB431542 significantly reduced PLX4032-induced collagen expression (Fig. 3B, $P < 0.0001$) but addition of exogenous TGFβ failed to rescue collagen expression (Fig. 3B, $P > 0.05$). We also measured collagen protein in cell lysates from parallel experiments, and found increased collagen protein in all cells treated with PLX4032, but virtually no increase in the cells treated with TGFβ alone (Fig. 3C). Specifically, no pre-pro-collagen band is present in the lysates from cells treated with TGFβ, unlike the cells treated with PLX4032. We attribute the difference in collagen mRNA and protein in response to TGFβ treatment to differences in the sensitivity of qRT-PCR and immunoblotting. Taken together, our findings suggest cooperativity between TGFβ signaling and PLX4032 in affecting collagen gene expression, but indicate that additional mechanisms are involved.

To confirm the role of TGFβ signaling in mediating an increase in collagen synthesis, we examined TGFβ protein levels in conditioned medium taken from these cells after 48 h (Table 1). Table 1 shows that after 48 h of culture in DMEM with 10% FBS, medium from DMSO treated cultures contained about 18 pg/ml TGFβ protein. Treating cells with PLX4032 increased TGFβ protein to about 48 pg/ml, suggesting that PLX4032 may mediate expression of TGFβ mRNA (Fig. 3A) as well as protein (Table 1). Treatment with SB431452 reduced TGFβ levels to undetectable, while medium from cells co-treated with the inhibitor and PLX4032 contained about 9 pg/ml (Table 1). Thus, the C4 cells secrete some TGFβ, and PLX4032 augments this by about 2.5-fold.

Evaluation of potential cross-talk between MAPK and TGFβ signaling

Cross-talk between MAPK (Ras-Raf-Mek-Erk) and TGFβ/pSmad signaling has been reported [30,36,38], and this cross-talk could implicate MAPKs in regulating collagen synthesis. Therefore, we examined whether TGFβ and pSmad signaling activated MAPK signaling by assessing pERK, pJNK, and p-p38 [30,36,38] expression after treatment of C4 cells with exogenous TGFβ or the TGFβ inhibitor, SB431452. Conversely, we examined if MAPKs could regulate TGFβ RI/Smads [30] by assessing pSmad2/3 and Smad7 after PLX4032 treatment. Accordingly, we treated the C4 melanoma cells with PLX4032, TGFβ, or SB431542, alone and in combination, and harvested cell lysates at time 0, 15, 45 and 90 min [33].

As expected, Fig. 4A demonstrates that pERK is constitutively expressed in C4 cells and that PLX4032 blocks this expression completely, within 15 min, while levels of pAKT and pJNK remain unaffected (Fig. 4A and data not shown). We also found that addition of exogenous TGFβ did not affect pERK or pAKT (Fig. 4A). In addition, pERK, but not pAKT expression was inhibited by 15 min with the addition of PLX4032 in combination with TGFβ alone or TGFβ and SB431452 (Fig. 4A). Similarly, pJNK was not affected by

PLX4032, TGFβ, and/or SB431452 (data not shown). These data suggest that, while PLX4032 affects pERK signaling, TGFβ does not affect pERK expression.

It is known that Smad 2 and 3 are receptor activated Smads that phosphorylate in response to TGFβ [39,40], while Smad 7 is an inhibitory Smad that can both inhibit and be inhibited by TGFβ [39,40]. As expected, we found that exogenous TGFβ increased expression of pSmad 2/3 within 15 min, but SB431452 blocked this increase (Fig. 4B). PLX4032 did not affect pSmad 2/3 expression (Fig. 4B), and Smad 7 expression was not changed by PLX4032, TGFβ or SB431452. These results suggest that MAPK signaling does not regulate TGFβ RI/Smads.

PLX4032 and TGFβ may cooperate to mediate p-p38 signaling in melanoma cells

We next measured the expression of p-p38 in lysates of C4 cells that had been treated with PLX4032 and/or TGFβ. While neither PLX4032 nor TGFβ, alone, activated p-p38, combined treatment of cells with TGFβ and PLX4032 resulted in an increase at 90 min (Fig. 5A). Thus, there appears to be cooperativity between PLX4032 and exogenous TGFβ in activating p-p38 MAPK [30] and perhaps, in affecting the expression of collagen.

To test the possible role of p-p38 in regulating collagen expression, we treated C4 cells with 10 μM of the p-p38 inhibitor, BIRB 796 (BIRB), 1 h prior to PLX4032 or TGFβ treatment. Similar to Fig. 5A, B demonstrates that a 2 h treatment of C4 cells with PLX4032 and TGFβ increases p-p38, and that pre-treatment with BIRB completely prevents phosphorylation of p-38.

After confirming the efficacy of BIRB, C4 cells were treated with BIRB in the presence or absence of PLX4032 and/or exogenous TGFβ for 24 h and assessed for expression of Col1A2 mRNA (Fig. 5C). No differences in collagen expression were found between BIRB and control/DMSO treated cells ($P > 0.05$), nor between cells treated with a combination of PLX4032 and BIRB and cells treated with PLX4032 alone (Fig. 5C, $P > 0.05$). However, when cells were treated with a combination of PLX4032, exogenous TGFβ, and BIRB, collagen expression significantly decreased (from 7.3 to 6.1 fold change relative to DMSO) compared to cells treated with just the PLX4032 and TGFβ combination (Fig. 5C, $P < 0.05$). These data suggest that PLX4032 and TGFβ may cooperate to mediate collagen expression through p-p38 signaling.

PLX4720 increases collagen deposition *in vivo*

To confirm that the increase in collagen synthesis seen *in vitro* in response to PLX4032 is paralleled in an *in vivo* environment, we administered PLX4720, a chemical analog of PLX4032 with favorable pharmaceutical properties in rodents, to mice in their chow (417 mg/kg chow [13,41]). Groups of 8 mice per experiment (2 experiments) were injected intradermally with 2×10^5 C4 cells, with each mouse receiving 2 injections, one on each flank. When the tumors reached ~5 mm diameter, 4 mice were fed chow containing PLX4720 and 4 mice were fed control chow for 10 days. As expected, PLX4720 significantly ($P < 0.005$) halted tumor growth in mice fed PLX4720 chow compared to those fed control chow (Fig. 6A, tumor size of mice fed control chow ≅ 10 mm by 10 days post treatment; tumor size in mice fed PLX4720 chow ≅ 5 mm diameter). At sacrifice, tumors

were harvested for histology and mRNA. While Masson Trichrome (blue staining) of the tumors revealed collagen deposition in C4 tumors from mice fed control chow (Fig. 6B), as was previously observed [28], C4 tumors from mice fed the PLX4720 chow had more collagen deposition (Fig. 6C). Similar growth kinetics and histology were seen in C9 and VMM12 flank tumors fed PLX4720 chow (data not shown). RT-PCR of mRNA from tumor tissue revealed increased mRNA for human Col1A2 mRNA and decreased MMP-1 and IL-8 mRNAs in mice fed chow with PLX4720 (Fig. 6D–F). Thus, our findings of increased synthesis of type I collagen *in vitro* in response to PLX4032 are mirrored in *in vivo* studies, suggesting that clinical use of vemurafenib may affect the extracellular matrix in the tumor microenvironment (TME).

Discussion

In this study, we made the unexpected finding that the BRAF inhibitor, PLX4032, and the MEK inhibitor, U0126, increase the synthesis of type I collagen by several human and murine melanoma cell lines. This increase was seen *in vitro* and *in vivo*, suggesting novel effects on melanoma behavior and on the tumor microenvironment. Importantly, the increase in collagen synthesis may not be limited to $BRAF^{V600E}$, since U0126, which blocks pMEK in $BRAF^{WT}$ cells also increased collagen production. Concomitantly, these same MAPK inhibitors decreased expression of two genes, the collagenase, MMP-1, and the cytokine IL-8. The facts that (a) the increase in collagen was seen in several cell lines and (b) the two genes that were down-regulated in our studies have also been reported to be down-regulated by others [11,29], indicate that our findings are not an artifact confined to a single cell line or a single gene.

In trying to identify a mechanism(s) by which blocking the Ras-Raf-Mek-Erk MAPK pathway increases collagen synthesis, we investigated a possible role for TGFβ, a well-known inducer of collagen [30,34–36]. Our data show that effects of PLX4032 and TGFβ on collagen production are additive, and that, while blocking TGFβ signaling slightly diminishes the expression of collagen, the effects of PLX4032 are largely independent of TGFβ. We did, however, detect some cross-talk between TGFβ and MAPK signaling, in keeping with a previous report [30]. We found that PLX4032 and TGFβ increase p-p38 and that when this increase is blocked with a specific p-p38 inhibitor, there is a slight decrease in collagen mRNA. Clearly, however, other mechanisms are involved.

What these other mechanisms are is still unclear. Regulation of collagen gene expression and matrix deposition are quite complex, involving TGFβ and its receptors [42], integrin signaling [42], the discoidin domain receptors (DDRs) [43], mRNA stability [23], hormonal influences [44], and effects of secreted products from Carcinoma Activated Fibroblasts (CAFs) [42] in the tumor microenvironment (TME), which may include TGFβ and other growth factors [42,44]. Indeed, CAFS found in the TME are a primary source of collagen stimulation [30,42], and perhaps they are contributing to matrix deposition seen *in vivo* with melanomas (Fig. 6B and C).

Nonetheless, our studies document that the melanoma cells, themselves, produce increased collagen in response to blocking the Ras-Raf-Mek-Erk pathway. Further, we showed that

PLX4032 increases TGFβ production by the melanoma cells (Fig. 3A, Table 1). Conceivably, secreted TGFβ could affect adjacent stromal fibroblasts to further enhance collagen production in the TME. Indeed, we have recently shown that secreted products from melanoma cells can activate stromal fibroblasts by increasing their expression of MMP-1 and inflammatory cytokines, and that treating the melanoma cells with PLX4032 indirectly subdues this activation by reducing the expression of secreted proteins by the melanoma cells [29]. However, in the present study, rather than subduing CAFs, PLX4032 would augment collagen production by adjacent fibroblasts due to increased TGFβ produced by PLX4032-treated melanoma cells. Thus, it is possible that PLX4032, although indirectly quieting some aspects of gene expression by CAFs [29], could stimulate matrix deposition by the stromal cells. Possibly, the increase in collagen seen in tumors harvested from mice fed PLX4720 in their chow results from the murine stromal cells as well as from the (human) tumor cells (Fig. 6).

Most studies have focused on the genes that are down-regulated by MAPK inhibitors [7,45]. PLX4032 is noted for its ability to suppress the expression of many genes [7,45], and expression of only three genes was found to be up-regulated in response to PLX4032 [45]. Two of these are predicted transcripts and the third is a transcriptional co-regulator, CITED-2. CITED-2 has been linked to the suppression of MMP-1 and MMP-13 *via* the TGFβ pathway. Further, depletion of CITED-2 has been associated with invasiveness of colon cancer *in vitro*, leading the authors to speculate that that down-regulation of CITED-2 by mutant $BRAF^{V600E}$ may play a role in the invasive potential of melanoma cells [45]. Conversely, the up-regulation of CITED-2 by PLX4032 suggests that an increase in its expression may facilitate tumor progression.

Although one report suggests that a collagen matrix may be therapeutically beneficial by decreasing melanoma proliferation [46], collagen deposition in tumors, including melanomas, has traditionally been associated with increased angiogenesis, tumor cell migration and activation of stromal fibroblasts [25,34,35,42,47]. Further, inhibiting collagen synthesis by pharmacologic intervention impairs angiogenesis, decreases bone metastasis and facilitates the distribution of therapeutic agents within a tumor [25–27,48], again suggesting that collagen deposition fosters tumor progression. Thus, the increase in collagen that we documented in response to PLX4032 treatment is seemingly at odds with its clinical efficacy. In addition, the increase in collagen synthesis was not limited to $BRAF^{V600E}$-specific inhibition, since U0126, which blocks pMEK in $BRAF^{WT}$ cells also increased collagen production. This suggests that increased collagen deposition could also be seen in melanoma patients with no BRAF mutation but who may be treated with broad-spectrum MAPK inhibitors, such as Sorafenib [49]. In conclusion, our findings clearly demonstrate that blocking MAPK signaling results in a substantial increase in the deposition of collagen, the major matrix protein in the melanoma TME. As yet, it is unclear whether increased collagen deposition in melanomas represents a therapeutic benefit or an unwanted “off target” effect of inhibiting the Ras-Raf-Erk-Mek pathway.

Materials and methods

Cell culture and reagents

The human vertical growth phase melanoma cell lines, C4, C9, and VMM12, have been described previously [11,28,50]. Cells were cultured as mono-layers in Dulbecco's Modified Eagle'sMedium (DMEM) (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS) and penicillin and streptomycin. In experiments where conditioned media was harvested, confluent cultures were washed three times with HBSS to remove traces of serum and then placed in serum-free DMEM with 0.2% lactalbumin hydrolysate (LH) for 24 or 48 h. The murine D4M.3A and D4M.7A cell lines have also been described previously [33]. Cells were cultured as monolayers in DMEM/F-12 advanced (Life Technologies, Carlsbad, CA) with 5% FBS, 1× penicillin/streptomycin, and 1× glutamine.

Stocks of PLX4032 (PLX) (Chemie Tek, Indianapolis, IN) and U0126 (Cell signaling, Beverly, MA) were prepared in DMSO, and diluted to final concentrations with media. Cells were treated with 0.3 μl/ml DMSO (equivalent to the percentage of DMSO in PLX and U0126 treated cells), 2 ng/ml Recombinant Human TGF-beta 1 (TGFβ) (R&D Systems, Minneapolis, MN), 100 ng/ml Recombinant Human IL-8 (Biolegend, San Diego, CA), 3 μM PLX, 10 μM U0126, 10 μM TGFβ R1 Kinase Inhibitor VI SB431542 (SB) (EMD Millipore, Billerica, MA), 10 μM BIRB 796 (BIRB) (Selleckchem, Houston, TX) for the specified time courses.

Analysis of mRNA

Total cellular RNA was purified using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was prepared by reverse transcription with 2 μg of total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules CA) as described by the manufacturer. Levels of mRNA were measured by qRT-PCR using the iQ SYBR Green Supermix (Bio Rad) as per the manufacturer. The primers used are as follows: human (h) Col1A2, Fwd: 5′-CTC AGA CCC AAG GAC TAT GAA-3′, Rev: GTT GCC CTC AGC AAC AAG TTC-3′; hMMP-1, Fwd: 5′-AGC TAG CTC AGG ATG ACA TTG ATG-3′, Rev: 5′-GCC GAT GGG CTG GAC AG-3′; hIL-8, Fwd: 5′-GAG TGG ACC ACA CTG CGC CAA-3′, Rev: 5′-TCC ACA ACC CTC TGC ACC CAG TT-3′; and hTGFβ1, Fwd: 5′-TGA ACC CGT GTT GCT CTC CCG-3′, Rev: 5′-CTG CCG CAC AAC TCC GGT GA-3′, mouse (m) Col1A2, Fwd: 5′-CCT GGT CTT ACT GGG AAC TTT-3′, Rev: 5′-CAG GTC CTT GGA AAC CTT GAG-3′;mMMP-13, Fwd: 5′-CAC TCC CTA GGT CTG GAT CA-3′, Rev: 5′-TTC ATC GCC TGG ACC ATA AAG-3′. All assays were carried out in triplicate, with machine duplicates. All samples were normalized to human β2microglobulin (β2M) [28] or mouse GAPDH [33] and relative fold change was calculated as $2^{-\Delta\Delta Ct}$.

Immunoblotting

Confluent cultures of C4, C9 and VMM12 cells in 6-well plates were placed in 1 ml fresh DMEM/10% FBS for times as indicated for specific experiments. Total cell lysates were prepared with RIPA buffer (Sigma Aldrich, St. Louis, MO) and were separated on 10% SDS-PAGE gels (Bio-Rad, Hercules, CA). Serum-free conditioned medium (from 48 h

cultures) was TCA precipitated, resuspended in 30 μl of 2× Laemmli buffer, and separated on 10% SDS-PAGE gels. Proteins were transferred to a PVDF membrane and visualized with anti-Col1A2 (1:200, Santa Cruz, CA), anti-MMP-1 (1:5000, Millipore), anti-pERK (9101), anti-total ERK (9102), anti-pAKT (4058), anti-total AKT (9272), anti-pSmad 2/3 (9520), anti-Smad 2/3 (5678), anti-total JNK (9252), anti-p-p38 MAPK (9215), or anti-p38 MAPK (8690) (1:1000 for each, Cell Signaling, Beverly, MA), anti-p-JNK (1:2000, Cell Signaling), or anti-Smad 7 (1:1000, Thermo Scientific, Rockford, IL), followed by the appropriate secondary antibody, donkey anti-rabbit HRP (1:20,000) or donkey anti-mouse (1:10,000). Signal was detected with Western Lightning Plus ECL (PerkinElmer, Waltham, MA, USA).

Measurement of TGFβ protein in medium of cultured cells

TGFβ protein was measured with a bioassay that used a construct of the plasminogen activator inhibitor-1 (PAI-1) promoter linked to the luciferase enzyme [51]. The construct was transfected into mink lung epithelial (MLE) cells, and the quantitative assay measured the ability of TGFβ (0.2 to >30 pM) in conditioned medium from melanoma cells to induce PAI-1 expression in the MLE cells, with dose dependent increases in luciferase activity. The conditioned medium was from C4 cells plated at 2×10^5 cells per well in 6-well plates. C4 cells were incubated overnight, treated with 0.3 μl/ml DMSO, 3 μM PLX4032 (PLX), 10 μM SB431542 (SB), or a combination of PLX + SB in serum-free media for 24 h, then conditioned medium was harvested. SB was added to the cells 1 h prior to PLX treatment. To measure total TGFβ, latent protein was acid activated and neutralized [51]. Recombinant TGFβ was used for a standard curve.

Orthotopic injections

C4, C9, and VMM12 melanoma cells were grown to confluence in DMEM 10% FBS. On the day of injection, cells were trypsinized, washed 3× with sterile PBS, counted and resuspended in sterile HBSS $2 \times 10^5 \times$ cells/50 μl were injected intradermally into the right and left flank of 6 week old female nude mice (strain nu/nu, Charles River, Wilmington, MA). In each experiment, eight mice were injected with C4 cells and each experiment was repeated twice. Eight mice were injected with C9 cells and four mice were injected with VMM12 cells. Tumor diameter was determined weekly with Vernier calipers (Fisher Scientific). When tumors reached ~5 mm diameter, the cohort of mice was split into two equal groups. Half the mice were fed chow containing the chemical analog of PLX4032 with favorable pharmaceutical properties in mice called PLX4720 (417 mg/kg chow [13,41]) (supplied under Materials Transfer Agreement by Plexxikon Inc. (Berkeley, CA)), while the other half of mice were fed control chow for 10 days. At that time, mice were sacrificed by inhalation of Isofluorane and cervical dislocation, and tumors were harvested. A portion of the tumor was processed for histology and staining for collagen with Masson Trichrome, and another portion was snap frozen at −70 °C for analysis of mRNA as described above. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Dartmouth College.

Statistical analyses

Growth curves and RT-PCR graphs were generated using GraphPad Prism software and statistical significance was evaluated with either a Student *t*-test or a one-way ANOVA. The Dunnett method was used to adjust p-values in multiple comparisons.

Supplementary Material

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References

1. Balch CM, Soong S-J, Atkins MB, Buzaid AC, Cascinelli N, Coit DG, et al. An evidence-based staging system for cutaneous melanoma. CA Cancer J Clin. 2004; 54(3):131–149. [quiz 182–4]. [PubMed: 15195788]
2. Smalley KSM, Lioni M, Herlyn M. Targeting the stromal fibroblasts: a novel approach to melanoma therapy. Expert Rev Anticancer Ther. 2005 Dec; 5(6):1069–1078. [PubMed: 16336098]
3. Vultur A, Villanueva J, Herlyn M. Targeting BRAF in advanced melanoma: a first step toward manageable disease. Clin Cancer Res. 2011 Apr; 17(7):1658–1663. [PubMed: 21447722]
4. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med. 2011 Jun; 364(26):2507–2516. [PubMed: 21639808]
5. Koya RC, Mok S, Otte N, Blacketor KJ, Comin-Anduix B, Tumeh PC, et al. BRAF Inhibitor Vemurafenib Improves the Antitumor Activity of Adoptive Cell Immunotherapy. Cancer Res. 2012:3928–3937. [PubMed: 22693252]
6. Shi, H.; Moriceau, G.; Kong, X.; Lee, M-K.; Lee, H.; Koya, RC., et al. Nat Commun. Vol. 3. Nature Publishing Group; 2012 Jan. Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance; p. 724
7. Joseph EW, Pratilas Ca, Poulikakos PI, Tadi M, Wang W, Taylor BS, et al. The RAF inhibitor PLX4032 inhibits ERK signaling and tumor cell proliferation in a V600E BRAF-selective manner. Proc Natl Acad Sci U S A. 2010 Aug; 107(33):14903–14908. [PubMed: 20668238]
8. Blackburn JS, Rhodes CH, Coon CI, Brinckerhoff CE. RNA interference inhibition of matrix metalloproteinase-1 prevents melanoma metastasis by reducing tumor collagenase activity and angiogenesis. Cancer Res. 2007 Nov 15; 67(22):10849–10858. [PubMed: 18006830]
9. Blackburn JS, Brinckerhoff CE. Matrix metalloproteinase-1 and thrombin differentially activate gene expression in endothelial cells via PAR-1 and promote angiogenesis. Am J Pathol. 2008 Dec; 173(6):1736–1746. [PubMed: 18988801]
10. Blackburn, JS.; Liu, I.; Coon, CI.; Brinckerhoff, CE. Oncogene. Vol. 28. Nature Publishing Group; 2009 Sep 7. A matrix metalloproteinase-1/protease activated receptor-1 signaling axis promotes melanoma invasion and metastasis; p. 4237-4248.
11. Huntington JT, Shields JM, Der CJ, Wyatt Ca, Benbow U, Slingluff CL, et al. Overexpression of collagenase 1 (MMP-1) is mediated by the ERK pathway in invasive melanoma cells: role of

BRAF mutation and fibroblast growth factor signaling. J Biol Chem. 2004 Aug 6; 279(32):33168–33176. [PubMed: 15184373]

12. Boni A, Cogdill AP, Dang P, Udayakumar D, Njauw C-NJ, Sloss CM, et al. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. Cancer Res. 2010 Jul 1; 70(13):5213–5219. [PubMed: 20551059]
13. Khalili JS, Liu S, Rodríguez-Cruz TG, Whittington M, Wardell S, Liu C, et al. Oncogenic BRAF(V600E) Promotes Stromal Cell-Mediated Immunosuppression Via Induction of Interleukin-1 in Melanoma. Clin Cancer Res. 2012 Oct 1; 18(19):5329–5340. [PubMed: 22850568]
14. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature. 2012 Jul; 487(7408):500–504. [PubMed: 22763439]
15. Noll WW, Belloni DR, Rutter JL, Storm CA, Schned AR, Titus-Ernstoff L, et al. Loss of heterozygosity on chromosome 11q22–23 in melanoma is associated with retention of the insertion polymorphism in the matrix metalloproteinase-1 promoter. Am J Pathol. 2001 Feb; 158(2):691–697. [PubMed: 11159206]
16. Airola K, Karonen T, Vaalamo M, Lehti K, Lohi J, Kariniemi AL, et al. Expression of collagenases-1 and -3 and their inhibitors TIMP-1 and -3 correlates with the level of invasion in malignant melanomas. Br J Cancer. 1999 May; 80(5–6):733–743. [PubMed: 10360651]
17. Walker RA, Woolley DE. Immunolocalisation studies of matrix metalloproteinases-1, -2 and -3 in human melanoma. Virchows Arch. 1999 Dec; 435(6):574–579. [PubMed: 10628799]
18. Hofmann UB, Westphal JR, Waas ET, Zendman AJ, Cornelissen IM, Ruiter DJ, et al. Matrix metalloproteinases in human melanoma cell lines and xenografts: increased expression of activated matrix metalloproteinase-2 (MMP-2) correlates with melanoma progression. Br J Cancer. 1999 Nov; 81(5):774–782. [PubMed: 10555745]
19. Nikkola J, Vihinen P, Vuoristo M-S, Kellokumpu-Lehtinen P, Kähäri V-M, Pyrhönen S. High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. Clin Cancer Res. 2005 Jul 15; 11(14): 5158–5166. [PubMed: 16033831]
20. Meierjohann S, Hufnagel A, Wende E, Kleinschmidt MA, Wolf K, Friedl P, et al. MMP13 mediates cell cycle progression in melanocytes and melanoma cells: in vitro studies of migration and proliferation. Mol Cancer. 2010 Jan.9(1):201. [PubMed: 20667128]
21. Zigrino, P.; Kuhn, I.; Bäuerle, T.; Zamek, J.; Fox, JW.; Neumann, S., et al. J Investig Dermatol. Vol. 129. The Society for Investigative Dermatology, Inc; 2009 Jun 11. Stromal Expression of MMP-13 Is Required for Melanoma Invasion and Metastasis; p. 2686-2693.
22. Brinckerhoff CE, Matrisian LM. Matrix metalloproteinases: a tail of a frog that became a prince. Nat Rev Mol Cell Biol. 2002 Mar; 3(3):207–214. [PubMed: 11994741]
23. Stefanovic B. RNA protein interactions governing expression of the most abundant protein in human body, type I collagen. Wiley Interdiscip Rev RNA. 2013; 4(5):535–545. [PubMed: 23907854]
24. Fang M, Yuan J, Peng C, Li Y. Collagen as a double-edged sword in tumor progression. Tumour Biol. 2014 Apr; 35(4):2871–2882. [PubMed: 24338768]
25. Van Kempen LCLT, Rijntjes J, Mamor-Cornelissen I, Vincent-Naulleau S, Gerritsen M-JP, Ruiter DJ, et al. Type I collagen expression contributes to angiogenesis and the development of deeply invasive cutaneous melanoma. Int J Cancer. 2008 Mar 1; 122(5):1019–1029. [PubMed: 17957794]
26. Juárez P, Mohammad KS, Yin JJ, Fournier PGJ, McKenna RC, Davis HW, et al. Halofuginone inhibits the establishment and progression of melanoma bone metastases. Cancer Res. 2012 Dec 1; 72(23):6247–6256. [PubMed: 23002206]
27. Javelaud D, Mohammad KS, McKenna CR, Fournier P, Luciani F, Niewolna M, et al. Stable overexpression of Smad7 in human melanoma cells impairs bone metastasis. Cancer Res. 2007 Mar 1; 67(5):2317–2324. [PubMed: 17332363]
28. Croteau W, Jenkins MH, Ye S, Mullins DW, Brinckerhoff CE. Differential mechanisms of tumor progression in clones from a single heterogeneous human melanoma. J Cell Physiol. 2013 Apr; 228(4):773–780. [PubMed: 23001823]

29. Whipple, CA.; Brinckerhoff, CE. Br J Cancer. Vol. 111. Cancer Research UK; 2014 Oct 14. BRAF(V600E) melanoma cells secrete factors that activate stromal fibroblasts and enhance tumourigenicity; p. 1625-1633.
30. Leivonen S-K, Kähäri V-M. Transforming growth factor-beta signaling in cancer invasion and metastasis. Int J Cancer. 2007 Nov 15; 121(10):2119–2124. [PubMed: 17849476]
31. Berking C, Takemoto R, Schaider H, Showe L, Satyamoorthy K, Robbins P, et al. Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling. Cancer Res. 2001 Nov 15; 61(22):8306–8316. [PubMed: 11719464]
32. Unemori EN, Amento EP, Bauer EA, Horuk R. Melanoma growth-stimulatory activity/GRO decreases collagen expression by human fibroblasts. Regulation by C-X-C but not C-C cytokines. J Biol Chem. 1993 Jan 15; 268(2):1338–1342. [PubMed: 8419336]
33. Jenkins MH, Steinberg SM, Alexander MP, Fisher JL, Ernstoff MS, Turk MJ, et al. Multiple murine BRaf(V600E) melanoma cell lines with sensitivity to PLX4032. Pigment Cell Melanoma Res. 2014 May; 27(3):495–501. [PubMed: 24460976]
34. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol. 2012 Feb 20; 196(4):395–406. [PubMed: 22351925]
35. Yin M, Soikkeli J, Jahkola T, Virolainen S, Saksela O, Hölttä E. TGF-β signaling, activated stromal fibroblasts, and cysteine cathepsins B and L drive the invasive growth of human melanoma cells. Am J Pathol. 2012 Dec; 181(6):2202–2216. [PubMed: 23063511]
36. Busse A, Keilholz U. Role of TGF-β in melanoma. Curr Pharm Biotechnol. 2011 Dec; 12(12): 2165–2175. [PubMed: 21619542]
37. Inman GJ, Nicolás FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol. 2002 Jul; 62(1):65–74. [PubMed: 12065756]
38. Imamichi Y, König A, Gress T, Menke A. Collagen type I-induced Smad-interacting protein 1 expression downregulates E-cadherin in pancreatic cancer. Oncogene. 2007 Apr 5; 26(16):2381–2385. [PubMed: 17043655]
39. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003 Oct 9; 425(6958):577–584. [PubMed: 14534577]
40. Feng X-H, Derynck R. Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol. 2005 Jan.21:659–693. [PubMed: 16212511]
41. Yang H, Higgins B, Kolinsky K, Packman K, Go Z, Iyer R, et al. RG7204 (PLX4032), a selective BRAFV600E inhibitor, displays potent antitumor activity in preclinical melanoma models. Cancer Res. 2010 Jul; 70(13):5518–5527. [PubMed: 20551065]
42. Miles FL, Sikes RA. Insidious changes in stromal matrix fuel cancer progression. Mol Cancer Res. 2014 Mar; 12(3):297–312. [PubMed: 24452359]
43. Fu H-L, Valiathan RR, Arkwright R, Sohail A, Mihai C, Kumarasiri M, et al. Discoidin domain receptors: unique receptor tyrosine kinases in collagen-mediated signaling. J Biol Chem. 2013 Mar 15; 288(11):7430–7437. [PubMed: 23335507]
44. Ponticos M, Smith BD. Extracellular matrix synthesis in vascular disease: hypertension, and atherosclerosis. J Biomed Res. 2014 Jan; 28(1):25–39. [PubMed: 24474961]
45. Packer LM, East P, Reis-Filho JS, Marais R. Identification of direct transcriptional targets of (V600E)BRAF/MEK signalling in melanoma. Pigment Cell Melanoma Res. 2009 Dec; 22(6):785–798. [PubMed: 19682280]
46. Henriet P, Zhong ZD, Brooks PC, Weinberg KI, DeClerck YA. Contact with fibrillar collagen inhibits melanoma cell proliferation by up-regulating p27KIP1. Proc Natl Acad Sci U S A. 2000 Aug 29; 97(18):10026–10031. [PubMed: 10944199]
47. Goldstein LJ, Chen H, Bauer RJ, Bauer SM, Velazquez OC. Normal human fibroblasts enable melanoma cells to induce angiogenesis in type I collagen. Surgery. 2005 Sep; 138(3):439–449. [PubMed: 16213896]
48. Diop-Frimpong B, Chauhan VP, Krane S, Boucher Y, Jain RK. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. Proc Natl Acad Sci U S A. 2011 Feb 15; 108(7):2909–2914. [PubMed: 21282607]

49. Augustine CK, Toshimitsu H, Jung S-H, Zipfel PA, Yoo JS, Yoshimoto Y, et al. Sorafenib, a multikinase inhibitor, enhances the response of melanoma to regional chemotherapy. Mol Cancer Ther. 2010 Jul; 9(7):2090–2101. [PubMed: 20571072]

50. Yamshchikov GV, Mullins DW, Chang C, Ogino T, Thompson L, Presley J, et al. Sequential immune escape and shifting of T cell responses in a long-term survivor of melanoma. J Immunol. 2005 Jun 1; 174(11):6863–6871. [PubMed: 15905528]

51. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal Biochem. 1994 Mar 1; 216(2):276–284. [PubMed: 8179182]

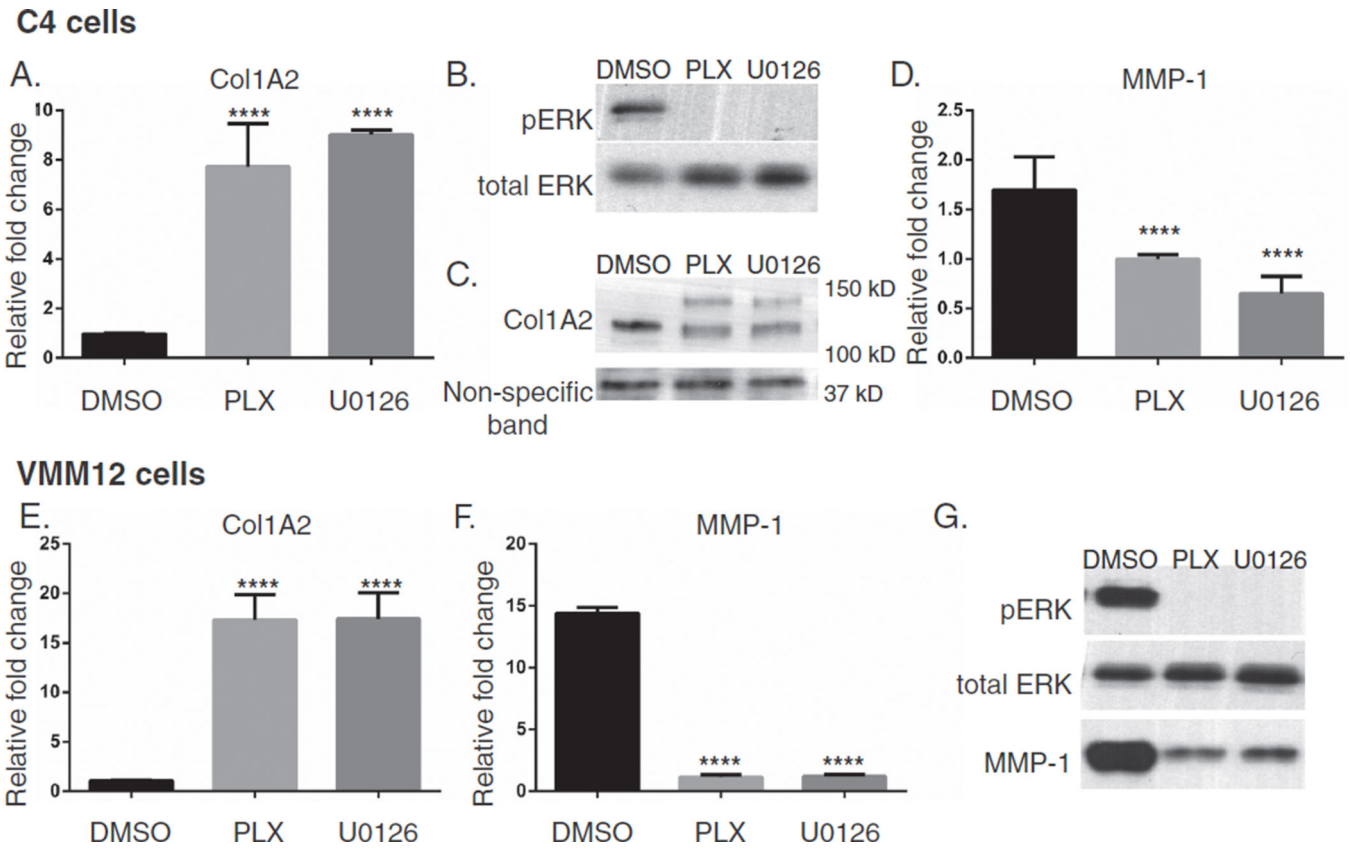

Fig. 1.

MAPK inhibition induces collagen synthesis in human melanoma cells. C4 or VMM12 cells were plated on 6-well plates at a density of 0.5×10^5 cells per well and treated the following day with 0.3 μl/ml DMSO, 3 μM PLX4032 (PLX) or 10 μM U0126 for 48 h. (A) RT-PCR of Col1A2 expression in mRNA from C4 cells. Fold change was calculated relative to cells treated with DMSO. Immunoblots probing for (B) pERK and total ERK (6 μg protein) and (C) Col1A2 and a non-specific band (22 μg protein) in C4 cell lysates. Pre-pro-collagen (upper band) and pro-collagen (lower band) are found between 150 kD–100 kD (after 1 min exposure), the non-specific band was found at 37 kD (after 15 min exposure). (D) RT-PCR of MMP-1 in cDNA from C4 cells. Fold change was calculated relative to cells treated with PLX. Expression of mRNA in VMM12 cells, (E) Col1A2 and (F) MMP-1, fold change was calculated as above. (G) Immunoblots of pERK and total ERK (top two panels — 6 μg protein) in VMM12 cell lysates, and (bottom panel) immunoblot of MMP-1 in VMM12 cell supernatants from the same serum-free culture (30 μl of TCA precipitate). One-way ANOVAs were performed on the relative fold changes compared to DMSO controls, data were pooled from 3 separate experiments (****$P < 0.0001$).

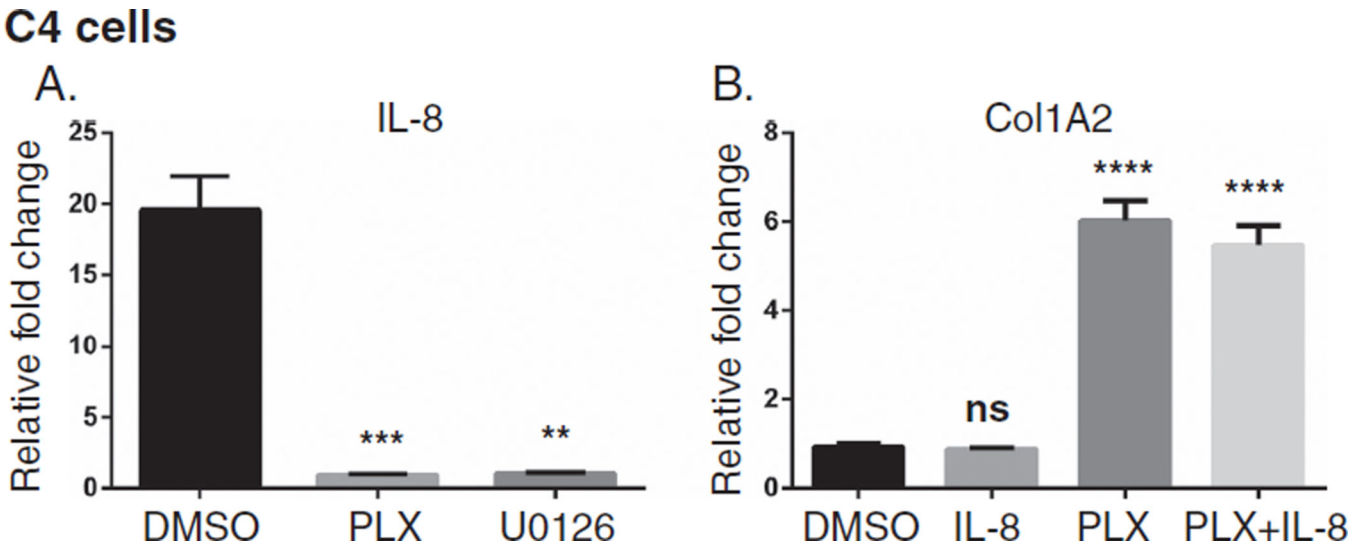

Fig. 2.

MAPK inhibition decreases IL-8 expression in human melanoma cells. (A) Expression of IL-8 as measured by RT-PCR of cDNA from C4 cells. Cells were plated on 6-well plates at a density of 0.5×10^5 cells per well and treated with 0.3 μl/ml DMSO, 3 μM PLX4032 (PLX) or 10 μM U0126 for 48 h. Fold change was calculated relative to cells treated with PLX, data pooled from at least 2 separate experiments with 3 technical replicates per experiment. One-way ANOVA was performed on fold changes compared to DMSO (**P < 0.005, ***P < 0.001). (B) Expression of Col1A2 in C4 cells measured by RT-PCR. Cells were plated as above and treated with DMSO, exogenous IL-8 (100 ng/ml), PLX, or a combination of PLX and IL-8. Fold change was calculated relative to cells treated with DMSO and a one-way ANOVA was performed on fold changes compared to DMSO (ns = not significant P-value, ****P < 0.0001).

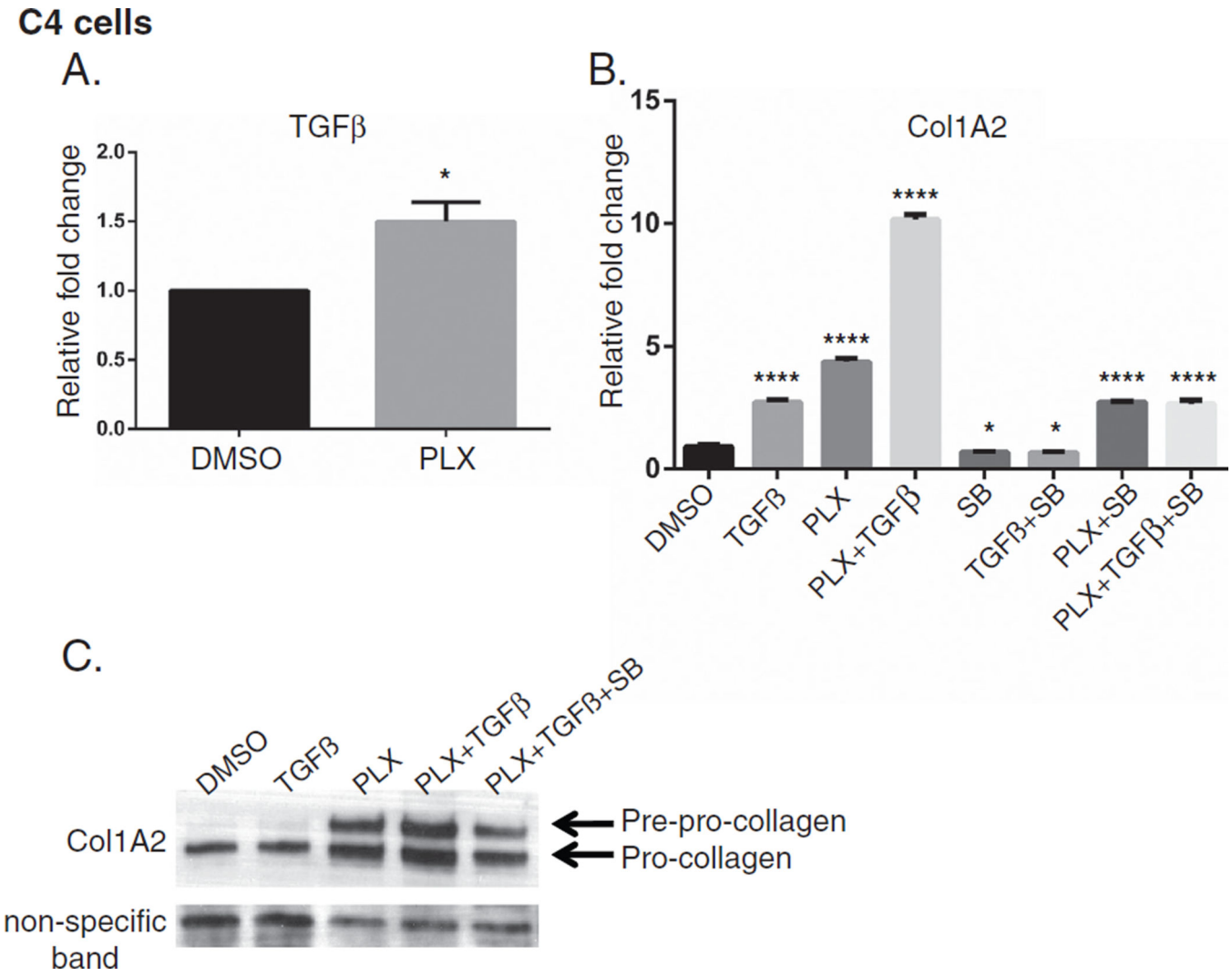

Fig. 3.

TGFβ and PLX4032 cooperate to mediate collagen expression. (A) RT-PCR analysis of TGFβ mRNA in C4 cells treated with 0.3 μl/ml DMSO or 3 μM PLX4032 (PLX) for 48 h. Fold change was calculated relative to cells treated with DMSO and a *t*-test was performed (*P < 0.05) (B) Expression of Col1A2 mRNA in C4 cells treated with 0.3 μl/ml DMSO, 2 ng/ml exogenous TGFβ, 3 μM PLX4032 (PLX), combination of PLX + TGFβ, 10 μM SB431542 (SB), TGFβ + SB, PLX + SB, or PLX + TGFβ + SB for 48 h. Fold change was calculated relative to cells treated with DMSO, and an ANOVA was performed (*P < 0.05, ****P < 0.0001). RT-PCR data was pooled from at least 2 separate experiments with 3 technical replicates per experiment. (C) Immunoblot of Col1A2 expression in C4 cell lysates from a parallel experiment (22 μg protein). Pre-pro-collagen and pro-collagen are found between 150 kD–100 kD (after 1 min exposure, top blot), the non-specific band was found at 37 kD (after 15 min exposure, bottom blot).

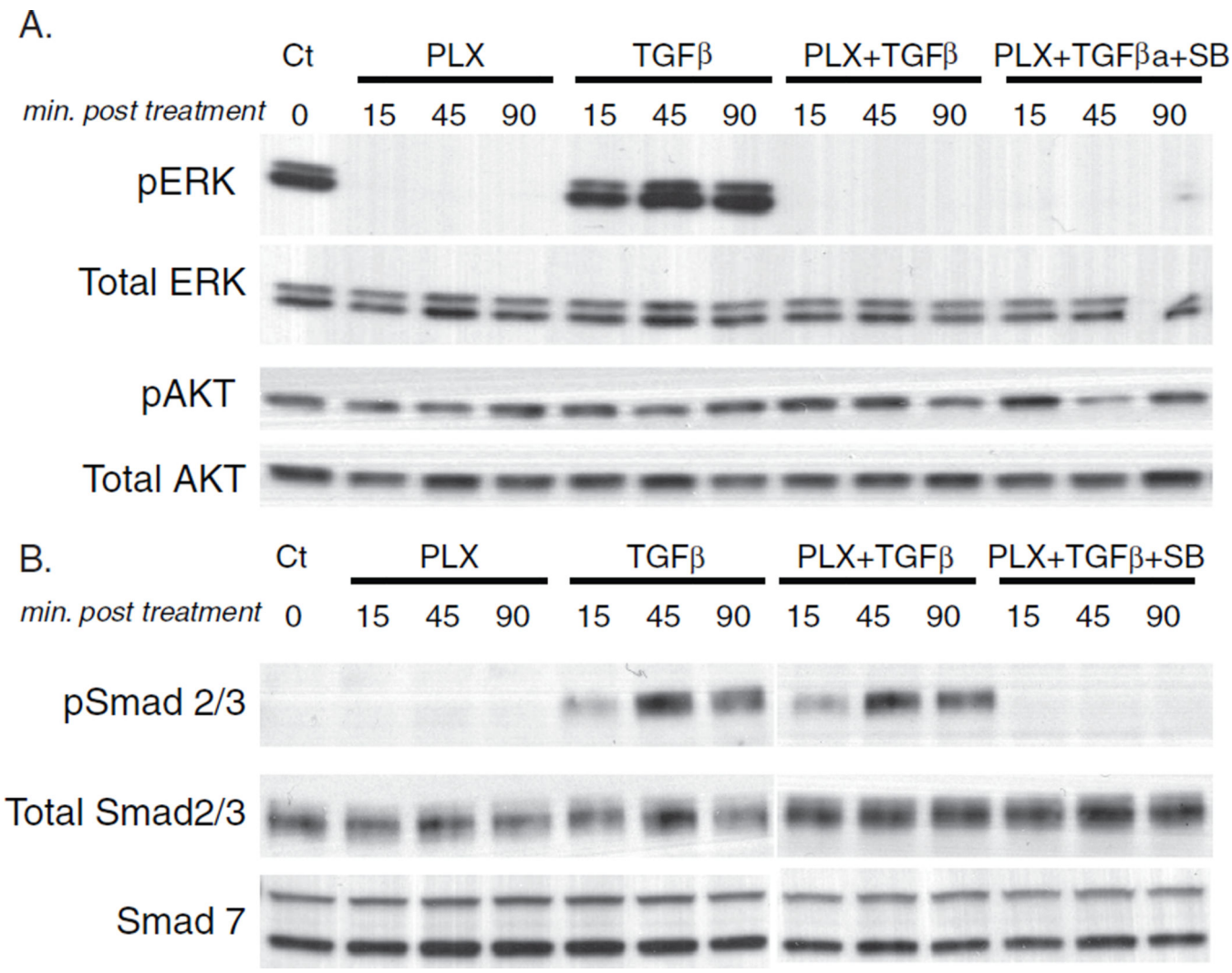

Fig. 4.

Potential cross-talk between MAPK and TGFβ signaling. C4 cells in DMEM/10% FBS were treated with 3 μM PLX4032 (PLX), 2 ng/ml exogenous TGFβ, combination of PLX + TGFβ, or combination of PLX + TGFβ + 10 μMSB431542 (SB) for 0 (Ct), 15, 45, or 90 min. Cell lysates were harvested at each time point. Immunoblot analyses of (A—top two blots—6 μg protein) pERK and total ERK, (B—bottom two blots—6 μg protein) pAKT and total AKT, (C—top two blots—44 μg protein) pSmad 2/3 and total Smad 2/3, and (B—bottom blot—44 μg protein) Smad 7. (A) Samples from each blot/row were run on the same gel. (B) Samples were run on different gels (Ct, PLX, and TGFβ on one, the rest of the samples on another gel), but the gels were loaded with the same amount of protein, processed simultaneously, and treated and exposed the same way.

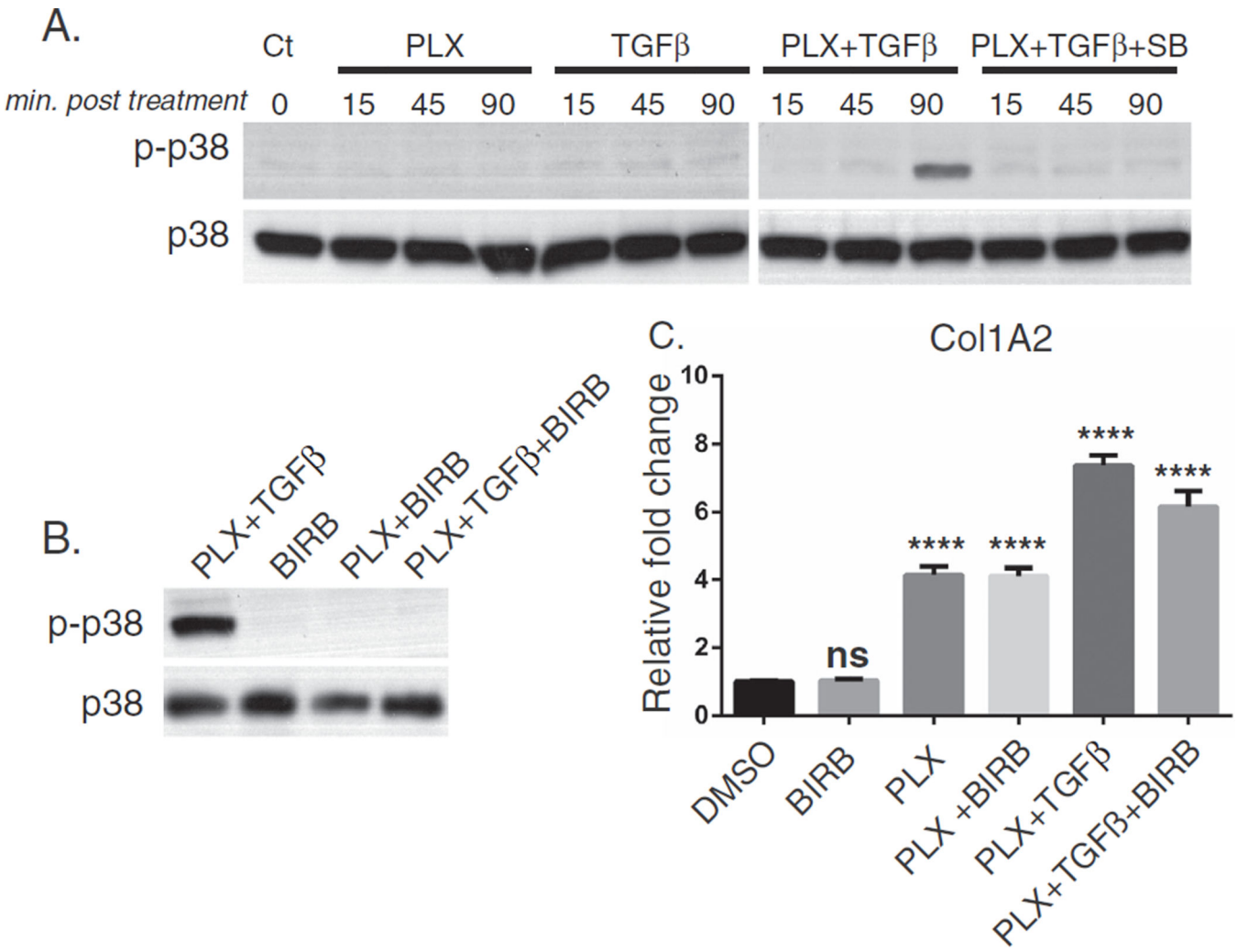

Fig. 5.

Collagen expression is partially mediated through p-p38 signaling. (A) Immunoblot of (top) phospho-p38 (p-p38) and (bottom) total p38 for 44 μg C4 cell lysates. C4 cells were treated with 3 μM PLX4032 (PLX), 2 ng/ml exogenous TGFβ, combination of PLX + TGFβ, or combination of PLX + TGFβ + 10 μM SB431542 (SB) for 0 (Ct), 15, 45, or 90 min. Cell lysates were harvested at each time point. (B) Immunoblot of (top) p-p38 and (bottom) total p-38 for 44 μg C4 cell lysates. C4 cells were treated with PLX + TGFβ, BIRB 796 (BIRB), PLX + BIRB, or PLX + TGFβ + BIRB. BIRB was administered 1 h prior to other treatments. Cells were then lysed after a 2 h treatment with DMSO, PLX, or TGFβ. (C) RT-PCR analysis of Col1A2 mRNA in C4 cells treated with DMSO, 10 μM BIRB, PLX, PLX + BIRB, PLX + TGFβ, or PLX + TGFβ + BIRB. Cells were treated with BIRB 1 h prior to treatment with PLX or TGFβ. Cells were then incubated for 24 h and harvested for RNA. Fold change was calculated relative to DMSO-treated cells and a one-way ANOVA was performed comparing fold changes to DMSO (ns = not significant, ****$P < 0.0001$).

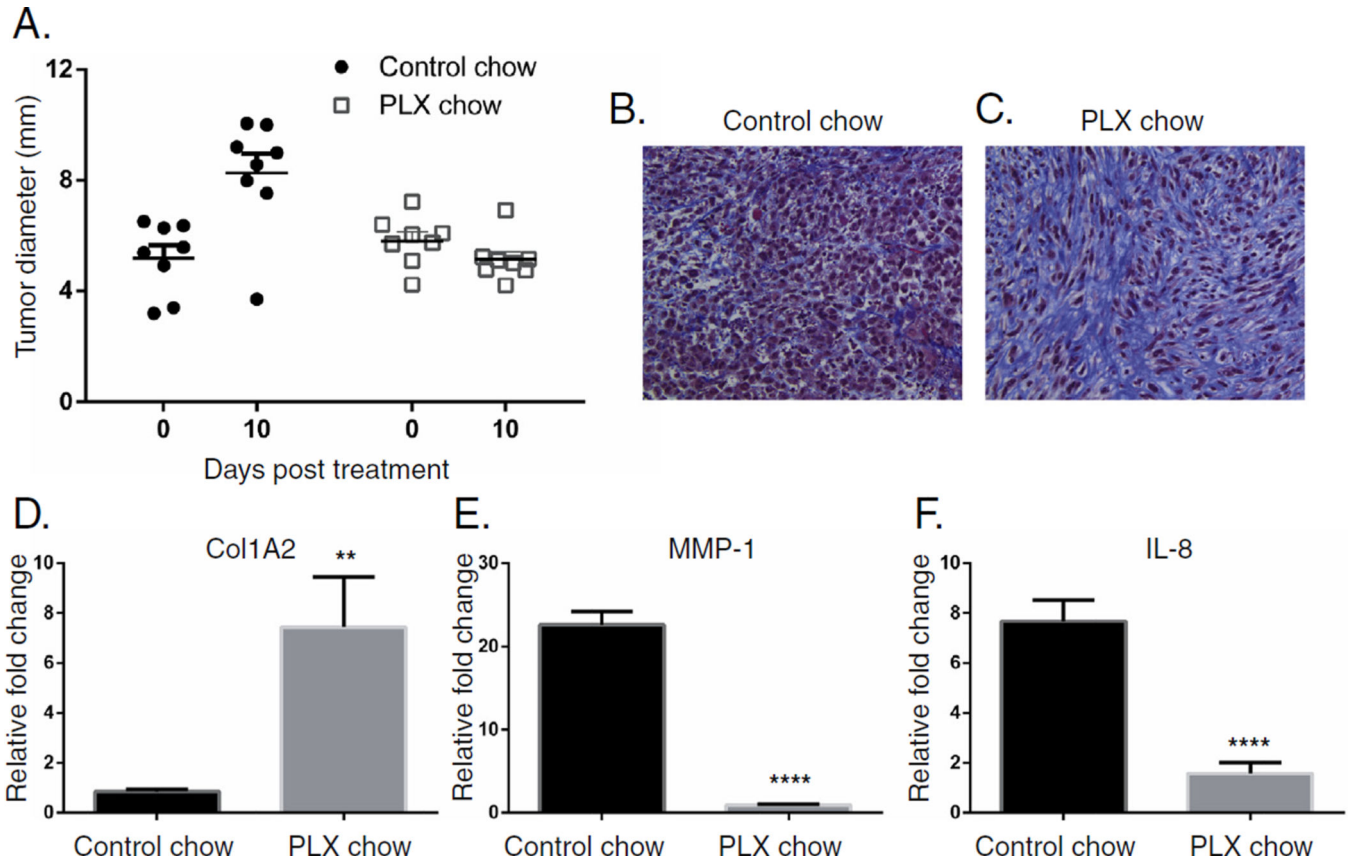

Fig. 6.

PLX4720 increases collagen deposition *in vivo*. (A) Diameter of flank tumors in *nu/nu* mice injected with 2×10^5 C4 cells in each flank. Tumors were grown to approximately 5 mm in diameter (day 0) before being fed either control chow or chow containing PLX4720 (8 tumors per group). Tumor diameter was measured again at 10 days and mice fed PLX chow had significantly smaller tumors than those fed control chow ($P < 0.005$). Data are representative from 2 separate experiments, with standard error bars. (B) Masson Trichrome staining (blue) of representative tumors from mice fed control chow (left) or PLX chow (right), 10× objective. (C–E) RT-PCR of mRNA from C4 tumor tissue; (C) Col1A2, (D) MMP-1, (E) IL-8. Data was pooled from 2 separate injection experiments (16 mice total). Fold change was calculated relative to (D) control chow fed mice, and (E–F) PLX chow fed mice, and t-tests were performed (**$P < 0.005$, ****$P < 0.0001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

TGF-β protein levels in the conditioned media of C4 cells 24 h post treatment.

TGF-β protein levels (pg/ml)		
Treatment	**Mean ± SEM**	**P-value**
DMSO	18.4 ± 0.3	
PLX	48.4 ± 4.1	**
SB	0.0 ± 0.0	****
PLX + SB	9.2 ± 1.5	**

P-values were calculated with a *t*-test comparing individual treatments with the DMSO control treatment (**P < 0.005, ****P < 0.0001).