EGFL7 is expressed in bone microenvironment and promotes angiogenesis via ERK, STAT3 and integrin signaling cascades

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Running title: EGFL7 regulates endothelial cell activities

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Abstract

Angiogenesis plays a pivotal role in bone formation, remodeling and fracture healing. The regulation of angiogenesis in the bone microenvironment is highly complex and orchestrated by intercellular communication between bone cells and endothelial cells. Here, we report that EGF-like domain 7 (EGFL7), a member of the epidermal growth factor (EGF) repeat protein superfamily is expressed in both the osteoclast and osteoblast lineages, and promotes endothelial cell activities. Addition of exogenous recombinant EGFL7 potentiates SVEC (simian virus 40-transformed mouse microvascular endothelial cell line) cell migration and tube-like structure formation in vitro. Moreover, recombinant EGFL7 promotes angiogenesis featuring web-like structures in ex vivo fetal mouse metatarsal angiogenesis assay. We show that recombinant EGFL7 induces phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3), and focal adhesion kinase (FAK) in SVEC cells. Inhibition of ERK1/2 and STAT3 signaling impairs EGFL7-induced endothelial cell migration, and angiogenesis in fetal mouse metatarsal explants. Bioinformatic analyses indicate that EGFL7 contains a conserved RGD/QGD motif and EGFL7-induced endothelial cell migration is significantly reduced in the presence of RGD peptides. Moreover, EGFL7 gene expression is significantly upregulated during growth plate injury repair. Together, these results demonstrate that EGFL7 expressed by bone cells regulates endothelial cell activities through integrin-mediated signaling. This study highlights the important role that EGFL7 expressed in bone microenvironment plays in the regulation of angiogenesis in bone.
Introduction
The vasculature in bone is pivotal for skeletal development during the embryonic stage, postnatal growth and bone remodeling. During endochondral bone formation, the vascular supply provides critical input allowing for the establishment of ossification centers within mesenchymal condensation. Vascular invasion allows recruitment of osteoblast precursors and deposition of bone within the cartilaginous diaphysis to form the primary ossification center. In postnatal life, secondary ossification centers are established within cartilaginous epiphyses by a similar mechanism. Once formed, bone is continuously undergoing remodeling to maintain mechanical integrity (Burkus et al., 1993; Carlevaro et al., 2000; Gerber and Ferrara, 2000). Communication between vascular endothelial cells, bone building osteoblasts and bone-resorbing osteoclasts is central to remodeling. This could be facilitated by multiple regulatory proteins that incorporate autocrine and/or paracrine modes of signaling to mediate the recruitment, survival, proliferation and differentiation of bone cells and vascular endothelial cells (Brandi and Collin-Osdoby, 2006; Chim et al., 2013; Eriksen et al., 2007). It has been demonstrated that osteoclasts, osteoblasts and osteocytes (terminally differentiated osteoblasts embedded within bone matrix) produce angiogenic factors such as VEGF, bFGF and EGFL6 (Chim et al., 2011; Thi et al., 2010; Utting et al., 2010). In contrast, endothelial cells produce regulatory factors that influence bone cells such as macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor kappa-B ligand (RANKL) and chemokines (Brandi and Collin-Osdoby, 2006; Collin-Osdoby et al., 2001).

Bone remodeling takes place in a specialized vascularised structure called the bone remodeling compartment (BRC), which is closely linked with capillaries (Andersen et al., 2009; Hauge et al., 2001). The canopy of the BRC provides a closed microenvironment for bone remodeling by separating the BRC from open bone marrow spaces. This allows multiple regulatory factors to reach a critical concentration and avoids them dispersing within the marrow (Eriksen et al., 2007). Both EGF and EGF-like proteins have been implicated in angiogenesis (Kim et al., 2003; Leker et al., 2009; Mehta and Besner, 2007; Penta et al., 1999; Schmidt et al., 2007). Recently, we identified several EGF-like family members, EGFL2, EGFL3, EGFL5, EGFL6, EGFL7, EGFL8 and EGFL9, that are differentially expressed in osteoclasts and osteoblasts. In addition, we demonstrated that EGFL6 is secreted by osteoblasts and mediates angiogenesis via a
paracrine mode of action (Chim et al., 2011). However, the specific roles of the other individual EGF-like family members in mediating angiogenesis in the bone microenvironment remains to be elucidated.

EGF-like family members mediate a wide range of biological activities such as migration, adhesion, proliferation and differentiation (Singh and Harris, 2005). The human and mouse EGFL7 genes map to chromosomes 9 and 2 respectively. EGFL7 is a secreted protein that contains an N-terminal cysteine-rich domain (EMI domain), followed by two EGF repeat domains. EGFL7 gene expression has been previously reported in endothelial progenitors, endothelial cells and neurons (Fitch et al., 2004; Parker et al., 2004; Schmidt et al., 2009). EGFL7 regulates tubulogenesis during embryogenesis, migration of endothelial cells, and spatial organization in angiogenic sprouts (Campagnolo et al., 2005; De Maziere et al., 2008; Parker et al., 2004; Schmidt et al., 2007). It has been suggested that EGFL7 acts as an antagonist to Notch and regulates ligand-mediated Notch receptor signaling (Nichol et al., 2010; Schmidt et al., 2009). EGFL7 can also regulate human hepatocellular carcinoma cell motility through EGFR-mediated FAK signaling (Wu et al., 2009). However, the role of EGFL7 in bone microenvironment is hitherto unknown.

In this study, we show that EGFL7 is expressed in osteoclasts, osteoblasts and bone tissues. Moreover, exogenous recombinant EGFL7 protein is capable of promoting endothelial cell migration, angiogenesis and integrin-mediated signaling including the phosphorylation of ERK1/2, STAT3 and FAK. EGFL7-induced endothelial cell migration was significantly blocked in the presence of RGD peptides. Furthermore, we observed up-regulation of EGFL7 gene expression during the angiogenic and repair phase in a rat model of injured growth plate bony repair. We propose that EGFL7 mediates endothelial cell activities in the bone local environment.
Materials and Methods

Cells and Cell Culture

Primary mouse bone marrow macrophages (BMM) were isolated from C57BL/6J mice as previously described (Wu et al., 2014). Briefly, bone marrow cells were collected from femora and tibia of mice by flushing the bone with α-modified Eagle’s medium (α-MEM; Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 1X penicillin-streptomycin (Gibco)(complete α-MEM), followed by filtration through a 100μm mesh. BMM were maintained in complete α-MEM in a humidified incubator at 37°C and 5% CO2. BMM were cultured in the presence of 50ng/ml RANKL and 10ng/ml MCSF (R&D Systems) for 5 days to form mature osteoclasts. RANKL recombinant proteins were expressed and purified as previously described (Xu et al., 2000). The cells were fixed and stained for Tartrate-resistant acid phosphate activity (TRACP) to identify osteoclasts using TRACP staining kit (Sigma Aldrich). Primary mouse osteoblasts were prepared from the calvariae of neonatal C57BL/6J mice by enzymatic digestion according to published protocols (Bakker and Klein-Nulend, 2012). For osteoblast differentiation, primary calvarial osteoblastic cells were seeded onto 12-well plates at a cell density of 4x10^4 cells/well and grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1X penicillin-streptomycin (Gibco)(complete DMEM) until reaching confluence. Cell medium was then replaced with osteogenic differentiation medium, complete DMEM containing 50μg/ml ascorbic acid (Sigma Aldrich), 10mM β-glycerophosphate (Sigma Aldrich), and 10nM dexamethasone (Sigma Aldrich). The medium was replaced every two to three days with fresh differentiation medium up to 21 days. Osteoblast mineralization was confirmed by alizarin red staining as previously described (Guo et al., 2012). COS-7 and SVEC cells were maintained in complete DMEM.

Semi-quantitative Reverse Transcription (RT)-PCR

Total cellular RNA was isolated from cultured cells using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer’s protocol. For RT-PCR, single-stranded cDNA was reverse transcribed from 2μg total RNA using reverse transcriptase with an oligo-dT primer. All PCR was carried out using 1μl of each cDNA using cycling parameters 94°C, 40secs; 55°C, 40secs; 72°C, 40secs for 30 cycles with primers designed against the following mouse sequences: EGFL7  (forward:5’-AGTGGGGATCCCCACTTACAATGCAGAC-3’; reverse:5’-
TCTTCTAGAAGATCTTTTTTCAGGAGCAG-3’), CTR (forward:5’-CGGACTTTTGACACAGCAGAA-3’; reverse:5’-CAGCAATCGACACAGGAGGTGA-3’), TRAP (forward:5’-CAGCAGCCAAAGAGGACTAC-3’; reverse:5’-ACATAGCCCACACCGTTC-3’), DC-STAMP (forward:5’-CTTGCAACCTAAGGGCAAAG-3’; reverse:5’-TCAACAGCTCCTGTCGACC-3’), EGFL6 (forward:5’-AAGCTTGGATCCGAATTCAGTATGCGACCAGAAGCAG-3’; reverse:5’-CTCGAGTCTAGAAGATCTACCTGACATAAAAAGT-3’), ALP (forward:5’-AECTGCTGGCCCTTGACCCCT-3’; reverse:5’-TCCTGCTCCTCCTCCACCAGCA-3’), OCN (forward:5’-GCGCTCTGTCTCTCGTGACCT-3’; reverse:5’-ATAGATGCGTTTGAGGC-3’), 18s (forward:5’-ACCATAAACGATGCCGACT-3’; reverse:5’-TGTCATCTCTGTCGTGTC-3’). PCR samples were analyzed by DNA agarose gel electrophoresis.

**Western Blotting**

Total cellular proteins were extracted from cultured cells using RIPA lysis buffer as previously described (Chim et al., 2011). Briefly, lysates were cleared by centrifugation at 16,000g for 20 mins at 4°C and post-nuclear supernatants were collected. For immunoblotting, equivalent amounts of extracted proteins diluted in SDS-sampling buffer with 5% β-mercaptoethanol and incubated at 95 °C for 5 mins. Samples were resolved by SDS-PAGE gels and then electroblotted onto nitrocellulose membranes. Following transfer, membranes were blocked with 5% skim milk for 1hr and then probed with primary antibodies for 2hrs. Primary antibodies used were mouse monoclonal anti-c-myc (Sigma Aldrich), goat polyclonal anti-EGFL7 (R&D Systems), mouse monoclonal anti-p-ERK, rabbit polyclonal anti-p-ERK5 (Santa Cruz Biotechnology), rabbit polyclonal anti-ERK (Promega), rabbit monoclonal anti-p-STAT1, rabbit polyclonal anti-STAT1, rabbit polyclonal anti-p-STAT3, rabbit monoclonal anti-STAT3, rabbit polyclonal anti-p-FAK and rabbit polyclonal anti-pEGFR (Cell Signaling Technology), mouse monoclonal anti-β-actin (JLA20; DSHB University of Iowa). All antibodies were used at the concentrations recommended by the suppliers. Membranes were washed and then incubated with HRP-conjugated secondary antibodies (Sigma Aldrich) for 1 hr. Immunoreactivity was visualized using the Western Lighting Ultra (PerkinElmer) and FujiFilm LAS-4000 Gel Documentation System (FujiFilm). Signal intensities were quantified by NIH imageJ software. To examine EGFL7 protein expression during bone development, proteins were extracted from long bones at
different time points of mouse development (E18.5, Week 1, Week 7 and Week 16). The dissected samples were snap frozen in liquid nitrogen and ground using a mortar and pestle. Proteins were then extracted using RIPA lysis buffer as above.

Preparation of Conditioned Medium Containing EGFL7
Conditioned medium containing EGFL7 was produced according to protocol described previously (Chim et al., 2011). Briefly, COS-7 cells were cultured overnight in a 6-well plate in complete DMEM at a density of 4x10^5 cells/well. The following day, the culture medium was transfected with EGFL7 expression vector pcDNA3.1-EGFL7-c-myc/His or empty pcDNA3.1 vector using lipofectamine 2000 (Invitrogen). After 6 hrs, cells were washed twice and incubated with Opti-MEM reduced serum medium (Gibco). EGFL7-enriched supernatant was harvested at 24 hrs, centrifuged at 2000 rpm for 10 mins to remove cell debris and snap frozen in aliquots at -80°C. The presence of EGFL7 in the supernatant was examined by Western blotting using anti-c-myc and anti-EGFL7 antibodies. The concentration of EGFL7 in conditioned medium was estimated to be ~211ng/ml (supplementary Fig. S1). Recombinant human EGFL7 was purchased from OriGene Technologies.

Scratch-Wound Healing Assay
The migration assay was performed as previously described (Chim et al., 2011). Briefly, the confluent SVEC cell monolayer was serum starved overnight prior to wounding. Cells were incubated at 37°C for 16 hrs with the conditioned medium containing EGFL7, or vehicle control. Human bFGF (PeproTech Inc) was used as a positive control. Time-lapse images were captured at 0 and 16 hr time points in the same position using a Nikon Eclipse TE2000-5 microscope. Five selected fields of view were captured in each sample and the wound areas are estimated by Nikon NIS-Elements computer software. For the scratch-wound healing assay with MEK1/2 inhibitor U0126 (Promega), STAT3 inhibitor Stattic (Sigma Aldrich) and RGD peptide (Sigma Aldrich), SVEC monolayers were treated with inhibitors for 1 hr prior to wounding.

Tube Formation Assay
The migration assay was performed as previously described (Chim et al., 2011). Briefly, cells were seeded onto the layer of Geltrex™ matrix (Invitrogen) and cultured with conditioned
medium containing EGFL7 or vehicle control for 24 hrs. Five random selected fields of view were captured using a Nikon Eclipse TE2000-5 microscope. Tube formation was quantified by measuring the length of tube-like structures by Nikon NIS-Elements computer software.

**STAT3 Luciferase Reporter Assay**

STAT3-responsive luciferase kit was purchased from Qiagen and used to measure the EGFL7-induced activation of STAT3. Briefly, SVEC were transfected with STAT3 Firefly luciferase reporter plasmid and control Renilla luciferase plasmid using Lipofectamine 2000. Twenty-four hours after transfection, cells were treated with conditioned medium containing EGFL7 or recombinant human EGFL7 (OriGene) for an additional 24 hours. Luciferase activity was determined using a Dual-Luciferase Reporter Assay kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. The STAT3-responsive activity was presented as fold change against controls.

**Mouse Fetal Metatarsal Angiogenesis assay**

The metatarsal angiogenesis assay was performed as previously described (Deckers et al., 2001). Briefly, metatarsals were dissected from E17.5 embryos. The isolated metatarsals were cultured in 24-well plates in complete α-MEM for 72 hours. The explants were cultured with human EGFL7 (200ng/ml) in the presence or absence of inhibitors for 14 days, then fixed and stained for CD31 (rabbit polyclonal, Abcam). Human VEGF (PeproTech Inc) (50ng/ml) was used as a positive control. Images were captured using a Nikon Eclipse TE2000-5 microscope and quantified by NIH imageJ software. Cultures were performed in sextuplicate.

**Growth Plate Injury Repair Time Course**

Eight-week-old male Sprague Dawley rats were subjected to experimental growth plate injury in the proximal tibia of both hind legs as previously described (Xian et al., 2004). All protocols followed the Australian code of practice for the care and use of animals, and were approved by the Animal Ethics Committee of the SA Pathology, South Australia. Under anaesthesia, an incision was made to expose the anterior-medial aspect of the proximal tibial bone of both hind-limbs. A 2-mm surgical drill was then used to make a cortical window in the metaphyseal bone on the medial side. A central disruption of the growth plate (about 30% area) was then induced
by inserting the drill through the cortical window and perpendicular through the growth plate cartilage. Rats (n=8 per group and per time point) were sacrificed for specimen collection at days 6, 14 and 28 post-surgery, three time-points found suitable to observe the fibrogenic, osteogenic and remodeling repair phases respectively (Xian et al., 2004). A normal group of rats (n=8) were sacrificed on day 14 and used as non-injured controls. Both tibias were dissected, cleared of soft tissue and samples collected, with the left tibia fixed, decalcified and paraffin-embedded for collecting 4-μm sections for histological studies and the right tibia used to collect the repair tissue within the growth plate injury site only for gene expression studies as described (Chung et al., 2013).

*Isolectin-B<sub>4</sub> Labelling of Endothelial Cells*

To investigate vascularization during growth plate injury repair, Isolectin-B<sub>4</sub> labelling – (a known marker for endothelial cells) was conducted (Grossmann et al., 2002). Isolated from *Griffonia simplicifolia* (Bandeiraea), isolectin-B<sub>4</sub> has previously been used to identify neovascular structures in tumor networks (Niethammer et al., 2002). Briefly, sections were deparaffinised and endogenous peroxidase quenched using 0.3% H<sub>2</sub>O<sub>2</sub> in methanol. Sections were blocked for 30 mins with 1% BSA/PBS solution and then incubated with biotinylated isolectin-B<sub>4</sub> (Vector Labs) (1:100 in PBS) for 30 mins. After washes, sections were incubated with avidin-biotin complex reagent (Dako) for 30 mins and then liquid DAB Plus (Dako) for colour development. Replacement of biotinylated isolectin-B<sub>4</sub> with 1% BSA in PBS was used as a negative control.

*Gene Expression of EGFL7, OCN and VEGF at the Injured Growth Plate*

Real-time quantitative RT-PCR assays were carried out to examine expression of EGFL7, osteocalcin (OCN) and VEGFA in injured growth plate in rat, respectively. Total RNA from growth plate injury site samples was extracted using the Aurum Total-RNA mini-kit (Bio-Rad) and purified through a column (with on-column DNase treatment). cDNA was then synthesized from 1μg RNA using iScript cDNA synthesis kit (Bio-Rad). Relative real-time PCR was carried out using gene specific primer pairs for EGFL7 (forward 5’- TGTGGGATGGCAGGGAGATA-3’ and reverse 5’-GGCACCAGTAACTTCCCACA-3’), Osteocalcin (forward 5’-ATTGTGACGAGCTAGCGGAC-3’ and reverse 5’- TCGAGTCCTGGAGAGTAGCC -3’),
VEGFA (forward 5’- ATCTTCAAGCCGTCTGTGTG-3’ and reverse 5’-ATCTTCAAGCCGTCTGTGTG-3’)
and Cyclophilin-A (CycA) (forward 5’-GAGCTGTTTGCAGACAAAGTTC-3’ and reverse 5’- CCCTGGCACATGAATCCTGG-3’). CycA was used as an internal control (Zhou et al., 2004). Gene expression analysis was expressed as fold change against non-injured controls.

Statistical Analysis and Data Presentation

All in vitro data shown represent one of at least three independent experiments. Data are expressed as means ± SD and statistics were performed using Students’ t-test with significance taken at p<0.05, except for real-time quantitative RT-PCR assays (mean ± SEM). For comparisons between time points, data were analysed using one-way ANOVA, and, when significance levels (p<0.05) were achieved, a post hoc analysis of groups was done using a Tukey’s test.
Results

Gene and protein expression of EGFL7 in bone microenvironment

In a previous study, we detected EGFL7 gene expression in both osteoclast and osteoblast lineages (Chim et al., 2011). We have now confirmed the gene expression of EGFL7 during primary osteoclast and osteoblast differentiation from BMM and calvariae; respectively by RT-PCR. Osteoclast differentiation was followed using the osteoclast marker genes calcitonin receptor (CTR), TRACP and dendritic cell-specific transmembrane protein (DC-STAMP), which were upregulated during osteoclast differentiation (Fig. 1A). Gene expression of EGFL7 was detected throughout osteoclast differentiation. Osteoblast differentiation was monitored by following the expression of osteoblast associated marker genes alkaline phosphatase (ALP) and OCN, and EGFL6 (known to be upregulated during osteoblast differentiation) (Chim et al., 2011). As shown in Figure 1A, unlike the osteogenesis markers whose expression was induced, EGFL7 gene levels were expressed at similar levels before and throughout osteoblast differentiation (Fig. 1A). Furthermore, Western blot analyses revealed that EGFL7 protein expression is present in developing and mature long bones (Fig. 1B). We also examined the tissue distribution of EGFL7 from selected mouse organs by semi-quantitative RT-PCR. It was revealed that EGFL7 was highly expressed in heart, kidney and lung (Fig. 1C).

Production of conditioned medium containing recombinant EGFL7

It has been reported that EGFL7 encodes a secreted protein (Campagnolo et al., 2005; Parker et al., 2004). We sought to produce conditioned medium containing exogenous recombinant EGFL7 proteins for further functional studies, as mouse recombinant EGFL7 protein expressed in a mammalian system is not commercially available. To aid this, we generated an expression construct of mouse full length EGFL7, pcDNA3.1-EGFL7-c-myc/His (Fig. 2A), and performed transient transfection in COS-7 cells. As shown in Figure 2B, the c-myc antibody specifically reacted to a major 34KDa and a minor 68KDa protein in EGFL7 transfected samples but not in vehicle transfected samples by Western blot analysis (Fig. 2B). The 68 KDa EGFL7 protein has not been previously described and might represent a homodimer or post-modified form of the major 34 KDa EGFL7 protein. The identity of these proteins was further confirmed by Western blot analysis using an anti-EGFL7 antibody (Fig. 2C).
Induction of cell migration and tube formation of SVEC endothelial cells and angiogenesis in fetal mouse metatarsal explants by EGFL7

EGFL7 is a chemoattractant for endothelial cells (Campagnolo et al., 2005) and regulates vascular development (Parker et al., 2004; Schmidt et al., 2007). As a first step to investigate the role of EGFL7 in angiogenesis, we examined the effects of recombinant EGFL7 protein on SVEC migration by a scratch wound healing assay. As shown in Figure 3A-B, conditioned medium containing recombinant EGFL7 proteins significantly enhanced endothelial cell migration. PBS and bFGF were used as negative and positive controls; respectively. This result was consistent with the chemotactic effects of EGFL7 on endothelial cells by a transwell migration assay (Campagnolo et al., 2005). As EGFL7-containing conditioned medium was shown to be active in angiogenic assays it was used for subsequent functional studies.

The organization of endothelial cells into a three-dimensional tube-like structure is important in angiogenesis. Next, we examined the effects of EGFL7 on angiogenesis in vitro by tube formation assays. As shown in Figure 3C-D, EGFL7-containing conditioned medium significantly enhanced tube-like structure formation after 24 hrs of culture compared with vehicle and PBS controls. bFGF was used as a positive control. We further investigated the effects of EGFL7 on angiogenesis in bone environment using an ex vivo fetal mouse metatarsal angiogenesis assay. As shown in Figure 3E-F, human recombinant EGFL7 significantly induced angiogenesis featuring web-like structures in cultured metatarsals.

Activation of ERK1/2, STAT3 and FAK signaling pathways by EGFL7

It has been suggested that EGFL7 mediates endothelial cell and neural stem cell activities through modulating Notch signaling (Nichol et al., 2010; Schmidt et al., 2009) and promotes hepatocellular carcinoma cell migration by activation of FAK through EGFR (Wu et al., 2009). In an effort to address the mechanism of action of EGFL7 on endothelial cells, SVEC cells were stimulated with conditioned medium containing recombinant EGFL7 or empty vehicle for 0, 5, 10, 20, 30 and 60 mins, and the cell lysates were analyzed by Western blotting for levels of phosphorylation of key signaling molecules involved in cell migration. As shown in Figure 4, EGFL7 (compared to vehicle control) induced the phosphorylation of ERK1/2 more than 2.5 fold at 5 and 10 mins, STAT3 more than 1.5 fold at 30 and 60 mins, and FAK more than 2 fold at 30
and 60 mins. However, EGFL7 did not affect phosphorylation of ERK5, STAT1 and EGFR. In addition, we examined the effects of EGFL7 on STAT3 transcriptional activity by STAT3-responsive luciferase assay in which a luciferase reporter was driven by a promoter containing STAT3 DNA-binding sequence. Luciferase reporter assays revealed that conditioned medium containing EGFL7 induced STAT3-responsive activity ~2 fold (Fig. 4B). STAT3-responsive luciferase activity was also induced by human EGFL7 in a dose dependent manner (Fig. 4C). The induction level of conditioned medium was similar to that of 200ng/ml human EGFL7 (Fig. 4B, 4C), which is consistent with the estimated concentration of EGFL7 conditioned medium. To further confirm that ERK1/2 and STAT3 pathways are involved in EGFL7-mediated endothelial cell activities, we examined the effects of U0126 (MEK1/2 inhibitor) and Stattic (STAT3 inhibitor) on EGFL7-induced endothelial cell migration. As shown in Figure 5A-B, U0126 and Stattic significantly inhibited EGFL7-induced endothelial cell migration.

**EGFL7-induced angiogenesis ex vivo is mediated through the activation of ERK1/2 and STAT3**

Having demonstrated that EGFL7 influences endothelial cell migration through ERK1/2 and STAT3 signalings, we next investigated whether EGFL7 regulates angiogenesis via the similar mechanisms by using *ex vivo* fetal mouse metatarsal angiogenesis assay. As shown in Figure 6A-B, human EGFL7 significantly enhanced blood vessel growth from metatarsals. Interestingly, EGFL7-induced angiogenesis was significantly inhibited by U0126 and Stattic. The inhibitors alone had no significant effect on angiogenesis as compared to PBS control (Figure 6).

**RGD Peptides Impaired EGFL7-induced Endothelial Cell Migration**

Bioinformatic analyses indicate that mouse and rat EGFL7 proteins contain a conserved QGD motif, and human EGFL7 an RGD motif, indicative of involvement in integrin signaling (Supplementary Fig. S2). It has been reported that the change of RGD to QGD maintains integrin binding ability (Elphick et al., 2009; Erb et al., 2001; Gresham et al., 1992). To determine whether EGFL7 signaling is mediated through its RGD/QGD functional domain, we examined the effect of RGD peptides on the EGFL7-induced endothelial cell migration. Cells were pretreated with RGD peptides for 1 hr prior to performing scratch wound healing assays. As shown in Figure 7, EGFL7-induced endothelial cell migration was significantly reduced in the presence of RGD peptides at concentrations of 250ng/ml and 500ng/ml. bFGF-induced
endothelial migration was not inhibited by RGD peptides. These results suggest that EGFL7 regulates angiogenic activities through its RGD/QGD domain and integrin receptor complex.

**Upregulation of EGFL7 gene expression during growth plate injury repair**- To further confirm the involvement of EGFL7 in the bone microenvironment *in vivo*, its expression during the bony repair time course was examined in a rat model of tibial growth plate injury repair, which is known to involve mesenchymal cell infiltration, osteoblastic differentiation and bone formation, angiogenesis, and osteoclast recruitment and remodeling (Chung et al., 2011; Chung et al., 2009). Haematoxylin and eosin (H&E) and alcian blue staining revealed an infiltration of mesenchymal-like tissue and other cells (Fig. 8A) at day 6 following injury. By day 14 post-injury, the cells within the injury site had undergone differentiation and tissues such as bone trabeculae were evident. Signs of bone remodeling were also evident with the presence of osteoblasts and osteoclasts surrounding the newly formed bony repair tissue (Fig. 8B). To investigate vascularization during growth plate injury repair and bony repair tissue formation, expression of isolectin-B4 was used to label endothelial cells during growth plate injury repair. Within the growth plate injury site itself, there was an increase in vessel number and size at day 14 compared to day 6 (Fig. 8C-D).

Quantitative RT-PCR gene expression analysis of the growth plate injury site showed increased EGFL7 expression post-injury peaking at day 14 during the repairing process (Fig. 8E). By day 28, however, levels of EGFL7 at the injury site had decreased to near or below the basal level. No significant changes were seen in OCN expression at days 6 and 14 (despite a trend of increase on day 14) until a significant 30-fold increase at day 28 in comparison to non-injured controls (Fig. 8F). Interestingly, compared to the normal growth plate cartilage, levels of VEGFA were significantly lower on days 6 and 14 followed by an increase back up to basal levels on day 28 (Fig. 8G). VEGFA levels at day 28 were more than 1.5 fold greater than at day 6 and 14.
Discussion

The crosstalk between bone cells and endothelial cells is important for normal bone growth, remodeling and fracture healing (Brandi and Collin-Osdoby, 2006; Chim et al., 2013; Eriksen et al., 2007; Fang et al., 2005). Identification of angiogenic factors produced by bone cells could enhance our understanding of intercellular communication in the BRC, which might help to develop new targets for the treatment of bone fracture and bone disorders such as osteoporosis and osteonecrosis. The present study demonstrates that EGFL7 is expressed in osteoclasts, osteoblasts and bone tissues, and that it regulates endothelial cell migration, angiogenesis and integrin-mediated signaling of ERK1/2, STAT3, and FAK.

EGFL7 is a secreted protein that contains an EMI domain at the N-terminus, followed by two EGF repeat domains. We have detected a 68 KDa EGFL7 protein which might represent a homodimer or post-modified form of EGFL7 protein. Future studies will be required to investigate the protein modification of EGFL7 and functions of the isoforms. Fitch et al. has suggested that EGFL7 is expressed in highly vascularized tissues including brain, heart, lung, kidney and ovary (Fitch et al., 2004). EGFL7 is also expressed in endothelial progenitors and endothelial cells (Fitch et al., 2004; Parker et al., 2004). Loss of EGFL7 function in zebrafish embryos results in a vascular tubulogenesis defect (Parker et al., 2004). Moreover, vasculature development in organs is reported to be delayed in EGFL7 knockout mice (Schmidt et al., 2007). These results suggest that EGFL7 regulates angiogenesis by an autocrine mechanism. Here we have demonstrated that EGFL7 is expressed by osteoclasts and osteoblasts allowing us to hypothesize that EGFL7, as an extracellular protein, regulates capillaries near the BRC by a paracrine mechanism.

Initially, the mechanism of EGFL7-mediated cellular activities was reported to involve the binding of EGFL7 to the extracellular domain of Notch, allowing it to act as an antagonist of Notch signaling in neural stem cells (Schmidt et al., 2009). A further study demonstrated that EGFL7 modulates endothelial cell activities by a similar mechanism (Nichol et al., 2010). Other studies have proposed that EGFL7 promotes human hepatocellular carcinoma cell migration by EGFR-mediated activation of FAK (Wu et al., 2009). Surprisingly, we could not detect any phosphorylation of EGFR by EGFL7 in endothelial cells when adding as an exogenous factor. It
is important to note that human EGFL7 protein contains an RGD motif whereas mouse and rat EGFL7 proteins consist of a QGD motif, raising an intriguing possibility that EGFL7 might interact with integrins. It has been reported the change of RGD to QGD maintains integrin binding ability (Elphick et al., 2009; Erb et al., 2001; Gresham et al., 1992). Consistent with this notion, we found that EGFL7-induced endothelial cell migration was significantly reduced in the presence of RGD peptides, attesting that EGFL7 could function via a RGD/QGD motif. Interestingly, a recent study demonstrated that EGFL7 interacts with integrin αvβ3 and regulates endothelial cell motility (Nikolic et al., 2013). The binding of ligand to integrin triggers intracellular signaling cascades (Kumar, 1998; Leveille et al., 2007; Shattil and Newman, 2004), including MAPK, STAT and FAK (Bauvois, 2012; Behera et al., 2010; Guo and Giancotti, 2004; Parsons, 2003), which are important in cell migration (Chim et al., 2011; Long et al., 2010; Mehta and Besner, 2007; Teng et al., 2009). In this study, we demonstrated that recombinant EGFL7 induced the phosphorylation of ERK1/2, STAT3 and FAK in endothelial cells for the first time. Moreover, inhibition of ERK1/2 and STAT3 signaling pathways blocks EGFL7-induced endothelial cell migration and angiogenesis. Taken together, these results suggest that EGFL7 as an exogenous factor regulates endothelial cell activities through its RGD/QGD functional domain and integrin-mediated signaling.

As a step to demonstrate the involvement of EGFL7 in bone remodeling in skeletal repair, EGFL7 was found upregulated during growth plate injury repair. The growth plate injury model involves an initial inflammatory response (day 1-3) followed by a fibrogenic response involving mesenchymal infiltration (day 3-7), trabecular bone formation (day 8-14) and remodeling (day 14 onwards) (Chung et al., 2011). Angiogenesis at the growth plate injury site takes place starting from the mesenchymal cell repair phase and is more prominent during the bone formation phase (day 14). It has been suggested that growth plate injury responses and bony repair mechanism are similar to the process of bone fracture healing (Chung et al., 2011). However, the processes of revascularization during growth plate injury healing and fracture healing remain unclear. Kilarski et al suggested that translocation of pre-existing vascular network is responsible for the rapid formation of vasculature in granulation tissues at early stages of tissue healing. The vasculature is then further remodeled by angiogenic splitting and sprouting during later stages of healing to fine-tune vascular networks (Kilarski et al., 2009). It has been demonstrated that neovessel
formation in granulation tissue is mediated by intussusceptive angiogenesis, a mechanism at relatively low energy costs as compared to vessel sprouting (Burri and Djonov, 2002; Kilarski et al., 2009). The current study showed that EGFL7 gene expression was markedly upregulated during the growth plate injury repair process at the period of time when VEGF expression was found relatively low. VEGFA/VEGFR2 signaling has been postulated to regulate angiogenic sprouting (Gerhardt et al., 2003). Consistently, Kilarski et al demonstrated that early steps of vascularization in tissue repair are independent of angiogenic sprouting (Kilarski et al., 2009). Recently, it has been demonstrated that inhibition of Notch signaling induces intussusceptive angiogenesis (Dill et al., 2012; Dimova et al., 2013). Given that EGFL7 is an antagonist of Notch signaling (Schmidt et al., 2009), EGFL7 could mediate intussusceptive angiogenesis in granulation tissues during the early stages of growth plate repair. Furthermore, EGFL7 might regulate sprouting angiogenesis during osteogenesis at the later stages of healing through integrin-mediated signaling of ERK1/2, STAT3, and FAK.

Recently, we have reviewed the expression of angiogenic factors in the bone microenvironment, and the complex interrelationship between angiogenesis and osteogenesis (Chim et al., 2013). The spatiotemporal regulation of molecules mediates different stages and types of angiogenesis in both physiological and pathological conditions (Arima et al., 2011; Chim et al., 2013; Clapp et al., 2009). EGF-like family members have been implicated in angiogenesis (Kim et al., 2003; Leker et al., 2009; Mehta and Besner, 2007; Schmidt et al., 2007). In the bone microenvironment, EGF, heparin binding-EGF, amphiregulin, betacellulin, and transforming growth factor-α are expressed in osteoclasts and osteoblasts and regulate angiogenesis (Nakamura et al., 2010; Qin et al., 2005; Yi et al., 2008). Our previous study and the current work have identified that EGFL6 and EGFL7 are both expressed in the bone local environment and have a regulatory role in angiogenesis (Chim et al., 2011). EGFL2, EGFL3, EGFL5, EGFL8 and EGFL9 are relatively newly identified EGF-like family members, and their cellular functions are still not fully understood. It is possible that EGF family members are expressed by different cell types in bone local environment, and regulate angiogenesis during fracture healing and bone remodeling. Future studies will be needed to address the specific roles of EGF family members in angiogenesis and their signaling cascades.
In summary, we have shown that EGFL7 is expressed in the bone microenvironment and has a role in promoting endothelial cell migration and tube-like structure formation through integrin-mediated pathways. This study highlights the important role that EGFL7 plays in the regulation of endothelial cell activities in the bone microenvironment, and might help to develop novel therapeutic approaches for bone fracture and bone disorders such as osteonecrosis.
Acknowledgments:

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References


Figure Legends

**Figure 1. The gene and protein expression of EGFL7 in bone microenvironment.** (A) RT-PCR amplification of EGFL7 showing that EGFL7 was expressed during osteoclast and osteoblast differentiation. Primers specific for osteoclast marker genes CTR, TRACP and DC-STAMP were included as markers for osteoclast differentiation. EGFL6, ALP and OCN were used as markers for osteoblast differentiation. TRACP staining for osteoclasts and alizarin red staining for osteoblast mineralization were also included in parallel experiments. Scale bar, 100 µm. (B) Western blot analyses showing that EGFL7 protein expression was present in developing and mature long bones. (C) Tissue expression profile of EGFL7 determined by RT-PCR analysis.

**Figure 2. Production of conditioned medium containing EGFL7 protein.** (A) An expression construct encoding mouse full length EGFL7, pcDNA3.1-EGFL7-c-myc/His, was generated. A conserved QGD motif is located between EGF repeats. (B-C) Detection of EGFL7 proteins, using anti-c-myc and anti-EGFL7 antibodies, in COS-7 cells transfected with empty vector or expression vector encoding c-myc/His-tagged EGFL7. EGFL7 was detected in both medium and cell lysates. Black arrows indicate the EGFL7 proteins at the positions of 34KDa and 68KDa; respectively.

**Figure 3. EGFL7 promotes endothelial cell migration, tube-like structure formation and angiogenesis.** (A) Representative microscopic views of scratch wound healing assays performed in SVEC cells treated with conditioned medium containing EGFL7 or vehicle control for 0 or 16 hrs. Scale bar, 100 µm. (B) Quantitative analysis of cell migration area after 16 hrs showing EGFL7 enhanced endothelial cell migration. (C) Representative photographs showing tube formation by SVEC cells cultured for 24 hrs in conditioned medium containing EGFL7 or vehicle control. Scale bar, 100 µm. (D) Quantitative analysis of tube length showing EGFL7 promoted tube-like structure formation. PBS and bFGF being used as a negative and positive control, respectively. (E) Mouse metatarsals were dissected from E17.5 embryos for an *ex vivo* metatarsal angiogenesis assay. Representative microscopic images showing that human EGFL7 (200 ng/ml) induced blood vessel growth from metatarsals. PBS and VEGF (50 ng/ml) were used as a negative and positive control; respectively. Scale bar, 250 µm. (F) Quantitative analysis
of vessel sprouting. **p<0.01, ***p<0.001.

**Figure 4. EGFL7 induced phosphorylation of ERK1/2, STAT3 and FAK.** (A) Conditioned medium containing EGFL7 induced the cellular phosphorylation levels of ERK1/2 at 5 and 10 mins, STAT3 and FAK at 30 and 60 mins compared to vehicle control. EGFL7 did not induce phosphorylation of ERK5, STAT1 and EGFR. β-actin was used as loading control. Signal intensities were quantified by imageJ software. Induction ratios at each time point were compared to 0 min, with p-ERK1/2 normalized to ERK1/2, p-STAT3 normalized to STAT3, and p-FAK normalized to β-actin. (B-C) Determination of STAT3 transcription activity in SVEC by STAT3-responsive luciferase reporter assays. EGFL7 conditioned medium and recombinant human EGFL7 significantly induced STAT3-responsive luciferase activities. Luciferase activity was compared to vehicle or PBS. *p<0.05, **p<0.01.

**Figure 5. EGFL7-induced endothelial cell migration was blocked by U0126 and Stattic.** (A) Representative microscopic views of scratch wound healing assays showing EGFL7-induced endothelial cell migration was inhibited in the presence of U0126 (5µM) and Stattic (1µM). Scale bar, 100 µm. (B) Quantitative analysis of cell migration area after 16 hrs showing U0126 and Stattic significantly inhibited EGFL7-induced endothelial cell migration. PBS and bFGF being used as a negative and positive control, respectively. *p<0.05.

**Figure 6. EGFL7-induced angiogenesis was blocked by U0126 and Stattic.** (A) Mouse metatarsals were dissected from E17.5 embryos for an ex vivo metatarsal angiogenesis assay. Representative microscopic images showing that EGFL7-induced angiogenesis was inhibited in the presence of U0126 (5µM) and Stattic (1µM). Inhibitors alone had no effects on angiogenesis as compared to PBS control. Scale bar, 250 µm. (B) Quantitative analysis of vessel sprouting. ***p<0.001.

**Figure 7. EGFL7-induced endothelial cell migration was blocked by RGD peptides.** (A) Scratch wound healing assays performed with the conditioned medium containing EGFL7 or vehicle control in the presence (250 or 500ng/ml) or absence of RGD peptides for 16 hrs showing that EGFL7-induced endothelial cell migration was reduced by RGD peptides. EGFL7-
induced endothelial cell migration was not inhibited by RGD peptides. Scale bar, 100 µm. (B) Quantitative analysis of cell migration area after 16 hrs showing that RGD EGFL7-induced endothelial cell migration was significantly inhibited by RGD peptides at the concentration of 250ng/ml and 500ng/ml. PBS and bFGF being used as a negative and positive control; respectively. *p<0.05.

**Figure 8. EGFL7 is upregulated during growth plate injury repair.** (A) Histology revealed a majority of mesenchymal repair tissue (Mes) at the injury site on day 6 post- growth plate injury. (B) By day 14, bone trabeculae repair tissue (Bt) surrounded by osteoblasts (OB) and osteoclasts (OC). (C-D) Isolectin-B4 positive endothelial cells at the growth plate injury site (C) 6 days and (D) 14 days post- injury. (E-G) Quantitative real-time RT-PCR expression data for (E) EGFL7, (F) OCN and (G) VEGFA are expressed as fold change to non-injured controls. Original scale bar= 125µm (applies to A-D). **p<0.01.
Figure 1

A) Osteoclast Differentiation

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Day0 vs Day5

Osteoblast Differentiation

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Day0 vs Day21

B) E18.5, Week1, Week7, Week16

EGFL7

β-actin

C) Ratio (Compared to 18s)

EGFL7

18s

Muscle, Kidney, Heart, Lung, Thymus, Liver, Spleen, Pancreas, Stomach, Intestine, Tibia
**Figure 2**

(A) Diagram of EGFL7-c-myc/His with QGD motif indicated.

(B) Western Blot (WB) showing c-myc expression in Medium and Lysate for Vehicle and EGFL7 conditions.

(C) Western Blot (WB) showing EGFL7 expression in Medium and Lysate for Vehicle and EGFL7 conditions.
Figure 3

A) Cell Migration Area

B) Cell Migrated Area

C) Tube Length

D) Tube Length

E) Vascular Density (%)

F) Vascular Density (%)

PBS, bFGF, Vehicle, EGFL7
Figure 5

(A) Images showing cell migration at 0hr and 16hr for different treatments: PBS, bFGF, Vehicle, EGFL7, Vehicle+U0126, EGFL7+U0126, Vehicle+Stattic, EGFL7+Stattic.

(B) Graph showing cell migrated area for different treatments: PBS, bFGF, Vehicle, EGFL7, Vehicle+U0126, EGFL7+U0126, Vehicle+Stattic, EGFL7+Stattic. The graph indicates significant differences (*p < 0.05) between treatments.
Figure 6
Figure 7

A

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B

![Graph showing cell migration area](image21.png)
Figure 8