CLINICAL CANCER RESEARCH | TRANSLATIONAL CANCER MECHANISMS AND THERAPY

EGFL7 Antagonizes NOTCH Signaling and Represents a Novel Therapeutic Target in Acute Myeloid Leukemia

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ABSTRACT

Purpose: EGF-like domain 7 (EGFL7) is a secreted protein and recently has been shown to play an important role in acute myeloid leukemia (AML); however, the underlying mechanism by which EGFL7 promotes leukemogenesis is largely unknown.

Experimental Design: Using an antibody interaction array, we measured the ability of EGFL7 to bind directly approximately 400 proteins expressed by primary AML blasts. Primary patient samples were stimulated *in vitro* with recombinant EGFL7 (rEGFL7) or anti-EGFL7 blocking antibody to assess alterations in downstream signaling and the ability to effect blast differentiation and survival. We treated three independent AML models with anti-EGFL7 or IgG1 control to determine whether anti-EGFL7 could prolong survival *in vivo*.

Results: We found EGFL7 significantly binds several signaling proteins important for normal and malignant hematopoiesis including NOTCH. Stimulation of AML blasts with rEGFL7 reduced NOTCH intracellular domain and NOTCH target gene expression while treatment with an anti-EGFL7 blocking antibody resulted in reactivation of NOTCH signaling, increased differentiation, and apoptosis. Competitive ligand-binding assays showed rEGFL7 inhibits DELTA-like (DLL) 4-mediated NOTCH activation while anti-EGFL7 combined with DLL4 significantly increased NOTCH activation and induced apoptosis. Using three different AML mouse models, we demonstrated that *in vivo* treatment with anti-EGFL7 alone results in increased survival.

Conclusions: Our data demonstrate that EGFL7 contributes to NOTCH silencing in AML by antagonizing canonical NOTCH ligand binding. Reactivation of NOTCH signaling *in vivo* using anti-EGFL7 results in prolonged survival of leukemic mice, supporting the use of EGFL7 as a novel therapeutic target in AML.

Introduction

EGF-like domain 7 (EGFL7) is a secreted protein and plays an important role in angiogenesis by regulating the growth, proliferation, and migration of endothelial cells (1–3). In solid tumors, it has been shown that *EGFL7* is overexpressed and associates with a more aggressive disease (4). We previously found that *EGFL7* is upregulated in primary acute myeloid leukemia (AML) blasts and that high *EGFL7* mRNA expression correlates with shorter event-free and overall survival in patients with AML. Moreover, we demonstrated that AML blasts are able to secrete EGFL7 protein and treatment of primary AML blasts with rEGFL7 *in vitro* leads to increases in leukemic blast cell growth (5). These data suggest an important clinical and biological role for EGFL7 in AML; however, the molecular mechanisms involving EGFL7-mediated leukemogenesis have not been thoroughly examined. EGFL7 has been shown to bind NOTCH receptors and can activate or antagonize canonical NOTCH signaling (2, 6, 7). Whether EGFL7 represses or activates NOTCH signaling in AML has not been determined.

NOTCH signaling is an evolutionarily conserved pathway and plays an important role in regulating cell-fate determination during development and maintenance of adult tissue homeostasis (8, 9). The NOTCH receptor is a single transmembrane protein composed of a functional extracellular (EC), transmembrane (TM), and intracellular (IC) domain. Signaling through NOTCH requires the activation of these receptors by binding canonical NOTCH ligands such as JAG1, JAG2, and DLL 1,3,4, resulting in intracellular cleavage of NOTCH protein to the truncated form (NICD), which then translocates to the nucleus to activate gene transcription (9–11). NOTCH signaling has been shown to be important for normal hematopoiesis and stem cell function (8, 12–15). Mutations in NOTCH receptors can result in NOTCH signaling hyperactivation, a well-known driver of leukemogenesis for a subset of T-cell acute leukemia (8). However, NOTCH signaling has been shown to have the opposite role in AML (16, 17). Several groups have shown that NOTCH signaling is silenced in AML and that reactivation of NOTCH signaling results in blast differentiation and disease elimination (16, 17). However, the mechanism underlying this NOTCH inactivation in AML has not been determined (16–18).

Here, we found that EGFL7 is able to bind many different signaling proteins important for regulating hematopoiesis including NOTCH. We confirmed these finding using coimmunoprecipitation assays and found that EGFL7 binds NOTCH receptors on primary AML blasts and cell lines. Stimulation of blasts with rEGFL7 protein results in decreases in the levels of NICD and NOTCH target genes. Conversely, blasts treated with an anti-EGFL7 blocking antibody showed reactivation of NOTCH activity resulting in increases in expression of

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Translational Relevance

Our previous work showed that EGFL7 is overexpressed in AML and associated with poor outcome. Here, we further dissect the possible mechanisms underlying EGFL7 leukemogenesis. We found that binding of EGFL7 to NOTCH receptors antagonizes canonical NOTCH ligand binding, resulting in down regulation of NOTCH target genes. Although NOTCH receptors are expressed at significant levels in AML blasts, NOTCH signaling is silenced. Reactivation of NOTCH signaling results in blast differentiation and apoptosis. More importantly, inhibiting EGFL7 using an anti-EGFL7 blocking antibody (parsatuzumab) results in reactivation of the NOTCH pathway resulting in blast differentiation, apoptosis, and prolonged survival in three independent mouse model. Because parsatuzumab has already been shown to be safe in phase I and II clinical trials for solid tumors, it has the potential to be used alone or in combination with current FDA-approved drugs for AML.

downstream target genes, NICD levels, and apoptosis. Primary blasts treated with a γ-secretase inhibitor blocked anti-EGFL7–induced NOTCH activation, substantiating our findings that NOTCH signaling is downstream of EGFL7 activity in AML. We also found the NOTCH ligands DLL1 and DLL4-mediated activation of NOTCH signaling can be inhibited by rEGFL7, while anti-EGFL7 treatment enhanced the activation of NOTCH by these ligands. Overall, we demonstrate that EGFL7 silences NOTCH signaling in AML by antagonizing canonical NOTCH ligand binding and that anti-EGFL7 therapy represents a rational, novel targeted therapy with low toxicity (19). Although we show biological activity as a single agent, we are also currently investigating the use of anti-EGFL7 therapy in combination with other currently approved drugs for AML in hope of a more effective, low toxic cure for patients with AML.

Materials and Methods

Reagents and cells

Recombinant human DLL4 (Fc chimera, ab108557) and natural human IgG Fc fragment proteins (ab90285) were obtained from Abcam. Anti-human EGFL7 therapeutic antibody (parsatumumab) was obtained from Creative Biolabs. Normal human immunoglobulin (Immune Globulin, 3036917) was purchased from Grifols. γ-secretase inhibitor [Avagacestat (AVA), BMS-708163] was obtained from Selleckchem. OP9 and OP9-DLL1 mouse stromal cells were cultured in MEMα/GlutaMAX medium (Gibco, Thermo Fisher Scientific) supplemented with 20% FBS.

Primary AML blasts and AML cell lines

THP-1, K-562, and Kasumi-1 cells were newly purchased from ATCC. EOL-1 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Thawed cells were passaged not more than three times before the experiments were performed. AML cell lines were cultured in RPMI1640 (Gibco) supplemented with 10%–20% FBS (Gibco). AML blasts used in the experiments were obtained from apheresis blood samples collected from patients treated at The Ohio State University (OSU, Columbus, OH) and stored in the OSU Leukemia Tissue Bank. Patients provided written informed consent, and protocols were in accordance with the Declaration of Helsinki and approved by the Institutional Review Boards. Primary blasts from patients with AML were maintained in SFEM (StemCell Technologies) medium supplemented with 10% FBS and 1× StemSpan CC100 cytokine cocktail (StemCell Technologies) unless otherwise noted.

AML mouse models

All studies using animals were carried out in accordance with the OSU institutional guidelines for animal care and under protocols approved by the OSU Institutional Animal Care and Use Committee. All mouse experiments were unblinded. All mice used in the experiments were between 8 and 10 weeks of age.

EOL-1 mouse model

Female NOD-*scid* IL2Rg^{null} (NSG) mice were transplanted with 1×10^7 EOL-1 cells. Two weeks posttransplant, mice were treated with anti-EGFL7 or IgG1 control (four mice per group; dosage 50 mg/kg body weight intraperitoneal), three times per week. Mice were monitored closely for clinical signs of leukemia such as weight loss and hind limb paralysis.

Murine AML model

We used our previously established *Mll*$^{PTD/WT}$;*Flt3*$^{ITD/WT}$ dKI mouse model that is maintained on a pure C57Bl/6J background (5). In the primary transplant, lethally irradiated (10 Gy) BoyJ (C57Bl/6J-CD45.1) were intravenously injected through the tail vein with 2.0×10^6 leukemic *Mll*$^{PTD/WT}$;*Flt3*$^{ITD/WT}$ BM cells along with 5.0×10^5 BM cells from a normal BoyJ (C57Bl/6J-CD45.1) control mouse. Two weeks posttransplantation, mice were treated with anti-EGFL7 or IgG1 control (eight mice per group; dosage 50 mg/kg body weight i.p.), two times per week. Mice were monitored closely for clinical signs of leukemia such as weight loss and hind limb paralysis. Two mice (one per group) were excluded from the study because they were identified as statistical outliers using the "interquartile range" method.

Patient-derived xenograft mouse model

Female NOD-*scid* IL2Rg^{null} (NSG) mice were conditioned with busulfan (30 mg/kg body weight, i.p.). After 24 hours, the mice were transplanted with 2×10^6 AML cells from cytogenetically normal AML patient 5. Four weeks posttransplant, mice were treated with anti-EGFL7 or IgG1 control (four mice per group; dosage 50 mg/kg body weight i.p.), two times per week. Mice were monitored closely for clinical signs of leukemia such as weight loss and hind limb paralysis. The experiment was stopped after 12 weeks of treatment.

RNA expression analysis

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using SuperScript III reagents (Invitrogen) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using commercially available TaqMan Gene Expression Assay primers and the 7900HT Fast Real-Time PCR System (Applied Biosystems, Invitrogen). The expression levels of target genes were normalized to *β-ACTIN*. Relative expression was calculated by using the comparative $2^{-\Delta\Delta C_t}$ method.

Detection of EGFL7-interacting proteins by antibody array

Potential EGFL7-interacting proteins were probed with Signal Transduction Antibody Array containing antibodies against 400 proteins from Hypomatrix (catalog No. HM3000) according to the manufacturer's protocol (see also Supplementary Materials). In brief, primary patients' cells ($5–10 \times 10^6$) were lysed in 500 μL of Pierce Co-

Immunoprecipitation lysis buffer supplemented with protease and phosphatase inhibitors (Roche). Cell lysis was performed on ice for 20 minutes and the cell lysate was sonicated for 5 seconds three times. After centrifugation at 14,000 × g at 4°C for 15 minutes, the cell lysate supernatant was diluted to 3 mL in lysis buffer containing 0.1% dry milk and 10 nmol/L recombinant human EGFL7 protein (PeproTech), added to the antibody array membrane previously blocked with 5% dry milk in tris-buffered saline with 0.05% Tween 20 (TBST) buffer for 2 hours, and incubated at 4°C for 2 hours on a flat shaker with slow shaking. Following washing five times with TBST buffer, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-EGFL7 antibody (Bioss) at room temperature for 2 hours and developed using ECL Select Western Blotting Detection Reagent (GE Healthcare).

Protein coimmunoprecipitation

Cells (1–5 × 10^6) were lysed in 400 μL of Pierce Co-Immunoprecipitation lysis buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche). Cell lysis was performed on ice for 20 minutes and the cell lysate was sonicated for 5 seconds three times. After centrifugation at 14,000 × g at 4°C for 15 minutes, the cell lysate supernatant was transferred to new tubes immediately and added to 50% Protein G Plus/Protein A agarose beads (EMD Millipore) with a ratio of 100 μL for a 1-mL sample solution. After incubation with rotation at 4°C for 1 hour, the mixture was centrifuged at 14,000 × g for 10 minutes at 4°C. The supernatant was transferred to new tubes, 2–20 μg antibody was added and incubated on a rotating shaker at 4°C overnight. The anti-NOTCH1 [(D1E11) XP Rabbit mAb No. 3608] and anti-NOTCH2 [(D76A6) XP Rabbit mAb No. 5732] antibody, were obtained from Cell Signaling Technology. Both antibodies recognize the full length and NICD of NOTCH1 and NOTCH2, respectively, according to manufacturers' product descriptions. The anti-MYC (71D10; Rabbit mAb No. 2278) and anti-FLAG (DYKDDDDK; (D6W5B) Rabbit mAb No. 2278) antibodies were obtained from Cell Signaling Technology. After centrifugation, the supernatant was incubated with 50-μL Protein G Plus/Protein A agarose beads at 4°C with rotation for 2 hours. The agarose beads were washed five times with ice-cold Pierce Co-Immunoprecipitation (co-IP) lysis buffer. Then, 20-μL 2× Laemmli sample buffer was added, boiled for 5 minutes, and subjected to Western blotting. The corresponding normal antibodies were applied as negative controls for co-IP.

Western blot analysis

Cells were lysed in 100 μL of Pierce RIPA buffer (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors (Roche) on ice for 20 minutes. After centrifuging at 14,000 × g for 15 minutes at 4°C, the supernatant was transferred into a new tube, mixed with 20 μL of 5× Laemmli sample buffer containing 5% β-mercaptoethanol and heated at 95°C for 5 minutes. Equal volume of samples (10–30 μL) were separated in precast SDS gel (Bio-Rad) and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was blocked by freshly prepared 5% dry milk made in TBST buffer (Bio-Rad) at room temperature for 1 hour and then incubated overnight at 4°C in a primary antibody. It was then washed three times with TBST buffer and incubated with secondary HRP-conjugated antibody at room temperature for 1 hour. Immunoreactive bands were detected using ECL Select Western Blotting Detection Reagent (GE Healthcare). Mouse anti-EGFL7 (LS-C153302) and rat anti-EGFL7 (LS-C40134) antibodies were purchased from LifeSpan BioSciences; goat anti-EGFL7 (sc-34116) and rabbit anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology; rabbit anti-NOTCH1 [(D1E11) XP Rabbit mAb No. 3608)], NOTCH2 [(D76A6) XP Rabbit mAb No. 5732], NICD [(D3B8) Rabbit mAb No. 4147], β-TUBULIN [(9F3) Rabbit mAb No. 2128], β-ACTIN [(13E5) Rabbit mAb No. 4970], and HES1 [(D6P2U) XP Rabbit mAb No. 11988] antibodies were obtained from Cell Signaling Technology. The NICD antibody detects only the NICD after cleavage of NOTCH1 and NOTCH2 proteins. HRP-conjugated sheep anti-mouse, goat anti-rat, donkey anti-rabbit, and donkey anti-goat secondary antibodies were bought from GE Healthcare.

Cell transfection

Transfection of K562 cells was conducted by Lonza transfection kit V following (Lonza Group Ltd) manufacturer's procedure. Briefly, 2.5 μg of an EGFL7 (FALG-DDK-tagged) pCMV3-SP-N-FLAG vector (HG11979-NF) from Sinobiological and 2.5 μg of a NOTCH2 (Myc-DDK-tagged) pCMV6-Entry vector (RC215107) from OriGene were cotransfected. Cells were cultured for 48 hours before co-IP was performed.

Cell apoptosis and differentiation

For the evaluation of apoptosis, cells were stained with Annexin V and 7-AAD as described previously (5, 20). For cell differentiation analysis, cells were stained with an anti-CD11B or anti-CD14 antibody (BD Biosciences) in the presence of DAPI and analyzed by flow cytometry. Only DAPI-negative (viable) cells were included in the differentiation analyses.

Statistical analysis

Two-tailed Student *t* tests were performed using GraphPad Prism version 7 (GraphPad Software) to analyze *in vitro* and *in vivo* experiments unless otherwise noted. *P* values < 0.05 were considered significant. Overall survival for the mouse AML xenograft model was calculated using the Kaplan–Meier method, and survival curves were compared by the log-rank test.

Results

EGFL7 interacts with NOTCH receptors in AML cells

Our previous studies demonstrate that *EGFL7* is upregulated in primary AML blasts and that high *EGFL7* mRNA expression correlates with poor outcome in patients with AML (5). However, the mechanism by which EGFL7 contributes to leukemogenesis is unknown. To obtain insights about potential mechanisms by which EGLF7 stimulates AML blast growth, we identified potential EGFL7-interacting proteins. Because other members of the EGFL family of proteins are known to have numerous potential binding partners (2, 21–23), we took an unbiased high-throughput approach by performing antibody interaction arrays. These arrays have 400 antibodies hybridized to a membrane. Protein lysates from AML blasts were first incubated with rEGFL7 protein, followed by incubation with the antibody array membranes. Then using an anti-EGFL7-HRP or anti-GST-HRP control antibody (**Fig. 1A**), the membranes were developed and densitometry of the individual spots were determined for both the anti-EGFL7 and anti-GST-HRP control blots. Array data were then $\log_2$ transformed and normalized by subtracting the mean of the background. Subsequently, a linear mixed model was used for analysis to compare the protein level between anti-EGFL7 antibody array and anti-GST antibody array. Using this strategy, we found

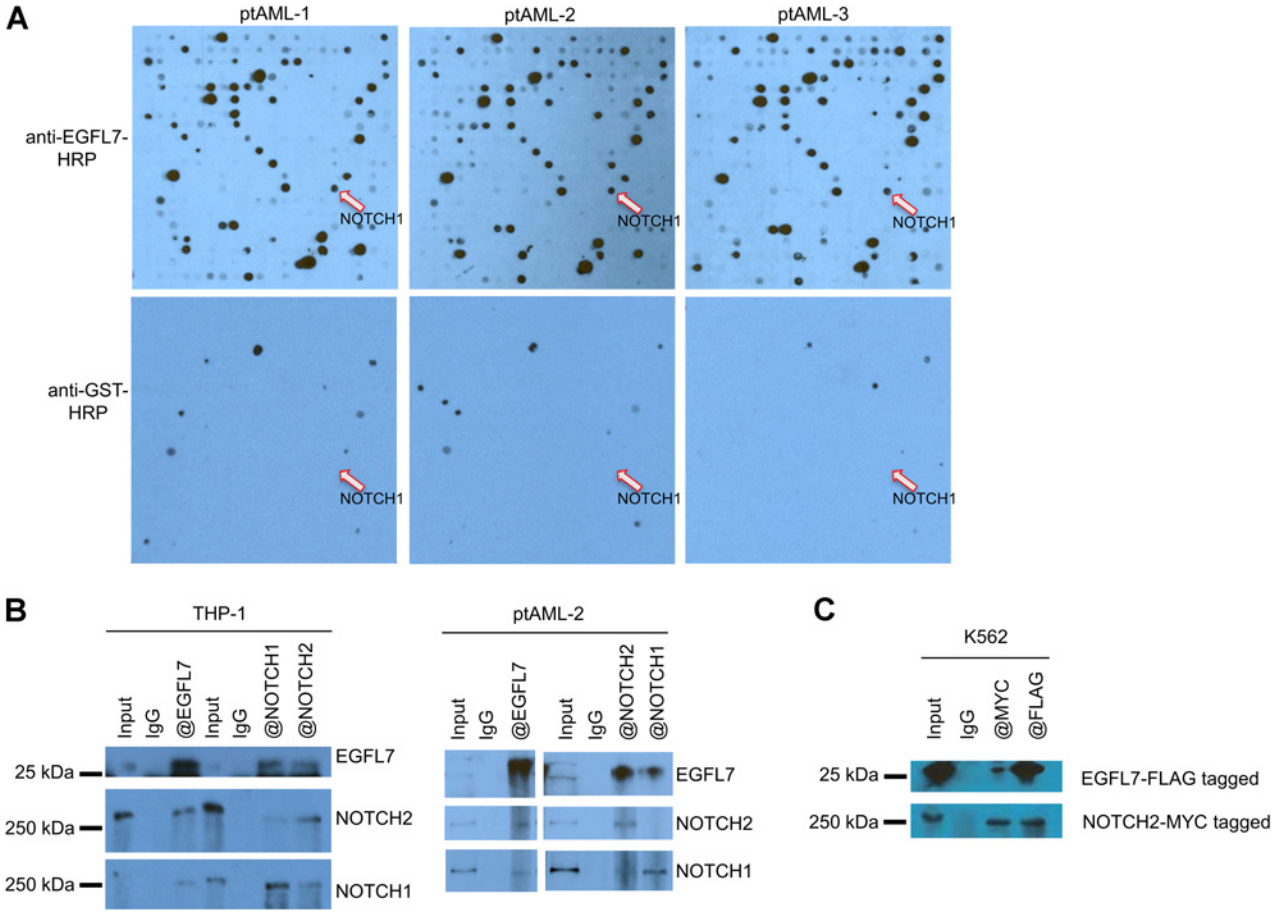

Figure 1.

A, Cell lysate of 5×10^6 primary AML blasts (ptAML; $n = 3$) was incubated with an antibody array containing antibodies against 400 signal molecules, followed by sequential incubation with 10 nmol/L human rEGFL7, HRP-conjugated anti-EGFL7 antibody (@EGFL7) or HRP-conjugated anti-GST antibody (control). The arrows show the identified NOTCH1 in the array membranes detected by HRP-conjugated anti-EGFL7 antibody but not HRP-conjugated anti-GST antibody. **B,** Cell lysate of 10×10^6 THP-1 cells or 5×10^6 primary AML blasts were subjected to co-IP with Protein G Plus/Protein A agarose beads. The eluate was analyzed by immunoblotting of EGFL7, NOTCH1, and NOTCH2 with the corresponding antibodies. **C,** Cell lysate of 1×10^7 K562 cells that were transfected with a MYC-tagged NOTCH2 and FLAG-tagged EGFL7 expression vectors, were subjected to co-IP with Protein G Plus/Protein A agarose beads. The eluate was analyzed by immunoblotting of EGFL7 and NOTCH2 with the corresponding antibodies.

that NOTCH1 was bound significantly to EGFL7 in all three samples ($P = 1.11 \times 10^{-06}$).

Next, we validated our screening results by assessing direct binding between EGFL7 and NOTCH receptors in both an AML cell line (THP-1) and primary AML blasts. Using the THP-1 (Supplementary Fig. S1) cell line that expresses EGFL7 and NOTCH receptors but exhibits dampened NOTCH activation (16, 17), we performed co-IP assays using an anti-EGFL7 antibody to pull down EGFL7-containing protein complexes. We then performed Western blotting to determine protein interactions using anti-NOTCH1 and 2 antibodies. Conversely, when we performed the pull-down using anti-NOTCH1 or NOTCH2 antibody, we found that both NOTCH1 and NOTCH2 could be coimmunoprecipitated as part of a complex with EGFL7 (**Fig. 1B**). These results were also confirmed using primary AML patient blasts (**Fig. 1B**). We further validated our results by transfecting K562 cells with EGFL7-FLAG- and NOTCH2-MYC–tagged expression plasmids. Co-IP experiments were then performed using a MYC-specific antibody to pull down NOTCH2, and subsequent Western blotting using an EGFL7-specific antibody. These experiments validate the interaction between the NOTCH (MYC-tagged) and EGFL7 protein. Concomitantly, using a FLAG antibody to pull down EGFL7, we found NOTCH2 to be coimmunoprecipitated with EGFL7 (**Fig. 1C**). Altogether these data demonstrate the direct interaction of EGFL7 with NOTCH receptors on AML cell lines and primary AML blasts.

EGFL7 suppresses NOTCH signaling in AML cells

Previous studies suggest that the interaction of EGFL7 with NOTCH receptors may lead to inhibition of NOTCH signaling (6, 24, 25). To determine whether EGFL7 binding of NOTCH results in alterations of NOTCH signaling in AML patient blasts, we examined the levels of the NICD produced in response to rEGFL7 stimulation of primary AML patient samples. We found that although the NICD1 antibody from Cell Signaling Technology (D3B8) was specific for the cleaved/activated form of NOTCH (i.e., NICD), we found that it was able to recognize both NICD from NOTCH1 and NOTCH2 (Supplementary Fig. S2). Because AML blasts express both

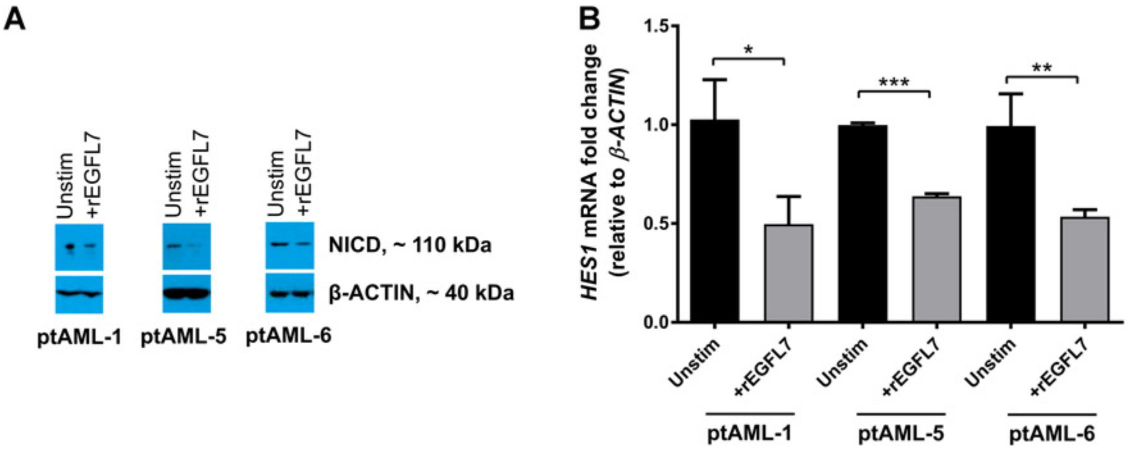

Figure 2.

A, Primary blasts (ptAML; $n = 3$) were cultured in serum-free expansion medium (SFEM) with 20% FBS and cytokines in the presence or absence (unstimulated control; Unstim) of 0.25 μmol/L rEGFL7 (+rEGFL7) for 4 hours. Total proteins were extracted for immunoblotting using an NICD-specific antibody with β-ACTIN as loading control. **B,** Total RNA was extracted for quantitative real-time (qRT-PCR) analysis of *HES1* mRNA normalized to *β-ACTIN* control ($n = 3$ individual AML patients after 24 hours of treatment; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; stimulated with +rEGFL7 vs. Unstim).

NOTCH1 and NOTCH2, we provide the endogenous *NOTCH1* and *NOTCH2* expression levels for each patient in Supplementary Figs. S3 and S4. Stimulation with rEGFL7 results in significant reductions in the levels of NICD in primary AML samples, indicating that EGFL7 inhibits NOTCH activation (**Fig. 2A**; Supplementary Fig. S5A). To test whether EGFL7-mediated downregulation of NOTCH activity results in decreased expression of NOTCH downstream target genes, we assessed one of the best characterized NOTCH target genes, *Hairy and Enhancer of Split-1* (*HES1;* refs. 13, 17). Primary AML patient samples stimulated with rEGFL7 for 24 hours results in decreased levels of *HES1* mRNA in primary AML blasts ($n = 3$, **Fig. 2B**).

Inhibition of EGFL7 results in NOTCH activation

Next, we examined whether NOTCH reactivation could be achieved by blocking EGFL7. We first measured activated NICD levels 4 hours after treatment with anti-EGFL7 and found significant increases in NICD levels in primary AML samples ($n = 3$, **Fig. 3A**; Supplementary Fig. S5B). We also found concomitant increases in the expression of *HES1* at 2 and 10 hours post anti-EGFL7 treatment (**Fig. 3B**). In

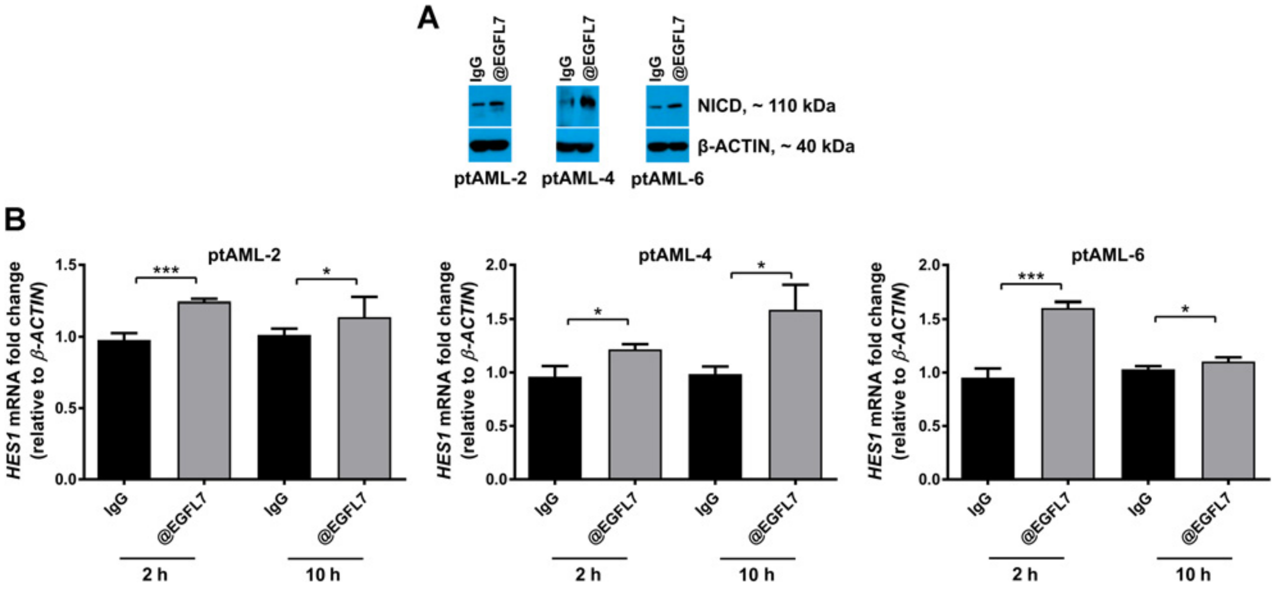

Figure 3.

A, Primary blasts (ptAML; $n = 3$) were cultured in presence of 100 μg/mL of normal IgG or 100 μg/mL anti-EGFL7 antibody (@EGFL7) for 4 hours. Total protein was extracted for immunoblotting of NICD with β-ACTIN as loading control. **B,** Primary blasts ($n = 3$) were cultured in SFEM medium with 100 μg/mL of normal IgG or anti-EGFL7 antibody for 2 hours and 10 hours. Total RNA was extracted for qRT-PCR analysis of *HES1* mRNA with *β-ACTIN* as internal control (*, $P < 0.05$; ***, $P < 0.001$ vs. IgG control).

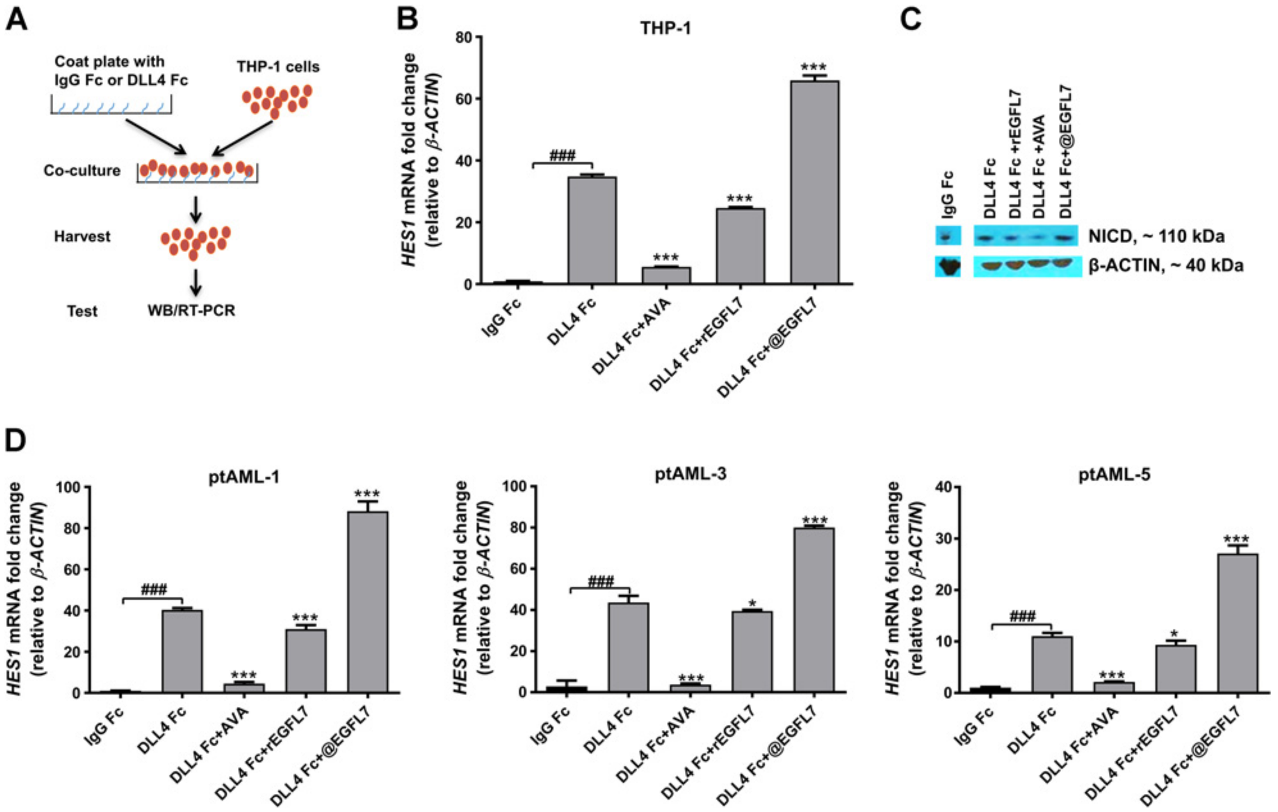

Figure 4.

A, Scheme of experiments using DELTA like (DLL) 4 Fc or IgG Fc control–coated plates to induce NOTCH activation. **B,** THP-1 cells were pre-treated with 10 μmol/L γ-secretase inhibitor [Avagacestat (AVA)], 250 μg/mL anti-EGFL7 antibody (@EGFL7) or 1.0 μmol/L rEGFL7 for 30 minutes, then transferred to IgG Fc or DLL4 Fc–coated plates. After 4 hours of incubation, total RNA from THP-1 cells was extracted for qRT-PCR analysis of *HES1* with *β-ACTIN* as internal control. ###, $P < 0.001$ IgG Fc versus DLL4 Fc; ***, $P < 0.001$ DLL4+AVA versus DLL4 Fc, DLL4+rEGFL7 versus DLL4 Fc, and DLL4+@EGFL7 versus DLL4 Fc. **C,** Total proteins of similar treated cells were extracted for immunoblotting of NICD with β-ACTIN as loading control. **D,** Primary AML blasts were pretreated with 10 μmol/L AVA, 100 μg/mL anti-EGFL7 antibody or 0.25 μmol/L rEGFL7 for 30 minutes, then transferred to IgG Fc or DLL4 Fc-coated plates. After 4 hours of incubation, total RNA was extracted for qRT-PCR analysis of *HES1* mRNA with *β-ACTIN* as internal control (###, $P < 0.001$ IgG Fc vs. DLL4 Fc; *, $P < 0.05$; ***, $P < 0.001$ DLL4+AVA vs. DLL4 Fc, DLL4+rEGFL7 vs. DLL4 Fc, and DLL4+anti-EGFL7 vs. DLL4 Fc).

addition, we found increases in two other well-known NOTCH targets, *HEY1* and *DTX1* mRNA (Supplementary Fig. S6). Altogether these data support the role of EGFL7 in blocking NOTCH activation in primary AML blasts.

EGFL7 prevents NOTCH ligand–induced NOTCH activation in AML cells

To determine whether EGFL7 inhibits NOTCH signaling in AML cells, we tested whether EGFL7 competes with canonical NOTCH ligands for receptor binding. To perform these experiments, we cultured THP-1 cells on DLL4-Fc and IgG-Fc (control)–coated plates to activate NOTCH signaling and then tested the effect of rEGFL7 and anti-EGFL7 on this DLL4-Fc–mediated NOTCH activation in THP-1 cells (**Fig. 4A**). We found that THP-1 cells cultured on DLL4-Fc–coated plates resulted in significant increases of NOTCH activation, assessed by *HES1* expression compared with cells cultured on IgG-Fc–coated control plates. Addition of γ-secretase inhibitor (AVA) significantly reduced DLL4-Fc–mediated NOTCH activation, while addition of an anti-EGFL7 blocking antibody enhanced DLL4-Fc–mediated NOTCH activation (**Fig. 4B** and **C**). Similar experiments were performed in primary AML blasts ($n = 3$). We found that DLL4-Fc pretreatment activated NOTCH and was enhanced by anti-EGFL7 antibody. Similar to the cell line, AVA treatment of primary AML patient blasts significantly prevented DLL4-Fc–mediated NOTCH activation. The treatment with rEGFL7 showed analogous results although the decrease was not as strong as chemical inhibition with AVA (**Fig. 4D**). To validate these results, we used another well-known NOTCH ligand DLL1 (see also Supplementary Materials). For these experiments, we performed co-culture of THP-1 cells with stromal cells (OP9) overexpressing membrane bound DLL1 (OP9-DLL1; ref. 26; Supplementary Fig. S7). We found that similar to DLL4-FC, THP-1 cells co-cultured with OP9-DLL1 also results in significant increases in NOTCH activation, assessed by *HES1* expression (Supplementary Fig. S7B, OP9 vs. OP9-DLL1, $P < 0.001$). Addition of rEGFL7 or AVA significantly reduced DLL1-mediated NOTCH activation (Supplementary Fig. S7B, OP9-DLL1 vs. OP9-DLL1 +rE7, $P < 0.01$). In addition, both rEGFL7 and AVA reduced NOTCH activation in THP-1 cells co-cultured with OP9 cells ($P < 0.01$ and $P < 0.05$, respectively).

Inhibition of EGFL7 results in increased blast cell apoptosis and differentiation

We have previously shown that anti-EGFL7 treatment of AML cells resulted in decreased blast growth and survival (5). To determine whether reactivation of NOTCH in AML cells leads to leukemic cell

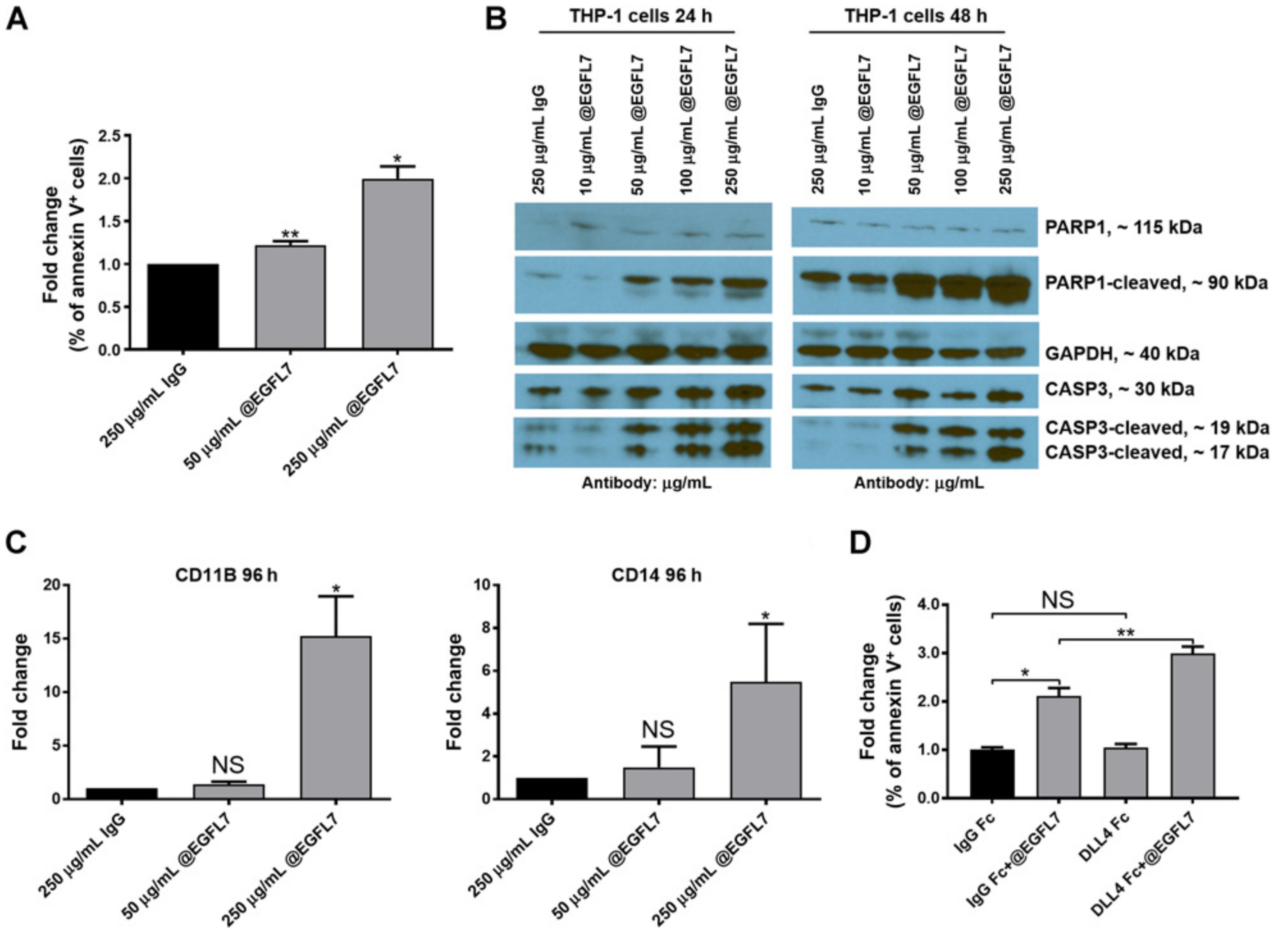

Figure 5.

A, THP-1 cells were treated at time 0 hours with the indicated concentration of anti-EGFL7 (@EGFL7). Cells were then assessed for apoptosis using AnnexinV$^+$ staining and flow cytometry at 48 hours posttreatment; *, $P < 0.05$; **, $P < 0.01$. **B,** Immunoblotting for full-length PARP1 (~115 kDa) and cleaved PARP1 (~90 kDa); full-length CASP3 (~30 kDa) and cleaved CASP3 (~17 and 19 kDa); GAPDH as loading control. **C,** Cell differentiation was assessed by CD11B and CD14 staining 96 hours post anti-EGFL7 treatment; *, $P < 0.05$; NS, not significant. **D,** To determine whether activation of NOTCH by DLL4 Fc could be accentuated by anti-EGFL7 treatment, we first treated THP-1 cells with anti-EGFL7 (100 µg/mL) and then cultured on DLL4 Fc or IgG Fc–coated plates and then assessed for apoptosis using AnnexinV$^+$ staining and flow cytometry (*, $P < 0.05$; **, $P < 0.01$, DLL4 Fc and anti-EGFL7; vs. DLL4 Fc +anti-EGFL7; NS, not significant).

differentiation and survival, we treated THP-1 cells with anti-EGFL7 and measured apoptosis and differentiation. We found an increase in apoptotic cells after anti-EGFL7 treatment (**Fig. 5A**; Supplementary Fig. S8A). These findings were supported by Western blots demonstrating an increase in PARP1 and CASP3 cleavage as a result of anti-EGFL7 treatment (**Fig. 5B**). To determine whether anti-EGFL7 also results in cell differentiation of THP-1 cells, we measured the levels of differentiation markers (CD11b and CD14) and found increases in both markers at 96-hours post anti-EGFL7 treatment (**Fig. 5C**; Supplementary Fig. S8B). To determine whether blocking EGFL7 and subsequent activation by DLL4 results in enhanced cell death of AML cells, we cultured THP-1 cells with anti-EGFL7 followed by NOTCH activation using DLL4-Fc or IgG-Fc control. We found significant increases in apoptosis in THP-1 cells treated with anti-EGFL7 + DLL4-Fc compared with either DLL4-Fc or anti-EGFL7 alone (**Fig. 5D**), suggesting that blocking EGFL7 may be a prerequisite for subsequent NOTCH activation in AML cells. In summary, these data demonstrate the ability of EGFL7 to block key NOTCH ligand binding and results in inhibition of canonical NOTCH signaling. Furthermore, EGFL7-mediated antagonism of NOTCH activation is reversible in AML, and can be reactivated by blocking EGFL7 with an mAb (Supplementary Fig. S9).

Anti-EGFL7 antibody activates Notch signaling *in vivo* and prolongs survival in AML mouse models

Because THP-1 xenografts do not develop a lethal leukemia, and both THP-1 and EOL-1 cells have significant levels of EGFL7 expression (Supplementary Fig. S1; ref. 5), we chose the EOL-1 xenograft AML model to test the feasibility of targeting EGFL7 *in vivo*. Briefly, 1×10^7 EOL-1 cells were transplanted into nonconditioned NSG recipient mice. Two weeks posttransplantation, mice were treated with 50 mg/kg body weight of anti-EGFL7 or IgG1 control antibody three times per week (**Fig. 6A**). We found that treatment with anti-EGFL7 prolonged survival compared with IgG1 controls ($P = 0.01$, **Fig. 6B**). Furthermore, we found *in vivo* anti-EGFL7 antibody treatment resulted in reactivation of NOTCH signaling as demonstrated by increased levels of HES1 protein via Western blots compared with IgG1 controls (**Fig. 6C**). To further strengthen these findings, we used

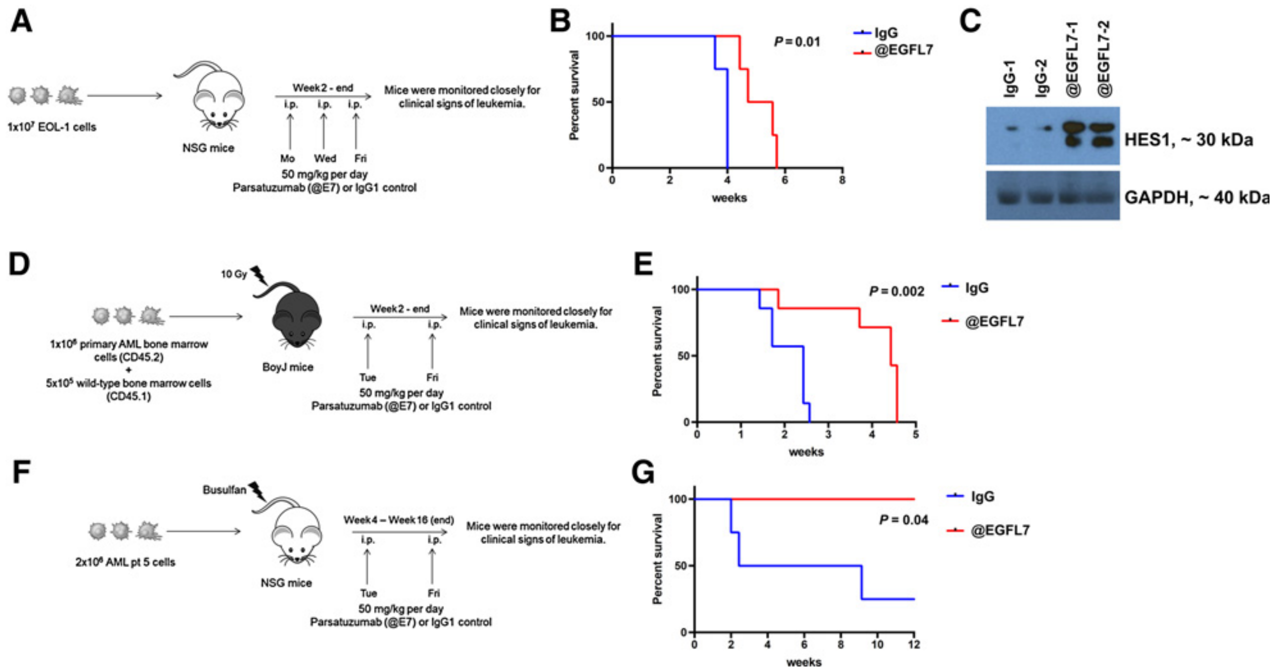

Figure 6.

A, Schematic representation of the experimental design using an EOL-1 xenograft AML mouse model. Briefly, 1 × 10^7 EOL-1 cells were transplanted into nonconditioned NSG recipient mice ($n = 4$ for each group). Two weeks posttransplantation, mice were treated with 50 mg/kg body weight of anti-EGFL7 or IgG1 control antibody 3 times per week. **B,** Treatment with anti-EGFL7 blocking antibody prolonged survival compared with IgG1 controls ($P = 0.01$). **C,** *In vivo* treatment using an anti-EGFL7 antibody treatment results in increased levels of HES1 protein via Western blots compared to IgG1 controls. **D,** Schematic representation of the experimental design using our *Mll*$^{\text{PTD/WT}}$ *Flt3*$^{\text{ITD/WT}}$ double knock-in mouse model. 1 × 10^6 primary AML bone marrow cells (CD45.2) together with 5 × 10^5 healthy wild-type bone marrow cells (CD45.1) were transplanted into lethally irradiated BoyJ (CD45.1) recipient mice ($n = 7$ for each group). Two weeks posttransplantation, mice were treated with 50 mg/kg body weight of anti-EGFL7 or IgG1 control antibody 2 times per week. **E,** Anti-EGFL7 treatment prolonged survival of *Mll*$^{\text{PTD/WT}}$ *Flt3*$^{\text{ITD/WT}}$ transplanted mice compared with IgG1 controls ($P = 0.002$). **F,** Schematic representation of the experimental design using a patient derived xenotransplant (PDX) mouse model. Briefly, 2 × 10^6 primary cells from a cytogenetically normal AML patient were transplanted into Busulfan-treated recipient mice ($n = 4$ for each group). Four weeks posttransplantation, mice were treated with 50 mg/kg body weight of anti-EGFL7 or IgG1 control antibody two times per week for 12 weeks. **G,** We found that treatment with anti-EGFL7 prolonged survival compared with IgG1 controls ($P = 0.04$).

our well-established *Mll*$^{\text{PTD/WT}}$ *Flt3*$^{\text{ITD/WT}}$ double knock-in AML mouse model (**Fig. 6D**; ref. 5). We found that treatment with the anti-EGFL7 antibody prolonged the life of the mice significantly compared with IgG1 control–treated mice ($P = 0.002$, **Fig. 6E**). Finally, using a PDX model derived from cells of AML patient 5 (**Fig. 6F**), we also observed prolonged survival of the anti-EGFL7–treated mice ($P = 0.04$, **Fig. 6G**). Overall, these results demonstrate that EGFL7-targeted therapy leads to NOTCH reactivation *in vivo* and represents a novel therapy for patients with AML.

Discussion

EGFL7 is a protein that is secreted by endothelial cells to promote cell growth and migration (2, 25, 27–29). Several other tissues also express *EGFL7* at low levels, but its expression can be induced during angiogenesis and in response to hypoxia, and vasculature injury (1, 23, 30). The Egfl7:eGFP transgenic mouse reporter strain demonstrated that *Egfl7* expression highly correlated with active sites of angiogenesis (1). Recently, the role of *EGFL7* overexpression has been shown to be clinically important and correlates with tumor grade in many solid tumors such as laryngeal squamous cell carcinoma, malignant glioma, breast cancer, and hepatocellular carcinoma (31, 32). In many cases, the mechanism of EGFL7-mediated tumorigenesis is believed to involve EGFL7′s role to induce vessel formation through regulation of endothelial cell functions such as: proliferation, survival, and/or migration (2, 25). Recently, we have described a role for EGFL7 in AML (5). We found that the levels of both EGFL7 mRNA and protein are increased in primary AML blasts compared with normal bone marrow cells. Moreover, high *EGFL7* mRNA expression associated with lower complete remission rates, shorter event-free and shorter overall survival in older (aged ≥60 years) and younger (aged <60 years) patients with cytogenetically normal AML. We further showed that AML blasts secrete EGFL7 protein, and that increased levels of EGFL7 protein are found in the sera from patients with AML compared with sera from healthy controls. Treatment of primary AML blasts with recombinant rEGFL7 *in vitro* leads to increases in leukemic blast cell growth (5). However, the molecular mechanisms underlying EGFL7-induced leukemogenesis has not been fully elucidated. Here, we demonstrate for the first time that EGFL7 is able to directly bind multiple proteins on primary AML blasts, including NOTCH. EGFL7 has previously been shown to bind NOTCH receptors and can both activate and repress NOTCH activation depending on the tissue cell type. Whether EGFL7 activates or represses NOTCH activity in AML has not previously been described. Our data now suggest that EGFL7 contributes to leukemogenesis, at least in part, through antagonizing NOTCH signaling (2, 25).

NOTCH signaling is important for normal hematopoiesis, and oncogenic activation of NOTCH signaling has been shown to contribute to leukemogenesis in T-ALL (33–37). However, there is still uncertainty when it comes to the exact role of NOTCH in different hematopoietic compartments. Previously, other groups have shown that the NOTCH signaling pathway is silenced in AML (17, 18). Lobry and colleagues demonstrated that NOTCH reactivation by DLL4 induced the expression of apoptosis- and differentiation-associated genes, including *HES1, BCL2, ADAMDEC1, CD11C, CD74*, and *CDKN1A* (17). Consequently, they found that NOTCH reactivation in AML blasts led to increased differentiation and apoptosis. However, the mechanism underlying the NOTCH inactivation in AML has remained to be further explained (16–18). It is possible that decreased NOTCH activation could be the result of decreased levels of NOTCH ligands, insufficient levels of NOTCH receptor expression, or inadequate cleavage and/or translocation of NICD to the nucleus to activate target gene transcription. Here, we propose one possible mechanism by which the NOTCH pathway remains inactive in AML is through EGFL7 antagonism of normal NOTCH ligand binding to NOTCH receptors on leukemic blasts (Supplementary Fig. S9). However, antagonism of NOTCH by EGFL7 binding might not be the only mechanism involving EGFL7-mediated silencing of NOTCH signaling. We found that in response to EGFL7 stimulation, primary AML blasts had decreased cleavage of NOTCH at 4 hours poststimulation. Interestingly, at 24 hours poststimulation, we found significant decreases in the levels of *NOTCH* mRNA. These results suggest that EGFL7 binding to NOTCH receptors may also lead to long-term transcriptional repression of *NOTCH*, creating a negative feedback loop involving EGFL7, NOTCH signaling inactivation, and *NOTCH* transcriptional repression. The exact mechanism by which EGFL7 stimulation leads to transcriptional repression of *NOTCH* mRNA is currently unknown, but future studies to determine how this is regulated could provide novel insights into NOTCH gene regulation.

Although the interaction of EGFL7 with a critical signaling pathway such as NOTCH is important for understanding the cross regulation of these pathways in AML, the results from our antibody array demonstrate the ability of EGFL7 to bind many proteins important for regulating diverse biological processes. Therefore, it seems likely that EGFL7 might be functioning in many different signaling pathways important for AML. Future studies examining how these pathways may be working in synergy in AML blasts will be important to determine the extent by which EGFL7 influences AML biology and the importance of developing anti-EGFL7 therapies to treat patients with AML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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