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The essential role of fibroblasts in esophageal squamous cell carcinoma-induced angiogenesis

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Abstract

Background & Aims—Esophageal squamous cell carcinoma (ESCC) is known to be a highly angiogenic tumor. Here, we investigate the role of the stromal fibroblasts in the ESCC-induced angiogenic response using a novel 3D model.

Methods—A novel assay was developed where co-cultures of ESCC and esophageal fibroblasts induced HMVEC cell vascular network formation in a 3D collagen gel. Biochemical studies showed that the ESCC-induced activation of the fibroblasts was required to induce vascular network formation via a TGF- β and VEGF dependent pathway.

Results—Conditioned media from a panel of 4 ESCC lines trans-differentiated normal esophageal fibroblasts into myofibroblasts via TGF- β signaling. The presence of fibroblasts was essential for efficient HMVEC network formation and the addition of ESCC cells to these cultures greatly enhanced the angiogenic process. The role of TGF- β in this process was demonstrated by the complete inhibition of network formation following TGF- β inhibitor treatment. Finally, we showed that ESCC-derived TGF- β regulates angiogenesis through the release of VEGF from the fibroblasts, and that the VEGF release was blocked following TGF- β inhibition.

Conclusions—This study demonstrates the essential role of fibroblasts in the ESCC angiogenic-induced response and suggests that the pharmacological targeting of the TGF- β signaling axis could be of therapeutic benefit in this deadly disease.

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Introduction

The tumor “organ” consists of a dynamic mixture of tumor cells, fibroblasts, endothelial cells and immune cells that all work together to drive tumor progression¹. Activated fibroblasts, also known as carcinoma-associated fibroblasts (CAFs)², have been identified at the leading edges of many solid tumors, including breast, colon and melanoma^{3–5}. The presence of CAFs within the tumor microenvironment is preceded by the chemoattraction and migration of precursor cells, which can either arise from the surrounding host fibroblasts or from circulating mesenchymal precursors/stem cells^{6, 7}. Once recruited, paracrine tumor-derived growth factors activate the CAFs which undergo a myofibroblastic transdifferentiation, defined by an elongated spindle shape, and the expression of contractile α -smooth muscle actin and vimentin⁸. CAFs are hypothesized to drive tumor progression through the deposition of extracellular matrix (ECM) proteins, the secretion of growth factors and the stimulation of invasion⁹.

One area that has been little explored is the potential role of CAFs in tumor angiogenesis. Much of the growth of solid tumors is dependent upon the ready supply of nutrients and oxygen from a local blood supply. As tumors grow beyond a few millimeters in size they readily outstrip the local supply of nutrients available through simple diffusion and stimulate the formation of their own tumor vasculature. Although it has been shown that stromal fibroblasts are an important source of the pro-angiogenic factor vascular endothelial cell growth factor (VEGF)¹⁰, it has been difficult to study the interaction of carcinoma cells, fibroblasts and endothelial cells in a physiologically relevant model. In the present study we have used a novel 3D *in vitro* model where the interaction of esophageal squamous cell carcinoma cells (ESCC) with fibroblasts drives vascular network formation in a 3D collagen gel. We show that ESCCs require the presence of stromal fibroblasts to stimulate vascular network formation, thereby suggesting that fibroblasts are the critical mediators of angiogenesis in this system. Mechanistic studies reveal that paracrine TGF- β from the ESCC leads to activation of the fibroblasts and that pharmacological inhibitors of TGF- β signaling can reverse both fibroblast activation and vascular network formation.

Materials and methods

Cell Lines

Esophageal cancer cells, TE cell lines (TE1, -8, -10, -11, -12) were cultured as previously described¹¹. Human esophageal keratinocytes EPC2 have been described previously^{11, 12}. Human microvascular endothelial cells HMVEC are available commercially through Cascade Biologics, Inc. (Portland, OR)¹³. Primary human esophageal fibroblasts designated as FEF3 were isolated from human fetal esophagus as described previously¹¹. FEF3 cells were stably transduced using ViraPower Lentiviral expression system containing the gene for GFP. GFP lentivirus was raised in our laboratory and the FEF3 cells were transduced in the presence of 6 μ g/ml polybrene. Forty-eight hours after transduction cells were selected in the presence of 10 μ g/ml blasticidin for 14 days.

Antibodies and Reagents

The following antibodies were used in this study: anti-human CD31 (Dako, Carpinteria, CA), anti- α -smooth muscle actin (Sigma-Aldrich, St. Louis, MO), anti-Von Willebrand Factor (Neomarkers, Fremont, CA), anti-fibroblast activation protein (EMD Biosciences, San Diego, CA), anti-TGF β RII (Santa Cruz, CA) and phalloidin-Texas Red (Molecular Probes, Eugene, OR). The Anti-smad2, anti-phospho-smad2 (Ser465/467), anti-phospho-smad3 (Ser423/425) and Smad1 (Ser463/465) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Recombinant human TGF- β 1 was purchased by R&D systems, Inc. (Minneapolis, MN). SB505124, GW-788388 and GW654652 were provided by GlaxoSmithKline (Collegeville,

PA). Bevacizumab (Avastin ®) was obtained from the Pharmacy of the Hospital of the University of Pennsylvania.

In vitro 3D Network Formation Assay and Fluorescence Imaging

Reconstruction of vessel-like structure in 3D collagen gels and subsequent fluorescent staining of networks/cords in whole-mount gels were performed as previously described¹³. Briefly, HMVEC were cultured as monolayers on bovine type I collagen-coated 24-well plates at 1.5×10^5 cells/well for 24 hour and overlaid with acellular collagen mixed in $10 \times$ Medium199 (Invitrogen) with heparin (100U/ml), vitamin C (50 μ g/ml), and FBS (1%). After polymerization of the collagen gels, the cells were further overlaid with a second collagen layer containing each $1.0\text{--}2.5 \times 10^5$ cells/ml FEF3, 0.5×10^5 cells/ml TE cells, or both cells. Wells were then filled with EBM-2 medium containing EGM-2MV. The reconstructs were incubated at 37°C for 7 days. To prepare for staining, medium was removed, and the collagen gels were fixed in Prefer (Anatech LTD, Battle Creek, MI) for 4 hours at room temperature. Gels were processed as whole-mounts. After blocking with 1% bovine serum albumin, gels were stained with monoclonal anti-CD31 antibody followed by Texas Red-conjugated secondary antibody. Gels were treated with VectaShield with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories), and staining of endothelial cells networks/cords of gels were photographed under a Nikon E600 fluorescent microscope.

The capillary-like networks were scored by counting of the number of CD31-stained branches. One branch was counted to be 3 cells thick or less (to discount disorganized masses of HMVECs) and at least 3 whole cells long. At least 5 randomly chosen low power fields were counted per sample (X10 magnification). Each figure shows one representative experiment. Data show the mean of at least three independent experiments.

Immunofluorescence Microscope

For co-cultures, FEF3 cells and TE cells mixed in a 1:1 ratio (2.5×10^4 cells per each) were seeded onto glass cover-slips in six-well plates and cultured in DMEM containing 10% FBS for 48 hours. Cells were then fixed and stained as described previously¹¹.

Western Blotting Analysis

Subconfluent cells were lysed and separated on a 4 to 12% sodium dodecyl sulfate-polyacrylamide gel, before being blotted as described previously¹¹.

Treatment of FEF3 cells with conditioned media

For preparation of conditioned medium, TE cells were cultured with DMEM containing 10% FBS overnight. Supernatants were removed and cells were washed with DMEM. Cells were cultured for 48 hours with fresh medium DMEM containing 2% FBS. FEF3 cells were cultured overnight with DMEM containing 10% FBS. Supernatants were replaced and cultured with fresh culture medium DMEM containing 2% FBS with or without TGF- β 1, or cultured with conditioned medium for each time.

TGF- β 1/2 ELISA

Cells were cultured overnight with DMEM containing 10% FBS. Supernatants of these cells were removed and cells were washed with DMEM basal medium. Cells were cultured for 48 hours with fresh culture medium DMEM, containing 2% FBS. These supernatants were measured as samples using each ELISA. ELISA kits for TGF- β 1, β 2 and VEGF were purchased from R&D systems. These assays were performed according to manufactures' instructions. For TGF- β 1 and β 2, samples were activated by adding 0.1 ml of 1M HCL for 10 min and

neutralized with 100 μ l of 1.2 M NaOH/0.5 M HEPES before assay to measure the total amount of TGF- β .

VEGF ELISA

For 2D co-cultures, fibroblasts and TE1 cells at a 1:1 ratio were seeded onto plate and cultured with or without SB-505124 compound overnight. Supernatants were removed and cells were washed with DMEM. Cells were cultured for 48 hours with fresh culture medium DMEM containing 2% FBS with or without the compound. These supernatants were measured as samples using VEGF ELISA. For 3D culture, each sample was cultured with or without SB-505124 compound for 48 hours and these supernatants were measured using the VEGF ELISA kit (R&D Systems).

Cell Proliferation Analysis

Cells were plated into a 96-well plate at a density of 2.5×10^4 cells per ml and left to grow overnight. Cells were treated with increasing concentrations of TGF- β , SB-505124 or GW788388 in triplicate. Proliferation was analyzed using the MTT assay as previously described ¹⁴.

Statistics

Unless otherwise stated, all experiments show the mean $\pm$ SD of at least three independent experiments. Statistical significance was measured using the Student's *t* test, where $P < 0.05$ was judged to be significant.

Results

The presence of fibroblasts is required for ESCC-induced angiogenesis

Studying the role of fibroblasts in ESCC-induced network formation *in vivo* is technically challenging. To overcome some of these issues, we developed a novel 3D model of ESCC-induced angiogenesis allowing us to study the role of CAFs in vascular network formation ¹³ (see supplemental figure 1 for scheme). In the model, factors derived from either fibroblasts or a mixture of fibroblasts and ESCC cause human microvascular endothelial cells (HMVECs) to detach from the tissue culture plate and migrate upwards into the 3D collagen gel, where they organize and form vascular networks. In an initial series of studies it was found that co-culture of the ESCC lines with the HMVECs (TE1, TE8 and TE11) did not lead to vascular network formation (Figure 1A). However, co-culture of the esophageal fibroblasts (FEF3) with the HMVECs led to endothelial cell migration and formation of moderately well differentiated vascular networks as shown by CD31 and Von-Willebrand Factor staining (Figure 1B and Figure not shown). An increase in the fibroblast concentration from 1×10^5 cells per ml to 1.5×10^5 and 2.5×10^5 cells per ml was associated with increased vascular network organization (Figure 1B). Addition of ESCC to the fibroblast/HMVEC co-cultures had the most striking effects upon vascular network formation, and led to the establishment of very organized capillary-like structures (Figure 1C). Increasing the numbers of both the fibroblasts and ESCC were associated with significantly more organized capillary networks (Figure 1B, D, E), demonstrating that the interaction of both the ESCC and fibroblasts was critical for efficient vascular network formation.

Co-culture of esophageal fibroblasts with ESCC leads to their activation and transdifferentiation into myofibroblasts

CAFs are typically in an activated state, having undergone transdifferentiation to a myofibroblast phenotype. Growing the human esophageal fibroblasts with the ESCC line TE1 for 48 hrs, led to a change in phenotype associated with the increased cytoplasmic expression

of the myofibroblast marker α -smooth muscle actin and fibroblast activation protein (FAP) (Figure 2A–B, and not shown). Three additional esophageal carcinoma lines were also noted to induce a similar degree of myofibroblast transdifferentiation (TE11, TE8, and TE12; Figures 2C–D). The induction of the myofibroblast phenotype was induced through soluble factors derived from the ESCC as demonstrated by the ability of conditioned media from a panel of ESCC lines to induce α -smooth muscle actin expression in the fibroblasts (Figure 2E). In contrast, conditioned media from primary human esophageal keratinocytes (EPC2) was unable to induce α -smooth muscle actin expression in the fibroblasts (Figure 2F).

Paracrine TGF- β secreted from ESCC is responsible for esophageal fibroblast transactivation

ELISA experiments demonstrated that all 4 ESCC lines secreted high levels of both TGF- β 1 and TGF- β 2 (Figure 3A). To rule out the role of other possible growth factors, further studies were performed revealing that the ESCCs secreted very little PDGF or insulin-like growth factor (IGF-I) (data not shown). In a similar manner to that of ESCC-conditioned media, exogenous TGF- β was also shown to induce esophageal fibroblast transdifferentiation associated with increased α -smooth muscle actin expression (Figure 3B). Stimulation of the fibroblasts with exogenous TGF- β was also accompanied by increased SMAD signaling, as shown by the rapid increase in phospho-SMAD2 (Figure 3B). Evidence for TGF- β 1 being the ESCC-derived fibroblast-activating factor came from the similar induction of nuclear SMAD3 accumulation seen to both exogenous TGF- β 1 and ESCC conditioned media (Figure 3C). Next, we investigated whether exogenous TGF- β would stimulate the fibroblasts to drive vascular network formation. Treatment of esophageal fibroblast/HMVEC co-cultures with 1 ng/ml TGF- β for 7 days led to a statistically significant increase in capillary network formation (Figure 3D) and demonstrated the essential role of TGF- β -induced fibroblast differentiation in the angiogenic process. To demonstrate the essential role of the fibroblasts in TGF- β -induced vascular network formation, we treated 3D mono-cultures of endothelial cells with TGF- β (1 ng/ml) and observed very little vascular network formation. Indeed, the endothelial cells remained attached to the plates and did not migrate upwards into the collagen (Supplemental Figure 2).

TGF- β plays a complex role in tumor progression and is known to be growth inhibitory to most epithelial cell types. To overcome the effects of TGF- β , most carcinoma cells escape by downregulating their expression of TGF- β receptors and instead secrete autocrine/paracrine TGF- β that recruit the surrounding stromal cells. In agreement with this idea, the ESCC lines tested were not found to express any TGF- β receptor subtype II protein, whereas esophageal fibroblasts and the parent esophageal keratinocyte line EPC2 maintained receptor expression (Figure 3E). The ESCC lines were shown to have escaped the inhibitory effects of TGF- β and proliferated normally in the presence of increasing concentrations of TGF- β (0.1 – 10 ng/ml), whereas the primary human esophageal keratinocyte line EPC2 was growth arrested (Figure 3F).

The TGF- β specific inhibitor SB505124 inhibits ESCC-induced fibroblast transdifferentiation

Pharmacological approaches that block tumor neoangiogenesis are an attractive therapeutic option. We next tested whether the specific inhibitor of TGF- β receptor kinase SB505124 blocked fibroblast transdifferentiation. Pre-treating the esophageal fibroblasts with increasing concentrations of SB505124 (0.01 – 10 μ M) successfully blocked the TGF- β induced increases in α -smooth muscle expression in the esophageal fibroblasts (Figure 4A). Maximal inhibition of the TGF- β induced fibroblast activation was seen at 1 μ M SB505124 and this concentration was selected for all subsequent experiments. Treatment of the cells with SB505124 also blocked α -smooth muscle actin induction and SMAD2 activation following treatment with ESCC conditioned media (Figure 4B–C). To investigate whether TGF- β inhibition was growth

inhibitory on any of the cell types used in the neoangiogenesis assay, endothelial cells (HMVEC), fibroblasts (FEF3) and 3 ESCC lines (TE1, TE8 and TE12) were treated with increasing concentrations of each of two structurally distinct small molecule inhibitors of TGF- β (SB505124 and GW788388) for 72 hrs. Measuring levels of proliferation using the MTT assay revealed that neither TGF- β inhibitor affected the growth of any of the cell types tested (Figure 4D–E).

The TGF- β inhibitors SB505124 and GW788388 inhibit ESCC-induced vascular network formation

We next investigated whether inhibition of fibroblast activation blocked ESCC-induced vascular network formation. Co-culture of fibroblasts with the HMVECs led to endothelial cell migration but very little differentiated vascular network formation (Figure 5A). Treatment of the fibroblast/HMVEC co-cultures with the TGF- β inhibitor GW788388 (1 μ M) did not significantly alter the level of vascular network organization (Figures 5A), demonstrating that there was only limited fibroblast activation in the absence of ESCCs. As before, addition of the ESCC lines TE1 and TE8 to the fibroblast/HMVEC co-culture dramatically increased the level of vascular network organization (Figures 5B and 5C). In contrast, treatment of the ESCC/fibroblast/HMVEC co-cultures with either SB505124 (1 μ M) or GW788388 (1 μ M) led to a complete reversal of vascular network formation (Figures 5B, 5C). These results demonstrate that inhibition of fibroblast activation by small molecule TGF- β inhibitors blocks ESCC-induced neoangiogenesis (Figure 5D).

Pharmacological inhibition of TGF- β inhibits VEGF-secretion from both esophageal fibroblasts and ESCC

VEGF is one of the most potent inducers of angiogenesis. To investigate the possible role of paracrine VEGF release from esophageal fibroblasts in ESCC-induced neoangiogenesis, we tested VEGF release from co-cultured and TGF- β stimulated esophageal fibroblasts using an ELISA. Unstimulated esophageal fibroblasts produce a basal level of VEGF under 2D cell culture conditions and this was significantly increased when the fibroblasts were grown under 3D conditions (Figure 6A–B). Treatment of the fibroblasts with the TGF- β inhibitor SB505124 did not reduce basal levels of VEGF under 2D cell culture conditions (data not shown), however it did inhibit basal VEGF release when the fibroblasts were grown under 3D cell culture conditions (Figure 6B). This demonstrated that there was some level of fibroblast activation when the cells were switched from the 2D to 3D cell culture conditions. Stimulation of the fibroblasts with exogenous TGF- β (1 ng/ml) led to a significant increase in the level of VEGF secreted (Figure 6A). In a similar manner, co-culture of the fibroblasts with the ESCC line TE1 also led to a significant increase in VEGF secretion under both 2D and 3D cell culture conditions (Figure 6A–B). Treatment of the ESCC/fibroblast co-cultures with SB505124 (1 μ M) led to a significant inhibition in the level of VEGF, demonstrating a possible mechanism by which ESCC-activated fibroblasts can induce vascular network formation. As a final test, we determined whether inhibition of VEGFR2 signaling via the selective inhibitor GW654652 could also block ESCC-induced vascular network formation. Co-culture of the HMVEC and fibroblasts led to some limited vascular network formation after 7 days of culture, and this was not affected by the presence of GW654652 (1 μ M) (Figure 6C). Treatment of the ESCC, fibroblast, HMVEC cultures with GW654652 (1 μ M) did not completely inhibit vascular network formation, but did lead to a reduction in the number of endothelial cell tubes formed (Figure 6C). Analysis of these results demonstrated a significant reduction ($P < 0.005$) in the extent of ESCC-induced vascular network formation following GW654652 treatment (Figure 6D). Similar results were also seen upon ESCC-induced vascular network formation following treatment with an antibody directed against VEGFR, Bevacizumab (Avastin®) (supplemental figure 3). A sample scheme demonstrating how ESCC cells stimulate fibroblasts to drive vascular network formation is shown in figure 7.

Discussion

Here we show for the first time, using a novel 3D *in vitro* model of the tumor microenvironment, the critical role of the host fibroblasts in directing ESCC-induced vascular network formation. This model differs from that in previous studies as it contains cancer cells, fibroblasts and endothelial cells. Co-culture of ESCCs with HMVECs did not stimulate vascular network formation in the absence of fibroblasts. Whereas the co-culture of fibroblasts with HMVECs induced some endothelial cell migration into the collagen, it was associated with large, poorly defined vascular networks with little organized branching. Truly organized vascular network formation was only seen when ESCCs were added to the fibroblast/HMVEC cultures. This suggested both that the fibroblasts were absolutely required for endothelial tube formation and that a prior fibroblast activation step, via factor(s) released from the ESCC were required for efficient network formation. Activated fibroblasts are often observed in the stroma associated with growing tumors^{15, 16}. In agreement with this observation, we noted that co-culture of ESCCs with esophageal fibroblasts led to their transdifferentiation into the myofibroblast phenotype. Myofibroblasts are known to be a good model for CAFs as the activated fibroblasts within the tumor milieu exhibit a phenotype that is virtually indistinguishable from that of myofibroblasts¹⁷.

One of the best known factors responsible for the phenotypic switch of fibroblast to myofibroblast is transforming growth factor (TGF)- β ¹⁸. Loss of TGF- β receptor function is a common event in cancer¹⁸ and ESCCs are no exception. They typically become insensitive to the growth inhibitory effects of TGF- β through either the loss of TGF- β receptor expression or the acquisition of mis-sense mutations in TGF- β RII^{19–21}. In agreement with these published studies we found that our panel of ESCC lines were refractory to the growth inhibitory effects of exogenous TGF- β , whereas primary human esophageal keratinocytes were strongly growth inhibited. Loss of TGF- β RII expression in the ESCC lines was also accompanied with the autocrine/paracrine production of both TGF- β 1 and TGF- β 2.

Both ESCC-conditioned media and exogenous TGF- β were similarly able to induce the myofibroblast phenotype and stimulate SMAD signaling, suggesting that TGF- β was the factor likely for the fibroblast activation observed. The requirement for TGF- β induced fibroblast activation in neoangiogenesis was demonstrated by the ability of exogenous TGF- β to enhance vascular network formation in the absence of the ESCC. Further evidence for the role of TGF- β in ESCC-induced fibroblast activation came from studies showing that a pharmacological inhibitor of TGF- β blocked the ability of ESCC conditioned media-induction of the myofibroblast phenotype in esophageal fibroblasts. Having demonstrated that ESCC secreted TGF- β is essential for fibroblast-induced activation, we next tested whether pharmacological inhibition of TGF- β signaling could inhibit vascular network formation. For these studies we used SB505124, a selective TGF- β receptor antagonist with very little activity against any other kinase tested²². Increasing concentrations of SB505124 were found to inhibit myofibroblast transdifferentiation induced both by TGF- β and ESCC conditioned media. The essential role of TGF- β induced fibroblast activation in vascular network formation was indicated by the fact that SB505124 and the structurally unrelated TGF- β inhibitor GW788388 significantly inhibited ESCC-induced neoangiogenesis. The requirement for both ESCCs and fibroblasts the angiogenic process was demonstrated by the fact that neither of the TGF- β inhibitors tested blocked the limited amount vascular network formation induced by fibroblasts alone.

ESCC-induced fibroblast activation was demonstrated to drive vascular network formation through the stimulation of vascular endothelial cell growth factor (VEGF) release. VEGF is a potent pro-angiogenic factor that stimulates endothelial cell migration and proliferation, as well as regulating microvascular permeability²³. There is compelling evidence that fibroblasts can be a major source of growth factors that may contribute to tumor progression and possibly

angiogenesis^{9, 17, 24}. Studies using transgenic mice expressing GFP under the control of VEGF promoter showed strong staining GFP staining in the stroma of spontaneously arising mammary tumors¹⁰. Here we demonstrate that fibroblasts activated either by exogenous TGF- β through co-culture with ESCCs secrete significantly higher VEGF levels through a mechanism involving SMAD signaling. The fact that a selective inhibitor of VEGFR signaling blocks efficient ESCC-induced vascular network formation provides the link between ESCC-induced fibroblast activation and angiogenesis in this model. In agreement with this, others have also shown that fibroblasts can release VEGF in response to hypoxic conditions^{25, 26} and exogenous TGF- β ^{27, 28}. It is unclear whether the ESCC are releasing latent VEGF from the fibroblasts, as has been suggested by others²⁹, or are instead driving *de novo* protein expression.

Here, we have shown that the stromal fibroblasts are essential for the angiogenic response in ESCC, opening up an intriguing new possibility for therapy. There is a growing body of evidence suggesting that therapies targeted against angiogenesis can lead to dramatic responses in diseases such as colorectal carcinoma, particularly when combined with established chemotherapeutic regimes³⁰. As ESCCs are typically detected at relatively advanced stages, optimized anti-angiogenic/chemotherapy combinations are ideal novel therapeutic candidates. The postulated role of CAFs in tumor invasion also makes targeting the stromal fibroblast activation through the inhibition of TGF- β signaling an appealing therapeutic option.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

ESCC, esophageal squamous cell carcinoma; CAF, carcinoma associated fibroblast; TGF, (transforming growth factor)- β ; HMVEC, human microvascular endothelial cell.

Figure 1. The interaction of ESCC and fibroblasts drive efficient vascular network formation in 3D culture

Immunofluorescence staining shows GFP-tagged FEF3 esophageal fibroblasts (Green), HMVECs (CD31: Red) and total nuclei (DAPI: Blue). CD31 staining as the inset. Incubation of the HMVECs with each of three ESCC lines (TE1, TE8, TE11) was not associated with any vascular network formation (A). Co-culture of the HMVECs with human esophageal fibroblasts (FEF3) led to increased vascular network formation (B). The addition of ESCCs to the fibroblast/endothelial cell co-culture markedly increased the organization of the vascular networks (C). Increasing the culture time to 14 days dramatically enhanced the organization of the vascular network. (D) ESCC number was also found to increase the degree of vascular network formation. (E) The extent of network formation under each of the culture conditions was scored, with the addition of increasing numbers of ESCC cells being found to significantly increase the level of vascular network formation. Images; scale bar = 200 μ m.

Figure 2. Co-culture of esophageal fibroblasts with ESCCs leads to their activation and transdifferentiation into myofibroblasts

(A) Control GFP-tagged fibroblasts (green) expressed very little α -smooth muscle actin. (B–D) Fibroblasts co-cultured with the ESCC lines (TE1, TE11, TE8, TE10 and TE12) for 48 hours stain strongly for stress fibers of α -smooth muscle actin (α -SMA) (red). (E) Western blot analysis showing that incubation of fibroblasts with conditioned media (CM) from ESCC lines for 47 hrs leads to increased α -SMA expression in esophageal fibroblasts. Blots were stripped and reprobed with anti- β -actin as a loading control. (F) Conditioned media from human esophageal keratinocytes does not induce α -smooth muscle actin expression in esophageal fibroblasts. Images; scale bar = 100 μ m.

Figure 3. ESCC lines secrete paracrine TGF-β that induces the transdifferentiation of esophageal fibroblasts

(A) ELISA assay showing secretion of total TGF-β1 and β2 by esophageal cancer cell lines (TE). TE cells were cultured for 48 h and secreted TGF was measured by ELISA. (B) Exogenous TGF-β1 induces α-smooth muscle actin (α-SMA) expression in fibroblasts and stimulates TGF-related signaling pathways. Exogenous rhTGF-β1 (48 hr) induces α-SMA expression in esophageal fibroblasts. (B) Exogenous rhTGF-β1 induces SMAD2 phosphorylation in human esophageal fibroblasts. (C) Both exogenous rhTGF-β1 and ESCC conditioned media induce SMAD3 activation in esophageal fibroblasts. Fibroblasts were treated with either rhTGF-β1 (1 ng/ml) or by conditioned medium of TE1 for 1 hr. Images show p-SMAD3 up-regulation and its subsequent nuclear translocation (green), cell morphology is indicated by phalloidin (Red) and nuclei by DAPI (Blue). (D) Exogenous TGF-β1 markedly enhances vascular network formation in the absence of ESCC. Fibroblasts and HMVECs were co-cultured in the presence of TGF-β1 (1 ng/ml), for 7 days. Vascular network formation was stained for CD31 (red), fibroblasts (GFP, green) and nuclei (DAPI, blue). Inset shows CD31 staining alone. Graph shows the significantly ($P<0.005$) increased numbers of micro-capillary networks per field treated by TGF-β1. (E) Western blot showing expression of TGFβRII expression in a panel of ESCC lines, fibroblasts and primary human esophageal

keratinocytes (EPC2). (F) Exogenous TGF- β 1 (72 hrs) reduces the growth of human esophageal keratinocytes (EPC2) but not ESCC lines. Cell proliferation was measured by the MTT assay. Images; scale bar = 50 μ m. (E); scale bar = 200 μ m.

Figure 4. Pharmacological inhibitors of TGF- β signaling block ESCC induced fibroblast transdifferentiation

(A) The TGF- β receptor kinase inhibitor SB505124 blocks TGF- β induced fibroblast transdifferentiation. Fibroblasts were cultured in the presence of TGF- β 1 (1 ng/ml) and increasing concentrations of SB505124 (0.01 – 10 μ M) for 48 hr followed by blotting for α -SMA expression. (B) SB505124 inhibits α -smooth muscle actin overexpression in fibroblasts stimulated by ESCC-conditioned medium (CM; from TE1 cells). Fibroblasts were cultured with CM as described in the methods in the absence or presence of SB505124 (1 μ M) for 48 h. (C) SB505124 inhibits phosphorylation of SMAD2 induced by ESCC-conditioned media. Fibroblasts were cultured with TGF- β 1 (1ng/ml) or conditioned medium as indicated and treated with SB505124 (1 μ M) for 1 h. (D) TGF- β inhibitors have little effect upon the proliferation of ESCCs, fibroblasts or HMVECs. Fibroblasts (FEF3), HMVECs and esophageal cancer cell lines (TE) were treated with increasing concentrations of TGF β RI inhibitors (either SB505124 or GW788388) for 72 hr before being subject to the MTT assay. The results were evaluated as a percentage of control absorbance.

Figure 5. Pharmacological inhibitors of TGF- β signaling block ESCC-induced vascular network formation

(A) TGF- β inhibition has little effect upon fibroblast-induced vascular network formation. Fibroblasts (FEF3; 1.5×10^5 cells/ml) were incubated with HMVECs in the absence or presence of one of two TGF- β inhibitors (SB505124 or GW788388, both 1 μ M) for 7 days. Cultures were stained for HMVECs (red: CD31), fibroblasts (GFP, green) and nuclei (DAPI, blue). (B, C) TGF- β inhibition completely blocks ESCC-induced vascular network formation. Addition of either SB505124 or GW788388 (both 1 μ M) led to complete inhibition of vascular network formation. (D) Bar graph shows mean data for vascular network formation in the absence and presence of either SB505124 or GW788388. All representative images are shown as 3-color merges, and original monochrome CD31 (white) are inset. Scale bar = 200 μ m.

Figure 6. Activated fibroblasts secrete VEGF leading to increased vascular network formation
 (A) Stimulation of esophageal fibroblasts with either TGF-β through co-culture with ESCCs leads to enhanced VEGF release in 2D adherent culture. Fibroblasts (FEF3) in monoculture or FEF3 and ESCCs (TE1) at the ratio 1:1 in co-culture were cultured in the absence or presence of SB505124 (1 μM) for 48 hr. Supernatants were harvested and quantified for VEGF expression using a specific ELISA. (B) Stimulation of esophageal fibroblasts with either TGF-β or ESCC conditioned media leads to enhanced VEGF release in 3D culture. Mono and co-cultures of esophageal fibroblasts were grown in a 3D collagen in the absence or presence of SB505124 for 48 hr. (C) The VEGF inhibitor GW654652 (1 μM) inhibits vascular network formation. (D) Bar graph shows mean data for vascular network formation in the absence and presence of GW654652. All representative images are shown as 3-color merges, monochrome images of CD31 are inset. Scale bar = 200 μm.

Figure 7. Schematic illustration showing the role of ESCC and fibroblasts in vascular network formation

Esophageal cancer cells produce TGF- β to activate stromal normal fibroblasts. Tumor stromal fibroblasts become transdifferentiated into myofibroblasts that secrete VEGF which in turn induces endothelial cells migration and the formation of a micro-capillary network.