

Inhibition of transforming growth factor- β signals suppresses tumor formation by regulation of tumor microenvironment networks

Tokizaki, Shiori; Podyma-Inoue, Katarzyna A.; Matsumoto, Takehisa; Takahashi, Kazuki; Kobayashi, Miho; Ibi, Haruka; Uchida, Shizuka; Iwabuchi, Sadahiro; Harada, Hiroyuki; Hashimoto, Shinichi; Miyazono, Kohei; Shirouzu, Mikako; Watabe, Tetsuro

Published in:
Cancer Science

DOI (link to publication from Publisher):
[10.1111/cas.16006](https://doi.org/10.1111/cas.16006)

Creative Commons License
CC BY-NC-ND 4.0

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

[Link to publication from Aalborg University](#)

Citation for published version (APA):
Tokizaki, S., Podyma-Inoue, K. A., Matsumoto, T., Takahashi, K., Kobayashi, M., Ibi, H., Uchida, S., Iwabuchi, S., Harada, H., Hashimoto, S., Miyazono, K., Shirouzu, M., & Watabe, T. (2024). Inhibition of transforming growth factor- β signals suppresses tumor formation by regulation of tumor microenvironment networks. *Cancer Science*, 115(1), 211-226. <https://doi.org/10.1111/cas.16006>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal -





Take down policy

If you believe that this document breaches copyright please contact us at vbn@aub.aau.dk providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from vbn.aau.dk on: July 04, 2025

ORIGINAL ARTICLE

Inhibition of transforming growth factor- β signals suppresses tumor formation by regulation of tumor microenvironment networks

Shiori Tokizaki^{1,2} | Katarzyna A. Podyma-Inoue²  | Takehisa Matsumoto³ | Kazuki Takahashi^{2,4} | Miho Kobayashi²  | Haruka Ibi^{1,2} | Shizuka Uchida⁵ | Sadahiro Iwabuchi⁶ | Hiroyuki Harada¹ | Shinichi Hashimoto⁶ | Kohei Miyazono^{7,8}  | Mikako Shirouzu³ | Tetsuro Watabe² 

¹Department of Oral and Maxillofacial Surgical Oncology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), Tokyo, Japan

²Department of Biochemistry, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), Tokyo, Japan

³RIKEN Center for Biosystems Dynamics Research, Yokohama, Japan

⁴Institute of Industrial Science, The University of Tokyo, Tokyo, Japan

⁵Center for RNA Medicine, Department of Clinical Medicine, Aalborg University, Copenhagen, Denmark

⁶Department of Molecular Pathophysiology, Wakayama Medical University, Wakayama, Japan

⁷Department of Applied Pathology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

⁸RIKEN Center for Integrative Medical Sciences, Yokohama, Japan

Correspondence

Katarzyna A. Podyma-Inoue and Tetsuro Watabe, Department of Biochemistry, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan.
Email: kapobch@tmd.ac.jp; t-watabe.bch@tmd.ac.jp

Funding information

Japan Agency for Medical Research and Development, Grant/Award Number: JP21cm0106253, JP22ama221001 and JP22ama221205; Japan Science and Technology Corporation SPRING, Grant/Award Number: JPMJSP2120; Japan Society for the Promotion of Science, Grant/Award Number: JP20H00513, JP20H03851, JP20J11926, JP20K10111, JP21J01294 and JP23H05486

Abstract

The tumor microenvironment (TME) consists of cancer cells surrounded by stromal components including tumor vessels. Transforming growth factor- β (TGF- β) promotes tumor progression by inducing epithelial-mesenchymal transition (EMT) in cancer cells and stimulating tumor angiogenesis in the tumor stroma. We previously developed an Fc chimeric TGF- β receptor containing both TGF- β type I (T β RI) and type II (T β RII) receptors (T β RI-T β RII-Fc), which trapped all TGF- β isoforms and suppressed tumor growth. However, the precise mechanisms underlying this action have not yet been elucidated. In the present study, we showed that the recombinant T β RI-T β RII-Fc protein effectively suppressed in vitro EMT of oral cancer cells and in vivo tumor growth in a human oral cancer cell xenograft mouse model. Tumor cell proliferation and angiogenesis were suppressed in tumors treated with T β RI-T β RII-Fc. Molecular profiling of human cancer cells and mouse stroma revealed that K-Ras signaling and angiogenesis were suppressed. Administration of T β RI-T β RII-Fc protein decreased

Abbreviations: DEG, differentially expressed gene; ECD, extracellular domain; EndoMT, endothelial-mesenchymal transition; EMT, epithelial-mesenchymal transition; EREG, epiregulin; GO, Gene Ontology; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HNSCC, head and neck squamous cell carcinoma; IL-1 β , interleukin-1 β ; MET, mesenchymal-epithelial transition; OSCC, oral squamous cell carcinoma; PAI-1, plasminogen activator inhibitor-1; PECAM-1, platelet endothelial cell adhesion molecule-1; qRT-PCR, quantitative RT-PCR; RNA-seq, RNA sequencing; SM22 α , smooth muscle 22 α ; TGF- β , transforming growth factor- β ; TME, tumor microenvironment; T β RI, TGF- β type I receptor; T β RII, TGF- β type II receptor; VEGF, vascular endothelial growth factor.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2023 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

the expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF), interleukin-1 β (IL-1 β) and epiregulin (EREG) in the TME of oral cancer tumor xenografts. HB-EGF increased proliferation of human oral cancer cells and mouse endothelial cells by activating ERK1/2 phosphorylation. HB-EGF also promoted oral cancer cell-derived tumor formation by enhancing cancer cell proliferation and tumor angiogenesis. In addition, increased expressions of IL-1 β and EREG in oral cancer cells significantly enhanced tumor formation. These results suggest that TGF- β signaling in the TME controls cancer cell proliferation and angiogenesis by activating HB-EGF/IL-1 β /EREG pathways and that T β RI-T β RII-Fc protein is a promising tool for targeting the TME networks.

KEYWORDS

epithelial-mesenchymal transition, heparin-binding epidermal growth factor-like growth factor, interleukin-1beta, transforming growth factor-beta, tumor microenvironment

1 | INTRODUCTION

Tumors develop in a complex and dynamic microenvironment (tumor microenvironment: TME), which consists of cancer cells and tumor stromal components.¹ Communication between these components of the TME activates various growth factor-related signaling pathways leading to the enhanced migration and proliferation of cancer cells and thus cancer progression.²

Transforming growth factor- β (TGF- β) present in the TME plays important roles during various stages of cancer formation and progression.³ There are three TGF- β isoforms, TGF- β 1, TGF- β 2, and TGF- β 3, which activate and transduce TGF- β signals through membrane-bound receptor complexes, including type I (T β RI) and type II (T β RII) receptors.⁴ Activation of TGF- β signaling results in various cellular responses such as induction of epithelial-mesenchymal transition (EMT) in epithelial cancer cells or stimulation of cancer cell motility.⁵ TGF- β also regulates the interactions between various components of the TME. TGF- β secreted by cancer cells promotes the formation of tumor blood vessels.⁶ It also induces endothelial-mesenchymal transition (EndoMT), a process similar to EMT, in endothelial cells^{7,8} and triggers the formation of cancer-associated fibroblasts.

Playing a pivotal role in cancer progression and mediating the interaction between TME constituents, TGF- β signaling has become an important target for the development of anticancer drugs. At present, various approaches to inhibit TGF- β signaling are based on low molecular weight compounds that target T β RI kinase activity^{9,10} and direct targeting of either TGF- β mRNAs by antisense oligonucleotides¹¹ or TGF- β protein by neutralizing monoclonal antibodies.¹² Although all approaches have been examined or are under investigation in clinical trials with various types of cancers, significant clinical outcomes have not been achieved thus far.^{13,14} The concept of interfering with the binding of TGF- β with its receptors by the use of ligand traps [extracellular domains

(ECDs) of TGF- β receptors fused with the Fc domain of IgG] has drawn much attention in recent years.¹⁵ It is speculated that such molecules can trap TGF- β secreted by various components of the TME and thus should effectively reduce ligand concentration in the extracellular environment. We have previously developed the chimeric Fc receptor, T β RI-T β RII-Fc, comprising the ECDs of T β RI and T β RII fused with the human IgG Fc domain.¹⁶ This chimeric receptor interacted with all TGF- β isoforms, suggesting that it was more effective than the previously developed T β RII-Fc, comprising only the ECD of T β RII and Fc region of human IgG, which could trap only TGF- β 1 and - β 3.¹⁷ We also showed that the expression of T β RI-T β RII-Fc significantly reduced primary tumor growth in subcutaneous murine xenograft models of oral squamous cell carcinoma (OSCC)¹⁶ and melanoma.¹⁸ In the present study, we elucidated the mechanisms underlying these phenomena and examined the changes in the TME following administration of recombinant T β RI-T β RII-Fc protein.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Human oral squamous carcinoma cell lines SAS and HSC-4 were obtained from the RIKEN BioResource Center Cell Bank and cultured as described previously.^{16,19} HEK-Blue TGF- β reporter cells (TGF- β responsive reporter cells derived from HEK293 cells) were purchased from InvivoGen. Expi293F and 293FT cells were obtained from Thermo Fisher Scientific. Mouse endothelial cell line MS1 was purchased from the ATCC. HEK-Blue cells were cultured in DMEM (Nacalai Tesque) supplemented with 10% FBS (Sigma-Aldrich), 100 μ g/mL streptomycin, and 100 units/mL penicillin (Nacalai Tesque) in a humidified incubator at 37°C, 5% CO₂. Expi293F cells were propagated in Expi293 Expression Medium (Thermo Fisher

Scientific). MS1 cells were cultured in α -MEM (Fujifilm Wako) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin.

TGF- β 1, - β 2 and - β 3 were purchased from PeproTech or R&D Systems and used at concentrations of 1 or 2 ng/mL depending on the experiment. All TGF- β isoforms were dissolved in 4 mM HCl containing 0.1% BSA. SB431542 (Fujifilm Wako) was prepared in DMSO and used at concentration of 10 μ M. When TGF- β and/or SB431542 was used, the same concentrations of each vehicle (0.1% BSA in 4 mM HCl or DMSO) were maintained in all samples. Treatment with TGF- β or TGF- β /recombinant Fc protein complexes was carried out as described previously.¹⁶ HB-EGF, IL-1 β , and EREG were obtained from R&D Systems and used at concentrations of 1 ng/mL.

2.2 | Xenograft mouse model

SAS cells (1×10^3 cells/100 μ L of Matrigel; Corning) were inoculated s.c. into the left inguinal region of BALB/c 6-week-old male immunodeficient nude mice. When tumor volume reached 50 mm³, mice were randomly divided into two groups: Control-Fc group ($n=15$) and T β RI-T β RII-Fc group ($n=15$). Recombinant Fc proteins, Control-Fc and T β RI-T β RII-Fc (100 μ g/50 μ L of PBS), were given twice a week by i.v. injection through the tail vein. Tumor growth was assessed by caliper measurement of minor and major axes and calculated using the following formula: tumor volume (mm³) = tumor length diameter (mm) \times square of tumor width (mm) \times 0.5. Harvested tumors were subjected to quantitative RT-PCR (qRT-PCR) analysis, immunohistochemistry and RNA sequencing (RNA-seq) as described in other sections. The experiment was repeated twice.

2.3 | Quantitative RT-PCR analysis

The qRT-PCR analysis was undertaken using SYBR Green (Roche) in a StepOne Plus Real-Time PCR System (Applied Biosystems) or QuantStudio 3 (Applied Biosystems). Expression data were normalized for the expression of β -actin (human/mouse), GAPDH (human), or Gapdh (mouse). The genes and the corresponding gene-specific primer sequences are listed in Table S1.

2.4 | Statistical analysis

Values are presented as mean \pm SD. Significant differences between means were determined using the unpaired Student's *t*-test and one-way or two-way ANOVA using Prism 9 software version 9.5.1 (GraphPad). A difference between means was considered statistically significant for *p* value < 0.05 .

Other procedures are described in Supporting Information.

3 | RESULTS

3.1 | T β RI-T β RII-Fc recombinant protein suppresses TGF- β signaling activated by all TGF- β isoforms and induction of EMT in oral cancer cells in vitro

Our previous study using cells expressing T β RI-T β RII-Fc indicated the potential application of Fc chimeric receptors in targeting TGF- β during pathological conditions.¹⁶ Inspired by these results, we performed large-scale purification of recombinant Fc proteins from the conditioned media of Expi293F cells expressing human IgG-Fc (hereafter, Control-Fc), T β RII-Fc, and T β RI-T β RII-Fc. The quality of purified recombinant Fc proteins was confirmed by immunoblotting with anti-human IgG-Fc antibody (Figure S1A) and the HEK-Blue TGF- β reporter system. Treatment of HEK-Blue cells with each TGF- β isoform upregulated TGF- β signaling (Figure S1B), which was not affected by the recombinant Control-Fc protein. Recombinant T β RII-Fc protein inhibited TGF- β signaling induced by TGF- β 1 and TGF- β 3 isoforms. In contrast, T β RI-T β RII-Fc protein suppressed TGF- β signaling induced by all TGF- β isoforms (Figure S1B).

Next, we confirmed the biological activity of recombinant T β RI-T β RII-Fc protein in vitro using the OSCC cell line, SAS. SAS cells show an intermediate state of EMT, so-called partial EMT, characterized by hybrid epithelial-mesenchymal phenotype,²⁰ and can respond to TGF- β as well as to the inhibitors of TGF- β signaling.^{16,21} We have previously reported that SAS cells treated with TGF- β undergo EMT, defined by increased expression of mesenchymal markers, vimentin and smooth muscle 22 α (SM22 α), and decreased expression of epithelial cell markers, claudin-1 and E-cadherin.¹⁶ The T β RI-T β RII-Fc protein and SB431542, a kinase inhibitor of T β RI, significantly suppressed the induction of EMT by all TGF- β isoforms, as revealed by the upregulation of claudin-1 (Figure 1A) and E-cadherin (Figures 1C and S2A) and downregulation of vimentin (Figure 1B,C) and SM22 α (Figure S2B) at RNA and protein levels. In contrast, the T β RII-Fc protein failed to suppress the EMT induced by TGF- β 2, which was consistent with our previous report.¹⁶ These results were confirmed with another OSCC cell line, HSC-4 (Figure S3). During EMT, cancer cells acquire motility. SAS cells treated with TGF- β 2 in the presence of Control-Fc increased their motility, which was significantly suppressed by T β RI-T β RII-Fc (Figure 1D). These results indicate that recombinant T β RI-T β RII-Fc protein can effectively inhibit the induction of EMT by suppressing TGF- β signaling.

3.2 | Recombinant T β RI-T β RII-Fc protein suppresses primary tumor growth in vivo

Next, we examined the efficacy of recombinant T β RI-T β RII-Fc protein in vivo in a subcutaneous murine xenograft model of SAS oral cancer cells. The mice bearing palpable tumors were intravenously administered with recombinant Fc proteins, Control-Fc or

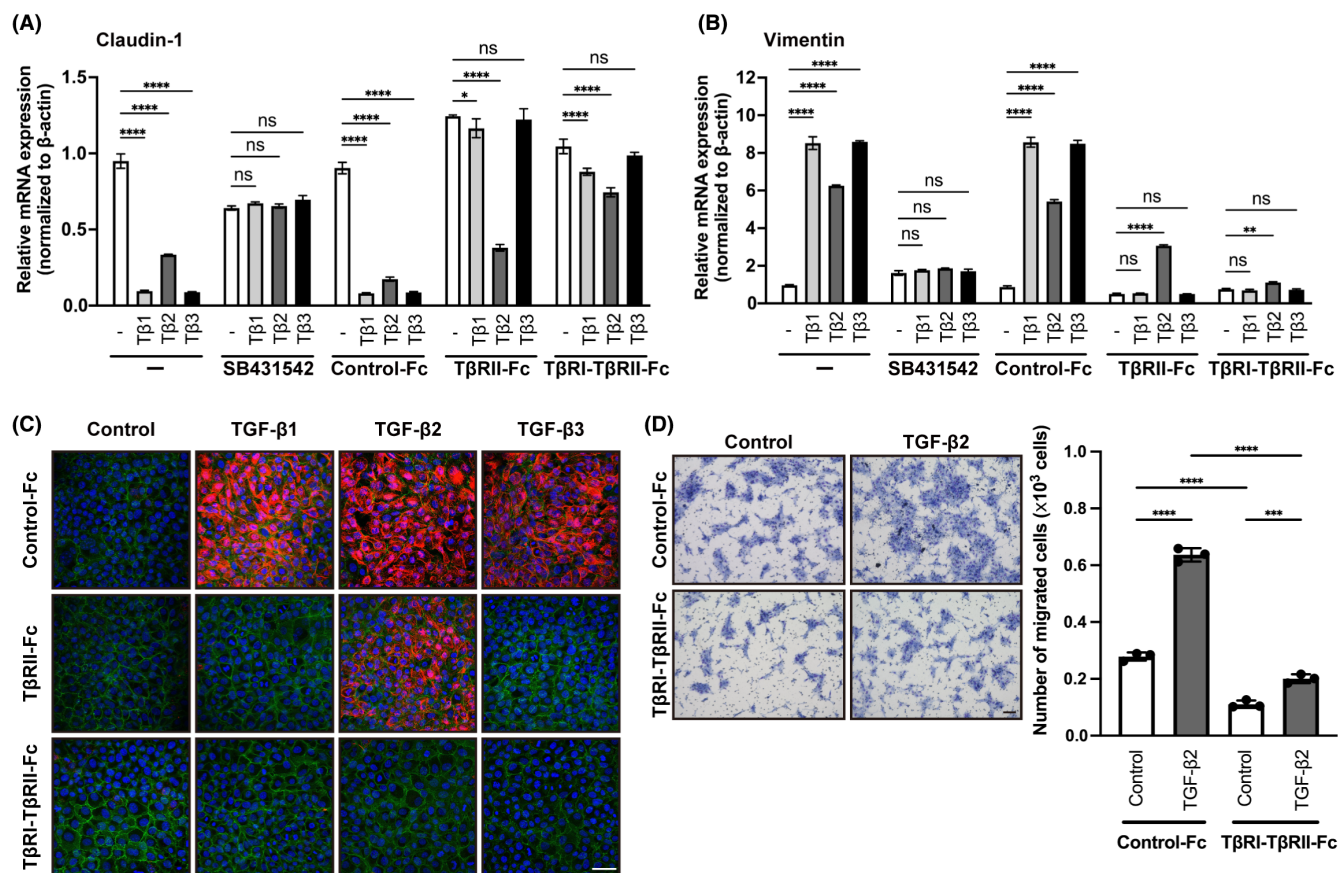


FIGURE 1 Recombinant transforming growth factor- β (TGF- β) type I and II receptor (T β RI-T β RII)-Fc protein inhibits TGF- β -induced epithelial-mesenchymal transition (EMT) and migration of SAS oral cancer cells. (A, B) Relative expression of (A) epithelial cell marker claudin-1 and (B) mesenchymal marker vimentin in SAS cells treated without (-) or with TGF- β 1 (T β 1), TGF- β 2 (T β 2), or TGF- β 3 (T β 3) (2 ng/mL) in the absence (-) or presence of TGF- β signal inhibitor SB431542 (10 μ M) or recombinant Fc proteins (Control-Fc, T β RII-Fc, or T β RI-T β RII-Fc) for 72 h. Induction of TGF- β -mediated EMT was defined by 50% decrease in claudin-1 and 200% increase in vimentin expression. All data are normalized to the β -actin expression. $n=3$. (C) Immunocytochemical analysis of cells treated without (Control) or with TGF- β 1, TGF- β 2, or TGF- β 3 (2 ng/mL) in the presence of recombinant Fc proteins (Control-Fc, T β RII-Fc, or T β RI-T β RII-Fc). Staining for E-cadherin (green), vimentin (red), and nuclei (blue). Representative images of cells cultured under the designated conditions. $n=3$. (D) Migration of SAS cells treated without (Control) or with TGF- β 2 (2 ng/mL) in the presence of Control-Fc or T β RI-T β RII-Fc proteins. Representative images and quantification of migrated cells. $n=3$. All data are shown as mean \pm SD. Scale bars: (C) 50 μ m; (D) 100 μ m. Statistical analyses: two-way ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. ns, not significant.

T β RI-T β RII-Fc. The volume of primary tumors developed in the T β RI-T β RII-Fc-treated mice was significantly smaller than that in the control group (Figure 2A). Importantly, there was no visible difference in the body weight between the two experimental groups (Figure 2B), suggesting that recombinant T β RI-T β RII-Fc proteins did not induce apparent severe side-effects.

3.3 | Treatment with T β RI-T β RII-Fc protein inhibits TGF- β signaling in the TME and induces mesenchymal-epithelial transition (MET) in primary tumors

To determine the mechanism underlying the growth-suppressive effects of the T β RI-T β RII-Fc protein, we examined T β RI-T β RII-Fc-mediated changes in the TME of primary tumors. The expression of the gene activated in the presence of TGF- β , that is, plasminogen

activator inhibitor-1 (PAI-1) encoded by SERPINE1, significantly decreased in primary tumors from T β RI-T β RII-Fc-treated mice when compared to that in the Control-Fc group (Figure S4A), suggesting effective inhibition of TGF- β signaling by the T β RI-T β RII-Fc protein in human oral cancer cells.

TGF- β is a potent inducer of EMT, thus, inhibition of TGF- β signaling in the TME would suppress induction of EMT or eventually reverse it, leading to MET. We showed effective inhibition of EMT (induction of MET) in vitro, in SAS cells cultured with T β RI-T β RII-Fc (Figure 1A-C). Therefore, we questioned whether the accumulation of recombinant T β RI-T β RII-Fc protein could also induce MET in primary tumors. A qRT-PCR analysis of T β RI-T β RII-Fc-treated tumors revealed higher levels of E-cadherin (Figure 2C) and claudin-1 (Figure 2D), and reduced expression of vimentin (Figure 2E) and fibronectin (Figure 2F) than the Control-Fc-treated group, suggesting an effective induction of MET in primary tumors developed by SAS cells. These results were also confirmed at the protein level.

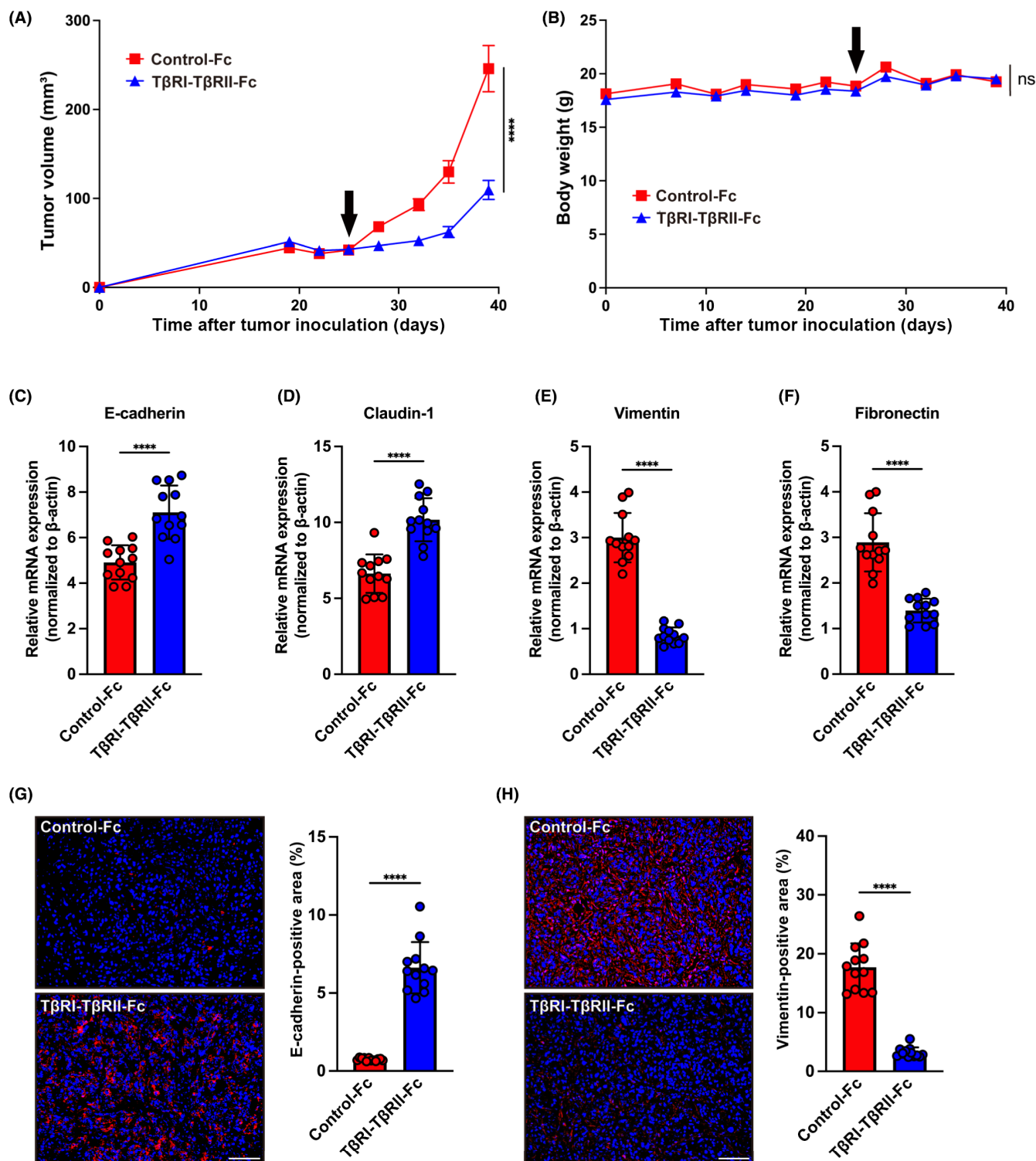


FIGURE 2 Treatment with recombinant transforming growth factor- β (TGF- β) type I and II receptor (T β RI-T β RII)-Fc protein inhibits growth of primary tumors and induces mesenchymal-epithelial transition. SAS cells were injected into the left inguinal region of BALB/c nude mice. The mice were treated i.v. twice a week with recombinant Fc proteins: Control-Fc or T β RI-T β RII-Fc (100 μ g/mouse) starting from day 25 postinjection (arrows). (A) Tumor growth curves. $n=15$. (B) Changes in body weight of mice with SAS cell xenotransplants. $n=15$. (C–H) Analysis of primary tumors excised on day 39 by quantitative RT-PCR (C–F) and immunohistochemistry (G, H). (C–F) Relative expression of (C) E-cadherin, (D) claudin-1, (E) vimentin, and (F) fibronectin in primary tumors. All data are normalized to β -actin expression. $n=12$. (G) Representative immunohistochemical images of staining for E-cadherin (red) and nuclei (blue) and quantitative analysis. Values represent E-cadherin-positive area (%) in each primary tumor. $n=12$. (H) Representative immunohistochemical images of staining for vimentin (red) and nuclei (blue) and quantitative analysis. Values represent vimentin-positive area (%) in each primary tumor. $n=12$. All data are shown as mean \pm SD. Scale bar, 100 μ m. Statistical analyses: one-tailed unpaired Student's t -test; **** $p < 0.0001$. ns, not significant.

There was a significant increase and decrease in E-cadherin-positive (Figure 2G) and vimentin-positive areas (Figure 2H), respectively, in primary tumors following treatment with T β RI-T β RII-Fc, further indicating its effectiveness in the induction of MET.

3.4 | Recombinant T β RI-T β RII-Fc protein reduces primary tumor growth by suppressing in vivo cancer cell proliferation and angiogenesis

TGF- β is known to suppress proliferation of epithelial cancer cells.^{16,22} As previously reported, the proliferation of SAS (Figure S4B) and HSC-4 (Figure S4C) cells was inhibited by all TGF- β isoforms, implying that inhibiting TGF- β signaling would likely enhance proliferation of oral cancer cells. Nevertheless, treatment with T β RI-T β RII-Fc protein inhibited primary tumor growth (Figure 2A). To elucidate the mechanism underlying these effects, we examined the expression of cell proliferation marker, Ki-67, in primary tumors. A significant decrease in the Ki-67 expression levels was observed in the T β RI-T β RII-Fc-administered group compared to that in the Control-Fc-treated group (Figure 3A), indicating that the inhibition of TGF- β signaling by T β RI-T β RII-Fc protein suppressed the in vivo proliferation of cancer cells. However, neither SB431542 nor recombinant Fc proteins had any effects on in vitro SAS cell proliferation (Figure S4D), suggesting that cancer cell growth suppression by T β RI-T β RII-Fc is observed only in the TME. As we observed a significant downregulation in the expression of mouse PAI-1, encoded by *Serpine1*, in primary tumors following treatment with T β RI-T β RII-Fc (Figure S5A), we hypothesized that its observed inhibitory effects on tumor growth might arise from changes in the cross-talk between the components of the TME.

As tumor vessels play important roles in the proliferation of cancer cells in tumors,¹ we examined the effect of T β RI-T β RII-Fc treatment on tumor angiogenesis using an antibody against platelet endothelial adhesion molecule-1 (PECAM-1), a vascular endothelial cell marker. Consistent with our previous findings,¹⁶ a significant decrease in PECAM-1 expression in the T β RI-T β RII-Fc-treated group was observed (Figure 3B), suggesting that the T β RI-T β RII-Fc protein might suppress the in vivo proliferation of cancer cells through its antiangiogenic effects. Notably, T β RI-T β RII-Fc protein administration also suppressed tumor lymphangiogenesis, which was revealed by immunostaining for the expression of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), a marker of lymphatic endothelial cells (Figure S5B). We previously reported that TGF- β signaling maintains the structure of lymphatic vessels and promotes tumor lymphatic metastasis,²³ which is consistent with the present findings.

In order to examine whether T β RI-T β RII-Fc directly influences endothelial cells, we performed in vitro proliferation (Figure S5C) and tube formation assays (Figure S5D) using mouse endothelial cells, MS1. However, T β RI-T β RII-Fc had no effect on either the cell number or tube formation in vitro, suggesting that the antiangiogenic effects of T β RI-T β RII-Fc are exerted through the regulation of angiogenic signals in the TME.

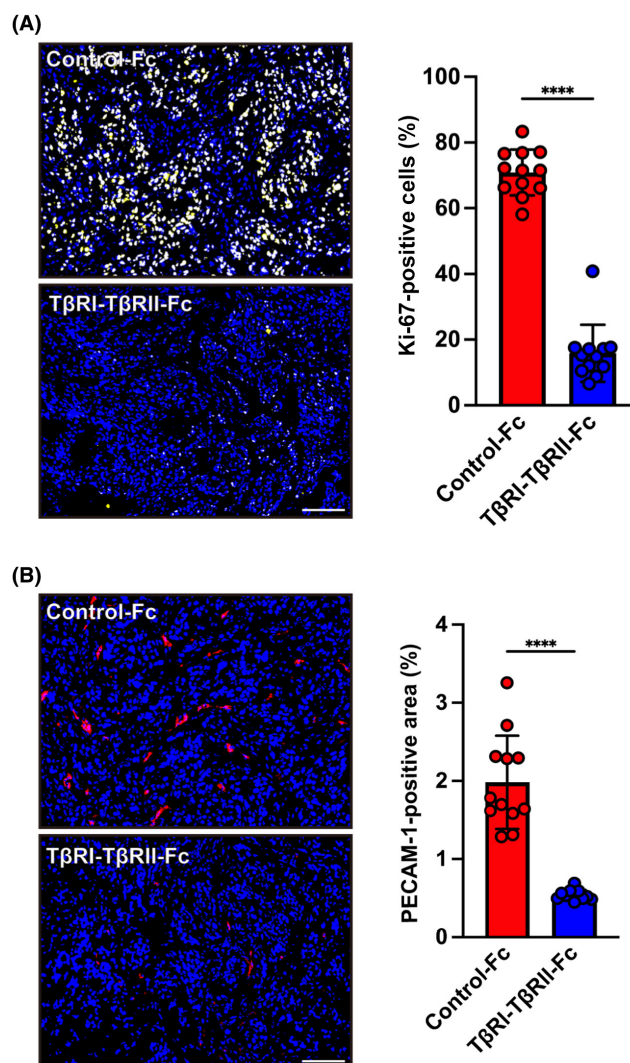
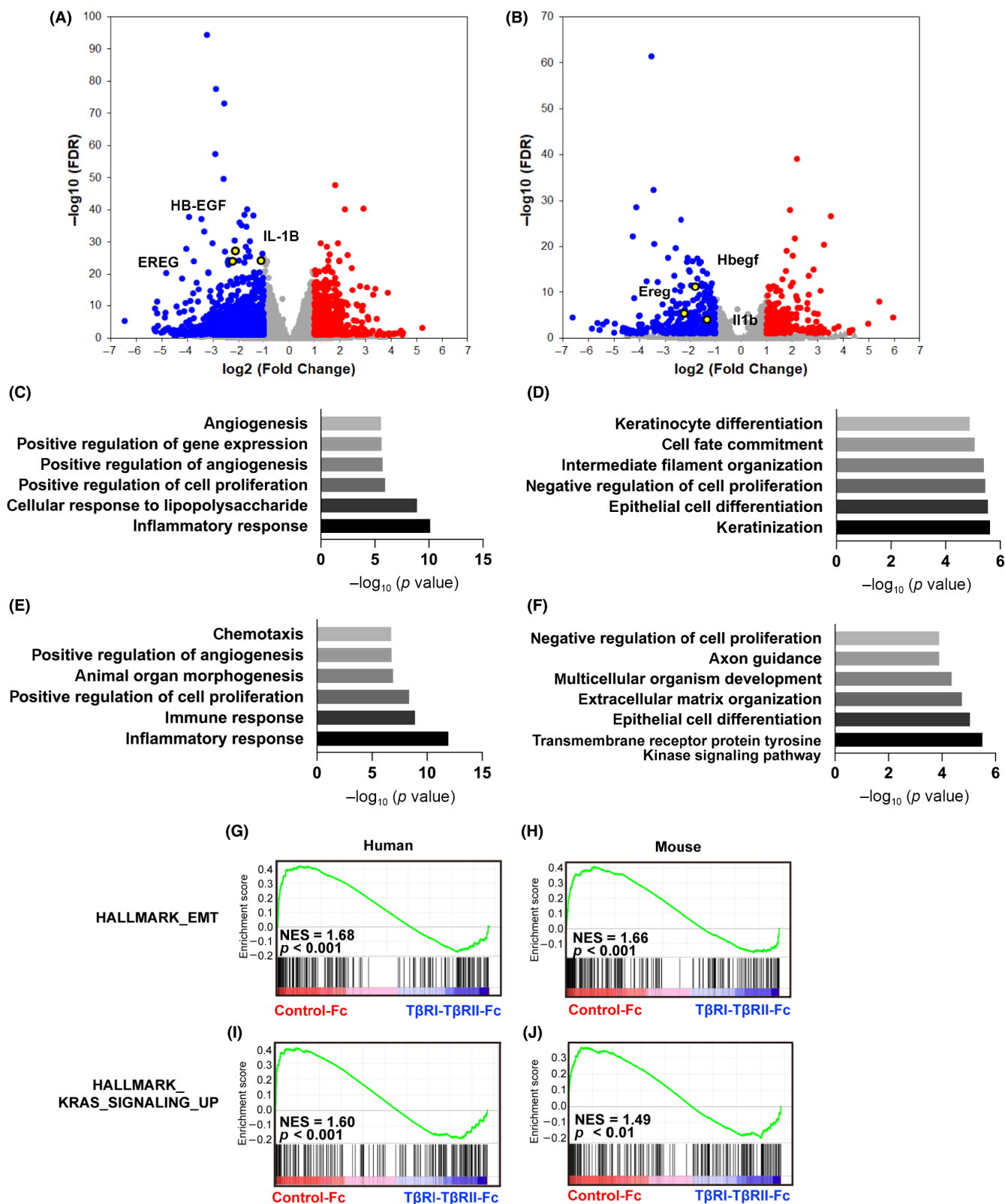


FIGURE 3 Treatment with recombinant transforming growth factor- β (TGF- β) type I and II receptor (T β RI-T β RII)-Fc protein reduces the expression of a proliferation marker and suppresses tumor angiogenesis in primary tumors. Primary tumors from mice treated with Control-Fc or T β RI-T β RII-Fc proteins were excised on day 39 postinjection and subjected to immunohistochemistry. (A) Representative immunohistochemical images of staining for proliferation marker Ki-67 (yellow) and nuclei (blue) and quantitative analysis. Values represent Ki-67-positive cells (%) in each primary tumor. $n=12$. (B) Representative immunohistochemical images of staining for endothelial marker platelet endothelial cell adhesion molecule-1 (PECAM-1; red) and nuclei (blue) and quantitative analysis. Values represent PECAM-1-positive area (%) in each primary tumor. $n=12$. All data are shown as mean \pm SD. Scale bar, 100 μ m. Statistical analyses: one-tailed unpaired Student's t -test; **** $p < 0.0001$.

3.5 | Recombinant T β RI-T β RII-Fc protein alters gene expression in multiple components of the TME of oral cancer cell-derived tumor tissues

Treatment with T β RI-T β RII-Fc inhibited cancer cell proliferation and vessel formation in tumor tissues in vivo (Figure 3), indicating its efficacy towards both human cancer cells and mouse



stromal endothelial cells. However, as T β RI-T β RII-Fc protein did not affect the cancer cell growth in vitro (Figure S4D), we hypothesized that observed effects of T β RI-T β RII-Fc in vivo might reflect the changes in various components of the TME. To test this hypothesis, we performed RNA sequencing (RNA-seq) analysis of T β RI-T β RII-Fc-treated or Control-Fc-treated tumor tissues

to determine changes in gene expression patterns in cancer cells (human component) and cells comprising the microenvironment, endothelium, and stroma (mouse components). Volcano plots of differentially expressed genes (DEGs) revealed 580 upregulated and 943 downregulated human-specific genes (Figure 4A), and 228 upregulated and 336 downregulated mouse-specific genes

FIGURE 4 Recombinant transforming growth factor- β (TGF- β) type I and II receptor (T β RI-T β RII)-Fc protein alters gene expression in multiple components of the tumor microenvironment. (A, B) Volcano plots showing downregulated and upregulated differentially expressed genes (DEGs) in primary tumor tissues from mice treated with Control-Fc or T β RI-T β RII-Fc proteins ($n=4$; two separate parts of tumors from two mice). (A) DEGs associated with cancer cells (human components). Dots represent 943 downregulated (blue) and 580 upregulated (red) human-specific genes. (B) DEGs associated with stroma (mouse components). Dots represent 336 downregulated (blue) and 228 upregulated (red) mouse-specific genes. (C–F) Gene Ontology (GO) functional enrichment analysis of downregulated and upregulated DEGs in primary tumor tissues from mice treated with Control-Fc or T β RI-T β RII-Fc proteins. (C, D) GO functional enrichment analysis of human genes (C) downregulated and (D) upregulated in cancer cells following treatment with T β RI-T β RII-Fc protein. (E, F) GO functional enrichment analysis of mouse genes (E) downregulated and (F) upregulated in stromal components following treatment with T β RI-T β RII-Fc protein. (G–J) Gene Set Enrichment Analysis (GSEA) of (G, H) epithelial–mesenchymal transition and (I, J) K-Ras signaling upregulation gene signatures, comparing RNA sequencing (RNA-seq) data from tumors isolated from mice treated with Control-Fc with RNA-seq data from tumors obtained following treatment with T β RI-T β RII-Fc proteins. EREG, epiregulin; FDR, false discovery rate; HB-EGF, heparin-binding epidermal growth factor-like growth factor; IL-1 β , interleukin-1 β ; NES, normalized enrichment score; p , empirical p value from GSEA.

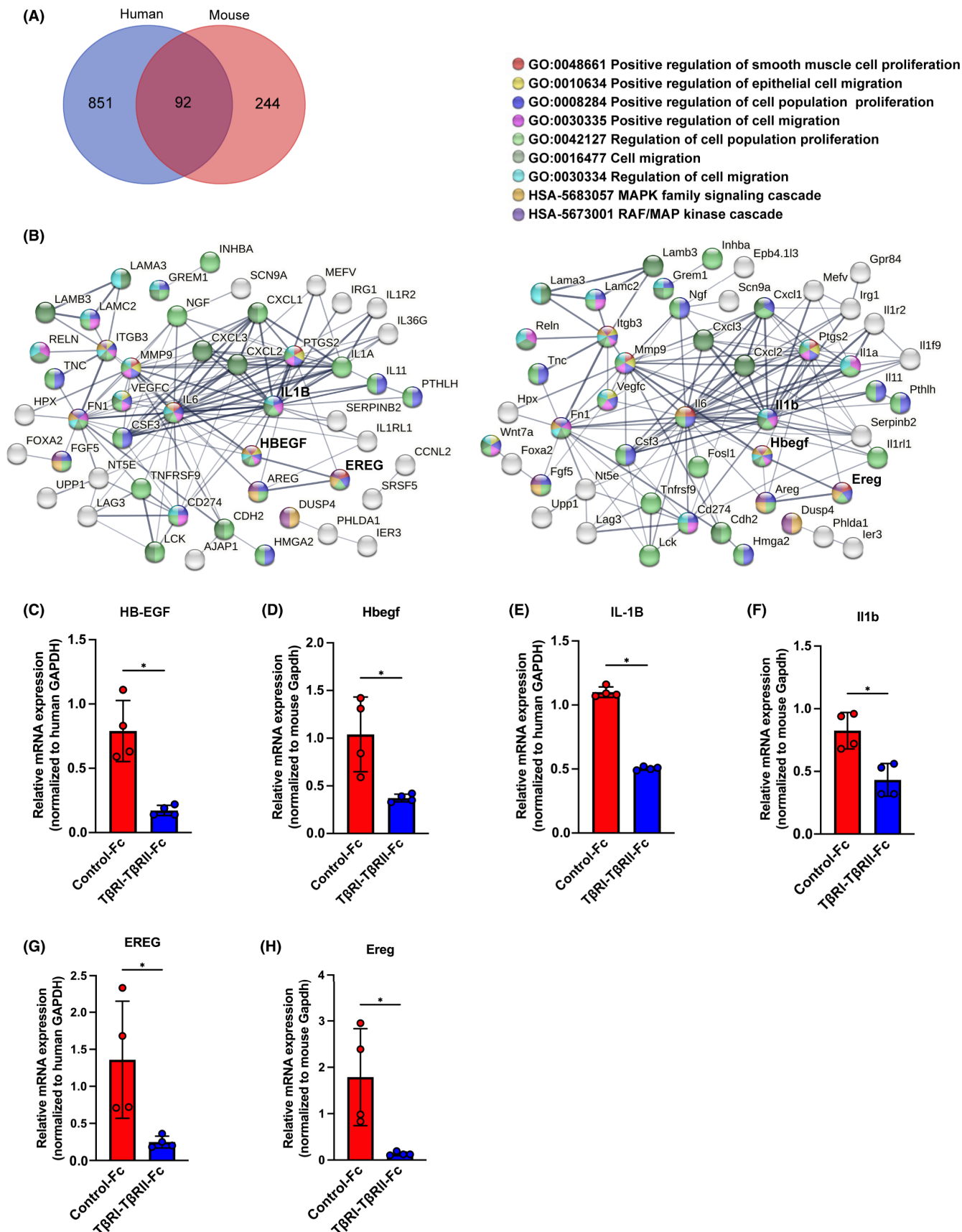
(Figure 4B) in tumor tissues. Gene Ontology (GO) functional enrichment analysis^{24,25} showed that human DEGs downregulated in cancer cells following treatment with T β RI-T β RII-Fc were enriched in GO terms related to the positive regulation of cell proliferation and angiogenesis (Figure 4C; Table S2A). Concomitantly, human DEGs upregulated in T β RI-T β RII-Fc-treated tumors were enriched in GO terms related to the negative regulation of cell proliferation and differentiation (Figure 4D; Table S2B). A similar tendency was observed in the stromal components of mouse origin (Figure 4E,F; Table S2C,D), suggesting that the administration of T β RI-T β RII-Fc protein affects various components of the TME. An unbiased Gene Set Enrichment Analysis (GSEA)²⁶ revealed the inhibition of EMT (Figure 5G,H), and inhibition of K-Ras signaling (Figure 4I,J; Table S3A,B) in the T β RI-T β RII-Fc-administered group in both human and mouse components of the TME. Since K-Ras signaling has been implicated in the proliferation of cancer cells via activation of Raf/MAPK/ERK kinase/ERK signaling pathway,²⁷ the downregulation of K-Ras signaling by T β RI-T β RII-Fc may lead to decreased proliferation of cancer cells in primary tumors derived from SAS cells.

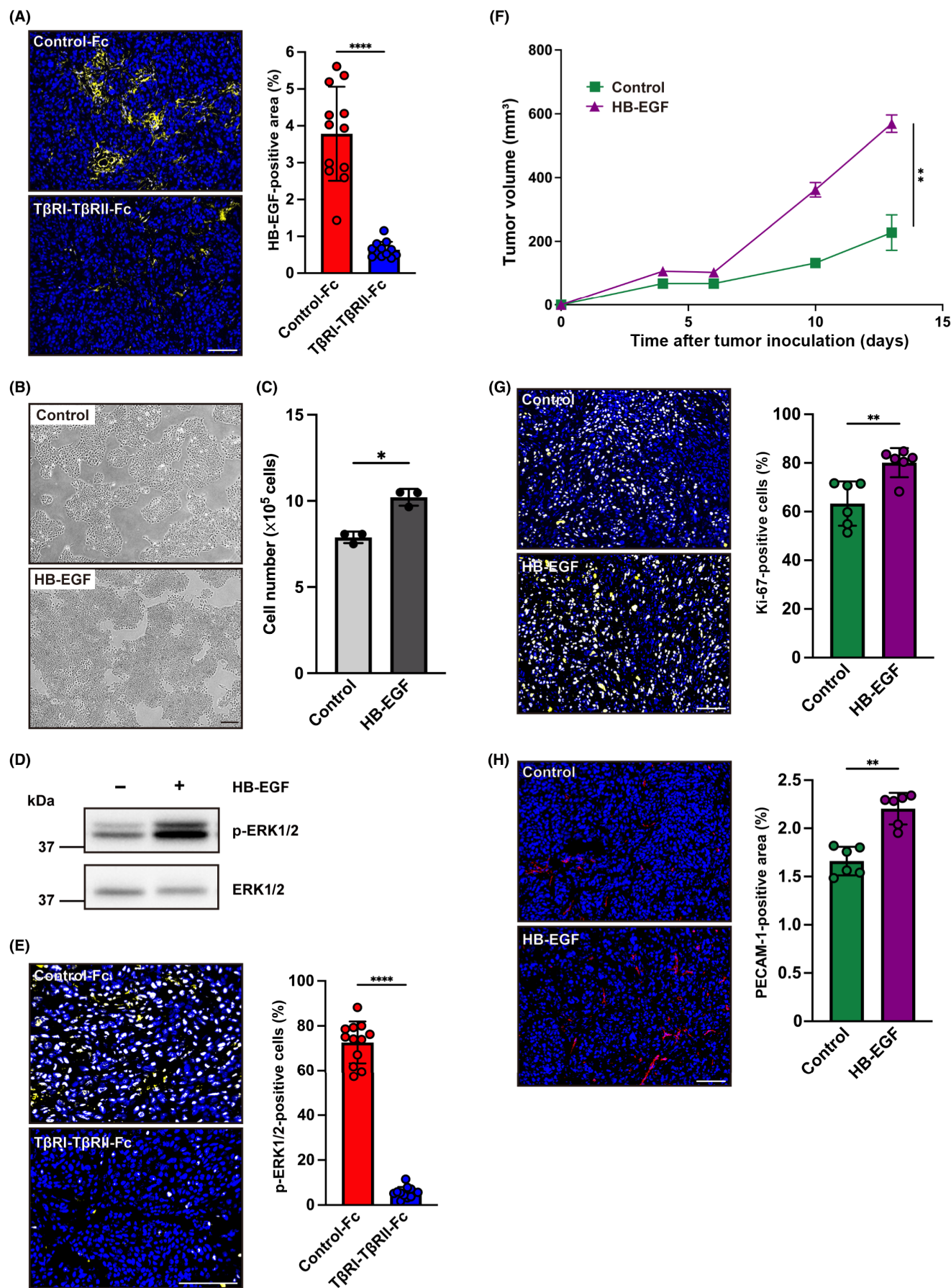
3.6 | Inhibition of TGF- β signals in the TME by T β RI-T β RII-Fc suppresses expression of HB-EGF, IL-1 β , and EREG

To further determine the molecular mechanisms regulating the cell proliferation in the tumors with suppressed TGF- β signaling

and to identify factors affecting human cancer cells and mouse stroma, we focused on the downregulated DEGs that were shared between human cancer cells and mouse stroma. Among 943 downregulated human-specific DEGs and 336 downregulated mouse-specific DEGs, we identified 92 genes shared between human and mouse components of the TME (Figure 5A; Table S4). We used the STRING database²⁸ to examine protein–protein interactions among these shared DEGs and identified several molecules, including growth factors, cytokines, and molecules regulating extracellular matrix or angiogenesis (Figure 5B). Moreover, GO analysis using these 92 downregulated DEGs revealed enrichment in annotations related to “positive regulation of cell proliferation” and “regulation of cell migration” (Table S5). Detailed GO functional cross-analysis of genes enriched in terms related to “positive regulation of cell proliferation” identified three molecules, heparin-binding epidermal growth factor-like growth factor (HB-EGF), interleukin-1 β (IL-1 β) and epiregulin (EREG), which overlapped with genes enriched in K-Ras signaling upregulation hallmark gene sets (Figure 5A,B; Tables S3A,B and S6). Previous reports have shown that all three candidate factors are involved in tumor progression through regulation of both cancer cells and stroma.^{29–35} To verify our selection, we examined their expression in primary tumor tissues. There was a significant decrease in the expression levels of HB-EGF (Figure 5C,D), IL-1 β (Figure 5E,F), and EREG (Figure 5G,H), encoded by HBEGF/Hbegf, IL1B/Il1b, and EREG/Ereg genes, respectively, following treatment with T β RI-T β RII-Fc protein, not only in human cancer cells (Figure 5C,E,G) but also in mouse stroma (Figure 5D,F,H).

FIGURE 5 Recombinant transforming growth factor- β (TGF- β) type I and II receptor (T β RI-T β RII)-Fc protein alters the expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF), interleukin-1 β (IL-1 β), and epiregulin (EREG) in multiple components of the tumor microenvironment. (A) Venn diagram showing number of differentially expressed genes (DEGs) downregulated in human cancer cells (blue) and mouse stromal components (light red) of primary tumors following treatment with T β RI-T β RII-Fc protein. The number of overlapping DEGs (dark red) is also shown. (B) STRING analysis of overlapped DEGs showing the interactions among 92 downregulated genes shared between human cancer cells (left panel) and mouse stroma (right panel). Functional enrichment in components associated with regulation of cell proliferation, cell migration, and the MAPK pathway is shown. Bold characters indicate molecules overlapping with genes enriched in hallmark gene sets related to the K-Ras signaling upregulation. (C–H) Quantitative RT-PCR analysis of candidate molecules involved in cross-talk between human cancer cells and mouse stroma. Relative expression of (C) human HBEGF, (D) mouse Hbegf, (E) human IL1B, (F) mouse Il1b, (G) human EREG, and (H) mouse Ereg. All data are normalized to human GAPDH (C, E, G) or mouse Gapdh (D, F, H) expression ($n=4$; two separate parts of tumors from two mice). All data are shown as mean \pm SD. Statistical analyses: one-tailed unpaired Student's t -test; * $p < 0.05$. GO, Gene Ontology.





3.7 | TGF- β signaling in the TME regulates HB-EGF expression, which maintains proliferation of cancer and endothelial cells by activating ERK1/2 phosphorylation

HB-EGF is a member of the EGF family. The binding of HB-EGF to its receptors activates the Ras/Raf/MAPK/ERK kinase/ERK signaling pathway and controls cell proliferation, migration, and angiogenesis.^{29–31} We found that HB-EGF expression in cultured SAS and HSC-4 oral cancer cells was upregulated by specific TGF- β isoforms (Figure S6A,B).

As there was a significant decrease in HB-EGF RNA expression in T β RI-T β RII-Fc-treated tumor tissues, in both human cancer cells and in mouse stroma cells, we examined the expression of HB-EGF protein in primary tumors by immunohistochemistry. There was a significant decrease in the HB-EGF-positive area in T β RI-T β RII-Fc-treated tumors (Figure 6A), suggesting that the TGF- β /HB-EGF axis is involved in regulating cell proliferation in SAS cell-derived tumors. To confirm this hypothesis, we examined the effect of HB-EGF on the in vitro proliferation of oral cancer cells and mouse endothelial cells. The treatment of SAS and HSC-4 cells with HB-EGF stimulated cell proliferation (Figures 6B,C and S7A,B), suggesting that HB-EGF is a putative regulator of cancer cell proliferation in the TME.

As HB-EGF transmits its signals by binding to the tyrosine kinase receptor and activating MAPK signaling pathways,^{30,36} we stimulated oral cancer cells with HB-EGF and examined the phosphorylation level of ERK1/2, a downstream effector of the tyrosine kinase signaling pathway. The treatment of oral cancer cells with HB-EGF resulted in increased phosphorylation levels of ERK1/2 (Figures 6D and S7C) suggesting that TGF- β -induced HB-EGF expression regulates cell proliferation in tumor tissues by activating the Ras/Raf/MAPK/ERK kinase/ERK signaling pathway. We also examined the phosphorylation level of ERK1/2 in primary tumors. Immunostaining with anti-phospho-ERK1/2 antibodies revealed a significant decrease in the number of phospho-ERK1/2-positive cells in T β RI-T β RII-Fc-treated

tumor tissues (Figure 6E), suggesting that cross-talk between TGF- β and ERK signaling in the TME regulates cancer cell proliferation in vivo. Notably, the number of MS1 cells, as well as the levels of phospho-ERK1/2, also increased following HB-EGF treatment (Figure S7D–F), suggesting that HB-EGF-related signals affect both cancer cells and the stromal components of the TME.

To further examine the effects of HB-EGF on cancer and endothelial cells in vivo, we performed tumor formation assay using SAS cells with altered HB-EGF expression. Primary tumors formed by the mice inoculated with SAS cells infected with lentivirus expressing HB-EGF were larger than the primary tumors developed by SAS cells infected with control lentivirus (Figure 6F). In addition, immunohistochemical analysis revealed an increased staining for Ki-67 (Figure 6G) and PECAM-1 (Figure 6H), suggesting that the protumorigenic effect of HB-EGF is related to enhanced cancer cell proliferation and tumor vessel formation in the TME.

To confirm the present findings, we also examined the effects of loss-of-function of HB-EGF on in vitro cancer cell proliferation and in vivo tumor formation. We first knocked down the HB-EGF expression in SAS cells using specific siRNAs (Figure S8A). Decreased expression of HB-EGF in SAS cells suppressed cell proliferation (Figure S8B), confirming that HB-EGF regulates in vitro cancer cell proliferation. To examine the effect of impaired HB-EGF expression in vivo, we established SAS cells with stable knockdown of HB-EGF using specific shRNA (Figure S8C). Decreased expression of HB-EGF reduced in vitro proliferation of SAS cells (Figure S8D) and suppressed the primary tumor growth (Figure S8E) in a murine xenograft model. Moreover, primary tumors that developed under impaired HB-EGF expression showed reduced cell growth and inhibition of tumor angiogenesis, as revealed by decreased staining for Ki-67 (Figure S8F) and PECAM-1 (Figure S8G), respectively. Taken together, our data suggest that TGF- β signaling in the TME regulates HB-EGF expression, leading to the enhanced cancer cell growth and stimulation of tumor angiogenesis.

FIGURE 6 Heparin-binding epidermal growth factor-like growth factor (HB-EGF) affects cancer cell proliferation and oral squamous cell carcinoma tumor formation through ERK1/2 phosphorylation. (A) Primary tumors from mice treated with Control-Fc or transforming growth factor- β (TGF- β) type I and II receptor (T β RI-T β RII)-Fc proteins were excised on day 39 postinjection and examined for the expression of HB-EGF. Representative immunohistochemical images of staining for HB-EGF (yellow) and nuclei (blue) and quantitative analysis. Values represent the HB-EGF-positive area (%) in each primary tumor. $n=12$. (B–D) Effects of HB-EGF on (B, C) cell proliferation and (D) ERK1/2 phosphorylation in SAS cells. (B) Representative images of SAS cells cultured in the absence (Control) or presence of HB-EGF (1 ng/mL) for 72 h. (C) Proliferation of SAS cells. $n=3$. (D) Effect of HB-EGF treatment on ERK1/2 phosphorylation in SAS cells. SAS cells were cultured in the absence (–) or presence of HB-EGF (+) for 10 min, followed by immunoblotting analysis with antiphospho-(p)-ERK1/2, and total anti-ERK1/2 antibodies. Representative blot of two independent experiments is shown. (E) Phosphorylation of ERK1/2 examined in primary tumors from mice treated with Control-Fc or T β RI-T β RII-Fc proteins. Representative immunohistochemical images of staining for p-ERK1/2 (yellow) and nuclei (blue) and quantitative analysis. Values represent p-ERK1/2-positive cells (%) in each primary tumor. $n=12$. (F–H) SAS cells infected with control lentivirus (Control) or lentivirus expressing HB-EGF (HB-EGF) were injected into the left inguinal region of BALB/c nude mice. Tumor growth was monitored from day 4 postinoculation until the end of the experiment. Primary tumors were harvested on day 13 and subjected to immunohistochemistry. (F) Tumor growth curves. $n=6$. (G) Representative immunohistochemical images of staining for Ki-67 (yellow) and nuclei (blue) and quantitative analysis. Values represent Ki-67-positive cells (%) in each primary tumor. $n=6$. (H) Representative immunohistochemical images of staining for endothelial marker platelet endothelial cell adhesion molecule-1 (PECAM-1; red) and nuclei (blue) and quantitative analysis. Values represent PECAM-1-positive area (%) in each primary tumor. $n=6$. All data are shown as mean \pm SD. Scale bars: (A, E, G, F) 100 μ m; (B) 300 μ m. Statistical analyses: one-tailed unpaired Student's t -test; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

3.8 | TGF- β signaling regulates expression of IL-1 β and EREG, which enhance tumor formation

Inhibition of TGF- β signaling in primary tumors by the T β RI-T β RII-Fc protein also affected the expression of IL-1 β (Figure 5E,F) and EREG (Figure 5G,H). IL-1 β is a multifunctional cytokine that was shown to regulate various steps of cancer progression: angiogenesis, cancer cell proliferation, migration, and metastasis.³² IL-1 β is associated with poor prognosis and was shown to induce both angiogenesis and EMT in OSCC.³³ Therefore, we examined the effect of IL-1 β on the proliferation of oral cancer cells *in vitro*. As expected, IL-1 β enhanced the proliferation of SAS and HSC-4 oral cancer cells (Figure S9A–D), confirming that IL-1 β is a potent regulator of cancer cell growth. As IL-1 β was also implicated in tumor angiogenesis,³⁷ we treated MS1 cells with IL-1 β . IL-1 β also enhanced the endothelial cell proliferation (Figure S9E,F), suggesting that IL-1 β regulates the multiple components of the TME.

We then examined the effect of IL-1 β on *in vivo* tumor formation using SAS cells infected with control lentivirus or lentivirus expressing IL-1 β . IL-1 β significantly enhanced the formation of SAS cell-derived tumors (Figure 7A). Furthermore, the analysis of tumor tissues with specific antibodies revealed significant increase in a number of Ki-67-positive cells (Figure 7B) and PECAM-1-positive area (Figure 7C) in primary tumors developed by SAS cells infected with lentivirus expressing IL-1 β compared to those in the control group, suggesting that, like HB-EGF, IL-1 β is involved in the regulation of tumor growth by affecting cancer cell proliferation and tumor angiogenesis.

REG is also one of the EGF receptor ligands and its elevated expression has been detected in various cancers, including head and neck squamous cell carcinoma (HNSCC).³⁴ High levels of EREG expression have been shown to correlate with the poor survival of patients with OSCC.³⁵ In addition, treatment with EREG stimulated spheroid formation in a 3D culture of HNSCC cancer cell lines.³⁴ Thus, we examined the effect of EREG on *in vitro* cultured oral cancer cells and *in vivo* tumor formation. The proliferation of SAS and HSC-4 oral cancer cells was enhanced by EREG (Figure S9G–J). Moreover, in a xenograft mouse model, SAS cells infected with lentivirus expressing EREG formed slightly bigger primary tumors than the control cells infected with control lentivirus (Figure 7D). Further analysis of tumor tissues with the antibody for Ki-67 revealed a significant increase in Ki-67-positive cells compared to that in the control group (Figure 7E), suggesting that EREG regulates tumor growth by affecting cancer cell proliferation in the TME.

4 | DISCUSSION

In the present study, we showed that recombinant T β RI-T β RII-Fc protein effectively inhibited EMT induced by all TGF- β isoforms *in vitro*, and demonstrated its efficacy *in vivo*. As TGF- β plays an important role in maintaining tissue homeostasis, complete inhibition of TGF- β signaling could result in adverse cytotoxic

effects.^{38,39} We did not observe any immediate side-effects following treatment with recombinant T β RI-T β RII-Fc protein, as previously reported for low molecular weight compounds.⁴⁰ Moreover, the presence of ECDs from T β RI and T β RII, which enables interaction with all TGF- β isoforms¹⁶ as well as the Fc region of human IgG that likely prolongs the plasma half-life time of recombinant protein, suggests the potential therapeutic value of T β RI-T β RII-Fc protein.^{41,42} However, as in the present studies we utilized immune-deficient mice, and as TGF- β plays important roles in tumor immune response,⁴³ further experiments are warranted to determine the effect of T β RI-T β RII-Fc protein on the TME and its efficacy against TGF- β -induced immunosuppression using a syngeneic mouse model.

TGF- β orchestrates the interaction between various components of the TME. It can be secreted by various cells in the TME network³⁸ and functions in both autocrine and paracrine manners.¹³ We previously reported that TGF- β 2, secreted by endothelial cells that have undergone EndoMT, induced EMT in oral cancer cells.⁴⁴ As such, administration of T β RI-T β RII-Fc that traps TGF- β 2 could potentially suppress EMT and EndoMT. Indeed, our transcriptomic analysis confirmed the induction of MET and inhibition of angiogenic signals following treatment with the recombinant T β RI-T β RII-Fc protein. We found that inhibition of TGF- β signals in the TME resulted in the suppression not only of tumor angiogenesis (Figure 3B) but also tumor lymphangiogenesis (Figure S5B). However, one of our previous reports showed that LY364947, a kinase inhibitor of T β RI, increased tumor lymphangiogenesis in a tumor xenograft model of BxPC3 pancreatic adenocarcinoma cells in the presence of vascular endothelial growth factor (VEGF-C).⁴⁵ This discrepancy might arise from the different experimental conditions such as the types of TGF- β inhibitors (Fc chimeric protein vs. small molecule inhibitor) used in the study, cancer cells, and presence of exogenous VEGF-C. In addition, we also reported that T β RII^{fl/fl};Prox1-Cre^{ERT2} mice with conditional deletion of T β RII in lymphatic endothelial cells showed reduced tumor lymphatic vessels in Lewis lung carcinoma cell-derived tumors.²³ These results suggest that the roles of TGF- β signals in tumor lymphangiogenesis are context-dependent and could be affected by various factors present in the TME. This point requires further study and will be examined in the future.

Mesenchymal-epithelial transition is implicated in the formation and stabilization of metastatic sites.⁴⁶ During MET, cancer cells with mesenchymal characteristics regain the epithelial characteristics and ability to proliferate that is necessary to form distant metastases. We showed that, despite MET induction, cell proliferation in the TME was suppressed by treatment with T β RI-T β RII-Fc (Figure 3A). This discrepancy could be derived from the difference in the primary signaling pathways that regulate cancer cell proliferation in certain types of tumors. Our data suggest that, in OSCC, there is a cross-talk between TGF- β and K-Ras signaling; therefore, activating these signaling pathways might be necessary to keep the proliferative state of cancer cells in the TME. This point warrants future investigation.

Recombinant T β RI-T β RII-Fc showed antitumorigenic effects *in vivo*. TGF- β suppresses the proliferation of epithelial cells,

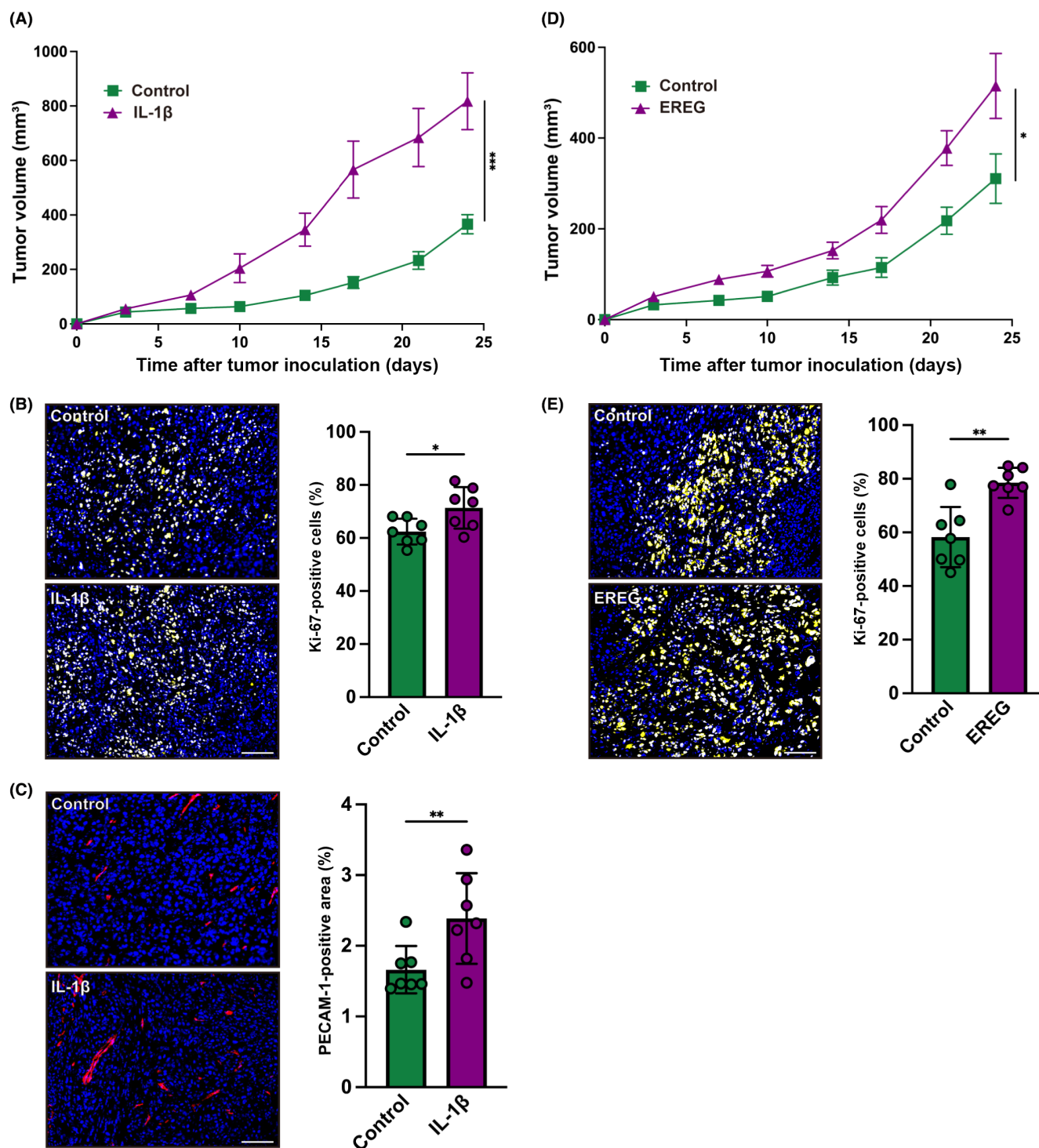


FIGURE 7 Interleukin-1 β (IL-1 β) and epiregulin (EREG) contribute to oral squamous cell carcinoma tumor formation. (A–C) SAS cells infected with control lentivirus (Control) or lentivirus expressing IL-1 β (IL-1 β) were injected into the left inguinal region of BALB/c nude mice. Tumor growth was monitored from day 3 postinoculation until the end of the experiment. Primary tumors were harvested on day 24 and subjected to immunohistochemistry. (A) Tumor growth curves. $n = 7$. (B) Representative immunohistochemical images of staining for Ki-67 (yellow) and nuclei (blue) and quantitative analysis. Values represent Ki-67-positive cells (%) in each primary tumor. $n = 7$. (C) Representative immunohistochemical images of staining for endothelial marker platelet endothelial cell adhesion molecule-1 (PECAM-1; red) and nuclei (blue) and quantitative analysis. Values represent PECAM-1-positive area (%) in each primary tumor. $n = 7$. (D, E) SAS cells infected with control lentivirus (Control) or lentivirus expressing EREG (EREG) were injected into the left inguinal region of BALB/c nude mice. Tumor growth was monitored from day 3 postinoculation until the end of the experiment. Primary tumors were harvested on day 24 and examined for expression of proliferation marker Ki-67 (E). (D) Tumor growth curves. $n = 7$. (E) Representative immunohistochemical images of staining for Ki-67 (yellow) and nuclei (blue) and quantitative analysis. Values represent Ki-67-positive cells (%) in each primary tumor. $n = 7$. All data are shown as mean \pm SD. Scale bar, 100 μ m. Statistical analyses: one-tailed unpaired Student's t -test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

therefore the inhibition of TGF- β signaling is expected to stimulate cell proliferation. Nonetheless, treatment with T β RI-T β RII-Fc resulted in a significant decrease in the expression of proliferation marker Ki-67. Mechanistically, we found that the inhibition of TGF- β signals in the TME downregulated the expression of K-Ras signaling-related genes (Figure 4I,J), suggesting that decreased cancer cell proliferation *in vivo* was caused by the downregulation of K-Ras signaling. K-Ras signaling plays an important role in the proliferation of various types of cancer cells.²⁷ A previous study reported that K-Ras signaling is also involved in TGF- β -induced EMT.⁴⁷ At an early stage of pancreatic cancer, activation of K-Ras signaling confers cancer resistance to an inhibitory effect of TGF- β on cell proliferation. Thus, it is possible that a similar mechanism also exists in OSCC, but this point requires further investigation.

We have identified HB-EGF as a factor regulating human and mouse components of the TME. HB-EGF stimulates cancer cell proliferation, migration, and invasion, and thus progression of various cancers, including HNSCC.^{29–31,34,35,48} It also affects the expression of proangiogenic factors VEGF-A and angiopoietin-like 4 through activation of the ERK/AKT signaling pathway.⁴⁹ Our results suggested a cross-talk between TGF- β and HB-EGF signaling in the TME. As such, inhibition of TGF- β signaling by treatment with T β RI-T β RII-Fc protein suppressed the expression of HB-EGF in the TME in both human cancer cells and mouse stroma (Figures 5C,D and 6A). We also showed that HB-EGF promoted *in vitro* and *in vivo* cancer cell proliferation and angiogenesis (Figures 6B,C,F–H and S8). It was reported that in normal rat intestinal epithelial cells, in response to injury, TGF- β upregulates the expression of HB-EGF mRNA⁵⁰ through activation of the protein kinase C pathway, suggesting a coordinated activity of TGF- β and HB-EGF. Upon injury, epithelial cells become motile and undergo EMT. Hence, HB-EGF might cooperate with TGF- β to control tumorigenesis during cancer progression.

Our RNA-seq analysis also identified IL-1 β and EREG as other potential factors regulating the components of the TME, human cancer cells, and mouse stroma (Figures 5B,E–H, 7, and S9). The expressions of IL-1 β (Figure 5E,F) and EREG (Figure 5G,H) were downregulated in primary tumors by recombinant T β RI-T β RII-Fc, implying cross-talk between signaling pathways mediated by TGF- β , HB-EGF, IL-1 β , and EREG during oral cancer progression.

Expression of IL-1 β has been shown to be associated with cancer progression. Lee and colleagues reported that IL-1 β contributes to OSCC aggressiveness by inducing angiogenesis and EMT.³³ In their study, IL-1 β secreted by oral cancer cell lines not only upregulated the expression of EMT-related factors in nonmetastatic oral cancer cells, but also induced tube formation by HUVECs.³³ In our study, IL-1 β affected not only cancer cell proliferation (Figure S9A–D) and primary tumor formation (Figure 7A), but also significantly enhanced endothelial cell proliferation (Figure S9E,F). As IL-1 β was also reported to induce VEGF production in tumors,³⁷ decreased IL-1 β expression by T β RI-T β RII-Fc might have suppressed tumor angiogenesis. A recent report suggested that during airway remodeling arising from asthma-related lung injury, the cooperative action of TGF- β 1 and IL-1 β activates ERK1/2, augmenting TGF- β -induced

EMT.⁵¹ Taken together with the previous findings, our data suggest that TGF- β signaling controls the expression of HB-EGF, EREG, and IL-1 β in the TME. All three molecules likely cooperate during OSCC tumor progression by affecting cancer cells and stromal components such as tumor vessels. The exact pathways and the detailed mechanisms by which these molecules orchestrate tumor progression will be elucidated in the future.

Numerous studies have shown that EREG induces cancer cell proliferation, metastasis, and tumor angiogenesis.^{35,52} EREG is expressed by tumor epithelial cells and stroma, and can act in an autocrine and paracrine manner. Previous reports describing the role of EREG in cancer development showed that at the early stage of OSCC, EREG activates JAK-STAT signaling in cancer cells and induces EMT.⁵² In our study, EREG affected oral cancer cell proliferation *in vivo* and *in vitro* (Figures 7D,E and S9G–J), suggesting that it contributed to the inhibitory effect of T β RI-T β RII-Fc on cancer cell proliferation. Taken together with the previous findings, our data suggest that TGF- β signaling controls the expression of HB-EGF, IL-1 β , and EREG in the TME. All three molecules likely cooperate during OSCC tumor progression by affecting cancer cells and stromal components such as tumor vessels. The exact pathways and the detailed mechanisms by which these molecules orchestrate tumor progression will be elucidated in the future.

In conclusion, we determined the therapeutic value of the recombinant T β RI-T β RII-Fc protein and highlighted the importance of the TME network in the regulation of tumor progression. Our data also suggest that the T β RI-T β RII-Fc protein is a promising tool for targeting TGF- β signaling in various cancers.

AUTHOR CONTRIBUTIONS

K.A.P.I., K.M., and T.W. conceived the concept of this study. S.T., K.A.P.I., T.M., K.T., M.K., S.U., M.S., and T.W. designed the experiments. S.T., T.M., K.T., H.I., and S.I. performed the experiments. S.T., K.A.P.I., K.T., M.K., H.I., S.U., S.I., and T.W. analyzed data and interpreted the results. S.T. and K.A.P.I. wrote an original draft. K.A.P.I., H.H., S.H., M.S., and T.W. supervised the whole project. All authors reviewed and edited the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Hiroyuki Miyoshi (deceased, formerly RIKEN) for providing plasmids, Miwako Hamagaki, Jin Lu, Megumi Naito (TMDU), and Dr. Kazuharu Hanada (RIKEN) for technical assistance, and Dr. Jun Ishihara (Imperial College London), Dr. Yasuyuki Michi, Dr. Kei Sakamoto, Dr. Kou Kayamori, Dr. Tohru Ikeda, Dr. Miki Yokoyama (TMDU), and members of the Department of Biochemistry at TMDU for insightful discussions.

FUNDING INFORMATION

This work was supported by research programs of the Japan Agency for Medical Research and Development (AMED) (JP21cm0106253 and JP22ama221205 to T.W., and JP22ama221001 to S.H. and M.S.), and in part by Grant-in-Aid for Scientific Research (A) (JP20H00513 to K.M. and K.A.P.I.), Scientific Research (B) (JP20H03851 to T.W.),

Scientific Research (C) (JP20K10111 to K.A.P.I.), Scientific Research (S) (JP23H05486 to K.M. and T.W.), JSPS Fellow (JP20J11926 and JP21J01294 to K.T.) from the Japan Society for the Promotion of Science (JSPS), and JST SPRING (JPMJSP2120 to S.T.). Immunocytochemical analysis was performed at the Research Core of Tokyo Medical and Dental University (TMDU).

CONFLICT OF INTEREST STATEMENT

Kohei Miyazono and Tetsuro Watabe are editorial board members of *Cancer Science*. No potential conflicts of interest were disclosed by the other authors.

ETHICS STATEMENT


Approval of the research protocol by an institutional review board: The experimental procedures for plasmid construction were approved by the Genetically Modified Organisms Safety Committee of Tokyo Medical and Dental University (registration number: G2019-026C9) and by the Director of Yokohama Branch of RIKEN (registration number: 2020-14 [19]).

Informed consent: N/A.

Registry and the registration no. of the study/trial: N/A.

Animal studies: The animal experiment procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (registration number: A2021-133C5 and A2023-133C3) and were performed according to the guidelines of the Animal Care Standards of Tokyo Medical and Dental University.

ORCID

Katarzyna A. Podyma-Inoue  <https://orcid.org/0009-0001-9720-0898>

Miho Kobayashi  <https://orcid.org/0000-0003-2548-4120>

Kohei Miyazono  <https://orcid.org/0000-0001-7341-0172>

Tetsuro Watabe  <https://orcid.org/0000-0001-5836-1309>

REFERENCES

- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013
- Jeon HM, Lee J. MET: roles in epithelial-mesenchymal transition and cancer stemness. *Ann Transl Med*. 2017;5(1):5. doi:10.21037/atm.2016.12.67
- Miyazono K, Katsuno Y, Koinuma D, Ehata S, Morikawa M. Intracellular and extracellular TGF- β signaling in cancer: some recent topics. *Front Med*. 2018;12(4):387-411. doi:10.1007/s11684-018-0646-8
- Heldin CH, Moustakas A. Signaling receptors for TGF- β family members. *Cold Spring Harb Perspect Biol*. 2016;8(8):a022053. doi:10.1101/cshperspect.a022053
- Katsuno Y, Derynck R. Epithelial plasticity, epithelial-mesenchymal transition, and the TGF- β family. *Dev Cell*. 2021;56(6):726-746. doi:10.1016/j.devcel.2021.02.028
- Ferrari G, Cook BD, Terushkin V, Pintucci G, Mignatti P. Transforming growth factor-beta 1 (TGF- β 1) induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis. *J Cell Physiol*. 2009;219(2):449-458. doi:10.1002/jcp.21706
- Yoshimatsu Y, Watabe T. Roles of TGF- β signals in endothelial-mesenchymal transition during cardiac fibrosis. *Int J Inflamm*. 2011;2011:724080. doi:10.4061/2011/724080
- Yoshimatsu Y, Watabe T. Emerging roles of inflammation-mediated endothelial-mesenchymal transition in health and disease. *Inflamm Regen*. 2022;42(1):9. doi:10.1186/s41232-021-00186-3
- Dituri F, Mazzocca A, Fernando J, et al. Differential inhibition of the TGF- β signaling pathway in HCC cells using the small molecule inhibitor LY2157299 and the D10 monoclonal antibody against TGF- β receptor type II. *PLoS One*. 2013;8(6):e67109. doi:10.1371/journal.pone.0067109
- Park SA, Kim MJ, Park SY, et al. EW-7197 inhibits hepatic, renal, and pulmonary fibrosis by blocking TGF- β /Smad and ROS signaling. *Cell Mol Life Sci*. 2015;72(10):2023-2039. doi:10.1007/s00018-014-1798-6
- Jaschinski F, Rothhammer T, Jachimczak P, Seitz C, Schneider A, Schlingensiepen KH. The antisense oligonucleotide trabedersen (AP 12009) for the targeted inhibition of TGF- β 2. *Curr Pharm Biotechnol*. 2011;12(12):2203-2213. doi:10.2174/138920111798808266
- Morris JC, Tan AR, Olencki TE, et al. Phase I study of GC1008 (fresolimumab): a human anti-transforming growth factor-beta (TGF β) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. *PLoS One*. 2014;9(3):e90353. doi:10.1371/journal.pone.0090353
- Teicher BA. TGF β -directed therapeutics: 2020. *Pharmacol Ther*. 2021;217:107666. doi:10.1016/j.pharmthera.2020.107666
- Metropulos AE, Munshi HG, Principe DR. The difficulty in translating the preclinical success of combined TGF β and immune checkpoint inhibition to clinical trial. *EBioMedicine*. 2022;86:104380. doi:10.1016/j.ebiom.2022.104380
- Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF- β inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest*. 2002;109(12):1551-1559. doi:10.1172/JCI15234
- Takahashi K, Akatsu Y, Podyma-Inoue KA, et al. Targeting all transforming growth factor- β isoforms with an Fc chimeric receptor impairs tumor growth and angiogenesis of oral squamous cell cancer. *J Biol Chem*. 2020;295(36):12559-12572. doi:10.1074/jbc.RA120.012492
- Yung LM, Nikolic I, Paskin-Flerlage SD, Pearsall RS, Kumar R, Yu PB. A selective transforming growth factor- β ligand trap attenuates pulmonary hypertension. *Am J Respir Crit Care Med*. 2016;194(9):1140-1151. doi:10.1164/rccm.201510-1955OC
- Kodama S, Podyma-Inoue KA, Uchihashi T, et al. Progression of melanoma is suppressed by targeting all transforming growth factor- β isoforms with an Fc chimeric receptor. *Oncol Rep*. 2021;46(3):197. doi:10.3892/or.2021.8148
- Ichijo H, Momose F, Miyazono K. Biological effects and binding properties of transforming growth factor- β on human oral squamous cell carcinoma cells. *Exp Cell Res*. 1990;187(2):263-269. doi:10.1016/0014-4827(90)90090-w
- Aggarwal V, Montoya CA, Donnenberg VS, Sant S. Interplay between tumor microenvironment and partial EMT as the driver of tumor progression. *iScience*. 2021;24(2):102113. doi:10.1016/j.isci.2021.102113
- Sakakitani S, Podyma-Inoue KA, Takayama R, et al. Activation of β 2-adrenergic receptor signals suppresses mesenchymal phenotypes of oral squamous cell carcinoma cells. *Cancer Sci*. 2021;112(1):155-167. doi:10.1111/cas.14670
- Zhang Y, Alexander PB, Wang XF. TGF- β family signaling in the control of cell proliferation and survival. *Cold Spring Harb Perspect Biol*. 2017;9(4):a022145. doi:10.1101/cshperspect.a022145
- Fukasawa K, Hanada K, Ichikawa K, et al. Endothelial-specific depletion of TGF- β signaling affects lymphatic function. *Inflamm Regen*. 2021;41(1):35. doi:10.1186/s41232-021-00185-4

24. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44-57. doi:[10.1038/nprot.2008.211](https://doi.org/10.1038/nprot.2008.211)
25. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res*. 2009;37(1):1-13. doi:[10.1093/nar/gkn923](https://doi.org/10.1093/nar/gkn923)
26. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. 2005;102(43):15545-15550. doi:[10.1073/pnas.0506580102](https://doi.org/10.1073/pnas.0506580102)
27. Upreti D, Adjei AA. KRAS: from undruggable to a druggable cancer target. *Cancer Treat Rev*. 2020;89:102070. doi:[10.1016/j.ctrv.2020.102070](https://doi.org/10.1016/j.ctrv.2020.102070)
28. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res*. 2019;47(D1):D607-D613. doi:[10.1093/nar/gky1131](https://doi.org/10.1093/nar/gky1131)
29. Dao DT, Anez-Bustillos L, Adam RM, Puder M, Bielenberg DR. Heparin-binding epidermal growth factor-like growth factor as a critical mediator of tissue repair and regeneration. *Am J Pathol*. 2018;188(11):2446-2456. doi:[10.1016/j.ajpath.2018.07.016](https://doi.org/10.1016/j.ajpath.2018.07.016)
30. Miyata K, Yotsumoto F, Nam SO, Kuroki M, Miyamoto S. Regulatory mechanisms of the HB-EGF autocrine loop in inflammation, homeostasis, development and cancer. *Anticancer Res*. 2012;32(6):2347-2352.
31. Taylor SR, Markesbery MG, Harding PA. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) and proteolytic processing by a disintegrin and metalloproteinases (ADAM): a regulator of several pathways. *Semin Cell Dev Biol*. 2014;28:22-30. doi:[10.1016/j.semcdb.2014.03.004](https://doi.org/10.1016/j.semcdb.2014.03.004)
32. Rébé C, Ghiringhelli F. Interleukin-1 β and cancer. *Cancers*. 2020;12(7):1791. doi:[10.3390/cancers12071791](https://doi.org/10.3390/cancers12071791)
33. Lee CH, Chang JS, Syu SH, et al. IL-1 β promotes malignant transformation and tumor aggressiveness in oral cancer. *J Cell Physiol*. 2015;230(4):875-884. doi:[10.1002/jcp.24816](https://doi.org/10.1002/jcp.24816)
34. Liu S, Wang Y, Han Y, et al. EREG-driven oncogenesis of head and neck squamous cell carcinoma exhibits higher sensitivity to erlotinib therapy. *Theranostics*. 2020;10(23):10589-10605. doi:[10.7150/thno.47176](https://doi.org/10.7150/thno.47176)
35. Shigeishi H, Higashikawa K, Hiraoka M, et al. Expression of epiregulin, a novel epidermal growth factor ligand associated with prognosis in human oral squamous cell carcinomas. *Oncol Rep*. 2008;19(6):1557-1564. doi:[10.3892/or.19.6.1557](https://doi.org/10.3892/or.19.6.1557)
36. Miyamoto S, Yagi H, Yotsumoto F, Kawarabayashi T, Mekada E. Heparin-binding epidermal growth factor-like growth factor as a novel targeting molecule for cancer therapy. *Cancer Sci*. 2006;97(5):341-347. doi:[10.1111/j.1349-7006.2006.00188.x](https://doi.org/10.1111/j.1349-7006.2006.00188.x)
37. Voronov E, Shouval DS, Krelm Y, et al. IL-1 is required for tumor invasiveness and angiogenesis. *Proc Natl Acad Sci USA*. 2003;100(5):2645-2650. doi:[10.1073/pnas.0437939100](https://doi.org/10.1073/pnas.0437939100)
38. Colak S, Ten Dijke P. Targeting TGF- β signaling in cancer. *Trends Cancer*. 2017;3(1):56-71. doi:[10.1016/j.trecan.2016.11.008](https://doi.org/10.1016/j.trecan.2016.11.008)
39. Huang CY, Chung CL, Hu TH, Chen JJ, Liu PF, Chen CL. Recent progress in TGF- β inhibitors for cancer therapy. *Biomed Pharmacother*. 2021;134:111046. doi:[10.1016/j.biopha.2020.111046](https://doi.org/10.1016/j.biopha.2020.111046)
40. Serova M, Tijeras-Raballand A, Dos Santos C, et al. Effects of TGF- β signalling inhibition with galunisertib (LY2157299) in hepatocellular carcinoma models and in ex vivo whole tumor tissue samples from patients. *Oncotarget*. 2015;6(25):21614-21627. doi:[10.18632/oncotarget.4308](https://doi.org/10.18632/oncotarget.4308)
41. Czajkowsky DM, Hu J, Shao Z, Pleass RJ. Fc-fusion proteins: new developments and future perspectives. *EMBO Mol Med*. 2012;4(10):1015-1028. doi:[10.1002/emmm.201201379](https://doi.org/10.1002/emmm.201201379)
42. Duivelshof BL, Murisier A, Camperi J, et al. Therapeutic Fc-fusion proteins: current analytical strategies. *J Sep Sci*. 2021;44(1):35-62. doi:[10.1002/jssc.202000765](https://doi.org/10.1002/jssc.202000765)
43. Seoane J, Gomis RR. TGF- β family signaling in tumor suppression and cancer progression. *Cold Spring Harb Perspect Biol*. 2017;9(12):a022277. doi:[10.1101/cshperspect.a022277](https://doi.org/10.1101/cshperspect.a022277)
44. Yoshimatsu Y, Wakabayashi I, Kimuro S, et al. TNF- α enhances TGF- β -induced endothelial-to-mesenchymal transition via TGF- β signal augmentation. *Cancer Sci*. 2020;111(7):2385-2399. doi:[10.1111/cas.14455](https://doi.org/10.1111/cas.14455)
45. Oka M, Iwata C, Suzuki HI, et al. Inhibition of endogenous TGF- β signaling enhances lymphangiogenesis. *Blood*. 2008;111(9):4571-4579. doi:[10.1182/blood-2007-10-120337](https://doi.org/10.1182/blood-2007-10-120337)
46. Yao D, Dai C, Peng S. Mechanism of the mesenchymal-epithelial transition and its relationship with metastatic tumor formation. *Mol Cancer Res*. 2011;9(12):1608-1620. doi:[10.1158/1541-7786](https://doi.org/10.1158/1541-7786)
47. Saitoh M. Epithelial-mesenchymal transition by synergy between transforming growth factor- β and growth factors in cancer progression. *Diagnostics*. 2022;12(9):2127. doi:[10.3390/diagnostics12092127](https://doi.org/10.3390/diagnostics12092127)
48. O-charoenrat P, Modjtahedi H, Rhys-Evans P, Court WJ, Box GM, Eccles SA. Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells. *Cancer Res*. 2000;60(4):1121-1128.
49. Yotsumoto F, Tokunaga E, Oki E, et al. Molecular hierarchy of heparin-binding EGF-like growth factor-regulated angiogenesis in triple-negative breast cancer. *Mol Cancer Res*. 2013;11(5):506-517. doi:[10.1158/1541-7786.MCR-12-0428](https://doi.org/10.1158/1541-7786.MCR-12-0428)
50. Bulus N, Barnard JA. Heparin binding epidermal growth factor-like growth factor is a transforming growth factor beta-regulated gene in intestinal epithelial cells. *Biochem Biophys Res Commun*. 1999;264(3):808-812. doi:[10.1006/bbrc.1999.1600](https://doi.org/10.1006/bbrc.1999.1600)
51. Zhang S, Fan Y, Qin L, et al. IL-1 β augments TGF- β inducing epithelial-mesenchymal transition of epithelial cells and associates with poor pulmonary function improvement in neutrophilic asthmatics. *Respir Res*. 2021;22(1):216. doi:[10.1186/s12931-021-01808-7](https://doi.org/10.1186/s12931-021-01808-7)
52. Cheng WL, Feng PH, Lee KY, et al. The role of EREG/EGFR pathway in tumor progression. *Int J Mol Sci*. 2021;22(23):12828. doi:[10.3390/ijms222312828](https://doi.org/10.3390/ijms222312828)

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Tokizaki S, Podyma-Inoue KA, Matsumoto T, et al. Inhibition of transforming growth factor- β signals suppresses tumor formation by regulation of tumor microenvironment networks. *Cancer Sci*. 2024;115:211-226. doi:[10.1111/cas.16006](https://doi.org/10.1111/cas.16006)