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CTGF is a therapeutic target for metastatic melanoma

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Abstract

Metastatic melanoma remains a devastating disease with a 5-year survival rate of less than five percent. Despite recent advances in targeted therapies for melanoma, only a small percentage of melanoma patients experience durable remissions. Therefore, it is critical to identify new therapies for the treatment of advanced melanoma. Here, we define connective tissue growth factor (CTGF) as a therapeutic target for metastatic melanoma. Clinically, CTGF expression correlates with tumor progression and is strongly induced by hypoxia through HIF-1 and HIF-2-dependent mechanisms. Genetic inhibition of CTGF in human melanoma cells is sufficient to significantly reduce orthotopic tumor growth, as well as metastatic tumor growth in the lung of severe combined immunodeficient (SCID) mice. Mechanistically, inhibition of CTGF decreased invasion and migration associated with reduced matrix metalloproteinase-9 expression. Most importantly, the anti-CTGF antibody, FG-3019, had a profound inhibitory effect on the progression of established metastatic melanoma. These results offer the first preclinical validation of anti-CTGF therapy for the treatment of advanced melanoma and underscore the importance of tumor hypoxia in melanoma progression.

Keywords

connective tissue growth factor (CTGF); melanoma; invasion; metastasis; therapeutic target; hypoxia

Introduction

Metastatic melanoma is a devastating disease with a five-year survival of less than five percent. The mortality rate for metastatic melanoma is particularly high due to resistance to traditional cancer therapies and a high propensity to metastasize to the central nervous system. Recent advances in our understanding of the biology of melanoma revealed

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alterations in several signaling pathways including NRas, BRaf, PTEN/PI3K and p16/Arf.[1–3] Initially, targeted therapies inhibiting these pathways, in particular BRaf and MEK inhibitors, showed clinical efficacy; however, 50% of patients treated with these inhibitors develop disease progression within 6–7 months after treatment.[4] Acquired resistance to targeted kinase therapies in melanoma is associated with the activation of survival signals redundant to those activated by the targeted kinase.[5] Thus, it is imperative that additional therapeutic agents targeting key effector molecules of melanoma metastasis and survival be identified for the treatment of advanced melanoma.

Hypoxia is a key microenvironmental factor driving melanoma tumor progression. It has been estimated that 50–60% of locally advanced solid tumors, including melanomas, contain areas of hypoxia.[6] Intratumoral hypoxia develops from an imbalance between oxygen supply and consumption in proliferating tumor cells, and is further exacerbated by a compromised tumor vasculature.[6] Clinically, hypoxia is not only associated with melanoma progression but is also associated with poor prognosis.[7] Hypoxia promotes tumor progression by selecting for cells with low apoptotic potential and increases genomic instability, allowing for rapid mutational adaptation.[8,9] Additionally, hypoxia directly increases the expression of genes involved in glucose transport, angiogenesis, anaerobic metabolism, cell survival, invasion and metastasis. All of these changes allow cells to adapt to oxygen-deprived conditions and permit cells to escape these conditions by establishing new blood supplies or by physically moving from an oxygen-poor environment to an oxygen-rich environment.[8,10,11]

The primary molecular mediators of hypoxic signaling are the hypoxia–inducible transcription factors HIF-1 and HIF-2. In the presence of oxygen, the alpha subunits of HIF-1 and HIF-2 are rapidly degraded through the cooperative actions of prolyl hydroxylase enzymes (PHD1, 2, and 3) and the E3 ubiquitn ligase substrate recognition component von Hippel-Lindau (VHL) protein.[12,13] Under hypoxia, HIF-1 and HIF-2 are stabilized and activate the expression of genes containing hypoxia response elements.[14] Over 200 genes are activated in response to HIF-1 and HIF-2 that allow cells to survive and adapt to low oxygen tensions.

In this study, we utilized complementary DNA microarray analysis to identify key molecular mediators of hypoxic responses in human melanomas. Here, we identify the secreted matricellular protein connective growth tissue factor, CTGF or CCN2, as a hypoxia and HIF-induced factor in metastatic melanomas. CTGF belongs to a family of secreted proteins that are multifunctional modulators of a number of cellular functions, as well as pathologic processes. In tumor cells, CTGF has been reported to regulate growth, migration, invasion, angiogenesis and anoikis.[15–17] However, the role of CTGF and the therapeutic efficacy of targeting CTGF in melanoma progression and metastasis remain unknown. Our studies show that CTGF expression is significantly unregulated by hypoxia through HIF-1 and HIF-2-dependent mechanisms in human melanoma cell lines. Clinically, CTGF expression correlates with melanoma progression and metastasis. Inhibition of CTGF, either genetically or with a specific anti-CTGF monoclonal antibody, significantly inhibits the ability of human melanoma cells to grow in the skin and establish distant metastases in the lungs of SCID mice. These studies identify CTGF as a key effector and therapeutic target of hypoxia-driven metastasis in melanoma and shows that a specific therapeutic agent targeting CTGF (FG-3019) is sufficient to significantly inhibit melanoma tumor progression.

Results

CTGF is hypoxia induced and regulated by HIF-1 and HIF-2 in human melanoma

In order to identify therapeutic targets of hypoxia-driven metastasis in melanoma, we performed complementary DNA microarray analysis on metastatic melanoma cells (K457) exposed to normoxia (21% oxygen) or hypoxia (2% oxygen) for 24 h. Data from scanned Human Exonic Evidence Based Oligo microarrays were entered into the Stanford Microarray Database for normalization and analysis.[18] Expression changes were analyzed using multicomponent significance analysis of microarrays to identify genes significantly changed among the small hairpin RNA (shRNA)-expressing cells. Genes with a false-discovery rate lower than 0.5% were extracted to calculate fold changes relative to the normoxic samples. Additionally, we compared the fold change in hypoxic gene expression profiles between K457 cells and those treated with shRNAs against HIF-1α or HIF-2α in order to identify genes that are induced by hypoxia in a HIF-dependent manner. Using this gene expression profiling, we identified HIF-regulated genes that were repressed or induced greater than 1.5-fold in hypoxic cells compared with normoxic cells. Our analysis identified a number of well-characterized HIF target genes including: PDK1, PGK-1, LOX-L2, BNIP3 and SMAD3 (Figure 1a, Supplementary Figure 1A).[13,19,20] The extracellular matrix protein, CTGF, was also identified as a putative HIF-regulated gene in metastatic melanoma cells (Figure 1a). CTGF belongs to a family of secreted matricellular proteins that are multifunctional modulators of a number of cellular functions, as well as pathologic processes. In tumor cells, CTGF has been reported to regulate growth, migration, invasion, angiogenesis and anoikis.[15–17] However, the role of CTGF and the therapeutic efficacy of targeting CTGF in melanoma progression and metastasis remain unknown.

To validate our screen, we first performed quantitative real-time PCR (qRT–PCR) analysis of CTGF expression in K457 cells exposed to normoxia (21% oxygen) and hypoxia (2% oxygen for 24 h). CTGF expression was significantly induced by hypoxia in K457 metastatic melanoma cells, whereas CTGF expression was not induced by hypoxia in shHIF-1 or shHIF-2 cells (Figure 1a). These findings indicate that CTGF expression is induced by hypoxia in a HIF-dependent manner. To determine whether the regulation of CTGF expression by hypoxia and HIF translates into increased CTGF protein, we examined CTGF protein levels in melanoma cells exposed to normoxia and hypoxia by western blot analysis. We confirmed CTGF protein was induced by hypoxia in a HIF-dependent manner in K457 cells (2% oxygen for 24 h, Figure 1b). Additionally, we extended these findings to a panel of melanoma cell lines isolated from both metastatic and primary melanomas. Induction of CTGF protein by hypoxia (2% oxygen) was observed in both metastatic (K457, 1676, V2386, S1273, 903, F6493) (Figure 1b and c) and primary melanomas (V2876, L2842II) (Figure 1c). The marked increase in CTGF expression was also observed in spheroids that form when the K457 cells are cultured in a hypoxic environment for several days (Figure 1d). Together, these data demonstrate that CTGF is a hypoxia–inducible factor in both primary and metastatic human melanoma cells. Consistent with our findings, a recent study identified CTGF as a HIF-1-regulated gene in human melanoma cells.[21]

CTGF Expression Correlates with Melanoma Progression and Metastasis

The association of CTGF expression and tumor progression was examined using tissue microarrays containing normal skin, cutaneous melanomas and metastatic melanomas screened with a CTGF-specific antibody. In normal skin tissue, 0/21 specimens exhibited medium-high to high levels of CTGF staining (Figure 2a). In contrast, CTGF expression increased with tumor stage and metastasis with 27/39 of stage 2, 8/8 of stage 3/4, and 31/41 of metastatic lesions expressing medium-high to high levels of CTGF (Figure 2a). Additionally, we mined the Oncomine database for CTGF expression and found a

significant increase in median CTGF expression in melanoma tumor metastases (n = 40) compared with primary skin tumors (n=42, Figure 2b). Furthermore, when sorting CTGF expression by skin cancer type, CTGF expression was highest in invasive and cutaneous melanoma cancers compared with melanoma *in situ*, basal cell carcinoma and squamous cell carcinoma (P<0.01, Figure 2c). The data obtained from this study was deposited in the Oncomine database by Riker *et al*.[22] These findings demonstrate that CTGF expression is significantly induced in advanced cutaneous melanoma and its expression is maintained at high levels in metastatic lesions.

CTGF is a critical factor for melanoma progression

To determine a functional role of CTGF in melanoma progression, we utilized both genetic and pharmacologic approaches to inhibit CTGF activity. First, three independent K457 cell lines with stable knockdown of CTGF were generated (Figure 3a, shCTGF #8–10). Second, we obtained a specific monoclonal anti-CTGF antibody (FG- 3019, Fibrogen (San Francisco, CA, USA)) that has previously shown efficacy in preclinical models of pancreatic cancer.[23] The effects of CTGF inhibition on biological activities associated with disease progression were measured through a series of *in vitro* assays including: growth in soft agar, wound healing, cell migration and invasion. Of note, little to no decrease in growth rate of CTGF-deficient cells was observed when grown on plastic. In contrast, inhibition of CTGF with either the anti-CTGF antibody (K457 cells + Ab) or stable knockdown (shCTGF8, shCTGF9 and shCTGF10) significantly decreased colony size and number when grown in soft agar (Figure 3b and c). Next, the effect of CTGF inhibition on cell migration was measured in a wound-healing assay with wound closure followed over time. Cells with stable knockdown of CTGF showed decreased wound-healing ability when compared with parental K457 cells (Figure 3d). Migration through fibronectin-coated transwells was also decreased in cells expressing the shCTGF RNAs (Supplementary Figure 2A). When the invasive capabilities of the cells were examined, we found that inhibition of CTGF expression decreased the ability of the cells to invade through matrigel-coated transwells (Figure 3e). The limited ability to invade the transwells by the shCTGF9-expressing melanoma cells was associated with a reduction in matrix metalloproteinase-9 expression (Figure 3f). Taken together, these *in vitro* experiments demonstrate that CTGF inhibition decreases key cellular activities associated with melanoma tumor progression including: growth in soft agar, motility and invasion.

To investigate the effect of CTGF inhibition on tumor growth and metastasis *in vivo*, we utilized an orthotopic mouse melanoma model and a metastatic lung metastasis assay. The orthotopic model allowed us to assess the role of CTGF on primary tumor growth over time. We observed a statistically significant decrease in primary cutaneous tumor growth of CTGF-deficient cells compared with CTGF wild-type cells (Figure 4a). As CTGF is hypoxia-responsive *in vitro*, we determined whether CTGF is localized to hypoxic regions within K457 orthotopic tumors. For this purpose, mice were injected with pimonidazole HCL (hypoxyprobe-1) before euthanizing. Pimonidazole forms adducts with thiol containing proteins in cells having an oxygen concentration <14 μM. Therefore, staining for pimonidazole is a marker for hypoxia *in vivo*. We observed an overlap between the regions that stained positive for pimonidazole and CTGF indicating that CTGF expression is induced by intratumoral hypoxia *in vivo* (Figure 4b). In addition, we utilized a cohort of mice in the orthotopic model to investigate the role of CTGF in spontaneous metastasis to the lung. At days 44–50 following orthotopic tumor injection, the lungs were harvested from mice injected with control (n=5) and shCTGF (n = 4) cells and analyzed by qRT–PCR analysis for human glyceraldehyde 3-phosphate dehydrogenase expression. Tumor burden in the lungs was decreased in mice injected with shCTGF cells compared with the mice injected with K457 control tumor cells (Supplementary Figure 3). These findings

demonstrate that in an orthotopic model of human melanoma, CTGF has a significant role in primary tumor growth and metastases.

In order to assess the role of CTGF in late stages of lung metastasis, we utilized the tail vein lung metastasis model. When control and CTGF-deficient K457 cells were injected into the tail vein of SCID mice, we observed a significant reduction in metastatic tumor burden in the lungs of mice injected with shCTGF cells compared with mice injected with parental K457 cells (Figures 4c and e). Histological analysis revealed significantly reduced tumor to lung area in mice injected with shCTGF9 compared with parental K457 cells (Figure 4c). Immunohistochemical staining for tyrosinase and CTGF confirmed the presence of melanoma derived cells expressing CTGF in metastatic lesions in the lung (Figure 4d). Consistent with these findings, we found a significant decrease in average lung weight and the appearance of macroscopic lung lesions in mice injected with CTGF-deficient cells (Figures 4c and e). qRT–PCR of lung samples show the ratio of human glyceraldehyde 3-phosphate dehydrogenase (a measure of the human melanoma tissue) to total 18S (both mouse and human tissue) is markedly lower in K457 shCTGF9 injected mouse lungs (Figure 4e). Importantly, we also observed that treatment with the anti-CTGF antibody 3 days after tumor seeding (40 mg/kg, twice a week, intraperitoneally) was sufficient to significantly reduce metastatic tumor burden in the lungs. Histological analysis of tumor to lung ratio and average lung weight was significantly reduced in mice treated with anti-CTGF antibody (Figure 4c). Purified polyclonal human IgG was used as a control and had no effect on the development of lung metastases (data not shown). Results from these *in vivo* models demonstrate that CTGF expression in melanoma cells has an important role in tumor growth and metastasis to the lung.

Discussion

In this report, we demonstrate that advanced melanoma and metastases strongly upregulate the expression of CTGF and are dependent on CTGF expression for both primary tumor growth and metastatic colonization in the lung. We show that intratumoral hypoxia is a key factor driving CTGF expression in melanoma and activates the expression of CTGF through HIF-dependent mechanisms. HIF may activate CTGF expression in human melanoma through multiple mechanisms. First, HIF may directly activate CTGF expression through direct binding to hypoxia response elements within the CTGF promoter or enhancer. In mice, a functional hypoxia response element has been identified where HIF-1 directly activates the expression of CTGF in hypoxic renal epithelial cells.[24] However, a functional hypoxia response element within human CTGF has not yet been described raising the possibility that HIF may activate CTGF through indirect mechanisms.[25] Indeed, we and others have found that hypoxia increases transforming growth factor-β and SMAD signaling, which is a well-characterized pathway for CTGF activation during fibrosis and tumorigenesis (Figure 1a).[26–28] In a study with human melanomas, Braig *et al*.[21] were not able to show regulation of CTGF by members of the transforming growth factor-β family; however, the effect of inhibiting transforming growth factor-β signaling in the presence of hypoxia was not examined. More recently, FOXO proteins have been identified as key transcriptional mediators of HIF-induced CTGF expression in endothelial cells.[29] Further elucidation of the mechanisms by which HIF signaling increases CTGF expression may reveal additional opportunities for therapeutic intervention in the treatment of melanoma.

We show that CTGF is a critical factor for malignant melanoma progression. CTGF is a cysteine-rich matricellular protein composed of four structural domains with homology to insulin-like growth factor-binding proteins (domain 1), a von Willebrand factor type C repeat (domain 2), a thrombospondin type 1 repeat (domain 3) and a cysteine-knot domain (domain 4). Each of these domains is proposed to have a distinct biological function,

although these domain specific functions have not rigorously been demonstrated to date.[30,31] CTGF is thought to primarily function through direct binding to cell adhesion receptors including integrins and heparan sulfate proteoglycans. Additionally, CTGF can bind to and modulate the activity of other growth factors and cytokines including transforming growth factor-β, bone morphogenetic protein-4, basic fibroblast growth factor and vascular endothelial growth factor.[32] CTGF modulates a number of cellular functions associated with pathologic processes including extracellular matrix production, survival, apoptosis, desmoplasia, tumor cell proliferation, adhesion, migration, angiogenesis, metastasis and vascular remodeling.[15–17] The role of CTGF during cancer development varies considerably and has just begun to be defined for melanomas.[21] Expression of CTGF has been associated with both tumor promotion and tumor suppression in different cancer types. Overexpression of CTGF correlates with decreased survival in patients with esophageal adenocarcinoma, glioblastoma, gastric cancer and adult acute lymphoblastic leukemia.[33–36] In contrast, CTGF expression levels also correlate with increased survival in patients with chondrosarcoma and lung cancer.[37,38] In pancreatic cancer, we have recently demonstrated a critical role for tumor-derived CTGF in tumor growth, and have demonstrated efficacy of anti-CTGF antibodies in preclinical models.[23,39] Here, we show that CTGF expression correlates with melanoma tumor progression and metastasis. We identify a critical role for tumor-derived CTGF in melanoma progression *in vivo* that is associated with its regulation of melanoma tumor cell invasion and migration.

Importantly, our studies demonstrate that single-agent anti-CTGF therapy is sufficient to suppress metastatic melanoma tumor progression *in vivo*. These findings have important clinical implications for the treatment of advanced melanoma. The importance of identifying novel therapeutic targets for melanoma therapy is evident from the myriad of reports on the development of resistance to targeted therapies against mutant BRAF and the limited treatment options available for patients with melanomas that lack BRAF alterations. Clinically, we found that CTGF is highly expressed in the majority of advanced primary melanomas and metastases, whereas CTGF is not expressed at high levels in normal skin. While the status of BRAF mutations in our tissue microarray specimens is unknown, we have found increased expression of CTGF in melanoma cell lines with BRAF mutations, as well as in those without BRAF mutations. In the majority of our studies, we used the K457 cell line, which is derived from a metastatic abdominal mass expressing the $BRAF^{V600E}$ mutation. In these cells, anti-CTGF therapy by FG-3019 effectively inhibited metastatic colonization in the lung indicating that anti-CTGF therapy may be clinically effective in both BRAF mutant and wild-type patient populations. Our studies indicate that by inhibiting CTGF directly, we are targeting the downstream cellular/effector function critical for tumor progression and metastatic spread and not the upstream signaling pathway in which blocking $BRAF^{V600}$ has triggered resistance through the activation of redundant pathways. Importantly, anti-CTGF therapy using anti-CTGF monoclonal antibodies has entered clinical trials for the treatment of pancreatic cancer and normal tissue toxicity has not been detected in the phase I trials indicating that anti-CTGF therapy may be a safe and effective therapy for the treatment of melanoma.[23,39]

In summary, our preclinical studies provide strong evidence for clinical evaluation of targeting CTGF in melanoma patients. Inhibition of CTGF represents a new therapeutic target that may be used alone or in combination with current treatments for melanoma. These findings have direct clinical implications for the treatment of melanoma where current therapies have limited efficacy.

Materials and Methods

Cell culture

Cell lines were established between 1987 and 1998 at the University of Arizona Cancer Center from primary and metastatic human melanomas as described previously.[40] Human melanoma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS), 1% glutamine and 1% penicillin–streptomycin. Cells were grown in 21% O_2 and 5% CO_2 at 37°C. shRNA clones in pLKO.1 lentiviral vector from Open Biosystems (Lafayette, CO, USA) specific for human CTGF (RHS4533-NM-001901 set, Open Biosystems) were used for these studies. The sequences used for HIF-1α and HIF-2α shRNA were previously described.[41,42]

Expression microarray analysis

Sample preparations and hybridizations were carried out with Stanford Human Exonic Evidence Based Oligo arrays according to established protocols (http://cmgm.stanford.edu/pbrown/protocols/index.html). Data from scanned microarrays were entered into the Stanford Microarray Database for normalization and analysis.[18] Expression changes were calculated by averaging data from duplicate samples and calculating fold change in expression compared with K457 controls. For profiling the effect of *HIF-1α or HIF-2α* shRNA knockdown in K457 cells exposed to 2% oxygen for 24 h, RNA was amplified using the Amino Allyl MessageAmp II aRNA kit (Ambion). Samples from independent triplicate experiments were labeled with Cy5 and hybridized to common reference complementary DNA from untreated K457 cells labeled with Cy3. Data were analyzed using multicomponent significance analysis of microarrays to identify genes significantly changed among the shRNA-expressing cells. Genes with a false-discovery rate lower than 0.5% were extracted to calculate fold changes relative to the normoxic samples. Fold change was calculated to identify genes repressed or induced greater than 1.5-fold by hypoxia.

Western blots

Equal numbers of cells were plated and culture media was collected or protein lysates were generated using 9 M Urea, 0.075 M Tris buffer (pH 7.6), quantified using the Bio-Rad assay (Bio-Rad Labs., Hercules, CA, USA), and subjected to reducing SDS–PAGE using standard methods. Westerns were probed with anti-CTGF (sc-14939, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-HIF-1α (A300-286A, Bethyl Laboratories, Montgomery, TX, USA and NB100-131, Novus Biologicals, Littleton, CO, USA) and anti-HIF-2α (NB100-122, Novus Biologicals). For CTGF double bands around 36–38 kDa can be detected, which is due to post-translational modifications, as has been reported previously.[43] Densitometry was done using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Spheroid formation

Melanoma cells (1×10^5) were cultured into T25 ventilated TC flask in DMEM/F12 medium supplemented with 8% FBS. Cell cultures were incubated in a 37 °C, 5% CO_2, 21% O_2 environment. Formation of K457 spheroids were stimulated by transferring the cultures to a low oxygen incubator (2% O_2, 5% CO_2, 37 °C) for 4–5 days. For immunohistochemistry of spheroids, the cell clusters were collected, pelleted and resuspended in liquefied HistoGel (Richard-Allan Scientific, Kalamazoo, MI, USA). HistoGel cell pellets were embedded in paraffin, sectioned and evaluated using IHC.

Immunohistochemistry and tissue analyses

Primary antibodies, anti-CTGF (sc-14939, Santa Cruz Biotechnology; AF660, R&D Systems, Minneapolis, MN, USA) and anti-Tyrosinase (Pep7, Bethyl Labs, Montgomery,

TX, USA), were used on paraffin sections. For secondary antibodies, Vector Labs (Burlingame, CA, USA): ImmPRESS Anti-Rabbit IgG and Anti-Goat IgG Polymer Detection Kits were used. To visualize the proteins, the slides were treated with 3,3′-diaminobenzidine (DAB) ((Vector Peroxidase Substrate kit SK-41000) or 3-amino-9-ethylcarbazole (AEC)) (Vector Labs) and counter stained with hematoxylin. Paraffin embedded tissue slides were deparaffinized with xylene, rehydrated and unmasked followed by standard histochemical methods. Negative controls for all samples were tissue sections treated with the secondary antibodies alone.

Tissue arrays (ME804, ME482t) were purchased from Biomax (Rockville, MD, USA). CTGF was detected in the tissues by incubation with anti-CTGF antibody (R&D Systems) at 1:100 and counter stained with Hematoxylin as described. CTGF was visualized using both DAB and AEC (Vector Labs). Staining was evaluated by two independent observers blinded from core details. Normal skin samples were used to establish the ‘light’ category. A combined score, based on both the intensity of staining and the corresponding percent of area that stained for each intensity within each tumor core, was assigned. The tumor samples and staining scores were put into categories: low, medium, medium-high and high. The data was summarized by stages of progression and staining intensity. Comparisons were made with normal skin and benign mole samples. Melanin in the tissue sections was clearly distinguishable from the CTGF staining reaction.

Soft agar

1×10^4–5×10^4 K457 stable cells were plated in triplicate in six-well dishes in 0.7% noble agar on top of solidified 1.4% agar, both mixed with an equal volume of DMEM + 20%FBS. Normal DMEM + 10% FBS was added on top of the solidified agar. Cells were maintained at 37 °C in 5% CO_2 for 14–20 days. Antibody treatment concentration was 100 μg/plate. Multiple images were taken using a Nikon Eclipse TS-100 (Nikon Instruments, Melville, NY, USA) at × 10 magnification and colony number was counted, averaged, and graphed with s.e.m. Additional images of the soft agar colonies were collected and ten fields were recorded per plate. Total image size was determined using Adobe Photoshop software and pixels/inch determined. These parameters were then entered into ImageJ to standardize the measurement scale for the area of the colonies.

Wound-healing (scratch) assay

Cells were plated to confluence in six-well dishes in DMEM plus 10% FCS. A 10 μl pipette tip was used to make a scratch wound through the confluent cell layer, and fresh DMEM plus 10% FCS were then added to the cultures. Images of initial scratch (0 h) and time points were recorded. Percent wound closure was determined using Adobe Photoshop to compare initial wound size to those at various time points.

Invasion and migration assays

5.0×10^4 cells were seeded in duplicate on Matrigel-coated inserts (BD BioCoat Matrigel Invasion Chambers, BD Biosciences, Bedford, MA, USA) and uncoated control inserts (BD Falcon, Franklin Lakes, NJ, USA) and BD BioCoat 8 μm control inserts (BD Biosciences, Bedford, MA, USA), then inserts were placed in chambers with 750 μl of 10% FBS as a chemoattractant and incubated under 21% oxygen at 37 °C for 18–24 h. Non-invading cells were removed, membranes were stained with three-Step Stain (Thermo Scientific Richard-Allan Scientific, Rockford, IL, USA), and mounted. For each membrane, two to five random fields were imaged at × 10 magnification, and cells were counted. The percent invasion was calculated using the following equation: Average number of cells invading through Matrigel insert/average number of cells through control insert × 100. The results were compared against untreated parental cells, and significance was determined using Student's *t*-test

($P<0.05$). Migration through fibronectin-coated transwells was carried out as described above, except 8 μm cell culture inserts (BD Falcon) were coated with 50 μg/ml fibronectin (Sigma, St Louis, MO, USA) before assay. Migrated cells were fixed, stained and counted.

Tail vein metastasis and orthotopic xenograft assays

All procedures involving animals and their care were approved by the Institutional Animal Care and Usage Committee of Stanford University in accordance with institutional and NIH guidelines. Four- to six-week-old hairless SCID mice (Charles River Labs, Hollister, CA, USA) were used for the orthotopic xenograft studies. Five hairless SCID mice per experimental group were injected intradermally with 1×10^6 cells. Primary tumor size was measured using calipers in two dimensions and tumor volume calculated as $W^2 \times L \times 0.5$ (where W=shortest of two measurements). Mice were monitored and followed over a 9-week time course. To evaluate the ability of the human melanoma cells to form tumors in the lungs, five hairless SCID mice per experimental group underwent intravenous injection of 7×10^5 cells suspended in 100 μl sterile PBS. Starting 3 days post injection, mice were dosed with FG-3019 anti-CTGF antibody (FibroGen) twice a week over the 30–45 day time course after which they were euthanized and tissues were collected, fixed in 10% formalin and embedded in paraffin. Percent tumor/normal area was determined using hematoxylin and eosin sections and ImageJ.

Staining for hypoxia *in vivo* was done on mice bearing K457 scrambled shRNA orthotopic tumors using the Hypoxyprobe-1 Omni Kit (Hypoxyprobe Inc., Burlington, MA, USA) according to manufacturer's instructions. Immunohistochemistry was performed as described above, using anti-CTGF at 1:25 (sc-14939, Santa Cruz Biotechnologies) at 4°C overnight, secondary biotinylated anti-goat antibody (Vector) followed by strep-horseradish peroxidase , both at 1:150 for 30 min at room temperature.

Quantitative real-time PCR

RNA was reverse transcribed with the iScript complementary DNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Approximately 0.5% of each RT reaction was added to qRT–PCR reactions containing the following in a total volume of 10 μl: 5 μl 2 × SYBR Green mastermix (ABI, Foster City, CA, USA) and 0.2 μl each of 10 μM forward and reverse primers specific for human glyceraldehyde 3-phosphate dehydrogenase, CTGF, HIF-1α, HIF-2α, PGK-1, BNIP3, TBP and both mouse/human 18S. Detection was carried out with the ABIPRISM 7900 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) and expression levels were calculated using the relative standard curve method to determine RNA quantity, normalized to 18S or TBP.

Statistics

Significance was determined by two-tailed Student's *t*-test. A *P*-value of <0.05 was considered significant. Oncomine data was analyzed using the GEO database's GEO2R tool, and the indicated *P*-value (Figures 2b and c) or F-statistic, which combines the *t*-statistics for all pair-wise comparisons giving an overall test of significance (Figure 2c), was determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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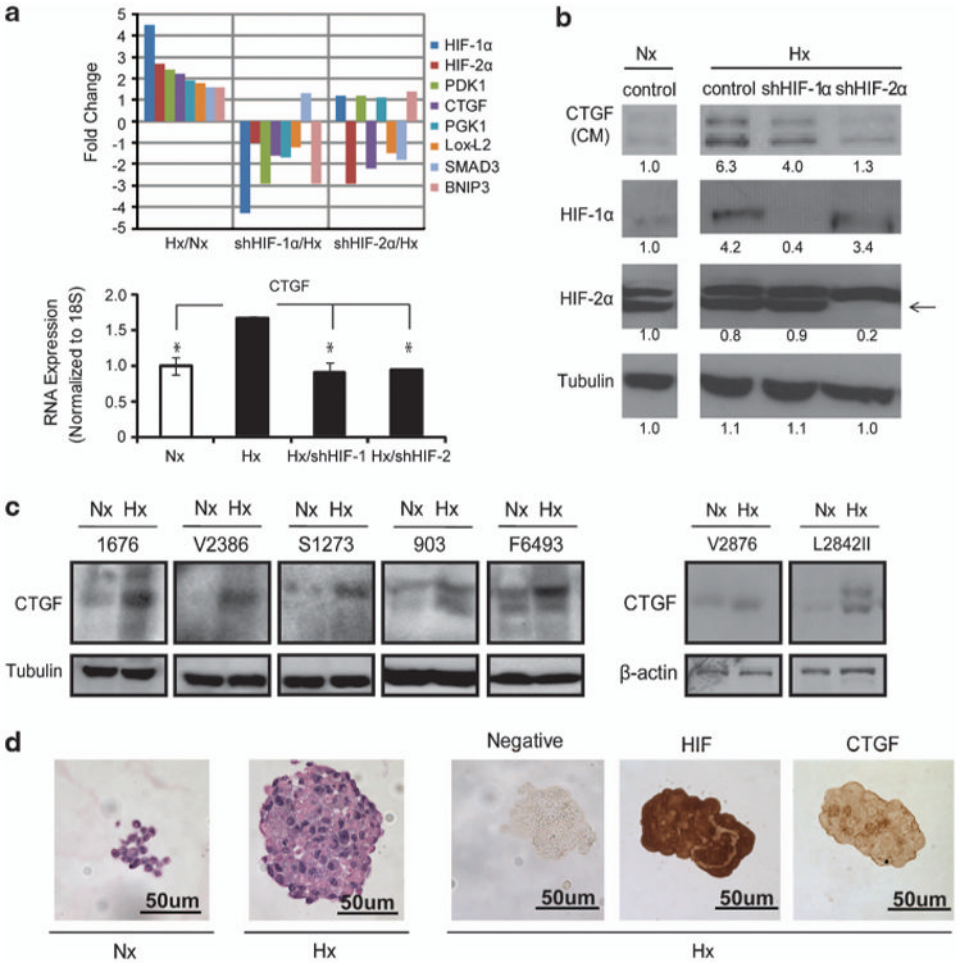

Figure 1.

CTGF is hypoxia-inducible and HIF regulated in melanomas. (**a**) Representative hypoxia-responsive genes associated with metabolism, survival and progression, detected in a complementary DNA expression microarray analysis of K457 metastatic melanoma cells and K457 cells-expressing shHIF-α RNAs, are included in the graph. qRT–PCR of CTGF RNA expression is shown below (*P<0.05, two-tailed t-test). CTGF is regulated by both HIF-1α and HIF-2α. (**b**) Western blot with K457 cells±stable shRNA HIF-α knockdowns, exposed to normoxia (Nx) or hypoxia (Hx, 2% O_2) were probed for CTGF in culture media (CM) and for HIF-1α and HIF-2α in cell lysates. The specific HIF-2α band is indicated by an arrow. Control is K457 shGFP cells. (**c**) A panel of low passage cultures of metastatic melanomas (1676, V2386, S1273, 903, F6493) and primary melanomas (V2876, L2842II) was examined by western analysis for CTGF expression. Hypoxic induction (2% O_2) of CTGF was observed in all cells. (**d**) K457 spheroids that form when exposed to hypoxia were stained with hematoxylin and eosin, anti-HIF-1α and anti-CTGF antibodies.

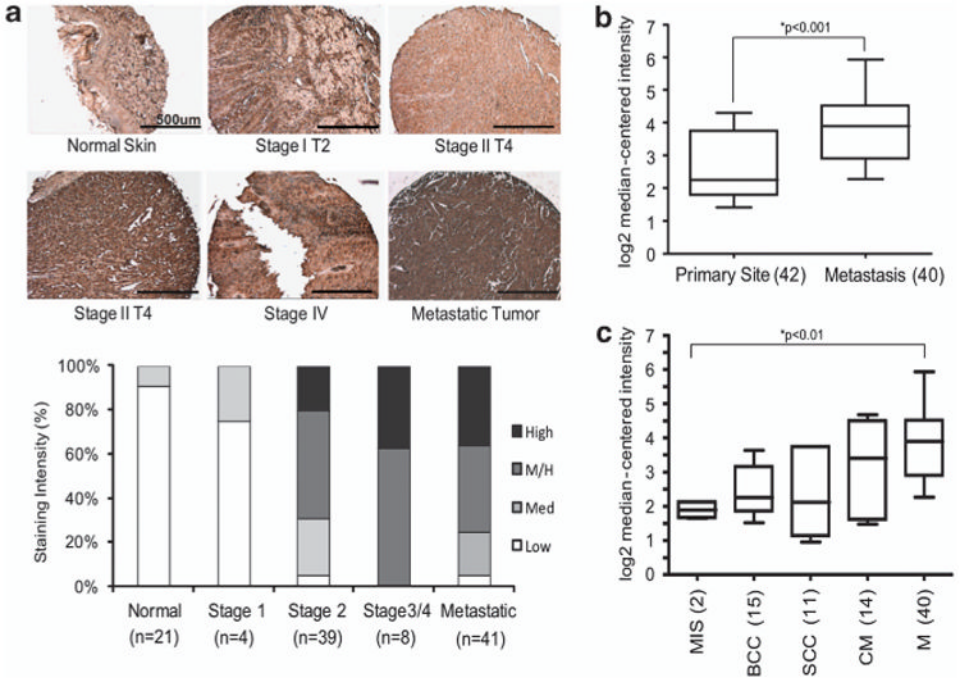

Figure 2.

CTGF expression is elevated in melanomas. (**a**) Tissue microarrays (ME804, ME482; Biomax) were stained for CTGF. Tumors were evaluated for staining intensity by stage. Scale bar = 500 μm. (**b**, **c**) Studies from Riker *et al.*[22] deposited in the Oncomine Database demonstrate elevated CTGF expression in metastatic melanomas versus primary tumors (**P*<0.001, *t*-test) (**b**) and elevated expression in more invasive (M) and cutaneous melanoma (CM) cancers compared with melanoma *in situ* (MIS), basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (F-statistic =7.03101, **P*<0.01) (**c**).

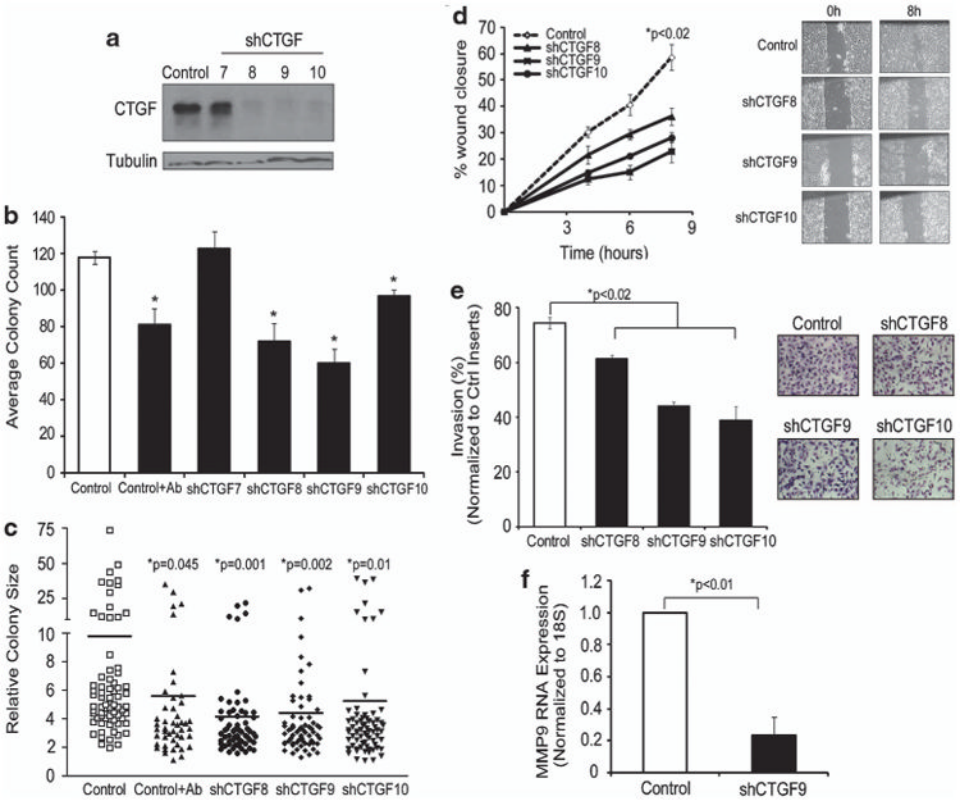

Figure 3.

Knockdown of CTGF decreases tumorigenic and metastatic behavior *in vitro*. (**a**) CTGF-specific shRNAs in K457 cells (shRNAs 8, 9 and 10) show significant reduction in CTGF protein expression. (**b**) Colony formation in soft agar is decreased in K457 cells with stable CTGF knockdown or by treatment with CTGF neutralizing antibody (FG-3109) (**P*<0.05, two-tailed *t*-test). (**c**) Colonies in the soft agar assay were analyzed based on size and results are shown graphically. Average colony size is represented with a horizontal bar. Significant differences were determined by a two-tailed *t*-test. (**d**) A wound-healing assay shows a decreased ability of K457 cells-expressing shCTGF8, shCTGF9 and shCTGF10 to migrate and close the wound (**P*<0.02, two-tailed t-test). (**e**) Cell invasion through matrigel-coated transwells is decreased with stable CTGF knockdown. Error bars are s.e. (**P*<0.02, two-tailed *t*-test). Photographs in **d** and **e** are representative images. (**f**) Expression of matrix metalloproteinase (MMP)9 was detected by qRT–PCR. K457 cells-expressing shCTGF9 show a significant reduction in MMP9 RNA compared with parental cells. Control represents K457 parental cells (**a–f**).

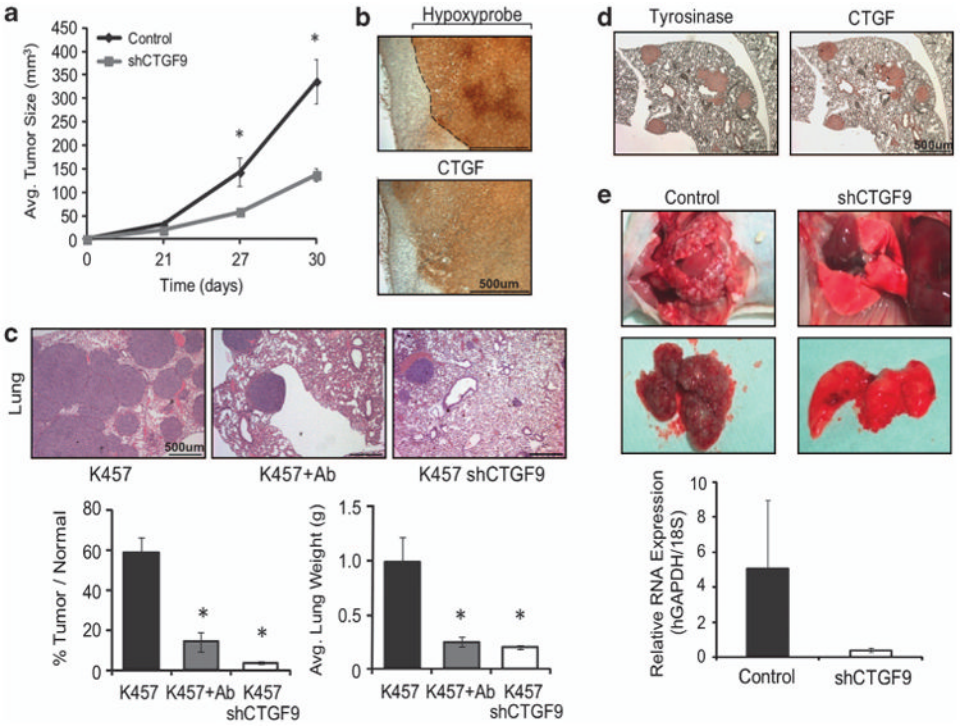

Figure 4.

Knockdown of CTGF decreases metastatic potential *in vivo*. (**a**) K457 cells (control)±stable knockdown of CTGF (shCTGF9) were injected intradermally and tumor volumes measured over time (**P*<0.03, two-tailed *t*-test). (**b**) IHC detecting overlapping regions of CTGF and hypoxia (Hypoxyprobe-1 Omni kit) in serial sections from K457 cutaneous tumors are shown in these representative images. (**c**) Sections of lungs from mice injected intravenously with K457, K457 shCTGF9 cells and K457 treated with anti-CTGF antibody (40 mg/kg, twice a week, intraperitoneal injection; F-3019, FibroGen) are presented. Metastases in mouse lungs were enumerated using Image J on hematoxylin and eosin sections. Percentage of tumor area to total mouse lung area was calculated (**P*<0.001, two-tailed *t*-test). (**d**) Adjacent sections of lungs from mice injected with K457 cells were stained for CTGF and tyrosinase, a melanoma marker. CTGF was observed in the metastatic tumors within the lungs. Scale bars=500 μm (**b–d**). (**e**) Images of lungs show markedly decreased numbers of tumors formed by the K457 shCTGF9 RNA-expressing cells. The graph indicates the ratio of human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH)/18S RNA from lungs of indicted mouse groups. hGAPDH primers detects only human RNA while 18S primers detect both mouse and human RNA.