Original Research *Highlight article*

Single-cell RNA sequencing reveals differential expression of EGFL7 and VEGF in giant-cell tumor of bone and osteosarcoma

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Impact Statement

Giant-cell tumor of bone (GCTB) is a benign locally aggressive tumor and osteosarcoma (OS) is the most commonly diagnosed pediatric bone malignancy. New, effective, and safe treatments are needed for these tumors as GCTB frequently recurs locally following resection and 30–40% of OS patients fail conventional multimodal therapy. This novel study using bioinformatic analyses of single cell data of GCTB and OS tumors reveals that pro-angiogenic factors, *EGFL7, VEGF-A*, *VEGF-B*, *VEGF-C*, and *VEGF-D* are differentially expressed (DE) in single GCTB and OS cells. The results presented here are of importance and allow us to better understand the cellular heterogeneity and genetic mechanisms underlying tumor development and progression in GCTB and OS.

Abstract

Dysregulation of angiogenesis is associated with tumor development and is accompanied by altered expression of pro-angiogenic factors. *EGFL7* is a newly identified antigenic factor that plays a role in various cancers such as breast cancer, lung cancer, and acute myeloid leukemia. We have recently found that *EGFL7* is expressed in the bone microenvironment, but its role in giant-cell tumor of bone (GCTB) and osteosarcoma (OS) is unknown. The aims of this study are to examine the gene expression profile of *EGFL7* in GCTB and OS and compare with that of *VEGF-A-D* and *TNFSF11* using single-cell RNA sequencing data. In-depth differential expression analyses were employed to characterize their expression in the constituent cell types of GCTB and OS. Notably, *EGFL7* in GCTB was expressed at highest levels in the endothelial cell (EC) cluster followed by osteoblasts, myeloid cells, and chondrocytes, respectively. In OS, *EGFL7* exhibited highest expression in EC cell cluster followed by osteoblastic OS cells, myeloid cells 1, and carcinoma associated fibroblasts (CAFs), respectively. In comparison, *VEGF-A* is expressed at highest levels in myeloid cells followed by OCs in GCTB, and in myeloid cells, and OCs in OS. *VEGF-B* is expressed at highest levels in chondrocytes in GCTB and in OCs in OS. *VEGF-C* is strongly enriched in ECs and *VEGF-D* is expressed at weak levels in all cell types in both GCTB and OS. *TNFSF11* (or RANKL) shows high expression in CAFs and osteoblastic OS cells in OS, and osteoblasts in GCTB. This study investigates pro-angiogenic genes in GCTB and OS and suggests that these genes and their expression patterns are cell-type specific and could provide potential prognostic biomarkers and cell type target treatment for GCTB and OS.

Keywords: Giant-cell tumor of bone, osteosarcoma, differentially expressed genes, single cell sequencing, EGFL7, VEGF

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Introduction

Giant-cell tumor of bone (GCTB) is a relatively rare benign neoplasm that results in significant bone destruction, increased fracture risk, and significant disability.[1–4] Although rarely fatal, GCTB is characterized by local invasion and occasional metastasis to the lung.[5–7] GCTB presents in young adults between the ages of 20 and 44 years with a higher incidence in females.[4] GCTB typically develops at the junction of the metaphysis and epiphysis of long bones and more commonly in the distal femur, proximal tibia, and distal radius.[5]

The histological characteristics of GCTB include the appearance of osteoclast-like multinucleated giant cells and the rapid proliferation of mononuclear stromal cells.[2,8] Osteosarcoma (OS) is the most common primary bone tumor with a peak incidence in adolescence and a secondary peak

in advanced age.[9,10] OS develops from malignant mesenchymal cells that produce pathological osteoid.[11] OS outcomes of survival in children and adolescence for a majority of the 1900s were only 20%.[9] Following the introduction of adjuvant chemotherapy in the 1970s, the predicted survival rate has increased dramatically to 65–70%.[9,12–14]

Angiogenesis is the fundamental biological phenomenon by which new capillary blood vessels develop from the remodeling of pre-existing vasculature.[15] Physiological angiogenesis occurs in bone development, growth, repair, and wound healing.[16–18] Pathological angiogenesis is critical to tumor development, progression, and metastasis in GCTB and OS.[19,20] The newly developed blood vessels that grow in these tumors remove metabolic waste from the tumor region and supply tumor cells with the nourishment and oxygen required for survival and proliferation.[21]

Investigations revealed that angiogenesis is tightly regulated by an equilibrium between endogenous stimulators and inhibitors.[22] Pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors promote angiogenesis.[23,24] Recently, epidermal growth factor-like domain-containing protein 7 (EGFL7), a secreted pro-angiogenic factor, was identified in bone local environments and is expressed in kidney, glioma, and colon cancer tissues.[17,25–27] However, little is known about gene expression levels and functions of *EGFL7* in GCTB and OS tumor cell types.

Single-cell RNA sequencing (scRNA seq) is a new technology that enables transcriptome profiling of individual cells.[28] This method can be used to determine whether a particular gene is expressed in a cell, the quantity of transcripts expressed in a cell, and differential splicing patterns.[29] The present study aimed to determine whether *EGFL7* is differentially expressed in identified single cells of GCTB and OS tumors by performing bioinformatic analyses of data obtained using scRNA seq. In this way, we will improve our understanding of the cellular and molecular basis of GCTB and OS by identifying critical GCTB and OS genes and contribute to the development of effective angiogenic strategies for GCTB and OS.

Materials and methods

scRNA seq data collection

To define the heterogeneity of GCTB and OS tumor cells at the transcriptional level, we performed bioinformatic analysis using results obtained from our previously published studies.[1,30] The mRNA expression data used in this study were downloaded from the NCBI Gene Expression Omnibus database. We refer to each dataset by the first author's last name and cancer type. The GCTB dataset GSE168664 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE168664) and the OS dataset GSE162454 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162454) were chosen for the analysis. In total, 8033 cells isolated from one primary tumor sample obtained from a patient diagnosed with GCTB and 29,278 cells isolated from six primary tumor samples obtained from six OS patients were included in this dataset.[1,30] We felt that these datasets were an excellent resource to investigate differentially expressed genes at the single-cell level. All data were obtained from a public database; therefore, ethics approval was not required.

Filtering and preprocessing

We used Seurat package (version 3.2.1) and Harmony package (version1.0; https://github.com/immunogenomics/Harmony) to perform quality control and remove batch effects in R language (http://www.rproject.org), as described.[1,30] For the OS dataset, cells with expression of genes (between 200 and 5000) and >10% mitochondrial genes were considered low-quality and discarded. For the GCTB dataset, cells with expression of genes (between 300 and 4500) and <10% mitochondrial genes were considered low-quality cells and discarded; 29,278 OS cells and 8033 GCTB cells passed quality control.

Cluster identification

Uniform manifold approximation and projection (UMAP) analysis was done in R using the Dimplot function with the following parameters (dim = 1:20 (OS), dim = 1:20 (GCTB), resolution = 0.10 (OS/GCTB)). To define the color the ggsci package (version 2.9) was used. The identified cell types were in accordance with our prior studies.[30]

Differential expression analyses

Then, after clustering gene expression distributions were expressed using the FeaturePlot function.

Processing of scRNA seq data

We transformed count values of single-cell data into TPM values using the following formula:

$$TPM_i = \frac{X_i}{\tilde{l}_i}\left(\frac{1}{\sum_j \frac{X_j}{\tilde{l}_j}}\right)\cdot 10^6$$

Pairwise sequence alignment and phylogenetic tree analysis

We constructed pairwise sequence alignment using ClustalW (ver. 1.83). The phylogenetic tree analysis was performed using MEGA7.0.

Statistical analyses

A $p < 0.05$ difference was statistically significant. Results were summarized and are displayed in Supplementary Tables 1–12.

Results

Cellular composition of GCTB and OS tumor tissue

To better understand the cellular diversity of GCTB and OS tumors, we performed UMAP clustering analysis. From 8033 GCTB cells, eight cell types were identified by unique marker genes: myeloid cells, osteoblasts, NK/T cells, osteoclasts,

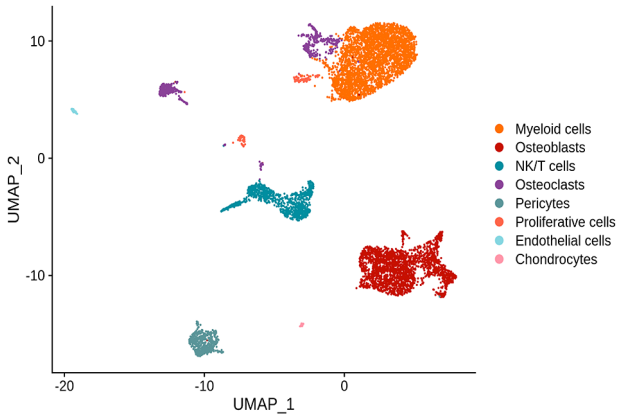

Figure 1. The eight diverse cell types identified in GCTB demonstrated by uniform manifold approximation and projection (UMAP) plot.

pericytes, proliferative cells, endothelial cells (ECs), and chondrocytes, suggesting intertumoral heterogeneity (Figure 1). From 29,278 available OS cells, nine malignant cell clusters were identified by unique marker genes: osteoblastic OS cells, NK/T cells, osteoclasts, carcinoma-associated fibroblasts, plasmocytes, ECs, B cells, and two subsets of myeloid cells, myeloid cells 1 and myeloid cells 2 (Figure 4).

Single-cell sequencing identifies *EGFL7*, *VEGF-A*, *VEGF-B*, *VEGF-C*, and *VEGF-D* differentially expressed in OS and cells of GCTB microenvironment

We have previously shown that *EGFL7* is expressed in GCTB tumor tissues.[1] Additional studies detected and localized expression of *EGFL7* in OS tissues.[31] *VEGF-A* was identified in both OS and GCTB tissues.[30] These studies, however, did not determine the precise expression profiles of these genes in single OS or GCTB cells. Given that OS and GCTB tumor cell populations have notable differences in gene expression, we selected a panel of genes (*EGFL7, VEGF-A-D*, and *TNFSF11*) for differential expression analysis. The average expression levels of each gene in each of the specific tumor cell types were determined. Then, the average expression levels for all genes were compared pairwise between cell groups from the same cancer type. Our results were consistent with previous studies that detected expression of *EGFL7* and *VEGF* in OS and GCTB tissues. In addition, we observed consistent significant differential expression of *EGFL7, VEGF-A-D*, and *TNFSF11* in single OS and GCTB cells, suggesting that the expression of *EGFL7, VEGF-*, and *TNFSF11* are cell-type specific (Figures 2(A) to (E) and 5(A) to (E)). The angiogenic factor *VEGF-A*, followed by *VEGF-B*, *EGFL7*, *VEGF-C*, *VEGF-D*, and *TNFSF11*, respectively, showed the most differential expression in OS cells. *VEGF-D* exhibited the most differential expression in GCTB cells, followed by *VEGF-A*, *VEGF-B*, *VEGF-C*, *EGFL7*, and *TNFSF11*, respectively.

EGFL7 is expressed primarily in ECs of cells of GCTB microenvironment

We quantified the relative abundance of *EGFL7* in various cells of the GCTB microenvironment and then pairwise compared average expression levels of *EGFL7* between GCTB tumor cell types to gain insight into the transcriptional complexity of GCTB. The gene expression levels of *EGFL7* were calculated using TPM. *EGFL7* was expressed in all eight major cell types (Figure 3(A)). We identified varied expression patterns of *EGFL7* in specific cells of the GCTB microenvironment (Figure 3(A)) and found differential expression of *EGFL7* in GCTB. The EC cluster stood out for its significantly high expression of *EGFL7*, followed by osteoblasts, myeloid cells, and chondrocytes (Figure 3(A)). Interestingly, *EGFL7* was expressed at low level OCs, NK/T cells, pericytes, and proliferative cells (Figure 3(A)).

We performed 28 pairwise comparisons of *EGFL7* gene expression levels between eight GCTB cell types (Supplementary Table 1). Of these, 13 pairwise comparisons of chondrocytes versus ECs, chondrocytes versus osteoblasts, ECs versus myeloid cells, ECs versus NK/T cells, ECs versus osteoblasts, ECs versus OCs, ECs versus pericytes, ECs versus proliferative cells, myeloid cells versus osteoblasts, NK/T versus osteoblasts, osteoblasts versus OCs, osteoblasts versus pericytes, and OCs versus proliferative cells revealed significant differential expression ($p < 0.05$) (Supplementary Table 1).

Fifteen pairwise comparisons of chondrocytes versus myeloid cells, chondrocytes versus NK/T cells, chondrocytes versus OCs, chondrocytes versus pericytes, chondrocytes versus proliferative cells, myeloid cells versus NK/T cells, myeloid cells versus OCs, myeloid cells versus pericytes, myeloid cells versus proliferative cells, NK/T cells versus OCs, NK/T cells versus pericytes, NK/T versus proliferative cells, OCs versus pericytes, OCs versus proliferative cells, and pericytes versus proliferative cells showed no significant differential expression ($p > 0.05$) (Supplementary Table 1).

VEGF-A is strongly expressed in myeloid cells, OCs, and proliferative cells of GCTB tumor

We next examined and pairwise compared the relative expression levels of *VEGF-A* in single GCTB cells. The gene expression levels of *VEGF-A* were calculated using TPM. *VEGF-A* was expressed in all eight major cell types (Figure 3(B)). Different expression levels of *VEGF-A* were reported in single GCTB cell types, indicating some differential expression of *VEGF-A* in GCTB (Figure 3(B)). The level of *VEGF-A* expression was highest in myeloid cells, followed by osteoclasts, proliferative cells, and osteoblast cell clusters, respectively (Figure 3(B)). We detected reduced expression levels of *VEGF-A* in the chondrocytes, pericytes, ECs, and NK/T cells clusters (Figure 3(B)). We performed 28 pairwise comparisons of *VEGF-A* gene expression levels between eight GCTB cell types. Of these, 20 pairwise comparisons of chondrocytes versus myeloid cells, chondrocytes versus NK/T cells, chondrocytes versus OCs, ECs versus myeloid cells, ECs versus osteoblasts, ECs versus OCs, ECs versus proliferative cells, myeloid cells versus NK/T cells, myeloid cells versus osteoblasts, myeloid cells versus pericytes, myeloid cells versus proliferative cells, NK/T versus osteoblasts, NK/T versus OCs, NK/T versus pericytes, NK/T versus proliferative cells, osteoblasts versus OCs, osteoblasts versus pericytes, OCs versus pericytes, OCs versus proliferative cells, and

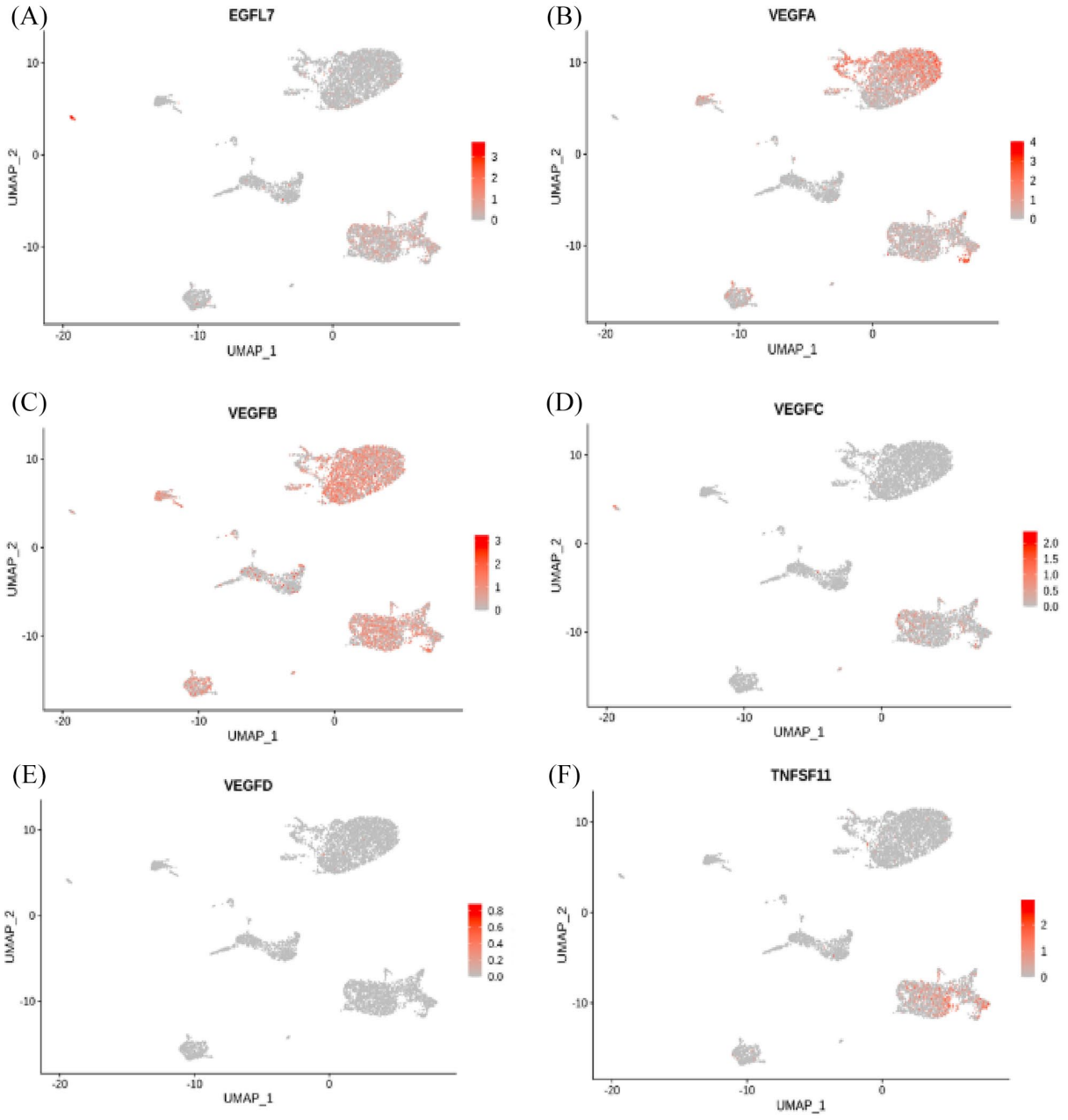

Figure 2. Uniform manifold approximation and projection (UMAP) plot of (A) *EGFL7*, (B) *VEGF-A*, (C) *VEGF-B*, (D) *VEGF-C*, (E) *VEGF-D*, and (F) *TNFSF11* expression in the eight main GCTB cell types.

pericytes versus proliferative cells revealed significant differential expression ($p < 0.05$) (Supplementary Table 2).

Eight pairwise comparisons of chondrocytes versus ECs, chondrocytes versus osteoblasts, chondrocytes versus pericytes, chondrocytes versus proliferative cells, ECs versus NK/T cells, ECs versus pericytes, myeloid cells versus OCs, and osteoblasts versus proliferative cells showed no significant differential expression (p 0.05) (Supplementary Table 2).

VEGF-B is strongly enriched in chondrocyte cells of GCTB tumor

We next quantified gene expression levels of *VEGF-B* in single cells of the GCTB microenvironment and measured differences in gene expression of this gene between GCTB cell types. The gene expression levels of *VEGF-B* were calculated using TPM. *VEGF-B* expression was detected in all eight cell types, and different expression levels of *VEGF-B* were found in specific GCTB cell types (Figure 3(C)). The expression of *VEGF-B* was highest in chondrocytes, followed by myeloid cells, osteoblasts, OCs, proliferative cells, pericytes, NK/T, and ECs (Figure 3(C)). We performed 28 pairwise comparisons of *VEGF-B* gene expression levels between eight GCTB cell types. Of these, 20 pairwise comparisons of chondrocytes versus ECs, chondrocytes versus NK/T cells, chondrocytes versus osteoblasts, chondrocytes versus OCs, chondrocytes versus pericytes, chondrocytes versus proliferative cells, ECs

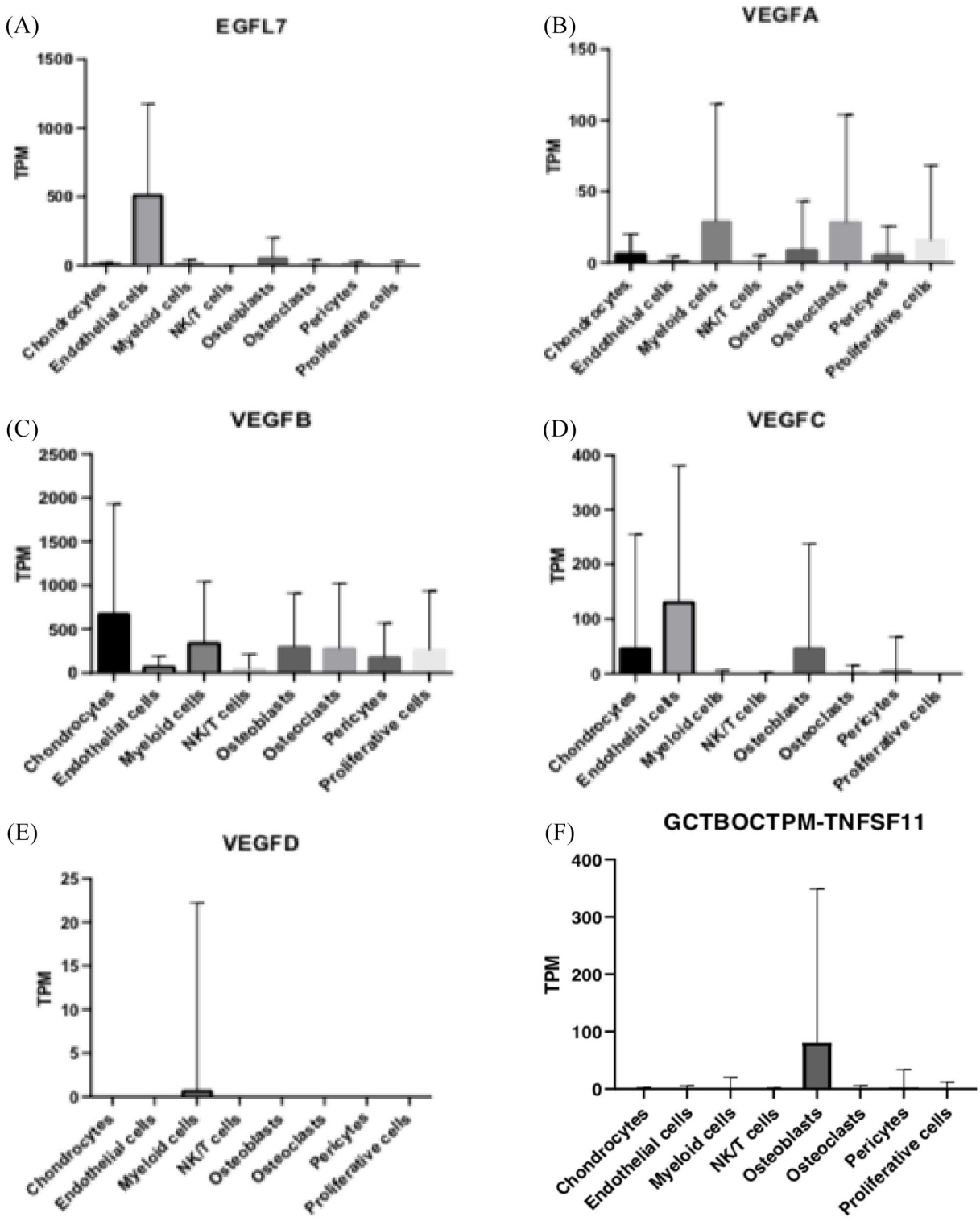

Figure 3. (A) Bar graph representing the relative expression levels of *EGFL7* in the eight major GCTB cell types. (B) Bar graph representing the relative expression levels of *VEGF-A* in the eight major GCTB cell types. (C) Bar graph representing the relative expression levels of *VEGF-B* in the eight major GCTB cell types. (D) Bar graph representing the relative expression levels of *VEGF-C* in the eight major GCTB cell types. (E) Bar graph representing the relative expression levels of *VEGF-D* in the eight major GCTB cell types. (F) Bar graph representing the relative expression levels of *TNFSF11* in the eight major GCTB cell types.

versus myeloid cells, ECs versus osteoblasts, ECs versus OCs, ECs versus proliferative cells, myeloid cells versus OCs, myeloid cells versus pericytes, myeloid cells versus proliferative cells, NK/T cells versus osteoblasts, NK/T versus OCs, NK/T versus pericytes, osteoblasts versus osteoclasts, and osteoblasts versus pericytes revealed significant differential expression ($p < 0.05$) (Supplementary Table 3).

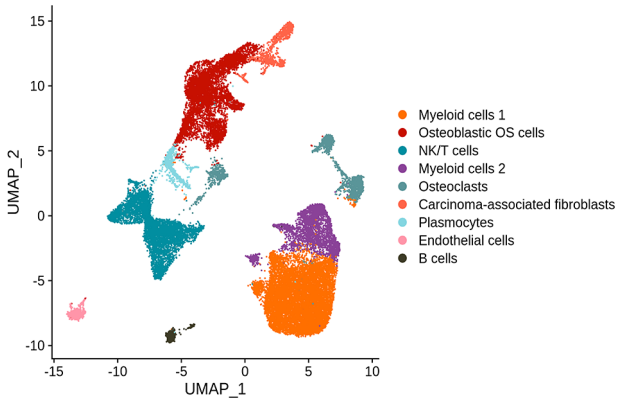

Figure 4. The nine diverse cell types identified in OS demonstrated by uniform manifold approximation and projection (UMAP) plot.

Eight pairwise comparisons of chondrocytes versus myeloid cells, ECs versus NK/T cells, ECs versus pericytes, myeloid cells versus osteoblasts, osteoblasts versus proliferative cells, OCs versus pericytes, OCs versus proliferative cells, and pericytes versus proliferative cells showed no significant differential expression ($p > 0.05$) (Supplementary Table 3).

VEGF-C is highly abundant in ECs, chondrocytes, and osteoblasts of GCTB tumor

We then measured and pairwise compared the gene expression levels of *VEGF-C* between clusters of GCTB cell types. The gene expression levels of *VEGF-C* were calculated using TPM. *VEGF-C* was expressed in all eight cell types examined (Figure 3(D)). Different expression levels of *VEGF-C* were reported in single GCTB cell types, indicating some differential expression of *VEGF-C* in GCTB (Figure 3(D)). We found that *VEGF-C* was most abundantly expressed in ECs followed by chondrocytes, osteoblasts, and pericytes cell clusters, respectively (Figure 3(D)). In comparison, *VEGF-C* was expressed at lower levels in four cell clusters: myeloid cells, NK/T cells, OCs, and proliferative cells (Figure 3(D)). We performed 28 pairwise comparisons of *VEGF-C* gene expression levels between eight GCTB cell types (Supplementary Table 4). Of these, 17 pairwise comparisons of chondrocytes versus ECs, chondrocytes versus myeloid cells, chondrocytes versus NK/T cells, chondrocytes versus OCs, chondrocytes versus pericytes, chondrocytes versus proliferative cells, ECs versus myeloid cells, ECs versus NK/T cells, ECs versus osteoblasts, ECs versus OCs, ECs versus pericytes, ECs versus proliferative, myeloid cells versus osteoblasts, NK/T versus osteoblasts, osteoblasts versus OCs, osteoblasts versus pericytes, and osteoblasts versus proliferative cells revealed significant differential expression ($p < 0.05$) (Supplementary Table 4).

Eleven pairwise comparisons of chondrocytes versus osteoblasts, myeloid cells versus NK/T cells, myeloid cells versus OCs, myeloid cells versus pericytes, myeloid versus proliferative cells, NK/T cells versus OCs, NK/T cells versus pericytes, NK/T cells versus proliferative cells, OCs versus pericytes, OCs versus proliferative cells, and pericytes versus proliferative cells showed no significant differential expression ($p 0.05$) (Supplementary Table 4).

Expression pattern differences of *VEGF-D* in various cells of GCTB microenvironment

Next, we compared average expression levels of *VEGF-D* in specific cells of GCTB microenvironment. The gene expression levels of *VEGF-D* were calculated using TPM. We identified the expression of *VEGF-D* in all eight cell types examined (Figure 3(E)). Varying expression levels of *VEGF-D* were found in single GCTB cell types (Figure 3(E)). Relatively low mean expression of *VEGF-D* was found in all eight cell types (Figure 3(E)). Slightly higher levels of *VEGF-D* expression were found in the myeloid cells cluster than in other cell types (Figure 3(E)). We performed 28 pairwise comparisons of *VEGF-D* gene expression levels between eight GCTB cell types (Supplementary Table 5). Of these, 28 pairwise comparisons of chondrocytes versus ECs, chondrocytes versus myeloid cells, chondrocytes versus NK/T, chondrocytes versus osteoblasts, chondrocytes versus OCs, chondrocytes versus pericytes, chondrocytes versus proliferative, ECs versus myeloid cells, ECs versus NK/T cells, ECs versus osteoblasts, ECs versus OCs, ECs versus pericytes, ECs versus proliferative cells, myeloid cells versus NK/T cells, myeloid cells versus osteoblasts, myeloid cells versus OCs, myeloid cells versus pericytes, myeloid cells versus proliferative cells, NK/T cells versus osteoblasts, NK/T cells versus OCs, NK/T cells versus pericytes, NK/T cells versus proliferative cells, osteoblasts versus OCs, osteoblasts versus pericytes, osteoblasts versus proliferative cells, OCs versus pericytes, OCs versus proliferative cells, and pericytes versus proliferative cells showed no significant differential expression ($p > 0.05$) (Supplementary Table 5).

Expression pattern differences of *TNFSF11* in various cells of GCTB microenvironment

We measured and pairwise compared gene expression levels of *TNFSF11* between clusters of GCTB cell types. The gene expression levels of *TNFSF11* were calculated using TPM. Varying expression levels of *TNFSF11* were detected in single GCTB cell types (Figure 3(F)). Strikingly, osteoblasts cells showed by far the highest expression of *TNFSF11* followed by pericytes, respectively (Figure 3(F)). The cell clusters chondrocytes, ECs, myeloid cells, NK/T cells, OCs, pericytes, and proliferative cells showed only weak expression of *TNFSF11* (Figure 3(F)). We performed 28 pairwise comparisons of *TNFSF11* gene expression levels between eight GCTB cell types (Supplementary Table 6). Of these, seven pairwise comparisons of chondrocytes versus osteoblasts, ECs versus osteoblasts, myeloid cells versus osteoblasts, NK/T cells versus osteoblasts, osteoblasts versus osteoclasts, osteoblasts versus pericytes, and osteoblasts versus proliferative cells revealed significant differential expression ($p < 0.05$) (Supplementary Table 6).

Twenty-one pairwise comparisons of chondrocytes versus ECs, chondrocytes versus myeloid cells, chondrocytes versus NK/T cells, chondrocytes versus osteoclasts, chondrocytes versus pericytes, chondrocytes versus proliferative cells, ECs versus myeloid cells, ECs versus NK/T cells, ECs versus osteoclasts, ECs versus pericytes, ECs versus

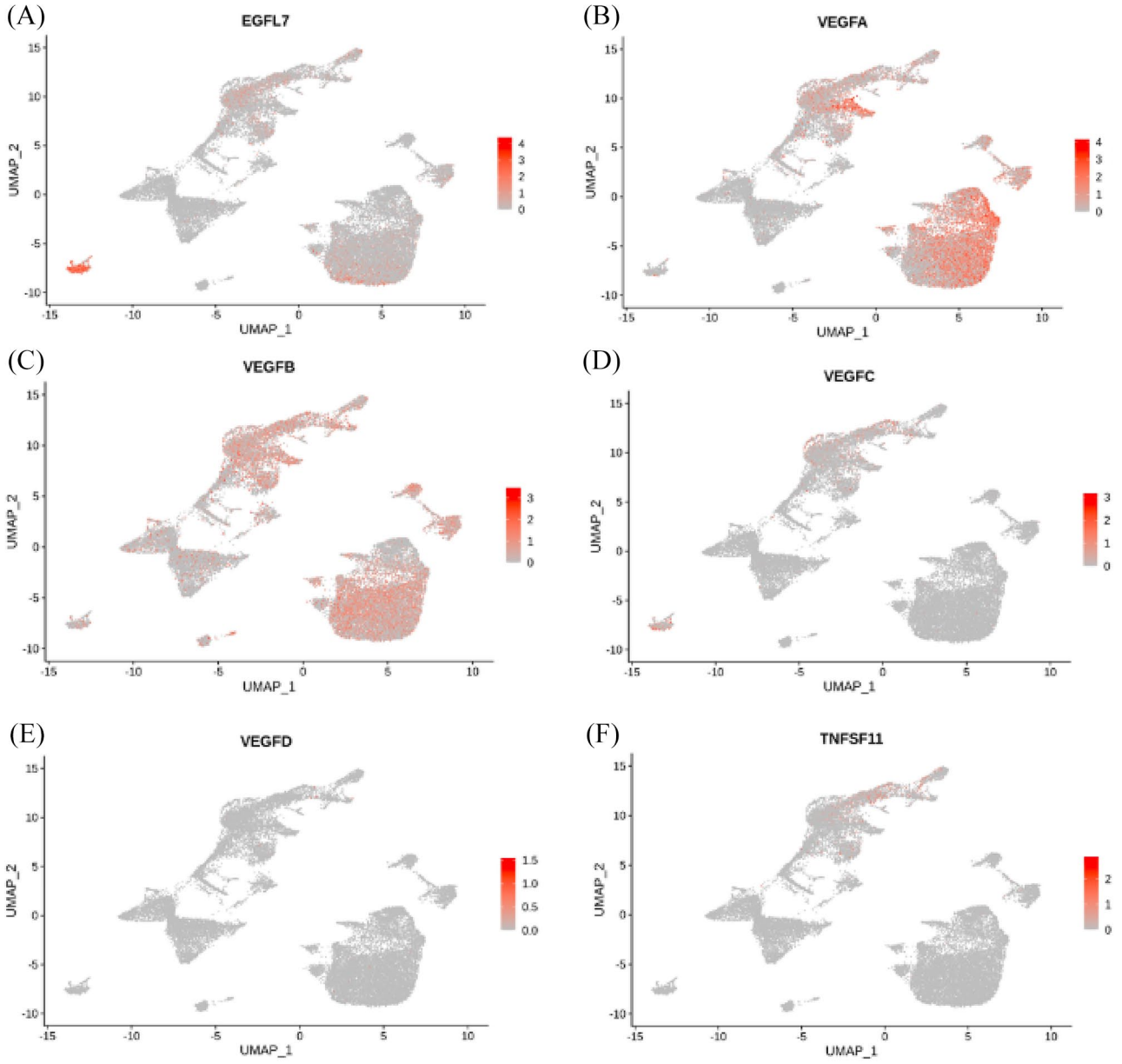

Figure 5. Uniform manifold approximation and projection (UMAP) plot of *EGFL7*, *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGF-D*, and *TNFSF11* expression in the nine major OS cell types.

proliferative cells, myeloid cells versus NK/T cells, myeloid cells versus osteoclasts, myeloid cells versus pericytes, myeloid cells versus proliferative cells, NK/T cells versus osteoclasts, NK/T cells versus pericytes, NK/T cells versus proliferative cells, OCs versus pericytes, OCs versus proliferative cells, and pericytes versus proliferative cells showed no significant differential expression ($p > 0.05$) (Supplementary Table 6).

EGFL7 is highly abundant in ECs of cells of OS microenvironment

We next sought to measure and compare average gene expression levels of *EGFL7* between various OS cell types. The gene expression levels of *EGFL7* were calculated using TPM. According to our data, *EGFL7* was expressed in all nine cell types (Figure 6(A)). *EGFL7* showed the highest expression in the ECs cell cluster, followed by osteoblastic OS cells, myeloid cells 1, and carcinoma-associated fibroblasts (CAFs) (Figure 6(A)). By contrast, low levels of *EGFL7* expression was found in B cells, myeloid cells 2, NK/T cells, osteoblastic OS cells, osteoclasts (OCs), and plasmocytes (Figure 6(A)). We performed 36 pairwise comparisons of *EGFL7* gene expression levels between nine OS cell types (Supplementary Table 7). Of these, 29 pairwise comparisons of B cells versus CAFs, B cells versus ECs, B cells versus myeloid cells 1, B cells versus osteoblastic OS cells, B cells versus OCs, CAFs versus ECs, CAFs versus myeloid cells 1, CAFs versus myeloid cells 2, CAFs versus NK/T cells, CAFs versus osteoblastic OS cells, CAFs versus plasmocytes, ECs versus myeloid cells 1, ECs versus myeloid cells 2, ECs versus NK/T cells, ECs versus osteoblastic OS cells, ECs versus OCs, ECs versus

plasmocytes, and myeloid cells 1 versus myeloid cells 2, myeloid cells 1 versus OCs, myeloid cells 1 versus plasmocytes, myeloid cells 2 versus NK/T cells, myeloid cells 2 versus osteoblastic OS cells, myeloid cells 2 versus OCs, NK/T cells versus osteoblastic OS cells, NK/T versus OCs, osteoblastic OS cells versus OCs, osteoblastic OS cells versus plasmocytes, and OCs versus plasmocytes revealed significant differential expression ($p < 0.05$) (Supplementary Table 7).

Seven pairwise comparisons of B cells versus myeloid cells 2, B cells versus NK/T cells, B cells versus plasmocytes, CAFs versus OCs, myeloid cells 1 versus osteoblastic OS cells, myeloid cells 2 versus plasmocytes, and NK/T cells versus plasmocytes showed no significant differential expression ($p > 0.05$) (Supplementary Table 7).

VEGF-A is highly expressed in myeloid cells 1 and myeloid cells 2 of cells of OS microenvironment

We further investigated and pairwise compared the relative abundance of *VEGF-A* in specific cells of OS microenvironment. The gene expression levels of *VEGF-A* were calculated using TPM. *VEGF-A* was expressed in all nine cell types (Figure 6(B)). The highest level of *VEGF-A* expression was found in the following cell clusters: myeloid cells 2, myeloid cells 1, OCs, and osteoblastic OS cells (Figure 6(B)). In addition, *VEGF-A* was expressed at low levels in five cell clusters: B cells, CAFs, ECs, NK/T cells, and plasmocytes (Figure 6(B)). We performed 36 pairwise comparisons of *VEGF-A* gene expression levels between nine OS cell types (Supplementary Table 8). Of these, 31 pairwise comparisons of B cells versus CAFs, B cells versus myeloid cells 1, B cells versus myeloid cells 2, B cells versus osteoblastic OS cells, B cells versus OCs, CAFs versus ECs, CAFs versus myeloid cells 1, CAFs versus myeloid cells 2, and CAFs versus NK/T cells, CAFs versus osteoblastic OS cells, CAFs versus OCs, CAFs versus plasmocytes, ECs versus myeloid cells 1, ECs versus myeloid cells 2, ECs versus osteoblastic OS cells, ECs versus OCs, myeloid cells 1 versus myeloid cells 2, myeloid cells 1 versus NK/T cells, myeloid cells 1 versus osteoblastic OS cells, myeloid cells 1 versus OCs, myeloid cells 1 versus plasmocytes, myeloid cells 2 versus NK/T cells, myeloid cells 2 versus osteoblastic OS cells, myeloid cells 2 versus OCs, myeloid cells 2 versus plasmocytes, NK/T cells versus osteoblastic OS cells, NK/T cells versus OCs, NK/T versus plasmocytes, osteoblastic OS cells versus OCs, osteoblastic OS cells versus plasmocytes, and OCs versus plasmocytes revealed significant differential expression ($p < 0.05$) (Supplementary Table 8).

Seven pairwise comparisons of B cells versus ECs, B cells versus NK/T cells, B cells versus plasmocytes, ECs versus NK/T cells, and ECs versus plasmocytes showed no significant differential expression ($p > 0.05$) (Supplementary Table 8).

Expression pattern differences of *VEGF-B* in various cells of OS microenvironment

We determined the relative abundance of *VEGF-B* and compared the expression levels of this gene among different OS tumor cell types. The gene expression levels of *VEGF-B* were calculated using TPM. *VEGF-B* was expressed in all nine main cell types and showed varied mean expression levels across the cell types (Figure 6(C)). *VEGF-B* was expressed at the highest levels in the OCs cell cluster, followed by myeloid cells 1, CAFs, osteoblastic OS cells, B cells, myeloid cells 2, plasmocytes, ECs, and NK/T cells. We performed 36 pairwise comparisons of *VEGF-A* gene expression levels between nine OS cell types (Supplementary Table 9). Of these, 30 pairwise comparisons of B cells versus myeloid cells 1, B cells versus NK/T, B cells versus OCs, B cells versus plasmocytes, CAFs versus ECs, CAFs versus myeloid cells 1, CAFs versus myeloid cells 2, CAFs versus NK/T cells, CAFs versus OCs, CAFs versus plasmocytes, and ECs versus myeloid cells 1, ECs versus myeloid cells 2, ECs versus NK/T cells, ECs versus osteoblastic OS cells, ECs versus OCs, myeloid cells 1 versus myeloid cells 2, myeloid cells 1 versus NK/T cells, myeloid cells 1 versus osteoblastic OS cells, myeloid cells 1 versus plasmocytes, myeloid cells 2 versus NK/T cells, myeloid cells 2 versus osteoblastic OS cells, myeloid cells 2 versus and OCs, myeloid cells 2 versus plasmocytes, NK/T cells versus osteoblastic OS cells, NK/T cells versus OCs, NK/T cells versus plasmocytes, osteoblastic OS cells versus OCs, osteoblastic OS cells v plasmocytes, and OCs versus plasmocytes revealed significant differential expression ($p < 0.05$) (Supplementary Table 9).

Seven pairwise comparisons of B cells versus CAFs, B cells versus ECs, B cells versus myeloid cells 2, CAFs versus osteoblastic OS cells, ECs versus plasmocytes, and myeloid cells 1 versus OCs showed no significant differential expression ($p > 0.05$) (Supplementary Table 9).

VEGF-C is highly expressed in ECs of cells of OS microenvironment

We next investigated the average expression levels of *VEGF-C* in specific cells of OS microenvironment and then pairwise compared the average expression levels of this gene between OS cell types. The gene expression levels of *VEGF-C* were calculated using TPM. *VEGF-C* was expressed in all nine cell types examined (Figure 6(D)). Varying expression levels of *VEGF-C* were found in single OS cell types (Figure 6(D)). Our results revealed that *VEGF-C* is expressed at the highest levels in the ECs cell cluster, followed by osteoblastic OS cells, CAFs, and myeloid cells 1 (Figure 6(D)). By contrast, *VEGF-C* was expressed at very low levels in five cell clusters: B cells, myeloid cells 2, NK/T cells, OCs, and plasmocytes (Figure 6(D)). We performed 36 pairwise comparisons of *VEGF-C* gene expression levels between nine OS cell types (Supplementary Table 10). Of these, 21 pairwise comparisons of B cells versus CAFs, B cells versus ECs, B cells versus osteoblastic OS cells, CAFs versus ECs, CAFs versus myeloid cells 1, CAFs versus myeloid cells 2, CAFs versus NK/T cells, CAFs versus osteoblastic OS cells, CAFs versus OCs, CAFs versus plasmocytes, ECs versus myeloid cells 1, ECs versus myeloid cells 2, ECs versus NK/T cells, ECs versus osteoblastic OS cells, ECs versus OCs, ECs versus plasmocytes, myeloid cells 1 versus osteoblastic OS cells, myeloid cells 1 versus osteoblastic OS cells, myeloid cells 2 versus osteoblastic OS cells, osteoblastic OS cells versus OCs, and osteoblastic OS cells versus plasmocytes revealed significant differential expression ($p < 0.05$) (Supplementary Table 10).

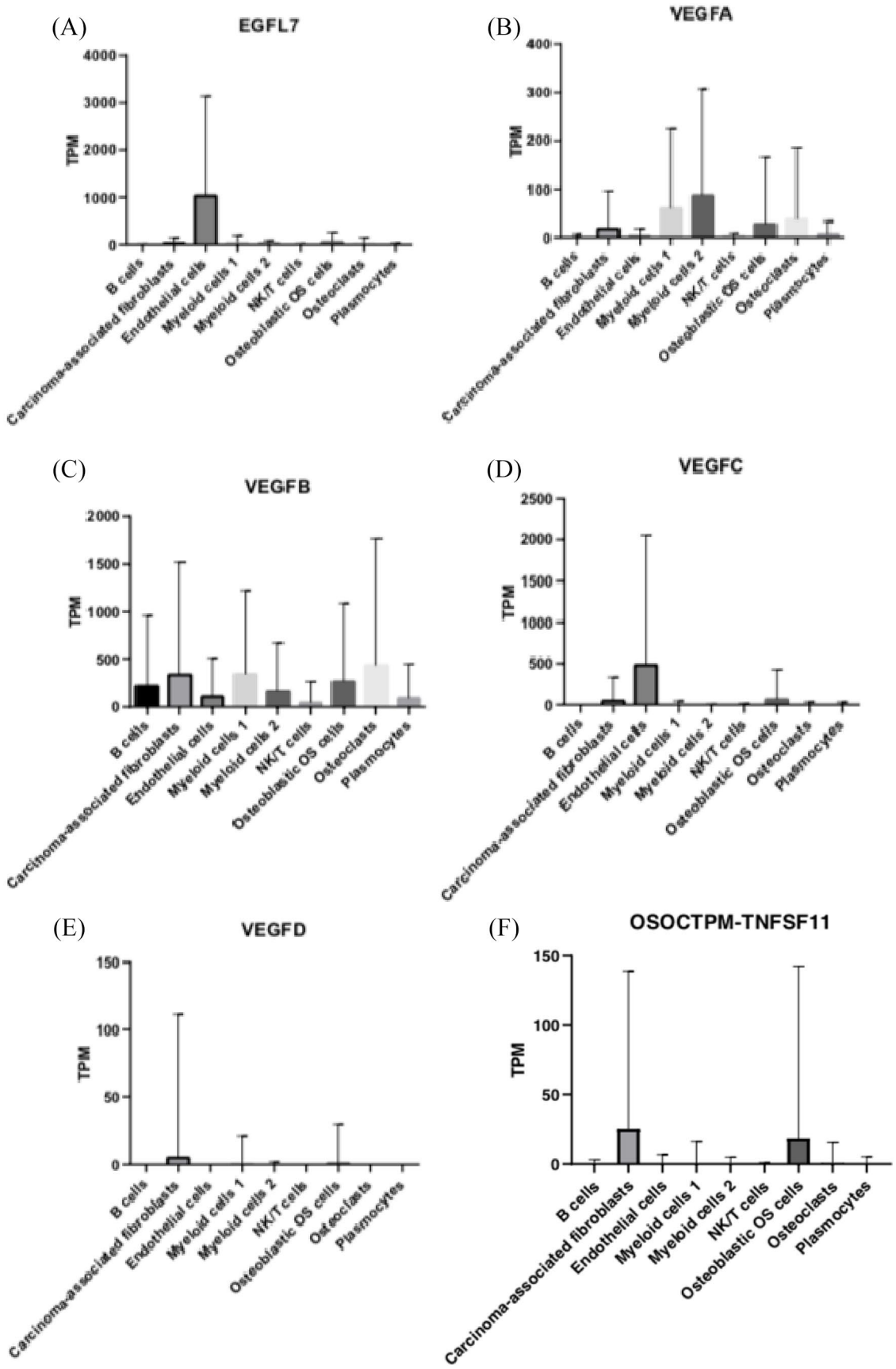

Figure 6. (A) Bar graph representing the relative expression levels of *EGFL7* in the nine major OS cell types. (B) Bar graph representing the relative expression levels of *VEGF-A* in the nine major OS cell types. (C) Bar graph representing the relative expression levels of *VEGF-B* in the nine major OS cell types. (D) Bar graph representing the relative expression levels of *VEGF-C* in the nine major OS cell types. (E) Bar graph representing the relative expression levels of *VEGF-D* in the nine major OS cell types. (F) Bar graph representing the relative expression levels of *TNFSF11* in the nine major OS cell types.

Fifteen pairwise comparisons of B cells versus myeloid cells 1, B cells versus myeloid cells 2, B cells versus NK/T cells, B cells versus OCs, B cells versus plasmocytes, myeloid cells 1 versus myeloid cells 2, myeloid cells 1 versus NK/T, myeloid cells 1 versus OCs, myeloid cells 1 versus plasmocytes, myeloid cells 2 versus NK/T cells versus, myeloid cells 2 versus OCs, myeloid cells 2 versus plasmocytes, NK/T cells versus OCs, NK/T cells versus plasmocytes, and OCs versus plasmocytes showed no significant differential expression ($p > 0.05$) (Supplementary Table 10).

Expression pattern differences of *VEGF-D* in various cells of OS microenvironment

Next, we measured the relative expression of *VEGF-D* in identified cells of OS microenvironment and pair versus wise compared these mRNA levels among various groups of OS cells. The gene expression levels of *VEGF-D* were calculated using TPM. *VEGF-D* was expressed in all nine cell types examined (Figure 6(E)). Varying expression levels of *VEGF-D* were found in single OS cell types (Figure 6(E)). The highest expression of *VEGF-D* and standard deviation were observed in the CAFs cell cluster followed by osteoblastic OS cells, myeloid cells 1, and myeloid cells 2, (Figure 6(E)). *VEGF-D* was expressed at very weak levels in B cells, ECs, NK/T cells, OCs, and plasmocytes (Figure 6(E)). We performed 36 pairwise comparisons of *VEGF-D* gene expression levels between nine OS cell types (Supplementary Table 11). Of these, eight pairwise comparisons of B cells versus CAFs, CAFs versus ECs, CAFs versus myeloid cells 1, CAFs versus myeloid cells 2, CAFs versus NK/T cells, CAFs versus osteoblastic OS cells, CAFs versus OCs, and CAFs versus plasmocytes revealed significant differential expression ($p < 0.05$) (Supplementary Table 11).

Twenty-eight pairwise comparisons of B cells versus ECs, B cells versus myeloid cells 1, B cells versus myeloid cells 2, B cells versus NK/T cells, B cells versus osteoblastic OS cells, B cells versus OCs, B cells versus plasmocytes, ECs versus myeloid cells 1, ECs versus myeloid cells 2, ECs versus NK/T cells, ECs versus osteoblastic OS cells, ECs versus OCs, ECs versus plasmocytes, myeloid cells 1 versus myeloid cells 2, myeloid cells 1 versus NK/T cells, myeloid cells 1 versus osteoblastic OS cells, myeloid cells 1 versus OCs, myeloid cells 1 versus plasmocytes, myeloid cells 2 versus NK/T cells, myeloid cells 2 versus osteoblastic OS cells, myeloid cells 2 versus OCs, myeloid cells 2 versus plasmocytes, NK/T cells versus osteoblastic OS cells, NK/T cells versus OCs, NK/T cells versus plasmocytes, osteoblastic OS cells versus OCs, osteoblastic OS cells versus plasmocytes, and OCs versus plasmocytes showed no significant differential expression ($p > 0.05$) (Supplementary Table 11).

Expression pattern differences of *TNFSF11* in various cells of OS microenvironment

Finally, we measured the relative abundance of *TNFSF11* in various cells of OS microenvironment and then pairwise compared these expression levels between various groups of OS cells. The gene expression levels of *TNFSF11* were calculated using TPM. *TNFSF11* was most enriched in CAFs followed by osteoblastic OS cells, respectively (Figure 6(F)). In contrast, low expression levels of *TNFSF11* were found in the following cell clusters: B cells, ECs, myeloid cells 1, myeloid cells 2, NK/T cells, OCs, and plasmocytes (Figure 6(F)). We performed 36 pairwise comparisons of *TNFSF11* gene expression levels between nine OS cell types (Supplementary Table 11). Of these, 15 pairwise comparisons of B cells versus CAFs, B cells versus osteoblastic OS cells, CAFs versus ECs, CAFs versus myeloid cells 1, CAFs versus myeloid cells 2, CAFs versus NK/T cells, CAFs versus osteoblastic OS cells, CAFs versus OCs, CAFs versus plasmocytes, ECs versus osteoblastic OS cells, myeloid cells 1 versus osteoblastic OS cells, myeloid cells 2 versus osteoblastic OS cells, NK/T cells versus osteoblastic OS cells, osteoblastic OS cells versus OCs, and osteoblastic OS cells versus plasmocytes revealed significant differential expression ($p < 0.05$) (Supplementary Table 12).

Twenty-one pairwise comparisons of B cells versus ECs, B cells versus myeloid cells 1, B cells versus myeloid cells 2, B cells versus NK/T cells, B cells versus OCs, B cells versus plasmocytes, ECs versus myeloid cells 1, ECs versus myeloid cells 2, ECs versus NK/T cells, ECs versus OCs, ECs versus plasmocytes, myeloid cells 1 versus myeloid cells 2, myeloid cells 1 versus NK/T cells, myeloid cells 1 versus OCs, myeloid cells 1 versus plasmocytes, myeloid cells 2 versus NK/T cells, myeloid cells 2 versus OCs, myeloid cells 2 versus plasmocytes, NK/T cells versus OCs, NK/T cells versus plasmocytes, and OCs versus plasmocytes showed no significant differential expression ($p > 0.05$) (Supplementary Table 12).

Phylogenetic relationship of VEGF-A, B, C, and D genes

Based on the sequence alignment results, a phylogenetic tree was constructed (Figure 7(A)). The human VEGF family tree consists of two branches that might have evolved from a common ancestor (Figure 7(A)). The first branch comprises VEGF-C and VEGF-D, and the second branch comprises VEGF-A and VEGF-B (Figure 7(A)). Our phylogenetic analysis showed that VEGF-C is more closely related to VEGF-D and that VEGF-A is more similar to VEGF-B (Figure 7(A)). A pairwise alignment of human VEGF-A and VEGF-B proteins sequences was performed (Figure 7(B)). We also performed a pairwise alignment of the sequences of human VEGF-C and VEGF-D (Figure 7(C)). We observed some similarities in the sequences, and high degrees of regional conservation were found.

Discussion

Differential gene expression (DGE) analysis is important for elucidating the molecular mechanisms underlying the genetic control of disease. This analysis is used to determine whether a gene is differentially expressed between two or more experimental conditions.[32] In the present study, bioinformatic DGE analysis of scRNA seq data generated on an Illumina platform was performed to identify changes in gene expression of *EGFL7*, *VEGF-A-D*, and *TNFSF11* in GCTB and OS cells. Using analysis, we identified and confirmed that, based on *p*-values, these pro-angiogenic factors were significantly differentially expressed in specific cells of the GCTB and OS microenvironment. We also described the extent of these expression differences. Genes that showed the

(A)

(B)

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SP|P15692|VEGFA_HUMAN MNFLLSWVHWSLALLLYLHHAKWSQAAPMA-EGGGQNHHEVVKFMDVYQRSYCHPIETLV 59
SP|P49765|VEGFB_HUMAN MSPLLRRL--LLAALLQLAPAQ----APVSQPDAPGHQRKVVSWIDVYTRATCQPREVVV 54
                      *. **  :   ** ** *  *:    **::  ..  ::::**.::*** *: *:* *.:*

SP|P15692|VEGFA_HUMAN DIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHIGE 119
SP|P49765|VEGFB_HUMAN PLTVELMGTVAKQLVPSCVTVQRCGGCCPDDGLECVPTGQHQVRMQILMIRYP-SSQLGE 113
                       :  *  . :   : **** : ****** *:******* : :: ***: *:   ..::**

SP|P15692|VEGFA_HUMAN MSFLQHNKCECRPKKDRARQEKKSVRGKGKGQKRKRKKSRYKSWSVYVGARCC---LMPW 176
SP|P49765|VEGFB_HUMAN MSLEEHSQCECRPKKKDSAVKPDRAAT----PHHRPQPRSVPGWDSAPGAPSPADITHPT 169
                      **: :*.:*******. :  : . .       ::: :     .*.   ** .      *

SP|P15692|VEGFA_HUMAN SLPGPHPCGPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNERTCRCDKPRR 232
SP|P49765|VEGFB_HUMAN PAPGPSA--HAAPSTTSALTPGPAAAAADAAA-SSVAKGGA--------------- 207
                        ***     .:   .  :. .* :. ...   .* .*.
```

(C)

```
SP|P49767|VEGFC_HUMAN ----MHLLGFFSVACSLLAAALLPGPREAPAAAAAFESGLDLSDAEPDAGEATAYASKDL 56
SP|O43915|VEGFD_HUMAN MYREWVVVNVFM----MLYVQLVQGSSNEH----------------GPVKRSSQSTLERS 40
                             ::..*     :* . *: *  :                  . .::  : :

SP|P49767|VEGFC_HUMAN EEQLRSVSSVDELMTVLYPEYWKMYKCQLRKGGWQHNREQANLNSRTEETIKFAAAHYNT 116
SP|O43915|VEGFD_HUMAN EQQIRAASSLEELLRITHSEDWKLWRCRLRLKSFTSMDS----RSASHRSTRFAATFYDI 96
                      *:*:*:.**::**: : : * **:::*:**  .:    .    .* :..: :***:.*:

SP|P49767|VEGFC_HUMAN EILKSIDNEWRKTQCMPREVCIDVGKEFGVATNTFFKPPCVSVYRCGGCCNSEGLQCMNT 176
SP|O43915|VEGFD_HUMAN ETLKVIDEEWQRTQCSPRETCVEVASELGKSTNTFFKPPCVNVFRCGGCCNEESLICMNT 156
                      * ** **:**::*** ***.*::*..*:* :**********.*:*******.*.* ****

SP|P49767|VEGFC_HUMAN STSYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRRSLPA-TLPQ 235
SP|O43915|VEGFD_HUMAN STSYISKQLFEISVPLTSVPELVPVKVANHTGCKCLPTAP--RHPYSIIRRSIQIPEEDR 214
                      ****:** ****:***:. *: * :..****.*:*:.______*:.:******:      :

SP|P49767|VEGFC_HUMAN CQAANKTCPTNYMWNNHICRCLAQEDFMFSSDAGDDSTDGFHDICGPNKELDEETCQCVC 295
SP|O43915|VEGFD_HUMAN CSHSKKLCPIDMLWDSNKCKCVLQEENPLAGTEDH-SH                      251
                      *. ::* ** : :*:.: *:*: **:  ::.  .. *

SP|P49767|VEGFC_HUMAN RAGLRPASCGPHKELDRNSCQCVCKNKLFPSQCGANREFDENTCQCVCKRTCPRNQPLNP 355
SP|O43915|VEGFD_HUMAN -------------------------LQEPALCGPHMMFDEDRCECVCKTPCPKDLIQHP 285
                                              *: ** :  ***: *:****  **::   :*

SP|P49767|VEGFC_HUMAN GKCAC-ECTESPQKCLLKGKKFHHQTCSCYRR------PCTNRQKACEPGFSYSEEVCRC 408
SP|O43915|VEGFD_HUMAN KNCSCFECKESLETCCQKHKLFHPDTCSCEDRCPFHTRPCASGKTACAKHCRFPKEKRAA 345
                       :*:* **.** :.*  * * ** :****  *      **:. :.**     : :*   .

SP|P49767|VEGFC_HUMAN -VPSYWKRPQMS 419
SP|O43915|VEGFD_HUMAN QGPHSRKNP--- 354
```

Figure 7. (A) Predicted evolutionary relationships between human VEGF proteins. (B) Pairwise sequence alignment of human protein VEGF-A and VEGF-B. (C) Pairwise sequence alignment of human protein VEGF-C and VEGF-D. (A color version of this figure is available in the online journal.)

most differential expression are considered the most biologically significant. Genes that were increased in specific cell types might be related to biological processes. Furthermore, hundreds of other genes are likely expressed at significantly different levels in GCTB and OS cells. To the best of our knowledge, this is the first comprehensive study to determine the differential expression of these genes in GCTB and OS cells. We discussed the relevant differentially expressed genes in cancer, specifically in GCTB and OS.

EGFL7, also known as vascular endothelial statin, is a gene highly conserved in vertebrates and a member of the epidermal growth factor (EGF)-like protein family.[31,33] The *EGFL7* gene encodes a protein of ~30 kDa produced by osteoblasts and secreted by blood vessel ECs that contain two epidermal growth factor-like domains.[10,34,35] Several studies in cancer biology showed that *EGFL7* is involved in tumor progression by regulating metastasis and proliferation.[10] The *EGFL7* protein is also suggested to mediate vascular tube formation in tumor angiogenesis.[25,36] Overexpression of *EGFL7* in cytogenetically normal acute myeloid leukemia is positively correlated with lower survival rates.[37] In gastric carcinoma, high expression of *EGFL7* advances tumor invasion and metastasis.[10,25] The highest levels of *EGFL7* are thought to be found in pancreatic, followed by renal, thyroid,

and testis cancer tissues (Supplementary Figure 1). In OS, *EGFL7* shows elevated expression in OS tissues.[38] Higher expression of *EGFL7* is found in advanced stage OS compared with early stage OS tumors.[38] These findings suggest that high levels of *EGFL7* could be used to predict poorer prognosis in OS.[38] The present study highlights that *EGFL7* is strongly enriched in ECs of both OS and GCTB tumors. However, the functional role of *EGFL7* in OS and GCTB is unclear and there are currently no studies that have established a functional link between OS and *EGFL7* and GCTB and *EGFL7*. It will be interesting to determine the function of *EGFL7* in GCTB and OS. The high expression of *EGFL7* in the EC clusters of GCTB and OS tumors suggests it may contribute to its cellular role in angiogenesis. Further studies are essential for determining the clinical value of this gene.

VEGF, also known as vascular permeability factor (VPF) are dimetric glycoproteins of 40 kDa.[39] There are seven members of the *VEGF* family; these are *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGF-D*, *VEGF-E*, *VEGF-F*, and placental growth factor (PLGF).[40] The various *VEGF* members mediate its function by binding to three structurally related receptor tyrosine kinases, known as VEGF receptor-1, -2, and -3.[40] *VEGF* is thought to be produced by cells, such as, tumor cells, macrophages, and platelets and acts on various cell types predominately ECs.[39,41] In normal physiological conditions, *VEGF* participates in angiogenesis during embryonic development and is crucial for adult wound healing in humans.[42] *VEGF* is also important in disease progression and is reported to promote tumor growth and metastasis by its involvement in pathological angiogenesis.[43] Specifically, *VEGF-A* and its receptors VEGFR-1 and VEGFR-2 have a key role in physiological and pathological angiogenesis, while VEGF-C/D and their receptor VEGFR-3 predominately regulate lymphangiogenesis.[44]

VEGF appears to be upregulated in multiple cancers.[45,46] Studies have demonstrated that *VEGF-A*, *VEGF-C*, and *VEGF-D* are involved in tumor progression in colorectal cancer.[47] In addition, it is reported that *VEGF-C* expression is associated with lymphatic spread in prostate, gastric, thyroid, and neuroblastoma tumors.[47] In renal cell carcinoma, high *VEGF* expression has been previously linked to higher malignant potential.[48] Renal, followed by glioma, thyroid, and lung cancer tissues, respectively, express the highest levels of *VEGF-A* (Supplemental Figure 2). *VEGF-B* is most highly expressed in melanoma followed by renal, ovarian, cancer tissues, respectively (Supplementary Figure 3). *VEGF-C* is most highly expressed in thyroid followed by head and neck, renal, and urothelial cancer tissues, respectively (Supplementary Figure 4). Lung followed by liver, stomach, and renal cancer tissues, respectively, express the highest levels of *VEGF*-D (Supplementary Figure 5). In OS, VEGF expression is implicated with pulmonary metastasis and poor survival in patients and could be used as a potential prognostic marker for OS.[49,50] According to the literature, *VEGF* expression in OS tissues may be associated with vascular permeability.[51] Our results show that in OS, *VEGF-A* is highly expressed in myeloid cells 1 and myeloid cells 2, *VEGF-B* is highly expressed in OCs, *VEGF-C* is highly expressed in ECs, and *VEGF-D* is primarily expressed at low levels in CAFs and at very low levels in all other cell types. In GCTB, VEGF is thought to be overexpressed in GCTB tissues and might function in invasion and metastasis of GCTB.[52] The overexpression of *VEGF* in GCTB could be linked to the advanced stage of the tumor.[53] According to this study, in GCTB *VEGF-A* was found to be highly expressed in myeloid cells, OCs, and proliferative cells. *VEGF-B* is highly expressed in chondrocytes, *VEGF-C* is highly expressed in ECs, and *VEGF-D* is expressed at low levels in myeloid cells and at very low levels in all other cell types (Figure 3(C) to (E)). The respective functional role and clinical value of *VEGF-A*, *VEGF-B*, *VEGF-C*, and *VEGF-D* in GCTB and OS is unknown and needs to be further investigated.

The receptor activator of NFκB ligand (RANKL) gene, also known as *TNFSF11*, encodes for a member of the tumor necrosis factor (TNF) family of cytokines.[54] In humans, *TNFSF11* comprises of five exons spanning 33.9 kb and binds to its receptor RANK.[55,56] *TNFSF11* appears to be expressed in various cell types and tissues in the human body.[54] Studies have reported that *TNFSF11* could be critically implicated in bone remodeling, lymph node formation, and mammary gland development during pregnancy.[57] *TNFSF11* is also thought to contribute to tumor progression by increasing tumor cell migration, inducing tumor neovascularization, and advancing metastasis.[54,58] The highest expression levels of *TNFSF11* are reported in pancreatic, followed by colorectal, stomach, and lung cancer tissues, respectively (Supplementary Figure 6). In OS, *TNFSF11* expression has been found in OS cells.[59] In addition, *TNFSF11* has been shown to be involved in the initiation of OS and associated with increased lung metastasis in OS.[59] In GCTB, immunohistochemical evidence suggests that *TNFSF11* is expressed in GCTB tumors at the protein level.[60] Several studies have further reported that *TNFSF11* is highly expressed in mesenchymal stromal cells of GCTB tumors resulting in excessive bone resorption by OCs.[7,61,62] Consistently, our results show that in OS *TNFSF11* is expressed at high levels in CAFs and osteoblastic OS cells, and in GCTB *TNFSF11* is highly expressed in osteoblast cells. Further research is needed to study its precise function and clinical value in OS and GCTB.

There were limitations in this study. First, the study is limited by the very small sample size of one patient in the GCTB scRNA seq dataset and six patients in the OS scRNA seq dataset, which could lead to inconsistencies and variability in our results. Further future studies with larger patient numbers are essential. Second, our study did not examine DGE at multiple time points, which indicates that gene expression levels of certain cell populations have not been studied. Third, this study suggests that these DE genes could be used as prognostic biomarkers; however, whether this is possible requires further research. Finally, the pairwise comparisons performed in this study were not corrected for multiplicity which may increase the chances of false significant findings. Nonetheless, despite these limitations, the results of this study clearly show differential expression profiles of angiogenic factors, EGFL7 and VEGF at a single-cell level of GCTB and OS.

Conclusions

This study presents evidence for the possible involvement and differential expression of various pro-angiogenic factors

in two tumors, OS and GCTB. In addition, it reveals the cellular composition of OS and GCTB tumors and provides novel insights into the distinct genetic constitution of several OS and GCTB cell populations. Our results suggest that mRNA levels of *EGFL7*, *VEGF-A*-D, and *TNFSF11* show considerable cell-to-cell variability in OS and GCTB. The activity of these genes likely plays a role in the heterogeneity in these cancers and could be essential for tumor progression, metastasis, and disease recurrence in OS and GCTB. More experimentation is needed to validate our results and determine the precise functional roles and biological significance of these differences in gene expression in OS and GCTB. Given that Denosunab is used in the treatment of GCTB and does not completely inhibit tumorigenesis, *TNFSF11* remains a promising therapeutic target that could be important to pursue.[1] *EGFL7* expression is significant and may be therapeutically targeted to overcome tumor immune escape mechanisms. Future studies might also correlate expression levels of these genes with various clinical outcomes of patients with OS and GCTB.

AUTHORS' CONTRIBUTIONS

MF and WF contributed to the preparation of the paper and the main figures and data analyses. MF contributed to the first draft of the article. ER, QW, HL, and DS assisted in data analysis. SK and DW discussed and revised the paper. YL and JX supervised the studies and data collections and revised the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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