Prognostic and biological significance of the proangiogenic factor EGFL7 in acute myeloid leukemia

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Epithelial growth factor-like 7 (EGFL7) is a protein that is secreted by endothelial cells and plays an important role in angiogenesis. Although EGFL7 is aberrantly overexpressed in solid tumors, its role in leukemia has not been evaluated. Here, we report that levels of both *EGFL7* mRNA and EGFL7 protein are increased in blasts of patients with acute myeloid leukemia (AML) compared with normal bone marrow cells. High *EGFL7* mRNA expression associates with lower complete remission rates, and shorter event-free and overall survival in older (age ≥60 y) and younger (age <60 y) patients with cytogenetically normal AML. We further show that AML blasts secrete EGFL7 protein and that higher levels of EGFL7 protein are found in the sera from AML patients than in sera from healthy controls. Treatment of patient AML blasts with recombinant EGFL7 in vitro leads to increases in leukemic blast cell growth and levels of phosphorylated AKT. EGFL7 blockade with an anti-EGFL7 antibody reduced the growth potential and viability of AML cells. Our findings demonstrate that increased EGFL7 expression and secretion is an autocrine mechanism supporting growth of leukemic blasts in patients with AML.

EGFL7 | acute myeloid leukemia | clinical outcome

Acute myeloid leukemia (AML) is a clonal hematopoietic disease characterized by the proliferation of immature blasts in the bone marrow (BM) and blood (1). Genetic alterations, including chromosomal translocations and deletions and gene mutations leading to aberrant downstream target gene expression, contribute to AML initiation and maintenance. Previously, our group demonstrated that increased miRNA-126-3p (miR-126) expression in patients with cytogenetically normal AML (CN-AML) correlated with shorter overall survival (OS). Furthermore, we found miR-126 to be essential for leukemia stem cell (LSC) homeostasis, and in vivo targeting of miR-126 in a patient-derived xenograft model resulted in prolonged survival in secondary bone marrow transplant (BMT) recipients (2). miR-126 is located within intron 7 of a protein-coding gene known as *Epithelial growth factor-like 7* (*EGFL7*) (3). Although we and others (2, 4, 5) have shown an important role for miR-126 in AML biology, we know of no studies that have been performed to understand the prognostic and functional implications of expression of its host gene, *EGFL7*, in AML.

EGFL7 is a secreted protein of ∼30 kDa and plays an important physiological role in angiogenesis (6–8). Unlike other angiogenic factors (e.g., VEGF), physiological EGFL7 expression and function has been restricted mainly to the endothelial cells where it regulates survival, migration, and differentiation (6). Aberrant expression of EGFL7 has been shown to be involved in tumor growth and disease progression of several solid tumors, including hepatocellular carcinoma, malignant glioma, and breast, lung, and pancreatic cancers (9), but its role in hematopoietic malignancies is currently unknown. Therefore, we investigated the prognostic and biological function of EGFL7 expression in AML.

We show that EGFL7 mRNA and protein expression is increased in patient AML blasts compared with normal BM mononuclear cells (NBM-MNCs) and that high *EGFL7* mRNA expression levels correlate with worse outcome in both younger (age <60 y) and older (age ≥60 y) patients with CN-AML. Furthermore, we demonstrate that AML blasts are capable of secreting EGFL7 protein, leading to enhanced leukemic blast growth. Our data suggest an independent role for EGFL7 in AML but also highlight the importance of this genetic locus in AML via up-regulation of both miR-126 and its host gene *EGFL7*.

Results

Pretreatment Features and Clinical Outcomes Associated with *EGFL7* Expression in Younger Adults with CN-AML. To evaluate the prognostic significance of *EGFL7* mRNA expression in CN-AML, we analyzed one cohort of younger adults ($n = 374$) and one of older

Significance

In this work we report on the previously uncharacterized clinical and biological role for EGFL7 in acute myeloid leukemia (AML). Patients with increased *EGFL7* mRNA expression had lower complete remission rates and shorter overall and event-free survival, demonstrating the clinical relevance of *EGFL7* expression in cytogenetically normal AML. Our results show that AML blasts are able to synthesize and secrete EGFL7 protein, promoting autocrine blast cell growth. Inhibition of EGFL7 results in decreased proliferation and induces apoptosis of AML cells. Taken together, our data provide the rationale for targeting EGFL7 using blocking antibodies as a therapy for patients with AML.

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The authors declare no conflict of interest.

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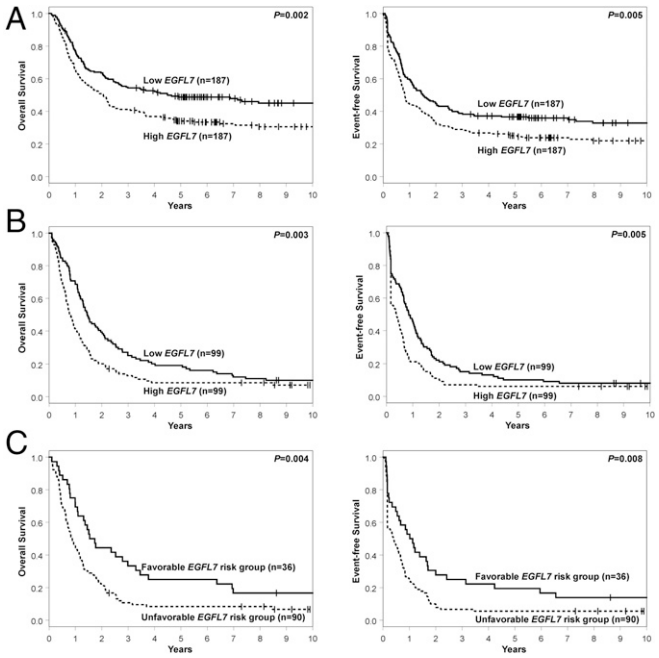

Fig. 1. Prognostic significance of *EGFL7* in younger and older CN-AML patients. (*A* and *B*) The association of *EGFL7* expression levels with OS and EFS of younger adult patients (age <60 y) (*A*) and older patients (age ≥60 y) (*B*). (*C*) OS and EFS according to *EGFL7* risk group in older CN-AML patients. The favorable risk group was comprised of patients with *EGFL7* low expression/high promoter methylation; the unfavorable risk group included the remaining patients (high expression/low promoter methylation, high expression/high promoter methylation, low expression/low promoter methylation). The median values of *EGFL7* expression and *EGFL7* promoter methylation were used as the high/low cut points.

patients ($n = 198$), for whom *EGFL7* expression was measured by RNA-sequencing (RNA-seq) and microarrays, respectively. The median expression value of *EGFL7* was used as a cut point to separate the analyzed cohorts into high and low *EGFL7* expressers.

Among younger adults, those with high *EGFL7* expression ($n = 187$) were more likely to present with lower platelet ($P = 0.002$) and WBC ($P = 0.001$) counts and higher percentages of blood blasts ($P < 0.001$) than patients with low *EGFL7* expression ($n = 187$). High *EGFL7* expressers were also less likely to have leukemic infiltration at extramedullary sites ($P = 0.02$). With regard to molecular characteristics, patients with high *EGFL7* expression more frequently harbored double *CEBPA* ($P < 0.001$) and *WT1* ($P = 0.02$) mutations and less frequently harbored *DNMT3A* ($P = 0.004$), *FLT3*-tyrosine kinase domain (*FLT3*-TKD; $P = 0.03$), *IDH2* ($P = 0.01$), and *NPM1* ($P < 0.001$) mutations. *EGFL7*-expresser status associated with significant differences ($P = 0.04$) in the risk stratification of patients according to the European LeukemiaNet (ELN) guidelines (10). Patients with high *EGFL7* expression were more frequently classified in the adverse risk group and less frequently in the favorable risk group than patients with low *EGFL7* expression. High *EGFL7* expression status associated with high expression of the *BAALC* ($P < 0.001$), *ERG* ($P < 0.001$), and *MN1* ($P < 0.001$) genes as well as high expression of miR-181a ($P < 0.001$) and miR-155 ($P = 0.008$). High *EGFL7* expressers were also more likely to express miR-3151 ($P < 0.001$) (Table S1). Because gene mutations frequently co-occur in CN-AML, we attempted to evaluate whether any mutational combinations are associated with *EGFL7* expression. Only the concomitant presence of *FLT3*-internal tandem duplications (*FLT3*-ITD) and *DNMT3A* and *NPM1* mutations (*FLT3*-ITD/*DNMT3A*mut/*NPM1*mut) were frequent enough for this analysis. Patients who harbored these three mutations ($n = 52$) had higher expression of *EGFL7* than patients who had WT *DNMT3A*, *NPM1*, and *FLT3* ($n = 82$; $P = 0.009$).

With regard to the clinical outcome, high *EGFL7* expression status associated with a lower complete remission (CR) rate (78 vs. 88%, $P = 0.01$). Patients with high *EGFL7* expression showed a trend for shorter disease-free survival (DFS) ($P = 0.09$, 5-y rates, 31 vs. 41%) (Fig. S1*A*) and had shorter OS ($P = 0.002$, 5-y rates, 34 vs. 49%) (Fig. 1*A*) than patients with low *EGFL7* expression. High *EGFL7* expressers also had shorter event-free survival (EFS) ($P = 0.005$, 5-y rates, 25 vs. 37%) (Fig. 1*A* and Table S2).

Pretreatment Features and Clinical Outcomes Associated with *EGFL7* Expression in Older Adults with CN-AML. Among older patients, those with high *EGFL7* expression more frequently harbored double *CEBPA* mutations ($P = 0.01$), *FLT3*-ITD ($P < 0.001$) and *RUNX1* mutations ($P < 0.001$), and less frequently harbored *NPM1* ($P < 0.001$) and *TET2* ($P = 0.001$) mutations. They were also less frequently classified in the favorable and more frequently in the intermediate or adverse risk group of the ELN classification than patients with low *EGFL7* expression ($P < 0.001$). High *EGFL7* expressers were more likely to have high expression of the *BAALC* ($P < 0.001$), *ERG* ($P < 0.001$), and *MN1* ($P < 0.001$) genes as well as miR-181a ($P = 0.02$) and miR-155 ($P = 0.05$) (Table S3).

With regard to mutational combinations, again only the *FLT3*-ITD/*DNMT3A*mut/*NPM1*mut mutational combination was frequent enough for analysis of its potential association with *EGFL7* expression in older CN-AML patients. However, in contrast to younger patients, *EGFL7* expression in older CN-AML patients with *FLT3*-ITD/*DNMT3A*mut/*NPM1*mut ($n = 21$) did not differ significantly from that in patients who had WT *DNMT3A*, *NPM1*, and *FLT3* ($n = 51$; $P = 0.79$).

Concerning clinical outcome, older CN-AML patients with high *EGFL7* expression were less likely to achieve a CR (58 vs. 76%, $P = 0.01$). High *EGFL7* expression status associated with shorter OS ($P = 0.003$, 5-y rates, 9 vs. 19%) (Fig. 1*B*) and EFS ($P = 0.005$, 5-y rates, 6 vs. 10%) (Fig. 1*B*), and in these patients there was a

Table 1. Multivariable analyses of outcome according to the *EGFL7* risk group in 126 older patients (age ≥60 y) with de novo CN-AML

	DFS		OS	
Variables in final models	HR (95% CI)	*P*	HR (95% CI)	*P*
EGFL7 risk group, favorable vs. unfavorable	0.57 (0.34–0.95)	0.03	0.45 (0.29–0.71)	<0.001
Extramedullary involvement, present vs. absent	1.99 (1.13–3.51)	0.02	—	—
miR-155, high vs. low*	—	—	2.47 (1.65–3.70)	<0.001
Platelets, continuous	—	—	1.22 (1.09–1.36)	<0.001

Hazard ratios (HR) greater than (or less than) 1.0 indicate higher (or lower) risk for relapse or death (in DFS) or for death (in OS) for the higher value of the continuous variables and for the first category listed in the categorical variables.

*The median expression value was used as the cut point.

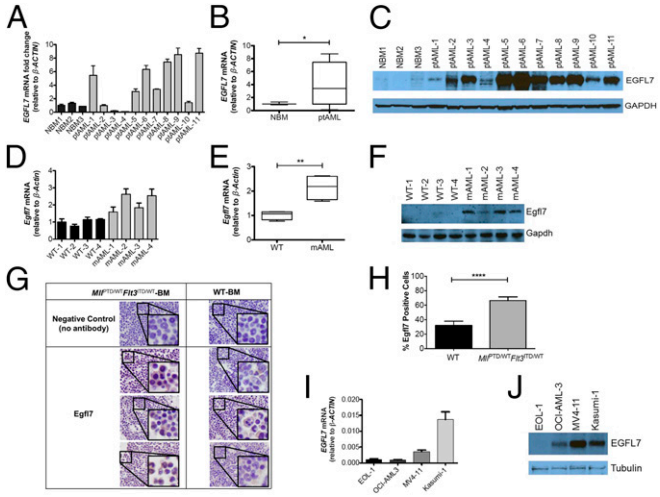

Fig. 2. EGFL7 is up-regulated in human and mouse AML cells. (*A*) NBM samples from healthy donors ($n = 3$) were compared with leukapheresis samples of AML patients (ptAML, $n = 11$). *EGFL7* levels were measured in AML samples by real-time RT-PCR, and the results were normalized to *β-ACTIN* RNA levels. (*B*) Mean ± SD of *EGFL7* mRNA expression levels between NBM and AML in aggregate. $*P < 0.05$. (*C*) EGFL7 protein levels in human NBM and leukapheresis samples of AML patients were determined by immunoblotting with GAPDH as loading control. (*D*) Normal BM from WT mice ($n = 4$) was compared with murine AML blasts of the $Mll^{PTD/WT}Flt3^{ITD/WT}$ mouse model (mAML, $n = 4$) for the detection of mouse *Egfl7* mRNA by RT-PCR with *β-Actin* as internal control. (*E*) Mean ± SD of *Egfl7* mRNA between murine NBM and murine AML blasts, in aggregate. $**P < 0.01$. (*F*) Mouse Egfl7 protein levels in WT murine controls ($n = 4$) and murine AML blasts from the $Mll^{PTD/WT}Flt3^{ITD/WT}$ mouse model (mAML) ($n = 4$) were assessed by immunoblotting using Gapdh as loading control. (*G*) Immunohistochemistry of Egfl7 in NBM of WT ($n = 3$) control mice vs. BM from $Mll^{PTD/WT}Flt3^{ITD/WT}$ leukemic mice ($n = 3$) using an Egfl7-specific antibody or no antibody controls. (Original magnification: 100×; *Insets* with same areas across the samples are magnified at same strength.) (*H*) Percent of $Egfl7^+$ cells in NBM of WT mice vs. BM from $Mll^{PTD/WT}Flt3^{ITD/WT}$ leukemic mice. $****P < 0.0001$. (*I* and *J*) *EGFL7* mRNA (*I*) and EGFL7 protein (*J*) expression levels in four human AML cell lines (EOL1, OCI-AML3, MV4-11, and Kasumi-1). The relative expression of *EGFL7* mRNA was measured with real-time RT-PCR normalized to *β-ACTIN*. For immunoblotting, β-Tubulin was used as loading control.

trend for the association of high *EGFL7* expression with shorter DFS ($P = 0.09$, 5-y rates, 11 vs. 13%) (Fig. S1*B* and Table S4).

Association of *EGFL7* Expression with Clinical Outcome in the Context of miR-126 Expression. As previously mentioned, *EGFL7* is the host gene for miR-126, which has been shown to associate independently with prognosis of CN-AML patients (2). Because *EGFL7* expression was also found to be prognostic, we decided to evaluate whether one of these two transcripts (i.e., *EGFL7* or miR-126) had a stronger association with clinical outcome than the other. We therefore studied 300 younger and 171 older CN-AML patients with available *EGFL7* and miR-126 expression data. In both age groups, we detected high correlation in the expression levels of *EGFL7* and miR-126 ($r = 0.84$ in younger CN-AML patients and $r = 0.64$ in older CN-AML patients). This high degree of correlation did not allow us to evaluate the association of *EGFL7* and miR-126 expression with prognosis in multivariable Cox-regression models, which include both covariates. Nevertheless, univariable analyses indicated a stronger association of *EGFL7* expression with outcome than for miR-126 for both younger and older CN-AML patients (Table S5).

Prognostic Significance of *EGFL7* Promoter Methylation Status. We hypothesized that the epigenetic regulation of *EGFL7* could also provide prognostic information, and thus we evaluated the *EGFL7* promoter methylation status in a subgroup of older CN-AML patients ($n = 126$) using a methylated DNA-capture technique, followed by next-generation sequencing (MethylCap-Seq), as described previously (11). We used the median value of *EGFL7* promoter methylation to dissect our cohort and found that patients with high *EGFL7* promoter methylation showed a trend toward higher CR rates (73 vs. 56%, $P = 0.06$) and had longer OS ($P = 0.05$) than patients with low *EGFL7* promoter methylation. There also was a trend for longer EFS in patients with high *EGFL7* promoter methylation ($P = 0.09$) but no significant association of DFS with *EGFL7* promoter methylation status.

Prognostic Significance of Integrated *EGFL7* mRNA Expression and *EGFL7* Promoter Methylation Status. The combination of high *EGFL7* promoter methylation with low *EGFL7* expression identified a subset of older CN-AML patients with better outcome ($n = 36$, hereafter referred to as the "*EGFL7* favorable risk group") compared with the remaining patients of the cohort ($n = 90$, the "*EGFL7* unfavorable risk group"). Patients in the *EGFL7* favorable risk group showed a trend for higher CR rates (78 vs. 59%, $P = 0.06$) and had longer DFS ($P = 0.05$) (Fig. S1*C*). Five years after diagnosis, 25% of these patients remained alive and leukemia-free, in contrast to only 9% of patients in the *EGFL7* unfavorable risk group. Patients in the *EGFL7* favorable risk group also had longer OS ($P = 0.004$, 5-y rates, 25 vs. 4%) and EFS ($P = 0.008$, 5-y rates, 19 vs. 6%) than those in the *EGFL7* unfavorable risk group (Fig. 1*C* and Table S6).

In multivariable analyses of older CN-AML patients, the *EGFL7* favorable risk group was shown to be an independent marker of longer DFS ($P = 0.03$) after adjusting for extramedullary involvement ($P = 0.02$) and of longer OS ($P < 0.001$) after adjusting for miR-155 expression status ($P < 0.001$) and platelet

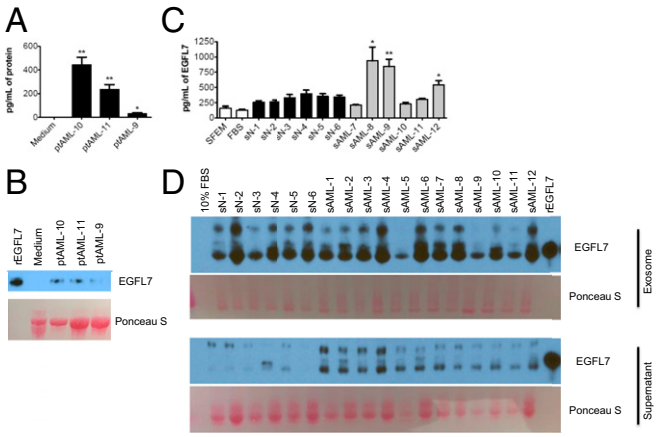

Fig. 3. EGFL7 is a secreted protein and is increased in the serum of some AML patients. (*A*) Blasts of AML patients (ptAML, $n = 3$) were cultured in SFEM medium + 10% FBS supplemented with cytokines for 24 h. The EGFL7 protein level in the cell-culture supernatant was detected by ELISA and was compared with that in medium from wells without cultured cells. $**P < 0.01$, $*P < 0.05$. (*B*) Blasts of AML patients ($n = 3$) were cultured in SFEM medium + 10% FBS supplemented with cytokines for 24 h. The EGFL7 protein in the cell-culture supernatant was assessed by immunoblotting with rEGFL7 as a positive control and medium from wells without cultured cells as a negative control. Ponceau S staining shows the loading control for protein. (*C*) EGFL7 protein levels in sera from normal healthy controls (sN, $n = 6$) and AML patients (sAML, $n = 6$) were determined by the EGFL7 ELISA kit. SFEM medium alone and 10% FBS serve as blank controls. $*P < 0.05$, $**P < 0.01$. (*D*) An equal volume of serum from AML patients (sAML, $n = 12$) or normal healthy donors (sN, $n = 6$) was subjected to the separation of exosomal vs. nonexosomal eluant using the ExoQuick kit (System Biosciences). EGFL7 protein levels in both isolated exosomes and the supernatant were determined by immunoblotting. SFEM containing 10% FBS served as a negative control, and rEGFL7 was used as a positive control. Ponceau S staining shows the loading of proteins.

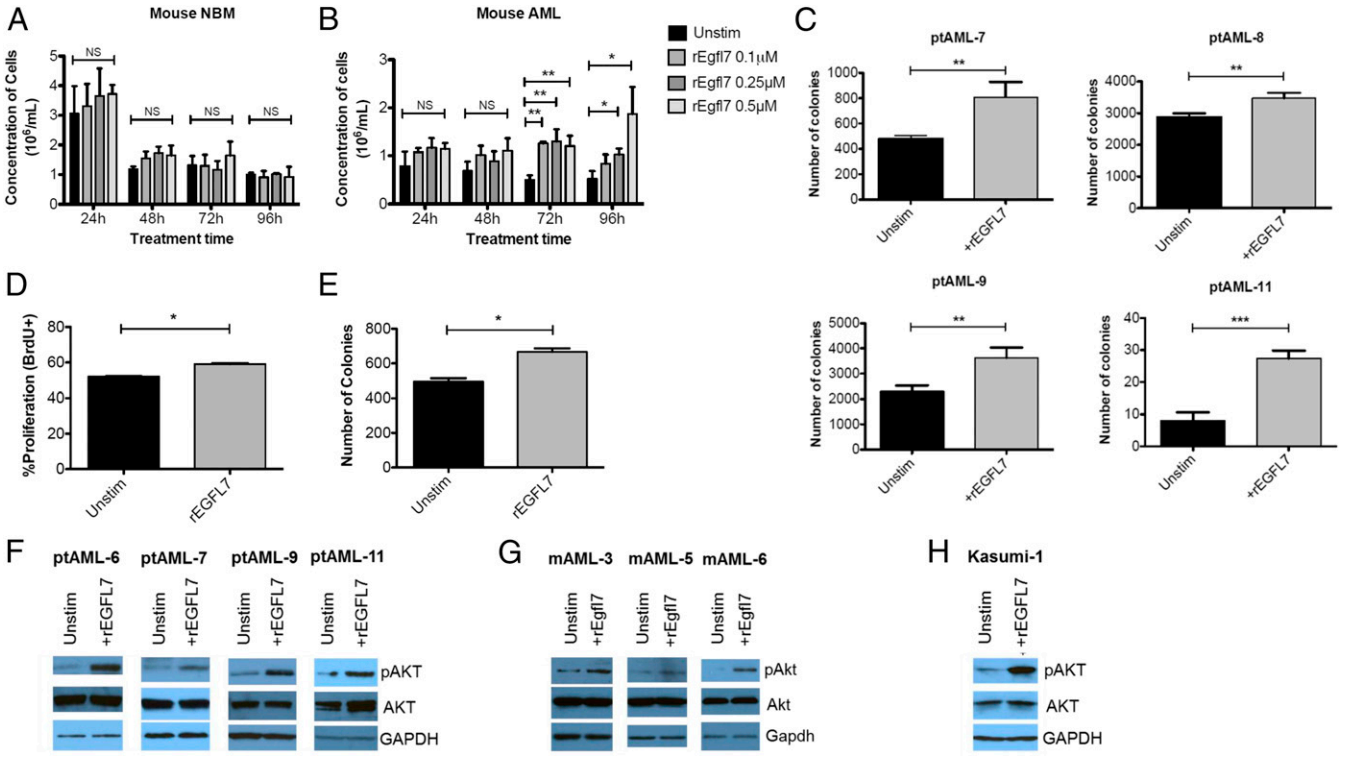

Fig. 4. EGFL7 stimulates the proliferation of human and mouse AML cells. (*A* and *B*) BM cells from WT (*A*) and *Mll*$^{PTD/WT}$*Flt3*$^{ITD/WT}$ (*B*) mice were treated without (Unstim) or with 0.1, 0.25, or 0.5 μM rEgfl7 in Iscove's Modified Dulbecco's Medium (IMDM) + 2% BSA for 24, 48, 72, or 96 h. At the indicated time points, the number of viable cells was determined by Trypan blue dye exclusion assay. Each condition was repeated in triplicate. *$P < 0.05$, **$P < 0.01$; NS, not significant. (*C*) Blasts of the indicated AML patients (20,000 cells) were mixed with methylcellulose medium in the absence or presence of 0.25 μM rEGFL7 and were plated onto 2-cm dishes for 10 d. Colonies with more than 50 cells were enumerated using a light microscope. Each condition for each patient ($n = 4$) was plated in triplicate; **$P < 0.01$, ***$P < 0.001$. (*D*) Kasumi-1 cells were stimulated with 100 nM rEGFL7 for 4 h in RPMI1640 with 10% FBS. Cell proliferation was assessed using APC-BrdU/7AAD staining coupled with flow cytometry; *$P < 0.05$. (*E*) Kasumi-1 cells (2,500 cells) were mixed with methylcellulose medium in the absence or presence of 100 nM recombinant human EGFL7 and were scored after 10 d. Each condition was plated in triplicate in three independent experiments. *$P < 0.05$. (*F*) Blasts from AML patients ($n = 4$) were cultured in SFEM + 2% BSA in the absence or presence of 0.25 μM rEGFL7 for 20 min. Total proteins were extracted for immunoblotting of pAKT-S473 and total AKT. GAPDH was used as loading control. (*G*) AML blasts from *Mll*$^{PTD/WT}$*Flt3*$^{ITD/WT}$ mice ($n = 3$) were cultured in IMDM medium + 2% BSA in the absence or presence of 0.25 μM rEgfl7 for 20 min. Total proteins were extracted for immunoblotting of pAkt-S473 and total Akt. Gapdh was used as loading control. (*H*) Exponentially growing Kasumi-1 cells were starved in serum-free RPMI1640 medium for 1 h, followed by the addition of 100 nM recombinant EGFL7 for 5 min. Total proteins were extracted for immunoblotting of pAKT-S473, total AKT, and GAPDH.

counts ($P < 0.001$) (Table 1). Because of the relatively small number of patients in the *EGFL7* favorable risk group, a final model could not be constructed for EFS. We could, however, generate three separate three-variable models, in which the association of favorable *EGFL7* status with longer EFS remained significant after adjusting for other covariables ($P = 0.002$, $P < 0.001$, and $P < 0.001$) (Table S7). With regard to CR, *EGFL7* risk group status was not significant in multivariable analysis.

EGFL7 Expression in AML. To validate the RNA-seq and microarray data further, we measured *EGFL7* mRNA and protein in patient AML blasts and NBM-MNCs using real-time RT-PCR and Western blotting, respectively (see Table S8 for cytogenetic and molecular characteristics of the patients). As shown in Fig. 2 *A–C*, higher levels of *EGFL7* mRNA and protein were observed in AML blasts from the majority of patients analyzed than in NBM-MNCs. We next investigated the *Egfl7* expression in our *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ mouse model of CN-AML (12) and compared it with *Egfl7* expression in WT murine controls. We found significant increases in *Egfl7* mRNA and Egfl7 protein in the BM of *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ leukemic blasts compared with WT NBM-MNCs (Fig. 2 *D–F*). To evaluate the expression of Egfl7 protein in AML further, we compared immunohistochemistry for Egfl7 in our *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ AML and in WT murine BM. We found substantial increases in Egfl7 in samples of *Mll*$^{PTD/WT}$*Flt3*$^{ITD/WT}$ leukemic BM compared with normal controls (Fig. 2 *G* and *H* and Fig. S2). We also measured *EGFL7* mRNA and EGFL7 protein expression in a panel of four human AML cell lines (EOL1, OCI-AML3, MV4-11, and Kasumi-1). We found Kasumi-1 cells to have the highest expression of *EGFL7* mRNA and MV4-11 cells to have the highest expression of EGLF7 protein (Fig. 2 *I* and *J*).

EGFL7 Expression and Secretion by AML Blasts. EGFL7 is normally expressed and secreted by endothelial cells to promote angiogenesis. We therefore sought to determine if AML blasts also acquired the ability to synthesize and secrete EGFL7 protein. Blasts from three patients with AML were cultured for 24 h in medium with 10% FBS and cytokines. EGFL7 levels were measured in the medium using an ELISA. As shown in Fig. 3*A*, we found significantly increased levels of EGFL7 protein in the medium from the AML blasts compared with the medium from wells without blasts. These results were confirmed using Western blotting (Fig. 3*B*). Next, we measured EGFL7 protein in the serum from healthy donors ($n = 6$) and AML patients ($n = 6$) using an ELISA. Significant increases in the level of EGFL7 were found in three of the six samples from the AML patients compared with normal controls (Fig. 3*C*). In our effort to validate these results using whole serum and Western blotting, we found that the serum was highly saturated with protein and interfered with resolution of the blot. When we separated the serum into the exosome-containing and supernatant fractions,

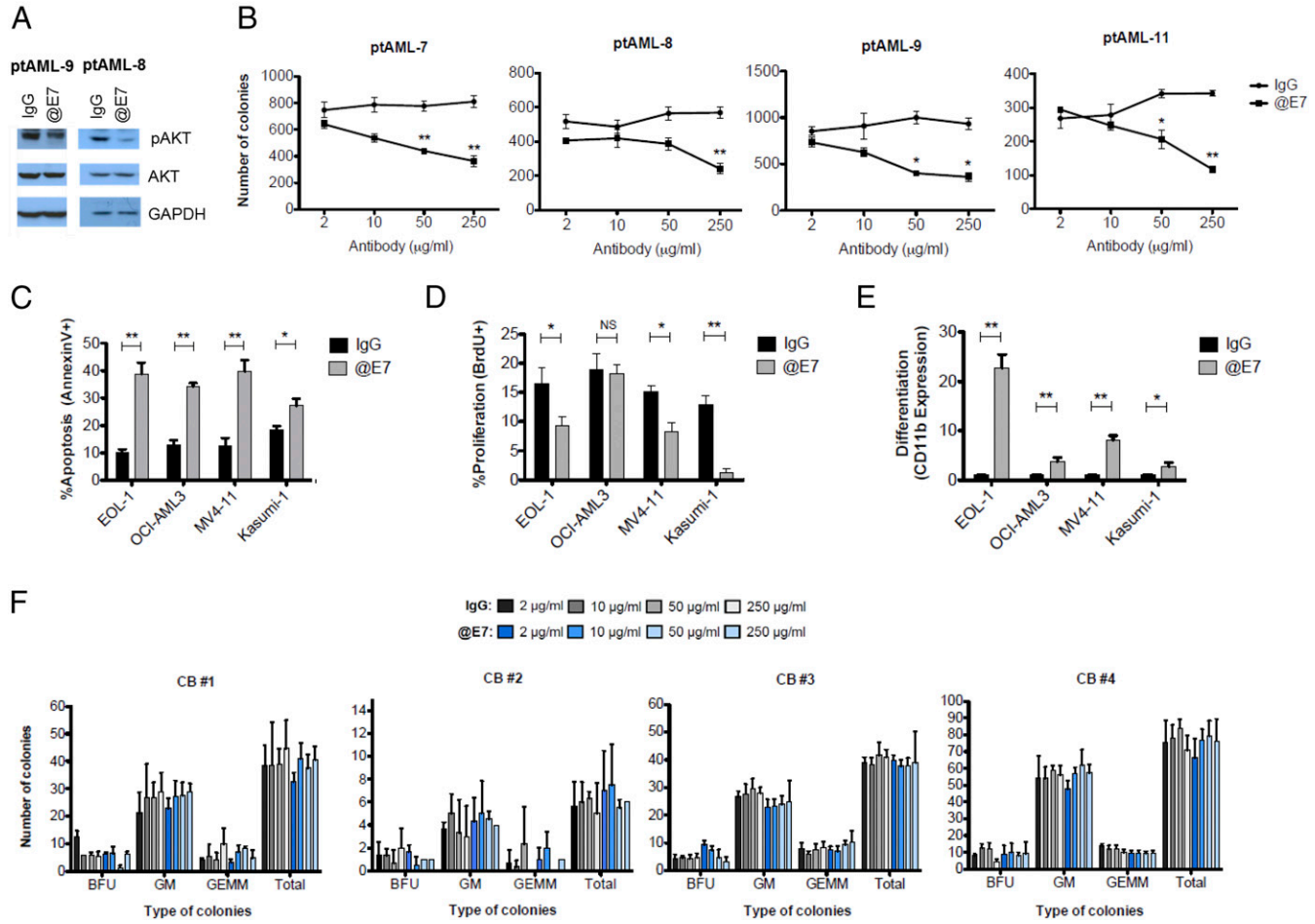

Fig. 5. EGFL7 inhibition results in decreased human AML cell growth without affecting normal hematopoietic cells. (*A*) Blasts from AML patients were cultured in SFEM with 10% FBS in the presence of 50 μg/mL of normal human IgG or anti-EGFL7 (@E7) antibody for 1 h. Total proteins were extracted for immunoblotting of pAKT-S473 and total AKT. GAPDH was used as loading control. (*B*) Human primary blasts (400,000) from AML patients ($n = 4$) were treated with 2, 10, 50, or 250 μg/mL of IgG control or anti-EGFL7 antibody in SFEM containing 10% FBS and cytokines for 2 h. Twenty thousand cells were plated in triplicate in methylcellulose medium and scored after 14 d of culture for mean ± SD colony numbers. $*P < 0.05$, $**P < 0.01$. (*C*) Forty-eight hours after IgG control vs. anti-EGFL7 antibody treatment (50 μg/mL) of AML cell lines, apoptosis was evaluated by Annexin V/7AAD staining, (*D* and *E*) Cell proliferation was measured using BrdU incorporation (*D*), and differentiation analysis was evaluated by CD11b expression (*E*). CD11b is depicted as the ratio of the CD11b expression value of the examined sample to the CD11b expression value of the corresponding IgG-treated control. $*P < 0.05$, $**P < 0.01$. (*F*) $CD34^+$ CB cells from four different donors were plated in methylcellulose medium in the presence of increasing concentrations (2, 10, 50, or 250 μg/mL) of human IgG or anti-EGFL7 and were scored after 14 d of culture. Along with total number of colonies, colony types [erythroid burst-forming units (BFU), granulocyte/monocyte (GM), or granulocyte/erythrocyte/monocyte/megakaryocyte colonies (GEMM)] were enumerated also.

we found variable high levels of EGFL7 in the exosomes in both normal and AML serum; however, there was substantially more "free" nonexosomal EGFL7 in the supernatant fraction from the AML patients than in the fraction from normal controls (Fig. 3*D*).

EGFL7 Stimulation Leads to Enhanced AML Blast Cell Growth. To compare the effect of Egfl7 protein on murine NBM-MNCs with its effect on murine AML blasts, we cultured blasts in minimal medium [Serum-Free Expansion Medium (SFEM; STEMCELL Technologies) + 2% BSA] and then stimulated them with recombinant Egfl7 (rEgfl7). Although the normal mouse BM did not expand at any time point (Fig. 4*A*), we found significant expansion of the murine AML blasts cells at 72 and 96 h poststimulation with rEgfl7 (Fig. 4*B*). We further validated the stimulatory effect of EGFL7 on growth of patient human AML blasts using CFU assays. We found an increase in the number of CFUs when rEGFL7 was added to the methylcellulose (Fig. 4*C*). In addition, we treated the Kasumi-1 AML cell line with rEGFL7, which we found, in concordance with previous reports (13), to express high levels of *EGFL7* mRNA. rEGFL7 treatment of these cells led to an increase in the fraction of proliferating cells, as measured by BrdU incorporation (percent of $BrdU^+$ cells in control vs. rEGFL7 treatment: 52 vs. 60, $P = 0.02$) (Fig. 4*D*) and by the number of colonies formed in CFU assays (Fig. S2*C*). In agreement with the increased cell growth in response to EGFL7, we found consistent increases in phosphorylated AKT (pAKT) in human (Fig. 4*E*) and mouse (Fig. 4*F*) AML cells, as well as in Kasumi-1 cells (Fig. 4*G*). These findings are in agreement with previous reports of increased pAKT levels in response to EGFL7 stimulation (14, 15).

To validate the role of the pAKT pathway in EGFL7-induced blast growth further, we tested whether inhibition of downstream targets of pAKT, such as the mTOR pathway, would abrogate the increased blast cell growth phenotype of rEGFL7. We found that although treatment with rEGFL7 consistently increased the number of AML primary blast colonies, this effect was abolished when the blasts were pretreated with the mTOR inhibitor rapamycin (Fig. S2), suggesting a role for AKT signaling in EGFL7-stimulated blast growth.

Differential Effect of EGFL7 Silencing on AML Blasts and Normal Hematopoietic Cells. To evaluate the effects and therapeutic potential of inhibiting EGFL7 in AML, we performed experiments with a commercially available antibody that binds to the EGFL7 protein and inhibits its downstream effects (16). Immunoblotting performed after treatment of patient AML blasts with the anti-EGFL7 antibody showed a decrease in the levels of pAKT (Fig. 5*A*). More importantly, treatment of human AML blasts with increasing concentrations of the anti-EGFL7 blocking antibody led to significant decreases in cell growth, assessed by the numbers of colonies in CFU assays, compared with normal IgG controls (Fig. 5*B*). In human AML cell lines (EOL-1, OCI-AML3, MV4-11, and Kasumi-1), EGFL7 inhibition led to significant decreases in the viability of leukemic cells in all cell lines (Fig. 5*C*), along with concomitant decreases in the fraction of proliferating cells in three of the four cell lines tested (i.e., EOL-1, MV4-11, and Kasumi-1) (Fig. 5*D*). We also evaluated the effect of anti-EGFL7 treatment on differentiation of AML cells by measuring CD11b expression. CD11b is a surface marker reported to be up-regulated upon differentiation of leukemic cell lines into macrophage-like cells (17, 18). We found that EGFL7 inhibition led to significant increases in CD11b expression in all four cell lines that were treated with the anti-EGFL7 antibody compared with treatment with human IgG control (Fig. 5*E*).

We hypothesized that dependency on EGFL7 signaling could be a feature that distinguishes leukemic blasts from healthy hematopoietic cells. To test this hypothesis, we treated CD34-selected umbilical cord blood cells ($CD34^+$ CBCs) from four separate donors with a range of anti-EGFL7 antibody concentrations and performed CFU assays. In contrast to the effect of anti-EGFL7 treatment on leukemic blasts, EGFL7 inhibition did not impede the proliferation/survival of colonies (measured by number of CFUs) or alter myeloid differentiation (measured by CFU types) of normal hematopoietic cells (Fig. 5*F*).

Discussion

EGFL7 is a secreted protein with a well-characterized role in the physiology of angiogenesis and the pathology of certain solid tumors (6–9). We and others have previously reported on the significance of miR-126 (2, 4, 5), which is located in intron 7 of the *EGFL7* gene, in AML, but an independent role of *EGFL7* in this disease has not been described to date. Here we analyzed a set of younger adults and a set of older patients with CN-AML to evaluate the prognostic and biologic significance of *EGFL7* expression. We found that high *EGFL7* expression associates with worse outcome in both studied cohorts. We also found that in the older patients the combination of high *EGFL7* promoter methylation and low *EGFL7* expression identified a subset of patients with favorable prognosis, independently of other prognostic covariables. In our effort to evaluate the functional role of EGFL7, we screened blasts from a number of AML patients and AML cell lines. In these samples, we found some discordance between the mRNA and the protein levels of EGFL7, indicating that additional, posttranscriptional or posttranslational mechanisms could be involved in the regulation of EGFL7.

Although it seems reasonable that the expression levels of miR-126 and *EGFL7* are regulated by the same mechanisms, including methylation, because they stem from the same transcript, mechanistic studies indicate that these genes have different effector functions. EGFL7 is a protein that is secreted by the AML blasts and is capable of directly inducing enhanced cell growth. Treatment of patient AML samples as well as cell lines with recombinant EGFL7 protein led to markedly increased levels of pAKT, a key regulator of cell proliferation. Concordantly, EGFL7 treatment increased the proliferating fraction as well as the number of colonies formed by AML cells. In contrast to this result, we observed no effects on cell proliferation when miR-126 expression was modulated in AML bulk blasts. Instead, we found miR-126 to be essential for LSC homeostasis (2). Whether EGFL7 also has a role in LSCs, dependent on or independent from miR-126, has not yet been determined.

Understanding the individual as well as combinatorial roles of EGFL7 and miR-126 in leukemogenesis could contribute significantly to more efficient therapeutic approaches in patients with aberrant activation of the *EGFL7* locus. Our data indicate that EGFL7 could represent a therapeutic target in AML. We found that treatment with an EGFL7-blocking antibody reduced pAKT levels and decreased AML blast growth. In addition, EGFL7 inhibition increased apoptosis, decreased proliferation, and induced differentiation in the majority of the AML cell lines that were tested, independently of their endogenous EGFL7 expression levels. In striking contrast, blocking EGFL7 did not affect growth or differentiation of normal $CD34^+$ umbilical CB cells. Thus, EGFL7-blocking antibodies may have a therapeutic effect, in particular in AML patients with increased EGFL7 expression, while preserving normal BM populations. In patients with concomitant aberrant overexpression of miR-126, EGFL7 blockade could be combined with therapeutic interventions to down-regulate miR-126, to target the LSC compartment additionally. We have previously shown the feasibility of therapeutic manipulation of miR-126 with nanoparticle-conjugated oligonucleotides (NP-antagomiR-126) in a preclinical model (2). In this sense, combining EGFL7-inhibition with NP-antagomiR-126 therapies may improve the treatment of AML patients, because the blocking the growth-promoting functions of EGFL7 on bulk blasts would be combined with the targeting of the therapy-resistant LSCs by the NP-antagomiR-126.

In conclusion, our results demonstrate the clinical and biological relevance of EGFL7 expression in AML. We found that expression levels of *EGFL7* are prognostic in CN-AML patients and that patient AML blasts are able to secrete EGFL7 protein and promote the leukemic cell growth in an autocrine fashion. Furthermore, our data indicate that targeting EGFL7 with a monoclonal blocking antibody could provide a therapeutic option for the treatment of AML.

Patients and Methods

All patients provided written informed consent, and all study protocols were in accordance with the Declaration of Helsinki and approved by the institutional review boards at each center. For details concerning the treatment protocols and the molecular profiling of the analyzed AML patients, as well as for details concerning the experimental procedures, see *SI Patients and Methods*.

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