**The role of EGFL7 as an angiogenesis regulator in cancer and skeletal system**[*]

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[*]abbreviation: AREG, amphiregulin; BLAST, Basic Local Alignment Search Tool; Dll4, Delta-like 4; DNMT1, DNA methyltransferase 1; DSL, Delta-Serrate-LAG-2; ECM, extracellular matrix; EGFL7, Epidermal growth factor-like domain-containing protein 7; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ERK1/2, extracellular signal-regulated kinase 1/2; ESCC, esophageal squamous cell carcinoma; FAK, focal adhesion kinase; GBM, glioblastoma multiforme; HCC, hepatocellular carcinoma; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; MALAT1, Metastasis associated lung adenocarcinoma transcript 1; MVD, microvessel density; NPNT, Nephronectin; NSCLC, non-small cell lung cancer; OSCC, oral squamouse cell carcinoma; PC, Pancreatic cancer; STAT3, signal transducer and activator of transcription 3; RhoA, Ras homolog gene family, member A; VCAM-1, vascular cell adhesion molecule-1; CASZ1, castor zinc finger 1; Flt3, Fms related tyrosine kinase 3.

**Abstract**

Epidermal growth factor-like domain-containing protein 7 (EGFL7), a member of epidermal growth factor (EGF)-like protein family, is a potent angiogenic factor expressed in many different cell types. EGFL7 plays a vital role in controlling vascular angiogenesis during embryogenesis, organogenesis and maintaining skeletal homeostasis. It regulates cellular functions by mediating the main signalling pathways-Notch, integrin and epidermal growth factor receptor (EGFR) cascades. Accumulating evidence suggests that *Egfl7* plays a crucial role in cancer biology by modulating tumour angiogenesis, metastasis and invasion. Dysregulation of *Egfl7* has been frequently found in several types of cancers, such as malignant glioma, colorectal carcinoma, oral and oesophageal cancers, gastric cancer, hepatocellular carcinoma, pancreatic cancer, breast cancer, lung cancer, osteosarcoma and acute myeloid leukemia. In addition, altered expression of miR-126, a microRNA associated with *Egfl7*, was found to play an important role in oncogenesis. More recently, our study has shown that EGFL7 is expressed in both the osteoclast and osteoblast lineages and promotes endothelial cell activities via ERK, STAT3, and integrin signaling cascades, indicative of its angiogenic regulation in bone microenvironment. Thus, understanding the role of EGFL7 may provide novel insights into the development of improved diagnostics and therapeutic treatment for cancers and skeletal pathological disorders, such as ischemic osteonecrosis and bone fracture healing.

Key words: EGFL7, EGF-like, angiogenesis, cancer, skeletal system, osteoblast

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## 1. Introduction

Members of the EGF-like protein family have been the subject of intense investigation owing to their fundamental role in Notch signal transduction and modulation of cellular functions. Importantly, they also play a critical role in angiogenesis [1], a complex and intricate process which involves endothelial cell proliferation and sprouting to form a functional vascular network to facilitate tissue growth and regeneration [2]. In recent years, *Egfl7* gene has emerged as an important angiogenic factor and a critical regulator of vascular development. *Egfl7* was first identified as a gene that is involved in the regulation of endothelium homeostasis during embryogenesis [3-5]. Subsequently, various studies have characterized EGFL7 as an angiogenic signaling molecule [4, 6] and an important regulator of cell membrane receptors, including tumour-associated EGFR [7], endothelial integrin receptors [8] and endothelial Notch receptors [9]. In addition, EGFL7 regulates placental vascularization and embryonic development [10], and has been implicated in the pathogenesis of pre-eclampsia by regulating placenta trophoblast cells via MAPK, PI3K and NOTCH signalling pathways [11]. EGFL7 also mediates thymogenesis via Fms related tyrosine kinase 3 (Flt3) signalling [12], and hyperoxia-induced lung injury of newborn rats [13].

More recently, in an attempt to understand the novel role of EGFL7 in the skeletal system, we have found that EGFL7 is involved in regulating angiogenesis in the bone microenvironment [6]. We demonstrated that EGFL7 may be involved in facilitating the repair process of growth plate injury, possibly via spatio-temporal regulation of angiogenesis in coordination with other prominent angiogenic factors. In addition to its physiological importance in the skeletal and other systems, accumulating evidence also suggest that EGFL7 is involved in cancer development. Many studies have demonstrated that EGFL7 plays a central role in tumour progression via mediating tumour metastasis, proliferation and angiogenesis [7, 14-18]. Furthermore, EGFL7 has also been associated with miR-126, a key microRNA that regulates tumour growth [19-21]. In this review, we discuss the molecular structures of EGFL7 protein and its gene expression profiling in different tissues. Furthermore, we explore the key mechanisms and signalling pathways of EGFL7 in mediating cellular functions. Finally, we summarize the role of EGFL7 in the skeletal system, and provide novel insights into the potential mechanisms of EGFL7 in the development of different cancers.

## 2. Molecular structure and expression profile of EGFL7

According to previous studies and genomic database from the National Center for Biotechnology Information, human *EGFL7* (also known as *VE-STATIN)* is located on chromosome 9 (9q34.3),

while mouse *Egfl7* on chromosome 2 (2A3) [3, 5]. Human and mouse *EGFL7* encode a 29.6 kDa protein (273 amino acids) and 29.8kDa (275 amino acids) protein, respectively [5]. This protein was first described to be expressed in developing vascular structures during embryogenesis [3, 5]. Structurally, EGFL7 protein contains an N-terminal signal peptide (blue), a cysteine-rich EMI domain (green), two centrally located EGF-like repeat domains (purple and dark red), a cell attachment site (RGD motif, orange) and coiled coil (light blue), as shown in Figure 1A [3-5]. Here, we performed 3D modeling to predict the structure of EGFL7, which shows the arrangement of different functional regions and the topology of domain architectures (Figure 1B).

Furthermore we performed bioinformatics analysis of *Egfl7* using a web-based Basic Local Alignment Search Tool (BLAST) from Uniprot. We found that human, mouse and rat EGFL7 share similar protein sequences (Figure 2). These proteins share domains that closely resemble those of secreted and extracellular matrix (ECM)–bound proteins, such as EMILIN and laminin [22, 23]. One of the EGF-like domains consisted of regions that is homologous to the Delta-Serrate-LAG-2 (DSL) domain, a sequence that is involved in protein interaction with Notch receptor, whereas the other EGF-like domain binds to ionic calcium and directs a structural change in the protein [3, 24]. In addition, gene expression profiling using BioGPS showed that human *EGFL7* is differentially expressed in cells, tissues and organs (Figure 3). In particular, the expression of *EGFL7* is the highest in $CD34^+$ cells, $CD105^+$ endothelial cells, cardiomyocytes, fetal and adult lung, fetal thyroid, smooth muscle, placenta and the heart.

The gene expression of EGFL7 is controlled by transcriptional regulatory mechanisms and tightly restricted to endothelium [3, 4, 9, 25]. The earliest evidence of this emerged in three independent studies, which examined the expression of *Egfl7* during embryogenesis [3-5]. In the studies conducted by Fitch (2004) and Soncin (2003), *Egfl7* mRNA expression was detected in the extra-embryonic mesoderm, where the first endothelial cells differentiate and which proves to be essential for the establishment of the vasculature [3, 5]. Furthermore, the expression of *Egfl7* coincided with the expression of *flk-1*, a specific marker for early vascular progenitor cells during embryonic development [5]. Interestingly, *Egfl7* expression is present in highly vascularized adult tissues such as the lung, heart, uterus and ovary, indicating its crucial role in the vascular system [3, 25]. These studies suggest that EGFL7 may play a critical role in mediating the formation of blood vessels. Indeed, subsequent studies revealed that EGFL7 can promote endothelial cell migration and vascular tube structure formation, sprouting, and invasion [4, 25, 26]. In addition, EGFL7 also plays a role in protecting endothelial cells from stress-induced apoptosis [27]. Furthermore, the secretion

of EGFL7 can also be influenced by other ECM proteins. For instance, fibronectin and type 1 collagen, ECM proteins that are encountered by nascent vessels when they invade new tissues, are capable of facilitating the deposition of EGFL7 [26]. The ability of EGFL7 to modulate vascular endothelial cell activities is consistent with its structural EGF-like domains, a key feature of many EGF-like proteins that are known to be angiogenic factors including, but not limited to, EGF, HB-EGF, TGFα and EGFL6 [1].

## 3. Molecular mechanisms and regulation of EGFL7 in angiogenesis

Following the discovery of EGFL7 as a critical angiogenesis mediator, several studies have subsequently proposed the molecular mechanisms by which EGFL7 regulates blood vessel formation. One of the key mechanisms in this delicate process is through the Notch signalling pathway. Notch is a highly conserved signaling pathway that plays a central role in regulating the formation and sprouting of endothelial tip cells in angiogenesis [28-31]. The main mechanism of Notch signalling involves the binding of Notch ligand Delta-like 4 (Dll4), a transmembrane ligand for Notch receptors on endothelial cells [30]. This interaction between Notch and Dll4 leads to the suppression of endothelial tip cell formation and vessel branching, as reported by several studies [28-30, 32]. Notch-Dll4 can be antagonized by other Notch ligands, such as Jagged 1, leading to a proangiogenic regulation [29]. EGFL7 can also act as an antagonist of Notch signalling, thereby modulating angiogenesis [9, 33]. It is proposed that EGFL7 physically binds to the Notch receptor and/or its ligand, leading to the disruption of the receptor-ligand interaction [9].

Another main signalling mechanism of EGFL7-mediated angiogenesis is through interaction with cell-surface integrins. Many integrins such as $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_5$ have been reported to be expressed on endothelial cells [34-36]. More recently, studies have shown that EGFL7 is a specific ligand for $\alpha_v\beta_3$ on endothelial cells and is capable of activating extracellular signal-regulated kinase 1/2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3) and integrin signalling cascades [6, 8]. Importantly, RGD peptides, which compete with EGFL7 for integrin binding, can attenuate EGFL7-induced migration of endothelial cells [6]. Indeed, many ECM proteins which contain RGD motifs are known to bind to endothelial cells and regulate angiogenesis. These proteins include vitronectin, fibronectin and fibrinogen, which form the main components of the provisional matrix secreted during angiogenesis [37]. Given EGFL7 also possesses a conserved RGD/QGD motif, it is unsurprising that it is involved in mediating angiogenesis in an integrin-dependent manner [6].

Interestingly, recent studies have proposed a new transcriptional regulatory pathway for *Egfl7*. This pathway involves the transcription factor called castor zinc finger 1 (CASZ1) and Ras homolog gene family, member A (RhoA) GTPase, collectively known as CASZ1/*Egfl7*/RhoA pathway [38]. CASZ1 was initially implicated to play an important role in cardiovascular development and possibly in hypertension [39, 40]. Interestingly, CASZ1 was found to directly regulate the transcription of *Egfl7* via downstream singaling molecule RhoA [41]. The activation of CASZ1/*Egfl7*/RhoA pathway could result in the modulation of endothelial cell adhesion, contractility and vessel assembly [38, 41, 42].

The regulatory effect of miR-126 on *Egfl7* transcription has recently been proposed. MicroRNAs are a class of non-coding RNAs with short nucleotide sequences that are capable of regulating gene expression [43]. Two independent studies have presented evidence that miR-126, which is located in intron 7 of *Egfl7*, is expressed in endothelial cells and regulates vascular integrity and angiogenesis [44, 45]. Disruption of miR-126 expression in mice induces a delay in retinal and cranial vascularization, possibly via VEGF-mediated Akt and ERK activation [46]. It is possible that miR-126 and *Egfl7* facilitate independent, but complementary mechanisms to regulate angiogenesis and maintain vascular integrity [47].

## 4. The emerging role of EGFL7 in the skeletal system

The EGF-like protein family, which includes EGFL7, is known to mediate embryonic skeletal development and postnatal bone remodeling [1]. For instance, well known regulatory factors such as EGF, HB-EGF, BTC, TGFα and amphiregulin (AREG) have been reported to be expressed by bone forming osteoblasts and bone resorbing osteoclasts [48-51]. Recently, our group has identified novel EGF-like family members such as *Egfl2, Egfl3, Egfl5, Egfl6, Egfl7, Egfl8* and *Egfl9* to be differentially expressed in the bone [52]. Importantly, EGFL7, along with EGFL6 and its homologue Nephronectin (NPNT), are secreted factors that promote endothelial cell migration and vascular tubulogenesis in the bone microenvironment [6, 52, 53]. This is in line with other findings that EGFL7 is a potent angiogenic factor and plays a critical role in embryonic vascular organization [26]. Furthermore, EGFL7 has also been reported to be present in the hypertrophic chondrocytes located in the perivascular regions of the cartilage, suggesting its potential role in modulating vessel infiltration and ossification of cartilage [54]. Interestingly, the expression of *Egfl7* and *Vegfa* was found to be regulated following the growth plate injury in rats [6]. This suggests that *Egfl7*, along with *Vegfa*, is involved in a spatiotemporal regulation of the growth plate injury repair process, possibly by modulating angiogenesis.

## 5. The role of EGFL7 in cancer development

Expression of *Egfl7* has been documented in several human malignancies including breast cancer, glioma, colorectal cancer, hepatocellular carcinoma, gastric cancer, lung cancer, prostate cancer, oesophageal cancer, ovarian cancer and renal cancer [55]. The elevated protein expression of EGFL7 in various cancers plays a central role in the pathological neoangiogenesis and tumour invasiveness. Specifically, the oncogenic potential of the EGFL7 involves ligand-receptor interaction, such as Notch and EGFR signalling [16, 56]. In this respect, EGFL7 could function as a tumour-secreted autocrine or paracrine regulator, in much the same way as VEGF [57]. Thus, the potential of targeting tumours for treatment via the inhibition of *EGFL7* and its signaling pathways is promising. To date, the expression and cellular mechanisms of *Egfl7* have been reported in many common, yet lethal cancers that possess high mortality rates. Here, we discussed the role of *Egfl7* in some of these cancers below.

### *5.1 Malignant glioma (brain cancer)*

Malignant gliomas (also known as ependymomas of the brain) are the most frequent and malignant human brain tumours, with very poor prognosis and survival rate [58]. Despite this, medical advances have been made to identify the key genetic factors that drive the development of malignant gliomas. Interestingly, *EGFL7* has been identified to be highly expressed in malignant glioma, but the expression is not detectable in normal brain tissues [59]. It was found that the levels *EGFL7* mRNA in glioma is elevated along with Ki-67 (a marker for tumour cell proliferation) expression and microvessel density (MVD), indicating a strong correlation between *EGFL7* expression and tumour angiogenesis/invasiveness [59]. To dissect the mechanism, Huang (2014) performed a co-culture system between human umbilical vein endothelial cells (HUVECs) and a glioma cell line (U251), and showed that vascular lumen formation and endothelial cell adhesion were inhibited following *EGFL7* silencing with siRNA [60]. Furthermore, a cohort cells microarray study revealed that *EGFL7* may regulate the development of glioma via PI3K/Akt and Ras/MAPK signaling pathways, possibly in an integrin or EGFR-dependent manner [61]. In line with this, it has been proposed EGFL7 may potentiate the oncogenicity of EGFR-positive glioblastoma multiforme (GBM) cells by binding to EGFR and activating Akt and ERK signalling pathways [14].

### *5.2 Colorectal carcinoma*

The cytoplasmic expression of EGFL7 in metastatic colorectal cancer tissues was first described by Hansen (2013) [15]. The same group further identified a positive correlation between *KRAS*

mutation in the tumours and higher median EGFL7 vessel area [62]. Although mechanistic evidence is currently lacking, it is possible that a constitutive activation of *KRAS* in tumour cells can regulate *EGFL7* expression, leading to increased tumour angiogenesis and cancer aggressiveness. Indeed, *KRAS* gene has been linked to angiogenesis in colon cancer in the past [63, 64]. In addition, another study by Hansen (2015) revealed that the intra-tumoural expression of EGFL7 is significantly higher in primary tumours from patients with recurrent disease compared to patients without relapse in stage II or III colorectal cancer [65]. Although these studies point to EGFL7 as a promising target for treatment of metastatic colorectal cancer, a randomized phase II clinical trial has found that parsatuzumab (a humanized anti-EGFL7 antibody) has failed to improve the conventional chemotherapy efficacy in these patients [66]. This surprising finding highlights the difficulty and challenges of developing an EGFL7-targeted therapy. Thus, future clinical studies may require an in-depth understanding of the mechanism and the optimization of the dosage and/or formulations of parsatuzumab in the development of effective anti-angiogenic drugs.

Interestingly, several studies have highlighted the regulation of miR-126, an *Egfl7*-related microRNA (refer to section 3 in this review) in colorectal cancer progression. For instance, reports have indicated that miR-126 expression is downregulated in colorectal cancer tissues compared to non-tumour tissues [67, 68]. Furthermore, silencing of miR-126 by DNA methylation in colorectal cancer could induce an upregulation of VEGF, leading to enhanced tumour invasiveness and angiogenesis [68]. Paradoxically, miR-126 is also known regulate embryonic angiogenesis by promoting VEGF signalling [44, 45]. It is possible that miR-126 may have diverse mechanisms in physiological and pathological conditions. This is especially important in the context of designing miR-126-targeted drugs, so that unintended side effects arising from the therapeutic treatment can be minimized or prevented.

### *5.3 Oral and oesophageal cancers*

Interestingly, EGFL7 and miR-126 have also been implicated in oral squamous cell carcinoma (OSCC) [69, 70] and esophageal squamous cell carcinoma (ESCC) [71]. Both *EGFL7* and miR-126 were shown to be downregulated in highly metastatic OSCC cells [69]. Similarly, Liu (2015) has shown that the downregulation of miR-126 in ESCC is induced by DNA methyltransferase 1 (DNMT1) -mediated hypermethylation of *EGFL7* promoter [71]. In OSCC, miR-126 was found to be a negative regulator of *VEGF-A*, a potent trigger for tumoural angiogenesis and tumour progression [69]. Intriguingly, Yang (2014) demonstrated that *EGFL7* is a direct target of miR-126, and that miR-126 could negatively regulate EGFL7 protein level [70]. This finding suggests that

although miR-126 is an intron-located miRNA and its host gene is *Eglf7*, it may be involved in suppressing EGFL7 expression at a post-transcriptional level.

### *5.4 Gastric cancer*

Gastric cancer is one of the leading causes of death from cancers worldwide [72]. *EGFL7* has been implicated in tumour invasion and metastasis of gastric cancer. A study conducted by Luo (2014) has shown that EGFL7 promotes metastasis and an epithelial-mesenchymal transition (EMT) , a process by which epithelial cells gain migratory and invasive properties to revert to mesenchymal stem cells, in gastric cancer via the activation of EGFR−Akt−Snail signalling pathway [16]. Importantly, Snail is a transcriptional repressor of E-cadherin [73], and the loss of E-cadherin is known to be associated with high tumour grade and invasiveness [74]. Furthermore, metastasis associated lung adenocarcinoma transcript 1 (MALAT1), a highly conserved non-coding RNA amongst mammals could regulate *EGFL7* expression by altering the level of H3 histone acetylation in malignant gastric cells [75].

### *5.5 Hepatocellular carcinoma*

A form of highly aggressive and malignant cancer originated from the liver, hepatocellular carcinoma (HCC) accounts for more than 700,000 deaths worldwide each year [72]. Importantly, EGFL7 is known to play a critical role in tumour progression and metastasis of HCC. For instance, it was found that *EGFL7* is overexpressed in HCC cells and this closely correlated with poor prognosis of HCC via enhancing HCC cell motility via EGFR activation of focal adhesion kinase (FAK) [7]. Another study by Campagnolo (2016) has found that the expression of *EGFL7* mRNA increases from well differentiated to less differentiated tumours, indicating that EGFL7 could be critical in promoting oncogenesis and angiogenesis in a more advanced metastatic phase [76]. Interestingly, by using an *in vivo* HCC xenograft mouse model, it was shown that miR-126 could inhibit tumour proliferation and angiogenesis by suppressing EGFL7 expression, possibly via binding to the UTR region of *Egfl7* mRNA [21]. This is consistent with the findings by Yang (2014) which showed that miR-126 regulates EGFL7 in OSCC as described previously [70].

### *5.6 Pancreatic cancer*

Pancreatic cancer (PC), with the most common form being pancreatic ductal adenocarcinoma, is one of the most lethal cancers with extremely poor prognosis and patient survival rate [77, 78]. Zhou (2014) revealed that EGFL7 is widely expressed in various PC cell lines and tumour samples, and is significantly associated with poor overall survival as a long term outcome [79]. Furthermore,

EGFL7 is capable of inducing PC cell invasion and tumour angiogenesis, although it does not appear to have an intrinsic effect on cell cycle progression, proliferation and colony formation [17]. More importantly, EGFL7 has been found to regulate an EMT change and cell migration in PC, the main drivers of carcinoma metastasis [80]. In addition, the suppression of *EGFL7* led to the downregulation of Snail and the upregulation of its transcriptional target E-cadherin, suggesting that the EGFR-Akt-Snail signalling pathway may be responsible for EGFL7-mediated EMT and tumour invasion. Interestingly, a recent study indicated that endothelial cell-derived EGFL7 may regulate the proliferation of pancreatic progenitors in a non-tumoural context [81]. Given that tumour vasculature is the main supporting factor in the growth of cancer, it is possible that tumour-associated endothelium may facilitate crosstalk with PC by secreting EGFL7. Therefore, further investigation is warranted in this area.

### *5.7 Breast cancer*

Breast cancer is a form of aggressive cancer caused by abnormal cell growth originated from breast lobules or ducts, affecting predominantly females with an estimated 1.7 million cases and 521,900 deaths in 2012 [72]. Similar to other human cancers, the role of *EGFL7* in breast cancer has been addressed by several studies. Philippin-Lauridant (2013) first investigated the expression of EGFL7 protein in breast tumours, and found that invasive tumour cells express higher EGFL7 levels compared to normal epithelial cells [82]. Furthermore, EGFL7 expression was found to be associated with an increase in tumour growth and microvessel density in mouse models bearing 4T1 tumour, a murine mammary carcinoma [18]. Strikingly, EGFL7 could suppress intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), two key endothelial adhesion molecules that facilitate immune cell attachment and infiltration [18, 83]. This suggests that EGFL7 can promote breast tumour evasion from the host immune system by modulating the activation of tumour endothelial cells. Interestingly, *Egfl7*-associated miR-126 was found to be able to inhibit lung metastatic colonization by human breast cancer cells in immune-deficient mice, implying its suppressive role in breast tumour metastasis [20].

### *5.8 Lung cancer*

Lung cancer, with the most common form being non-small cell lung cancer (NSCLC), is the most frequently diagnosed cancer and the leading cause of death among males in 2012 [72]. Azhikina (2011) first proposed the concept of heterogeneity of *EGFL7* promoter methylation as a crucial factor for carcinogenesis of lung cancer [84]. A subsequent study revealed that *EGFL7* is overexpressed in human lung cancer [55]. Notably, it has been revealed that miR-126 can target

EGFL7, thereby inhibiting the NSCLC cell proliferation and tumour growth [19]. To investigate the clinical implications of targeting EGFL7 as a treatment for NSCLC, anti-EGFL7 antibody was generated and evaluated in preclinical murine NSCLC models [27]. It was found that anti-EGFL7 improves the anti-angiogenic activity and enhances survival benefits derived from anti-VEGF therapy [27]. This suggests that EGFL7 could be a very promising target in combination with conventional chemotherapeutic cancer treatment in clinical settings, at least in the context of lung cancer.

### *5.9 Osteosarcoma*

More recently, several studies have pointed to the roles of *EGFL7* and related miR-126 in osteosarcoma, a rare but aggressive malignant neoplasm arising from mesenchymal origin [85]. This disease primarily affects the adolescent and elderly populations [86]. Given EGFL7 is produced by osteoblasts and plays a physiological role in angiogenesis of the skeletal system [6], and osteosarcomas are vascularized tumours, perhaps it is envisaged that the dysregulation of EGFL7 may represent a prime factor in regulating osteosarcoma metastasis. Indeed, EGFL7 expression level in osteosarcoma is found to be higher in advanced stage compared to early stage tumour [87]. Furthermore, the expression of EGFL7 in the tumour tissues is positively correlated to microvessel density [87]. This indicates that EGFL7 is upregulated in a tumour grade-dependent manner and may support angiogenesis in osteosarcoma. Interestingly, miR-126 has also been implicated in osteosarcoma. Studies have found that miR-126's expression is lower in osteosarcoma tissues compared to normal bone tissues [88, 89], and the low expression is correlated to shorter patient survival time following surgery [88]. Overexpression of miR-126 in osteosarcoma cell line MG-63 inhibits proliferation, migration, and invasion, and induces cellular apoptosis. These findings support the role of miR-126 as a tumour suppressor in osteosarcoma.

### *5.10 Acute myeloid leukemia*

Acute myeloid leukemia (AML) is the most common type of leukemia, accounting for approximately 25% of all leukemia in adults in the Western world with the lowest survival rate of all leukemias [90]. It is a clonal hematological malignancy characterized by the infiltration of bone marrow, blood, and other tissues by hematopoietic blasts which are highly proliferative, immature and poorly differentiated [91]. Interestingly, a study has shown that high *EGFL7* expression is associated with lower complete remission rates, lower event-free and overall survival in patients with cytogenetically normal AML (CN-AML) [92]. *EGFL7* mRNA and protein levels in patient AML blasts are significantly lower compared to normal bone marrow mononuclear cells (NBM-

MNCs) from healthy donors [92]. Crucially, these authors demonstrated that AML blasts secrete EGFL7, and that EGFL7 promotes leukemic blast growth and the phosphorylation of Akt. Additionally, a role for *EGFL7*-associated miR-126 has also been proposed. Li (2008) found that miR-126 can inhibit apoptosis and enhance the viability of AML cells [93]. Other studies have shown that miR-126 regulates the maintenance and self-renewal of leukemia stem cells (LSCs), quiescent primary stem cells that contribute to AML progression, recurrence and chemotherapy resistance [94, 95]. Inhibition of EGFL7 via miR-126 in LSCs and AML cells have yielded positive pre-clinical outcomes [92, 94]. This suggests that a combined EGFL7/miR-126-targeted therapy may be a feasible therapeutic option to improve the treatment of AML patients in the future.

### *5.11 Other EGFL7-associated cancers*

An emerging role of *EGFL7* has also been described in ovarian cancer [96], uterine cervical cancer [97], laryngocarcinoma [98], malignant pleural mesothelioma [99] and renal cell carcinoma [100]. Collectively, these studies indicate the potential involvement of EGFL7 in tumourigenesis and pathological angiogenesis. Further preclinical and clinical studies are required to examine the efficacy of EGFL7-targeted treatment in these cancers.

## 6. The model of EGFL7 signalling mechanisms

Three major signalling mechanisms have been proposed by which EGFL7 regulates the activity of endothelial and tumour cells (Figure 4). Firstly, EGFL7 interacts with integrins on the endothelial cell surface. Specifically, EGFL7 binds to integrin $\alpha_V\beta_3$ and promotes the adhesion of endothelial cells to extracellular matrix, thus contributing towards the blood vessel reconstruction and formation The integrin-dependent signalling model of EGFL7 is further reinforced by the findings that EGFL7 mediates integrin signalling cascades, leading to the activation of FAK and subsequent downstream signalling pathways such as MAPK/ERK, PI3K/Akt and JAK/STAT3 pathways [6, 8]. Crucially, our study has demonstrated that the EGFL7-integrin interaction is likely to be mediated by RGD binding motif [6], a motif that is present in many other extracellular matrix proteins involved in angiogenesis [37]. Therefore, the current literature strongly supports the model that EGFL7 regulates angiogenesis through integrin interaction.

Secondly, EGFL7 regulates angiogenesis by modulating Notch signalling in endothelial cells, either in an autocrine or paracrine manner [9, 33]. As described previously, Notch signalling is an important mechanism of vascular development whereby its ligand, Dll4, is involved in negatively regulating endothelial cell proliferation, migration, vessel branching and VEGF function [101]. In

this model, EGFL7 can act as antagonist of Notch receptor. Mechanistically, it is proposed that EGFL7 physically antagonizes Notch/Ligand interaction, leading to an inhibition of proteolytic cleavage and release of intracellular domain to the cell nucleus [33]. This results in the inhibition of CSL (CBF1/RBP-J in mammals, Su(H) inflies, LAG-1 in worms) transcription factor-mediated activation of Notch target genes, leading to the modulation of angiogenesis [33]. This model is strongly supported by the findings that Dll4 is a negative regulator of endothelial tip cell formation, angiogenic sprouting and branching [30, 32]. In fact, pharmacological inhibition of either Dll4 or Notch signalling stimulates angiogenesis, a notion that is consistent with EGFL7 promoting angiogenesis by antagonizing the Notch signalling pathway [30, 32]. Moreover, EGFL7 is also known to reduce proliferation and self-renewal of neural stem cells via decreasing Notch signalling [102]. This shows that the concept of EGFL7 as a regulator of Notch is not only restricted to the vascular system, but potentially in other physiological systems as well.

Thirdly, EGFL7 can physically interact with EGFR and activates downstream signalling pathways such as PI3K/Akt and MAPK/ERK pathways, thus promoting cellular regulation. The EGFL7-EGFR signalling model is extremely relevant especially in the context of many cancers, where pathological dysregulation of EGFL7 and EGFR are commonly detected. For instance, EGFL7 is known to promote the activation of AKT and ERK pathways in metastatic gastric cancer and glioma via EGFR interaction [14, 16]. In another study, EGFL7 promotes HCC metastasis by activating FAK phosphorylation through EGFR binding [7]. Furthermore, renal cell carcinoma is also found to induce endothelial cell migration and vascular tube formation by activating EGFL7-EGFR-FAK pathway, thus contributing to the progress of tumour growth [100]. Interestingly, a study has proposed that EGFL7 can regulate both the Notch and EGFR pathways in Jeg3 human choriocarcinoma cell line, resulting in an increased cell migration and invasiveness via the convergence of both pathways [103]. Overall, EGFL7-EGFR may play a key role in inducing intratumoural angiogenesis, tumour metastasis and invasion.

## 7. Conclusion

Angiogenesis plays a central role in mediating embryogenesis, organogenesis and maintaining tissue homeostasis. However, disruption in the regulation of angiogenic factors can often lead to abnormal angiogenesis. This is most commonly observed in cancer tissues, where uncontrolled tumour angiogenesis can provide nutrients and promote growth to the tumour tissues. Without early medical intervention, this would lead to the spread of cancer to nearby tissues/organs and the formation of secondary tumours, a prime cause of cancer mortality. Therefore, identification and

understanding of how angiogenic factors are regulated both in physiologically normal and cancerous conditions are critically important.

The emerging roles of EGF-like family members in angiogenesis have been described by different studies. In particular, EGFL7 is known to modulate endothelial cell activity and vasculature formation during embryogenesis. Recently, our group has identified EGFL7 as a vital angiogenic factor in the maintenance of skeletal homeostasis, and might serve as a therapeutic target for ischemic osteonecrosis and bone fracture healing. In addition, many pre-clinical and clinical studies have implicated EGFL7 in cancer development and metastasis, with high EGFL7 expression in many epithelial cancers commonly observed. Furthermore, EGFL7 is known to modulate tumour metastasis through EGFR-Akt-Snail pathway in gastric cancer [16] and pancreatic cancer [80], and immune evasion in breast cancer [18, 83]. Several studies have also shown the association between miR-126 and regulation of VEGF/EGFL7 in several metastatic cancers, thus revealing exciting molecular insights of tumourigenesis and metastasis in these cancers. However, it is important to note that miR-126 appears to constitute as an oncogene or tumour suppressor, depending on the type of tumours. While pre-clinical studies evaluating the therapeutic efficacy of EGFL7-targeted cancer treatment appear to be promising [27], a clinical trial where anti-EGFL7 was combined with conventional chemotherapeutic agents yielded no significant clinical benefits [66]. It is possible that the clinical response of EGFL7-targeted therapy is tumour-specific and that the maximum benefits will be observed in a certain group of patients, whilst conferring no favourable therapeutic outcome for others. Therefore, the design and implementation of future clinical experiments involving EGFL7 as a treatment target may require dosage optimization and pharmacodynamic biomarker development.

Overall, the emerging role of EGFL7 is of great interest and significance, especially in the context of skeletal and cancer biology. Thus, understanding the molecular mechanisms and the pathological role of EGFL7 is essential to harness the therapeutic potential, improve prognosis and provide better treatment outcomes for patients.

**Disclosure**

All authors state that they have no conflicts of interest.

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**Figure Legends**

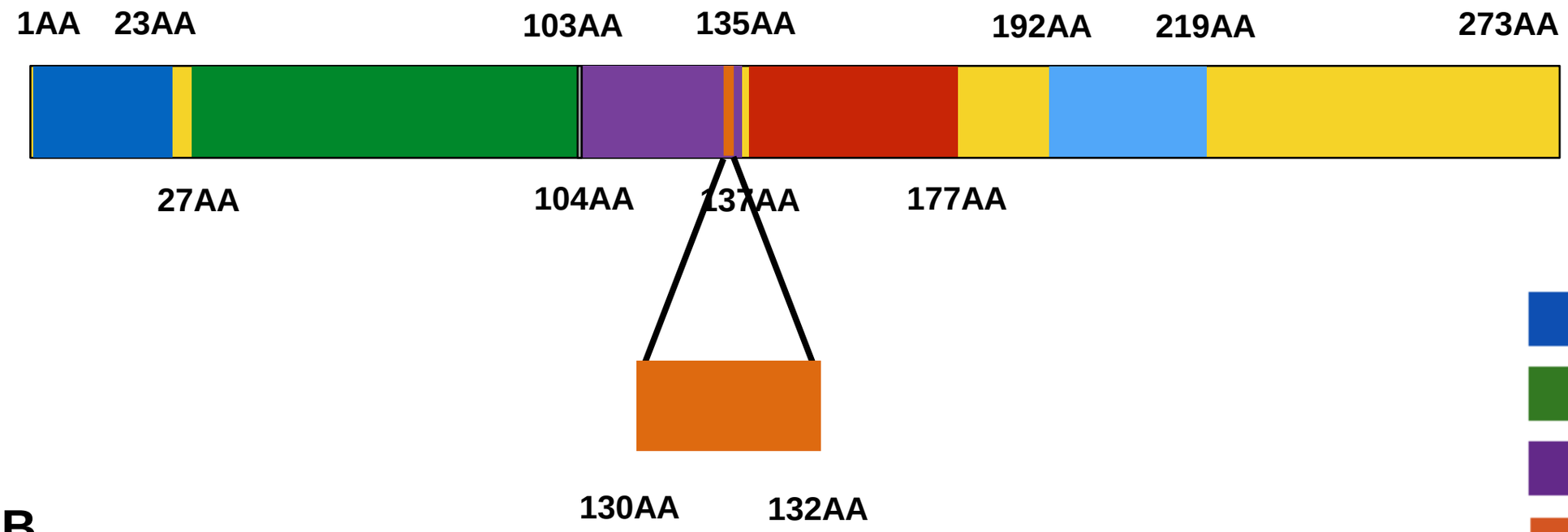
**Figure 1. The topology and predicted 3D structures of human EGFL7 protein.** (A) Distinct domains and structural motifs are emphasized by different colours. (B) Three different views highlighting various functional domains and structural motifs of EGFL7. Tertiary structure predictions were initially calculated by I-TASSER (UMICH), following by the refinement of DeepView Swiss-Pdb Viewer v4.1 (Swiss Institute of Bioinformatics) and the visualisation conducted via Protean 3D software 13.0.0 (DNASTAR).

**Figure 2. Sequence alignment of predicted EGFL7 proteins from human, mouse and rat.** Highlighted regions showing structurally conserved domains, including signal peptide, a cysteine-rich EMI domain and two centrally located EGF repeat domains.

**Figure 3. Predicted gene expression profile of human *Egfl7***. Prediction of expression in different tissues was performed using BioGPS program (http://biogps.org/).

**Figure 4. The signalling pathways of EGFL7 in angiogenesis and cancer metastasis.** EGFL7 binds to EGFR and integrin on cell membrane, which results in FAK-mediated activation of MAPK/ERK, PI3K/Akt and JAK/STAT3 signalling pathways, leading to the transcription of key genes that are crucial in regulating cancer metastasis and angiogenesis. In addition, EGFL7 acts as an antagonist of NOTCH receptor or DLL4 ligand, preventing the proteolytic cleavage and translocation of the Notch intracellular domain (NCID) into the nucleus. This results in the suppression of CSL transcription factor-mediated Notch target gene activation, leading to the modulation of angiogenesis.

A

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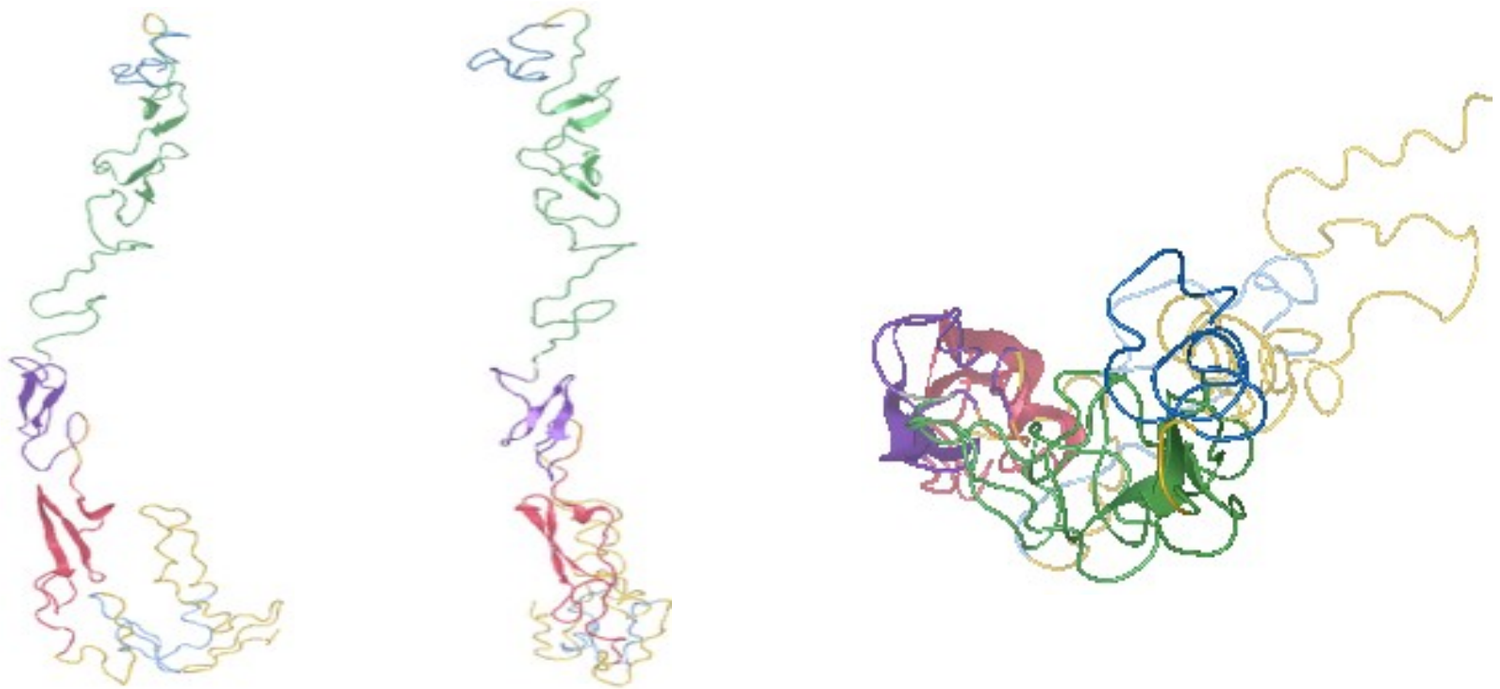

Anterior View  Lateral View  Superior View

Figure 1

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EGFL7_MOUSE  MWGSGELLVAWFLVLAADGTTEHVYRPSRRVCTVGISGGSISETFVQRVYQPYLTTCDGH  60
EGFL7_RAT    MWGSGELLVAWFLVLAAGGTTEHVYRPSRRVCTVGVSGGSISETFVQRVYQPYLTTCDGH  60
             * **  *:*:  *:****..* ***.***.*****:*    *.  :**:*********:*******

EGFL7_HUMAN  RACSTYRTIYRTA----YRRSPGLAPARPRYACCPGWKRTSGLPGACGAAICQPPCRNGG  115
EGFL7_MOUSE  RACSTYRTIYRTA----YRRSPGVTPARPRYACCPGWKRTSGLPGACGAAICQPPCGNGG  116
EGFL7_RAT    RACSTYRTIYRTAYRIAYRHSPGLTPSRPRYACCPGWKRTNGLPGACGAAICQPPCGNEG  120
             **************     **:***::*:***************.**************** * *

EGFL7_HUMAN  SCVQPGRCRCPAGWRGDTCQSDVDECSARRGGCPQRCVNTAGSYWCQCWEGHSLSADGTL  175
EGFL7_MOUSE  SCIRPGHCRCPVGWQGDTCQTDVDECSTGEASCPQRCVNTVGSYWCQGWEGQSPSADGTR  176
EGFL7_RAT    SCIRPGRCRCPVGWQGDTCQIDVDECSTGEARCPQRCVNTVGSYWCQCWEGQSPSADGVL  180
             **::**:****.**:***** ******: .. ********.****** ***:* ****.

EGFL7_HUMAN  CVPKGGPPRVAPNPT-GVDSAMKEEVQRLQSRVDLLEEKLQLVLAPLHSLASQALEHGLP  234
EGFL7_MOUSE  CLSKEGPSPVAPNPTAGVDSMAREEVYRLQARVDVLEQKLQLVLAPLHSLASRSTEHGLQ  236
EGFL7_RAT    CLPKEGPSPVAPSPTPGVDSVVREEVYKLQARVDVLEQKLQLVLAPLHSLASRSPEHGLQ  240
             *: * **  ***.** ****  :*** :**:***:**:****************:: ****

EGFL7_HUMAN  DPGSLLVHSFQQLGRIDSLSEQISFLEEQLGSCSCKKDS  273
EGFL7_MOUSE  DPGSLLAHSFQQLDRIDSLSEQVSFLEEHLGSCSCKKDL  275
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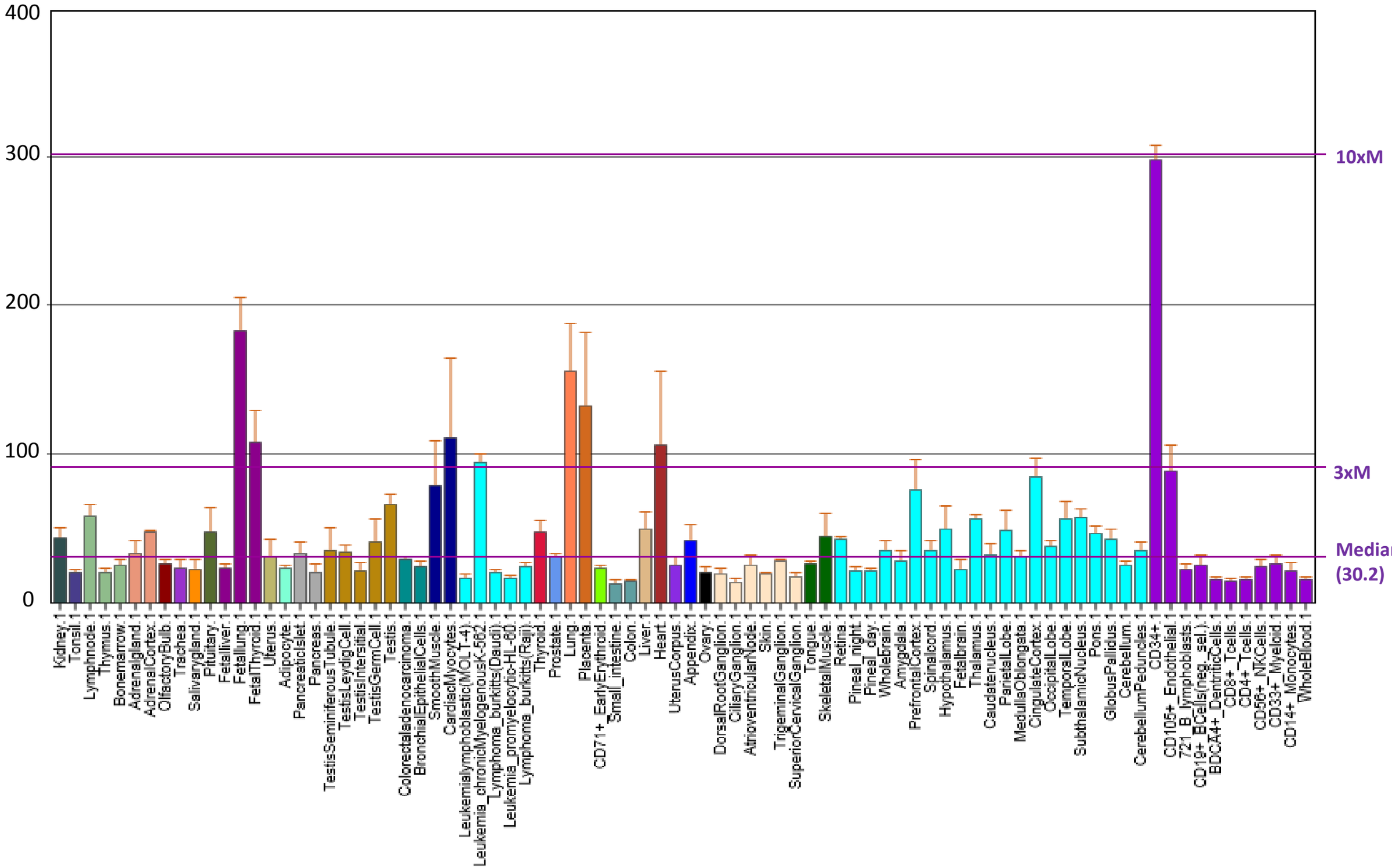
**Figure 2**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300
200
100
0
10xM
3xM
Median
(30.2)
Kidney.1
Tonsil.1
Lymphnode.1
Thymus.1
Bonemarrow.1
AdrenalGland.1
AdrenalCortex.1
OlfactoryBulb.1
Trachea.1
SalivaryGland.1
Pituitary.1
FetalLiver.1
FetalLung.1
FetalThyroid.1
Uterus.1
Adipocyte.1
PancreaticIslet.1
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TestisLeydigCell.1
TestisInterstitial.1
TestisGermCell.1
Testis.1
ColorectalAdenocarcinoma.1
BronchialEpithelialCells.1
SmoothMuscle.1
CardiacMyocytes.1
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LeukemiaChronicMyelogenousK-562.1
Lymphoma_burkitts(Daudi).1
Leukemia_promyelocytic-HL-60.1
Lymphoma_burkitts(Raji).1
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Prostate.1
Lung.1
Placenta.1
CD71+EarlyErythroid.1
Small_intestine.1
Colon.1
Liver.1
Heart.1
UterusCorpus.1
Appendix.1
Ovary.1
DorsalRootGanglion.1
CiliaryGanglion.1
AtrioventricularNode.1
Skin.1
TrigeminalGanglion.1
SuperiorCervicalGanglion.1
Tongue.1
SkeletalMuscle.1
Retina.1
Pineal_night.1
Pineal_day.1
WholeBrain.1
Amygdala.1
PrefrontalCortex.1
Spinalcord.1
Hypothalamus.1
FetalBrain.1
Thalamus.1
CaudateNucleus.1
ParietalLobe.1
MedullaOblongata.1
CingulateCortex.1
OccipitalLobe.1
TemporalLobe.1
SubthalamicNucleus.1
Pons.1
GlobusPallidus.1
Cerebellum.1
CerebellumPeduncles.1
CD34+.1
CD105+_Endothelial.1
721_B_lymphoblasts.1
CD19+_BCells(neg._sel.).1
BDCA4+_DentriticCells.1
CD8+_Tcells.1
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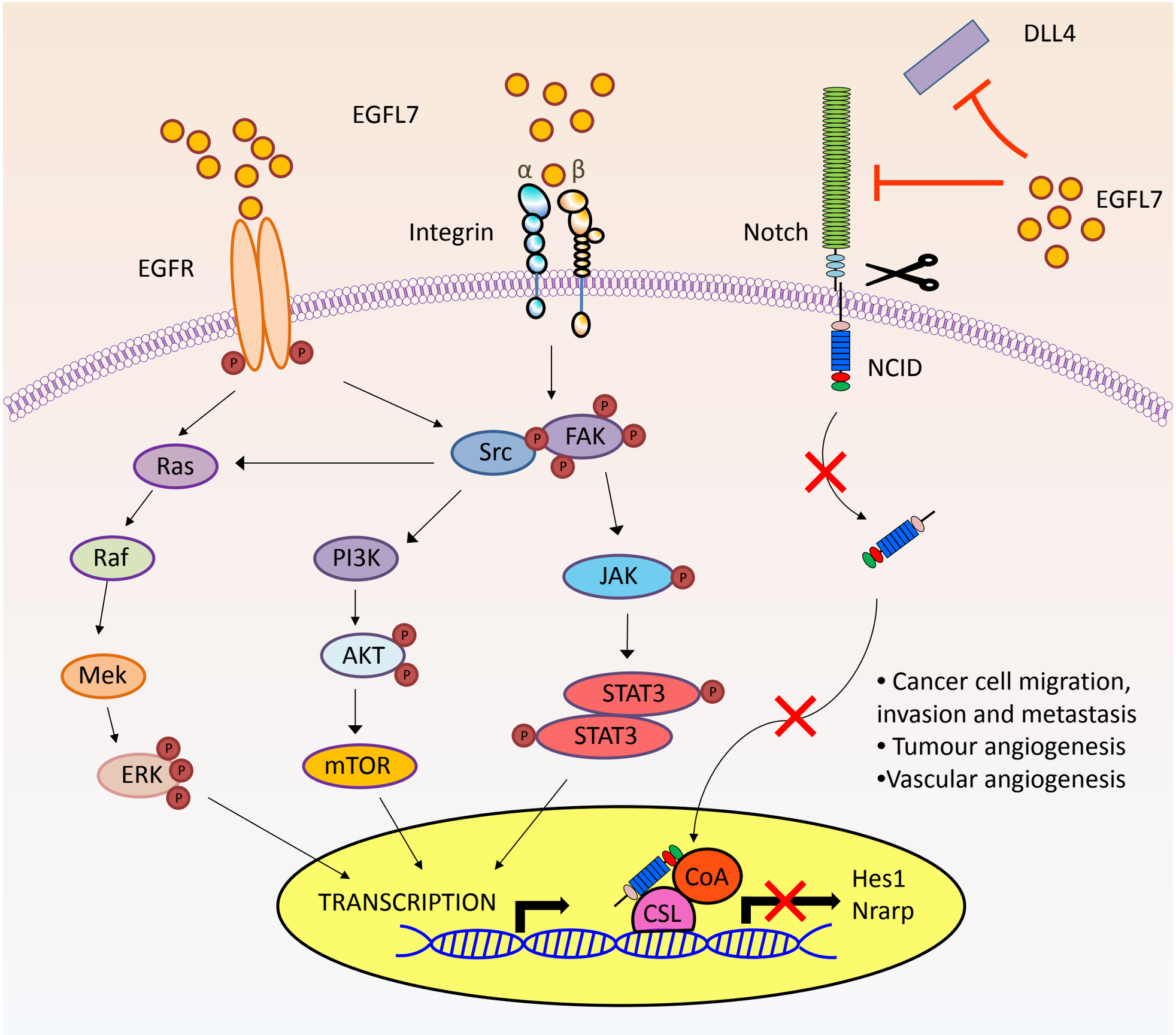
CD14+_Monocytes.1
WholeBlood.1

**Figure 3**


DLL4
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EGFL7
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β
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Notch
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P
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Src
FAK
Raf
PI3K
JAK
Mek
AKT
STAT3
ERK
mTOR
• Cancer cell migration, invasion and metastasis
• Tumour angiogenesis
•Vascular angiogenesis
TRANSCRIPTION
CoA
CSL
Hes1
Nrarp

**Figure 4**