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Key Words. Focal adhesion kinase • Leukemia • Splice variants • Stem cells • Wnt

ABSTRACT

Focal adhesion kinase (FAK) activity contributes to many advanced cancer phenotypes, but little is known about its role in human acute myeloid leukemia (AML). Here, we show that FAK splice variants are abnormally expressed in the primitive leukemic cells of poor prognosis AML patients. In the $CD34^+38^-123^+$ long-term culture-initiating cell-enriched leukemic cells of these patients, FAK upregulates expression of Frizzled-4 and phosphorylates Pyk2 to enable the required association of Pyk2 with the Wnt5a/Frizzled-4/LRP5 endocytosis complex and downstream activation of β-catenin, thereby replacing the Wnt3a-controlled canonical pathway used by normal hematopoietic stem cells. Transduction of primitive normal human hematopoietic cells with FAK splice variants induces a marked increase in their clonogenic activity and signaling via the Wnt5a-controlled canonical pathway. Targeting FAK or β-catenin efficiently eradicates primitive leukemic cells in vitro suggesting that FAK could be a useful therapeutic target for improved treatment of poor prognosis AML cases. STEM CELLS *2012;30:1597–1610*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The focal adhesion kinase (FAK) proteins (FAK and Pyk2) are involved in cell proliferation, survival, and migration but normally through separate pathways [1, 2]. FAK is a nonreceptor tyrosine kinase that is commonly activated downstream of integrins and growth factor receptors. In contrast, the closely related Pyk2 is activated primarily by stress and calcium signaling. Both the expression and activity of FAK have been found to be deregulated in cancer cells in association with the acquisition of increased metastatic activity, increased resistance to treatment, and a poor prognosis [3]. Interestingly, deletion of FAK in the epidermis prevents chemically induced skin tumor formation [4]. In addition, a report has linked FAK with breast cancer stem cell maintenance [5]. This finding suggests that FAK may play an important role in the genesis and growth of malignant populations.

The *FAK* gene encodes four variably spliced coding exons (13, 14, 16, and 31) [6]. None of these contribute to the standard protein termed FAK^0, which is thus distinguished from other variant FAK proteins [7]. Examples of the latter are FAK^{28}, FAK^6, and FAK^7, encoded by transcripts containing exons 13, 14, and 16, respectively. Retention of noncanonical *FAK* exons can occur independently and is under both developmental- and tissue-specific control [6]. FAK^0 is the most abundant transcript in many tissues, whereas expression of FAK^6 is normally restricted to the testis, heart, and adrenal gland and FAK^7 to the testis and brain. Alternative splicing dramatically increases the ability of FAK to undergo autophosphorylation at Y397, which is located in between the sequences encoded by exons 14 and 16 [7–9]. Increased pY397 favors the interaction of FAK variants with Src kinases, although all FAK isoforms have the same capacity to target focal adhesions. To date, nothing has been reported about the potential role of FAK variants in hematopoiesis or cancer.

Pyk2 is expressed in primitive normal hematopoietic cells, whereas both FAK and Pyk2 are expressed and play major functional roles in mature myeloid and lymphoid cells [10–13]. We previously reported that FAK is expressed in the leukemic

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blasts 46% of patients with acute myeloid leukemia (AML) and in these cells confers adhesion independency and enhanced migratory ability. We also showed that this elevated expression of FAK in human AML cells is associated with a hyperleucocytosis, resistance to therapy, and poor survival [14]. Expression of FAK was also found to induce Pyk2 phosphorylation [2, 14].

Given the evidence that human AML is a clonal disease sustained by a rare subset of leukemic stem cells (LSCs) with extensive self-renewal capacity [15], we next sought to investigate whether activated expression of FAK might contribute to the altered biology of primitive AML cells. Although it is clear that intrinsic changes are essential to the malignant properties of the LSCs in AML, the interactions of these cells with their microenvironment in vivo are also likely to be important. In normal hematopoietic stem cells (HSCs), activation of β_1-integrin signaling through a Pyk2/phosphoinositide 3-kinase (PI3K) pathway has been associated with the regulation of quiescence [16], and Wnt signaling has been implicated in the regulation of normal HSC self-renewal [17, 18]. Interestingly, β-catenin, a downstream effector of the Wnt canonical pathway, has been found to be crucial for the generation and maintenance of LSCs from mixed lineage leukemia (*MLL*)-mediated AML [19, 20] but not in other AML genotypes [21]. When deregulated, β-catenin is associated with a poor event-free survival (EFS) and shortened overall survival (OS) [22–24]. We now show that the expression of FAK variants is deregulated in the primitive AML cells of poor prognosis AML patients where they play a key and previously undescribed role in the maintenance of these cells by altering Wnt signaling and β-catenin activity.

Materials and Methods

Antibodies

FAK clone 4.47 was from Millipore (Billerica, MA, http://www.millipore.com). Pyk2 clone 11, β-catenin clone 14, CD34 clone 8G12/phycoerythrin (PE)-Cy7, CD38 clone HIT2/allophycocyanin (APC), and CD123 clone 9F5/PE were from BD Biosciences (San Jose, CA, http://www.bdbiosciences.com). pS473 Akt and pT183/Y185 JNK clone G9 were purchased from Cell Signaling Technologies (Danvers, MA, http://www.cellsignal.com). Frizzled-4 clone 145901/biotinylated and ROR2 clone 231512 were from R&D Systems (Minneapolis, MN, http://www.rndsystems.com). CD45 clone HI30/pacific blue was from Ozyme (St Quentin, France, http://www.ozyme.fr). pY579 Pyk2, pY881 Pyk2, secondary idiotypic anti-mouse or anti-rabbit antibodies conjugated to AlexaFluor-488, AlexaFluor-594, or AlexaFluor-647 were obtained from Invitrogen (Carlsbad, CA, http://www.invitrogen.com).

Primary Cells

Healthy donor bone marrow samples were obtained from discarded hip surgery fragments from hematological healthy patients (Orthopedic Department of Toulouse Purpan Hospital, Pr. Bonnevialle), and AML samples were obtained after informed consent and stored at the Hemopathies Inserm Midi-Pyrénées (HIMIP) collection. According to the French law, HIMIP collection has been declared to the Ministry for Higher Education and Research (DC 2008-307 collection 1) and a transfer agreement was obtained (AC 2008-19) after approval by the "Comité de protection des Personnes Sud-Ouest et Outremer II" (Ethical committee). Clinical and biological annotations of the AML samples have been declared to the CNIL (Comité National Informatique et Liberté, i.e., Data processing and Liberties National Committee). Normal bone marrow samples were collected anonymously.

Primary cells were thawed in Hank's balanced salt solution (HF) supplemented with 100 μg/ml DNAse and 5% 06-900 fetal calf serum (StemCell Technologies, Vancouver, BC, http://www.stemcell.com), and remaining cell clumps were eliminated using nylon mesh. After centrifugation, cells were resuspended in serum-free medium (SFM) (Iscove's Modified Dulbelcco's medium from Invitrogen supplemented with 10 mg/ml bovine serum albumin (BSA), 10 μg/ml insulin, 200 μg/ml transferrin, 100 units/ml penicillin, 100 μg/ml streptomycin all from StemCell Technologies and 10^{-4} M β-mercaptoethanol) at a cell density of 10^6 cells per milliliter. For Wnt treatment experiments, cultured cells were incubated for 8 hours in medium supplemented with 20 ng/ml recombinant Wnt3a or 0.3 μg/ml recombinant Wnt5a [24], unless indicated differently. Inhibition of all class I-A PI3K was obtained by treatment of cells with 25 μM of LY294002 or 50 nM wortmannin (Sigma-Aldrich, St Louis, MO, http://www.sigmaaldrich.com) for 4 hours. Inhibition of FAK and β-catenin was obtained by treatment of cells with increasing doses of, respectively, PF-573228 and PKF 115-584 (Tocris, Bristol, U.K., http://www.tocris.com) for 4 hours followed by a 24-hour incubation in SFM. For pharmacological assays, chemotherapy drugs (VP16 from Teva Pharma, Courbevoie, France, http://www.tevapharm.com, and ara-C from Upjohn, Paris, France) have been incubated with AML samples at 1 μM for 24 hours after FAK or β-catenin inhibition. Cells were then processed for apoptosis, cell cycle, and quiescence staining and analyzed by flow cytometry. Inhibition of clathrin- and caveolae-dependent endocytosis was obtained by a 30-minute pretreatment of cells with 200 μM monodansylcadaverin and 80 μM dynasore (Sigma-Aldrich), respectively, prior to Wnt stimulation.

Flow Cytometry and Cell Sorting

Cells were stained for extracellular antigens in HF supplemented with 0.3% BSA on ice for 30 minutes at a density of 1×10^6 per milliliter. After washing, secondary labeling reagents were added and incubated on ice for 30 minutes. After washing, the cells were fixed in 4% paraformaldehyde on ice for 15 minutes and rewashed. For intracellular staining, cells were transferred into HF with 0.3% BSA–0.1% saponin for 20 minutes. After centrifugation, cells were resuspended in a permeabilization buffer containing intracellular primary antibodies and incubated on ice for 30 minutes, washed, and incubated in permeabilization buffer containing secondary labeling reagents for 20 minutes, washed in phosphate buffer saline (PBS), filtered, and transferred into fluorescence-activated cell sorting (FACS) tubes to get analyzed or sorted. For staining controls, both single stained and fluorescence minus one controls were used. Cells were analyzed on a BD LSRII cytometer or on a FacsScan and sorted on a BD FACSAria Sorp (BD Biosciences) using FlowJo software from TreeStar to treat data.

For apoptosis assays, cells were stained for extracellular antigens and then stained with Annexin-V fluorescein isothiocyanate (FITC) (BD Biosciences) according to the manufacturer's protocol. Results were expressed as the average apoptotic percentage within the HSC and LSC populations isolated from three samples each and defined, respectively, as cells expressing a $CD45^{hi}CD34^{hi}CD38^{-/lo}$ or $CD45^{lo}CD34^{hi}CD38^{-/lo}CD123^{+}$ phenotype.

For G0/G1 analysis, cells were stained using Click-It Edu proliferation assay (Invitrogen) according to the manufacturer's protocol together with extracellular antigens. Results were expressed as the average percentage of G0/G1 LSCs of three different primary samples. For quiescence analysis, cells were stained using Hoechst 33342 (Molecular Probes, Eugene, OR, http://probes.invitrogen.com), Pyronin Y, and extracellular antigens as described in [25]. Quiescent LSCs were defined as $CD45^{lo}CD34^{+}CD38^{-/lo}CD123^{+}$ cells that were negative for Pyronin-Y after excluding cycling cells based on Hoechst staining. Results are expressed as the percentage of quiescent LSCs within the G0/G1 population from three primary samples.

RNA Extraction and Polymerase Chain Reaction

Total RNA was extracted with the Arcturus Picopure RNA isolation kit from Applied Biosystems (Life Technologies Corporation, Carlsbad, CA, http://www.lifetech.com). Control reactions were

performed in the absence of RNA. Polymerase chain reaction (PCR) was performed with the ABI Prism 7000 Sequence Detection System (Applied BioSystems, Foster City, CA, http://www.appliedbiosystems.com) using the manufacturer's protocol, and Platinium SYBR Green quantitative PCR (qPCR) SuperMix UDG (Invitrogen). Primer sequences used in this work are indicated in the Supporting information. The $\Delta\Delta C_t$ was calculated as following: $C_t = (C_{t_{target}} - C_{t_{\beta-ACTIN}})_x - (C_{t_{target}} - C_{t_{\beta-ACTIN}})_y$ where x is the sample to analyze and y is the sample arbitrarily chosen to normalize. Results are expressed as N-fold changes in target gene copies, according to the following equation: amount of target $= 2^{-\Delta_{\Delta(Ct)}}$. Determination of FAK variants' expression levels was performed as described in [6], by an experimenter unaware of the diagnosis, and normalized to *GAPDH*.

Transfection Experiments

For small interfering RNA (siRNA) experiments, 6×10^6 primary cells from AML or normal bone marrow samples were resuspended in Nucleofector V solution for electroporation (Lonza, Köln, Germany, http://www.lonza.com). A total of 10^6 cells were then mixed with 200 nM control, FAK, Pyk2 ON-TARGET plus SMARTpool siRNA (Dharmacon, Lafayette, CO, http://www.dharmacon.com; Sigma-Aldrich, Saint Quentin Fallavier, France), or Frizzled-4 three-pooled siRNA (Santa Cruz Biotechnology, Heidelberg, Germany, http://www.scbt.com) and immediately nucleofected with an Amaxa Nucleofector apparatus (program U-15). Cells were then immediately transferred into wells containing prewarmed culture medium at a density of 10^6 cells per milliliter. Assessment of protein decrease was performed by flow cytometry. For the T-cell factor (TCF) reporter assay, 6×10^6 AML cells were transfected with 2 μg of TOP-FLASH and FOP-FLASH luciferase reporter and incubated overnight before LSC sorting. After 24 hours of Wnt stimulation, TOP-FLASH and FOP-FLASH activities were assessed using the Dual Luciferase Kit (Promega, Madison, WI, http://www.promega.com), and results were expressed as a ratio of corrected TOP-FLASH/FOP-FLASH of three different primary samples.

For lentiviral infection, $CD34^+$ cells (>80% purity) were purified by immunomagnetic selection (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) from mononuclear cells obtained from healthy donor bone marrow samples. The FAK^0 and FAK^6 coding sequences were cloned from the pBK-CMV2 plasmids [7] into the pTRIP-internal ribosome entry site (IRES)-green fluorescent protein (GFP) lentiviral vector plasmid [26]. Vector particles were produced as previously described [26], and $CD34^+$ cells were transduced as follows: freshly purified $CD34^+$ cells (10^6 cells per milliliter) were incubated with the lentiviral vectors (multiplicity of infection (MOI) of 4–5, twice at 24-hour intervals) in SFM supplemented with cytokines (100 ng/ml stem cell factor (SCF), 60 ng/ml interleukin-3, 100 ng/ml Flt3, 10 ng/ml thrombopoietin) on fibronectin-coated wells. After 3 days, cells were washed, analyzed for transgene expression and vector integration by flow cytometry, and used for culture.

Immunofluorescence

A total of 10^5 sorted LSCs were plated on 0.01% (w/v) poly-L-lysine precoated slides for 7 minutes, then fixed with a 20-minute incubation in 4°C paraformaldehyde and then permeabilized using HF–0.3% BSA–0.1% saponin for 20 minutes. Cells were then incubated in a humid chamber with primary antibodies for 1 hour. After washing, anti-idiotypic secondary antibodies coupled with AlexaFluor-488 or AlexaFluor-594 were added for 30 minutes and then washed three times with PBS. Coverslips were mounted with Vectashield Hard Set mounting medium with 4′-6-diamidino-2-phenylnidole (DAPI) (Clinisciences, Montrouge, France, http://www.clinisciences.com), and all the preparations were analyzed by confocal laser scanning using Zeiss LSM710 (Leica Microsystems, Heerbrugg, Germany, http://www.leica.com) equipped with a 63× objective using Zen software. MetaMorph imaging software (Molecular Devices, Sunnyvale, CA, http://www.moleculardevices.com) was used to capture and quantify the fluorescence signal between nucleus and cytoplasm. Briefly, the nuclear compartment was delineated as stained with DAPI. The cytoplasmic region was identified as a disc beginning at the nuclear/cytoplasmic region and extending and ending at the plasma membrane. The values were background corrected. Cells were manually included or excluded by inspection to ensure that all cell regions used for scoring were well-defined. The statistic evaluated was the ratio of the average nuclear region intensity to the average cytoplasmic region intensity for each cell. At least 10 cells for each of three independent experiments were analyzed.

Colony-Forming Cell and Long-Term Culture-Initiating Cell

For long-term culture-initiating cell (LTC-IC) assays, sorted LSCs were treated with FAK or β-catenin inhibitors and then seeded in limiting dilution assays (from 700 to 70,000 cells per well) on MS5 feeder cells in Myelocult H5100 supplemented with 10^{-6} M hydrocortisone (StemCell Technologies). Half media exchanges were performed weekly. After 6 weeks, cells were harvested, and colony-forming cells (CFCs) present were assessed by plating in Methocult H4435 (StemCell Technologies). LTC-IC frequency was calculated from the proportion of negative wells (no CFCs present) 18 days after seeding, using L-Calc software (StemCell Technologies). Results are expressed as average LTC-IC frequencies from three different primary samples. IC_{50} was determined as the dose responsible for a 50% decrease in LTC-IC frequency using Prism software (GraphPad Software Inc., San Diego, CA, http://www.graphpad.com). Results were expressed as the average IC_{50} calculated from three different primary samples. For CFC assays, primary samples were treated with FAK or β-catenin inhibitors and then plated in Methocult H4435 in duplicate. Colonies were counted 18 days after seeding. Results are expressed as average CFC output from three different primary samples. A total of 5–10 individual CFC-derived colonies were analyzed cytogenetically to confirm their leukemic origin. Results were based in each case on an examination of at least two Giemsa-banded metaphases per individually processed CFC-derived colony.

Statistical Analysis

Pairwise comparisons between continuous variable distributions were carried out with the Kruskal–Wallis test and Fisher's exact test for categorical variables. The mean ± SEM was calculated for each group. Kaplan–Meier curves were plotted for OS, EFS, and length of clinical remission (LCR) and compared using the Mantel-Cox log-rank test. OS endpoints, measured from time of diagnosis, were death (failure) or survival at the last follow-up (censored). EFS endpoints, measured from time of diagnosis, were death or relapse (failure) and survival at the last follow-up (censored). LCR endpoints, measured from time of complete remission, were death or relapse (failure) and survival at the last follow-up (censored). Results were significant at $p < .05$.

Results

FAK Variant Expression Is a Feature of Poor Prognosis AML

A new survey of cells from 48 cases of AML showed that 52% of these samples expressed FAK (Supporting Information Table S1), a percentage close to that reported in our previous study [14]. Further analysis of FAK isoform expression in the 48 samples analyzed here led to their stratification into three groups: a FAK^- group, a FAK^0 group (positive only for FAK^0 isoforms), and a FAK^{6*} group (positive for FAK^6 and/or $FAK^{6,7}$ and/or $FAK^{6,28}$ isoforms) based on the main isoforms detected (Table 1; Supporting Information Table S1). The groups of AML patients thus identified did not show any

Table 1. Clinical and biological characteristics of AML patients classified along FAK variants' expression

	FAK^-	FAK^0	FAK^{6*}	*p*
Gender—total (%)				
Male/female	14 (60.9)/9 (39.1)	6 (66.6)/3 (33.4)	10 (55.6)/8 (44.4)	ns
Age				
Median (range)	49 (31–80)	59 (30–71)	62 (22–71)	ns
>60 years	18	5	8	
<60 years	5	4	10	
FAB—total (%)				
M0/M1	6 (27.3)	1 (14.3)	6 (33.3)	ns
M2	5 (22.7)	1 (14.3)	4 (22.2)	
M3	1 (4.5)	0	0	
M4	4 (18.2)	2 (28.6)	6 (33.3)	
M5	6 (27.3)	3 (42.8)	2 (11.2)	
n/a	1	2	0	
Cytogenetic risk—total (%)				
CBF	2 (10.5)	1 (12.5)	2 (12.5)	ns
Intermediate	17 (89.5)	6 (75.0)	12 (75.0)	
Unfavorable	0 (0)	1 (12.5)	2 (12.5)	
n/a	4	1	2	
Flt3-ITD—total (%)				
Negative	13 (61.9)	4 (50.0)	7 (41.2)	ns
Positive	8 (38.1)	4 (50.0)	10 (58.8)	
n/a	2	1	1	
Cell count—median (range)				
WBC $\times 10^9$ per liter	36.2 (1.3–226)	41.5 (4.4–109)	65.3 (1.2–322.5)	ns
Blast %	86 (44–95)	63 (26–94)	81 (30–98)	ns
n/a	5	3	5	
Clinical outcome				
% CR	84.2	85.7	58.8	ns
% Relapse	20	50	54.6	ns
% Death	26.3	57.1	81.3	<.01
% Early death	15.8	14.3	41.2	<.05
n/a	4	0	1	

AML patients were classified in three groups FAK^- (FAK negative), FAK^0 (positive only for FAK^0 isoforms), and FAK^{6}* (positive for FAK^6 and/or $FAK^{6,7}$ and/or $FAK^{6,28}$ isoforms) depending on the expression of the main isoforms in AML cells as measured by semiquantitative reverse transcription polymerase chain reaction. Main clinical and biological characteristics are represented. Patient's karyotypes were classified as favorable CBF, intermediate, and unfavorable (others, including complex karyotypes). Pairwise comparisons between continuous distributions were carried out with the Kruskal-Wallis test and Fisher's exact test for categorical variables.
Abbreviations: AML, acute myeloid leukemia; CBF, core binding factor; CR: complete remission; EFS: event-free survival; FAB: French American British classification; FLT3-ITD: FLT3 internal tandem duplication; FAK, focal adhesion kinase; LCR: length of clinical remission; OS: overall survival; WBC: white blood cells.

differences in age at diagnosis, sex distribution, presence or absence of a FLT3-internal tandem duplication (ITD) mutation or cytogenetic risk factors. However, expression of FAK^{6*} variants was associated with a decreased OS, EFS, and length of clinical remission as compared to FAK^- cases (Fig. 1). The FAK^{6*} group also showed an increased death rate (even in under 60-year-old patients, Supporting Information Table S1) and early death (i.e., death before a first complete remission was achieved, Table 1). Altogether, these data show that FAK^{6*} variant expression in the blasts from AML patients is independently associated with a poor outcome, whereas expression of FAK proteins that do not include contributions from alternative exons (FAK^0 proteins) had no significant prognostic impact. Subsequent experiments were thus performed on FAK^{6*} AML samples to delineate FAK and β-catenin signaling crosstalk, and on both FAK^0 and FAK^{6*} AML samples to compare their drug sensitivity (Supporting Information Table S2).

Deregulated FAK Expression in Primitive AML Cells Increases Their Expression of β-Catenin

We next asked whether and how FAK and Pyk2 expression may be deregulated in primitive cells from FAK variant$^+$ AML patients. As shown in Supporting Information Figure S1A, LTC-IC activity was mostly measured in the $CD45^{lo}CD34^{hi}CD38^{-/lo}CD123^+$ subpopulation suggesting its enrichment in LSCs [27, 28]. Initial examination of the $CD45^{hi}CD34^{hi}CD38^{-/lo}$ HSC-enriched population isolated from normal bone marrow samples confirmed the expression of Pyk2 and β-catenin in these cells in concert with an absence of expression of FAK (Supporting Information Fig. S1B). We then analyzed FAK and β-catenin expression in the corresponding $CD45^{lo}CD34^{hi}CD38^{-/lo}CD123^+$ population isolated from AML patients. As shown in Figure 1D, β-catenin and FAK variant expression levels were correlated in this subset that we will subsequently name LSCs given its potential enrichment in LSCs. LSCs from FAK^{6*} AML samples ($n = 8$) expressed the highest levels of β-catenin, with decreasing levels in the FAK^0 LSCs ($n = 3$) and the lowest levels in the FAK^- LSCs ($n = 5$), which were indistinguishable from β-catenin levels measured in normal HSCs.

To investigate the possibility of a causal relationship between FAK and β-catenin expression, we used a siRNA strategy (Supporting Information Fig. S1C). As shown in Figure 1E and Supporting Information Figure S1D, depletion of either FAK or Pyk2 had no effect on β-catenin expression in normal HSCs or FAK^- LSCs. In contrast, siRNA treatment

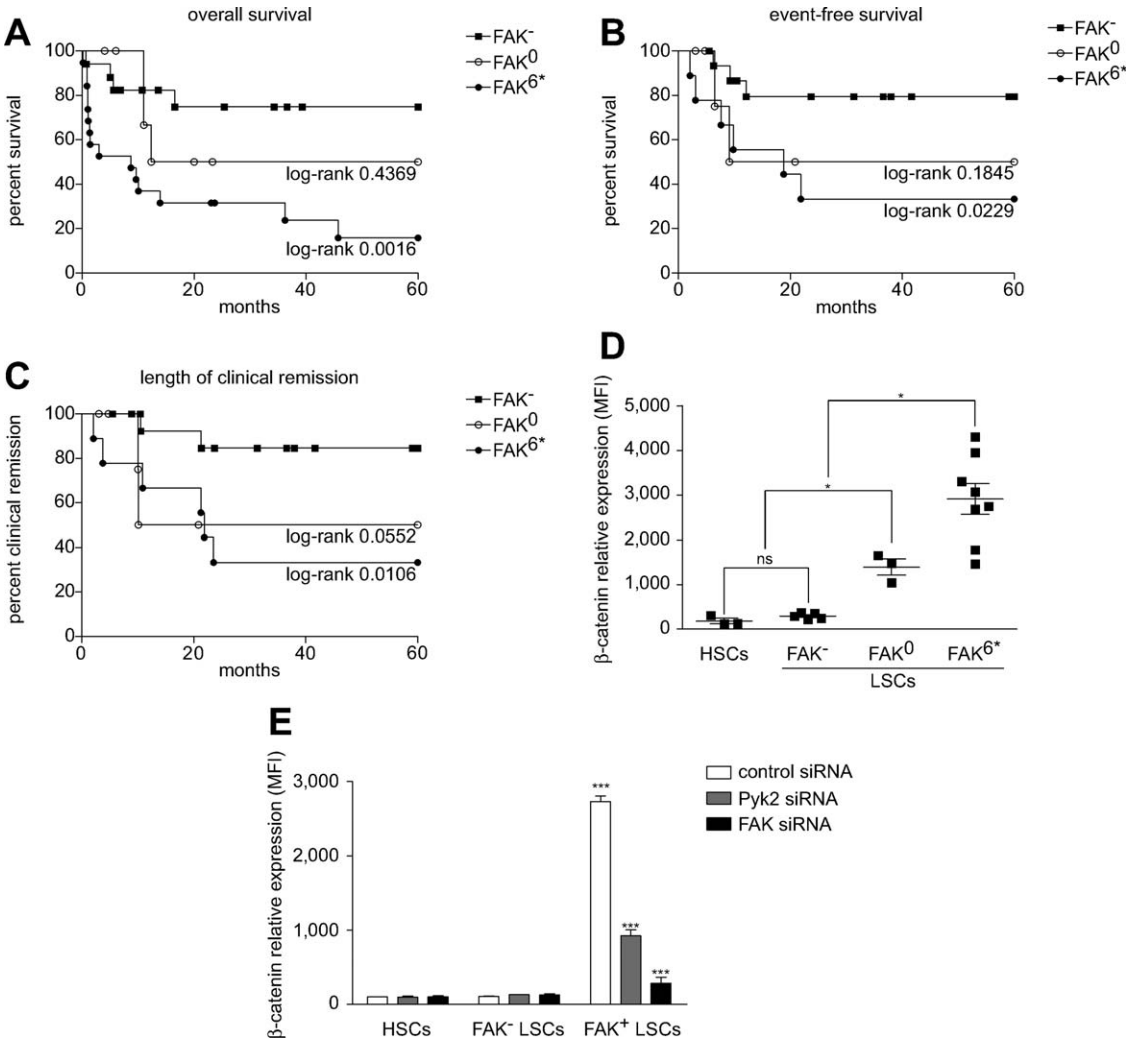

Figure 1. Correlation of FAK variant expression with acute myeloid leukemia (AML) clinical outcome and β-catenin overexpression. Kaplan-Meier analysis of overall survival **(A)**, event-free survival **(B)**, and length of clinical remission **(C)**, for AML patients grouped according to their FAK expression profiles (FAK^-: $n = 23$; FAK^0: $n = 8$; FAK^{6*}: $n = 17$). **(D):** β-Catenin relative expression (expressed as MFI—measured by flow cytometry) in normal HSCs or AML LSCs from FAK^-, FAK^0, and FAK^{6*} AML samples. Each dot represents one sample. **(E):** Relative expression of β-catenin (MFI) in HSCs from healthy donors and in AML LSCs from FAK^- and FAK^+ AML samples 48 hours after transfection with control, Pyk2, or FAK siRNA. Data are expressed as mean ± SEM of three different primary samples. Statistical comparison to HSCs, to control siRNA or as indicated (D, E): ns nonsignificant; *, $p < .05$; ***, $p < .005$. Abbreviations: FAK, focal adhesion kinase; HSC, hematopoietic stem cell; LSC, leukemic stem cell; MFI, mean fluorescence intensity; siRNA, small interfering RNA.

significantly decreased β-catenin expression in FAK^+ LSCs. Importantly, the FAK-dependent regulation of β-catenin expression was specific to the $CD45^{lo}CD34^{hi}CD38^{-/lo}CD123^+$ cells when compared to other subpopulations of AML cells from both FAK^0 and FAK^{6*} AML samples (Supporting Information Fig. S1E). qPCR analysis of *c-MYC*, *CYCLIN D1*, *VEGF*, *BCLXL*, and *BCL2* expression in sorted FAK^+ LSCs treated (or not) with FAK or β-catenin siRNAs demonstrated that expression of these genes is controlled by the activities of both FAK and β-catenin (Supporting Information Table S3).

FAK Increases β-Catenin Transcriptional Activity in Primitive AML Cells

We then asked whether FAK also increases β-catenin activity in FAK^+ primitive AML cells (from FAK^{6*} AML samples) by comparing the subcellular localization of β-catenin in sorted HSCs and LSCs (Fig. 2A) after siRNA-induced FAK or Pyk2 depletion. After transfection of these cells with control siRNA, β-catenin was mostly cytoplasmic in normal HSCs and FAK^- LSCs (Fig. 2B). In contrast, β-catenin was mostly nuclear in FAK^+ LSCs, but its location shifted to the cytoplasm after FAK or Pyk2 depletion in FAK^+ LSCs (Fig. 2B; Supporting Information Fig. S2A). To determine whether the nuclear localization of β-catenin seen in control FAK^+ LSCs is associated with an activation of transcription, a TCF reporter construct was cotransfected into the cells together with the FAK or Pyk2 or control siRNA. We found the reporter activity to be more than sixfold higher in control FAK^+ LSCs than in normal HSCs or FAK^- LSCs (Fig. 2C). This difference was eliminated by siRNA depletion of FAK or Pyk2 in the FAK^+ LSCs (Fig. 2C). In addition, we found that the transcription of *c-MYC*, another known target of β-catenin that contributes to HSC self-renewal [29], was higher in FAK^+ LSCs as compared to normal HSCs and FAK^- LSCs (Supporting Information Fig. S2B), and in a FAK- or Pyk2-dependent fashion (abolished by FAK or Pyk2 siRNA treatment; Supporting Information Fig. S2B). Taken together, these results show that β-catenin transcriptional activity is

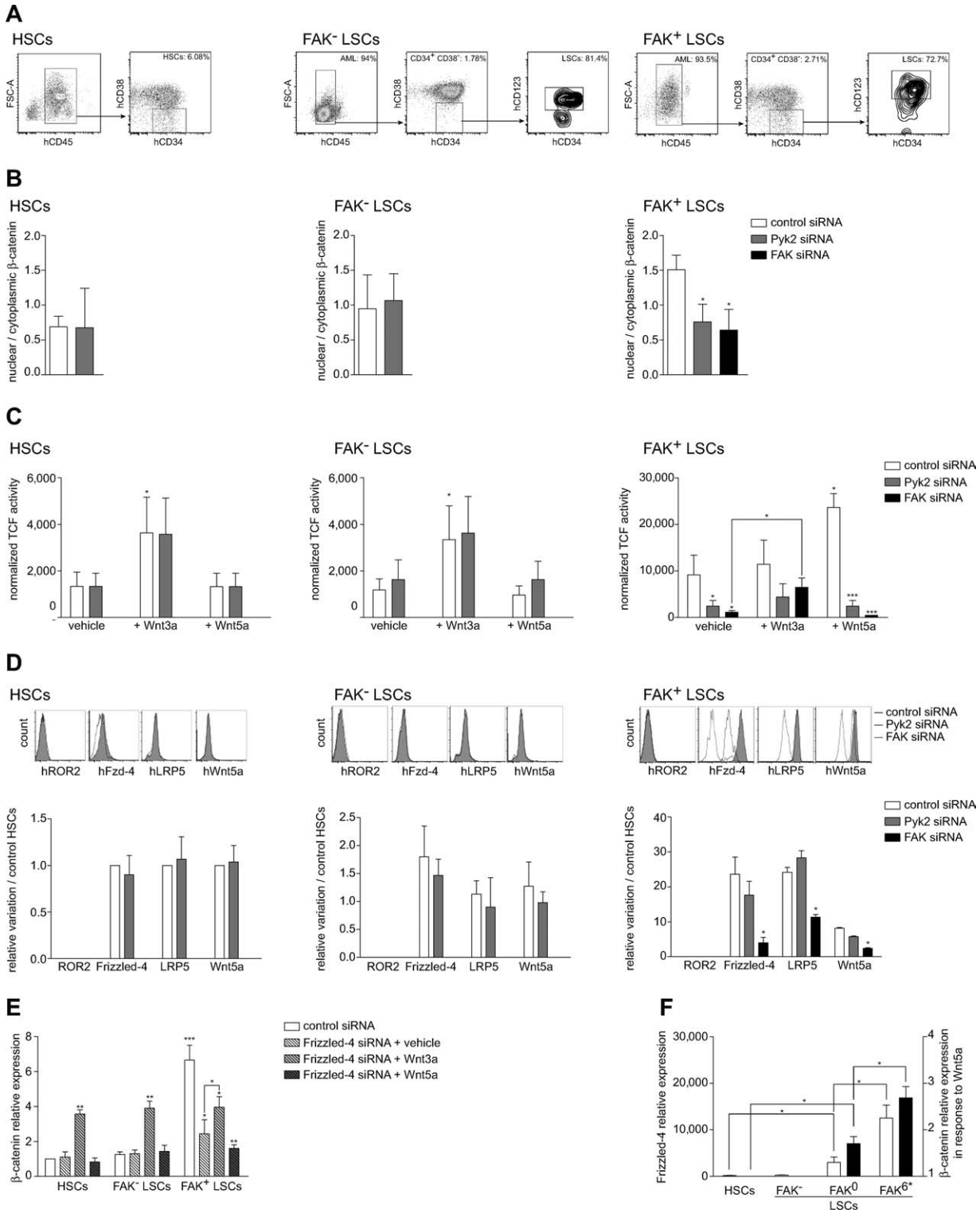

Figure 2. FAK-dependent β-catenin hyperactivation through Wnt5a/Frizzled-4/canonical signaling. Experiments were performed on sorted stem cell-enriched populations (**A**, $CD45^{hi}CD34^{hi}CD38^{-/lo}$ HSCs; $CD45^{lo}CD34^{hi}CD38^{-/lo}CD123^{+}$ FAK^{-} LSCs; $CD45^{lo}CD34^{hi}CD38^{-/lo}CD123^{+}$ FAK^{+} LSCs) whose representative gating schemes obtained by flow cytometry are shown. In some experiments, whole cell samples were transfected 48 hours before experiments by control, Pyk2, FAK, or Frizzled-4 siRNA and further treated by Wnt3a or Wnt5a. **(B, C):** FAK induces β-catenin nuclear accumulation and transcriptional activity, and canonical response to Wnt5a. Nuclear–cytoplasmic ratio of β-catenin and TCF activity (calculated as the average ratio of TCF renilia/control firefly luciferase luminescence) in the absence (vehicle) or presence of Wnt3a or Wnt5a are shown. **(D):** FAK induces overexpression of Frizzled-4, LRP5, and Wnt5a. Representative (upper) and average expression (lower, calculated as a fold-variation normalized to the measurement in HSCs arbitrarily set at one) of ROR2, Frizzled-4, LRP5, and Wnt5a. **(E):** Frizzled-4 is necessary for β-catenin overexpression and canonical response to Wnt5a. Relative expression of β-catenin (normalized to β-catenin mean fluorescence intensity [MFI] in control HSCs arbitrarily set at one) in HSCs and LSCs treated by Frizzled-4 siRNA and Wnt3a or Wnt5a. (**F):** Wnt5a-induced canonical response is conditioned by FAK variant expression. Frizzled-4 expression (MFI, open bars) and β-catenin accumulation in response to Wnt5a (MFI of β-catenin in Wnt5a-treated conditions normalized to vehicle-treated conditions, closed bars) in HSCs or LSCs from FAK^{-}, FAK^{0}, and FAK^{6*} acute myeloid leukemia. Data are expressed as mean ± SEM of three different primary samples. Statistical comparison to control siRNA (B), to vehicle or to control siRNA or as indicated (C), to control siRNA (D), to HSCs or to control siRNA or as indicated (E): *, $p < .05$; **, $p < .01$; ***, $p < .005$. Abbreviations: FAK, focal adhesion kinase; FSC, forward scatter; HSC, hematopoietic stem cell; LSC, leukemic stem cell; siRNA, small interfering RNA; TCF, T-cell factor.

enhanced in FAK^+ primitive AML cells via a mechanism that requires both FAK and Pyk2.

FAK^+ Primitive AML Cells Redirect Wnt Signaling to Deregulate β-Catenin Activation

The PI3K/Akt/GSK3β pathway is a major signaling pathway activated downstream of FAK and has been implicated in the regulation of β-catenin activity in malignant cells [30, 31]. Pharmacological inhibition of FAK in FAK^0 or FAK^{6*} AML samples decreased both phosphorylation of Akt and β-catenin expression, whereas PI3K inhibitors did not decrease β-catenin levels (Supporting Information Fig. S2C). Thus activation of the PI3K/Akt pathway is not responsible for the increased levels of β-catenin seen in FAK^+ primitive AML cells.

Since Wnt signaling is another regulator of β-catenin activity [24], we studied the expression and transcriptional activity of β-catenin in sorted HSCs and LSCs after their stimulation with either Wnt3a or Wnt5a (which are thought to activate canonical and noncanonical Wnt signaling pathways, respectively). We found that β-catenin expression and activity were both stimulated by Wnt3a but not Wnt5a in normal HSCs and FAK^- LSCs (Fig. 2C; Supporting Information Fig. S2D) as previously reported [32, 33]. However, we were surprised to find that Wnt3a did not significantly increase either the expression or activity of β-catenin in FAK^+ LSCs from FAK^{6*} AML samples, in spite of the ability of Wnt5a to elicit this response (Fig. 2C; Supporting Information Fig. S2D). This switch was accompanied by an increase in *c-MYC* mRNA level (as shown by reverse transcription PCR [RT-PCR]; Supporting Information Fig. S2E) without any change in JNK phosphorylation (Supporting Information Fig. S2F), consistent with activation of the Wnt canonical pathway. Importantly, this switch was also abolished and responsiveness to Wnt3a restored upon siRNA-mediated depletion of FAK or Pyk2 (Fig. 2C; Supporting Information Fig. S2D).

Wnt5a Activation of β-Catenin by FAK-Dependent Overexpression of Frizzled-4/LRP5

A role for Wnt5a in promoting the invasive properties of cancer cells has been attributed to the activation of a noncanonical ROR2-dependent signaling pathway [34–37]. However, Mikels and Nusse [38] have demonstrated that Wnt5a can also act as a canonical agonist when the Wnt receptors Frizzled-4 and LRP5 are coexpressed. Interestingly, evidence of elevated expression of Frizzled-4 in AML cells has been reported [39]. We therefore next investigated whether Frizzled-4 expression was also upregulated in primitive AML cells and, if so, how this might relate to FAK expression in the same cell populations.

Expression of ROR2 receptors, Frizzled-4, LRP5, and Wnt5a was low in normal HSCs and FAK^- LSCs, in contrast to FAK^+ LSCs, where much higher levels of Frizzled-4, LRP5, and Wnt5a were detected (Fig. 2D). Moreover, siRNA-mediated depletion of FAK (not of Pyk2) in FAK^+ LSCs from FAK^0 or FAK^{6*} AML samples decreased the upregulated expression of both Wnt5a and its receptors (Fig. 2D; Supporting Information Fig. S2G) indicating how FAK expression may specifically redirect Wnt signaling. This result also suggested that FAK is epistatic to Pyk2 in this regard. To confirm that Frizzled-4 is the receptor that enables Wnt5a to induce β-catenin signaling, we studied β-catenin levels in LSCs treated with Frizzled-4 siRNAs (Supporting Information Fig. S1F). This treatment abolished Wnt5a sensitivity and normalized β-catenin levels (Fig. 2E). Interestingly, Frizzled-4 downregulation was associated with a recovery of Wnt3a sensitivity, suggesting that FAK and Frizzled-4 expression are sufficient to deregulate β-catenin expression and activity (Fig. 2E).

Since we had found that β-catenin expression correlates with the expression of FAK variants, we then analyzed the impact of expression of different FAK variants on the stabilization of β-catenin in response to Wnt5a (Fig. 2F). We found the Wnt5a response to be highest in LSCs expressing FAK^{6*} variants, less in FAK^0 LSCs, and lowest and equivalent in HSCs and FAK^- LSCs (Fig. 2F). Of note, FAK^{6*} cells expressed higher levels of Frizzled-4 than FAK^0 cells (Fig. 2F).

Y579-Phosphorylated Pyk2 Facilitates the Endocytosis of the Wnt5a/Frizzled-4/LRP5 Complex

FAK-dependent activation of Grb2 has been shown to contribute to the activation of β-catenin [40] and phosphorylation of the Y881 residue on Pyk2 also activates Grb2. Moreover, pY881 Pyk2 has been shown to be a binding site for c-Abl that interferes with β-catenin signaling in leukemia [41]. Since we had previously found that Pyk2 in FAK^+ AML blasts exhibits specific Y881 phosphorylation [14], we undertook further experiments to determine whether and how Pyk2 phosphorylation might contribute to its role in Wnt signaling deregulation.

We first confirmed that Pyk2 is phosphorylated on Y881 in FAK^+ AML bulk cells from FAK^{6*} AML samples (Fig. 3A). In contrast, we found Y881 phosphorylation is specifically downregulated in FAK^+ LSCs as compared to FAK^- LSCs and normal HSCs (Fig. 3A; Supporting Information Fig. S3A). Conversely, Pyk2 phosphorylation on its Y579 residue, a residue substrate of Src kinase and involved in maximal activation of Pyk2 [1], was detected only in FAK^+ LSCs (Fig. 3A; Supporting Information Fig. S3A). To investigate whether these differences in Pyk2 phosphorylation are dependent upon FAK and/or early stages of Wnt signaling, we treated FAK^+ LSCs from FAK^{6*} AML samples with FAK and Frizzled-4-specific siRNAs and then assessed the effects on Pyk2 Y579 and Y881 phosphorylation. Pyk2 phosphorylation on Y579 was partially but specifically reversed and phosphorylation of Y881 residue was restored in LSCs treated with either siRNA (Fig. 3B; Supporting Information Fig. S3A). Since depletion of FAK or Frizzled-4 also abolished the sensitivity to Wnt5a-induced signaling, these results suggested that phosphorylation of Pyk2 Y579 might be involved in the mechanism by which Wnt5a triggers a canonical signaling response specifically in FAK^+ primitive AML cells.

Both clathrin- and caveloae-dependent endocytosis of Frizzled receptors have been shown to play a critical role in regulating the Wnt pathway [42, 43]. We therefore hypothesized that Pyk2 might be involved in regulating the endocytosis of the Wnt5a/Frizzled-4/LRP5 complex required for downstream activation of β-catenin. Immunoprecipitation experiments in a model cell line provided proof-of-principle evidence of an interaction of pY579 Pyk2 with Frizzled-4 (Supporting Information Fig. S3B). We then analyzed the phosphorylation and recruitment of pY579 Pyk2 by Frizzled-4 in FAK^+ LSCs from FAK^{6*} AML samples. Wnt5a but not Wnt3a stimulation was associated with a marked increase in Pyk2 Y579 phosphorylation (Fig. 3C; Supporting Information Fig. S3C). Analysis by confocal microscopy (Supporting Information Fig. S3C) showed that Frizzled-4 was localized homogeneously at the plasma membrane under steady-state conditions. However, 30 minutes after Wnt5a stimulation, Frizzled-4 was clustered and colocalized with pY579 Pyk2 both at the plasma membrane and in the cytoplasm. A measureable decrease in Frizzled-4 in the cell membrane following exposure of FAK^+ LSCs to Wnt5a, but not Wnt3a, was also

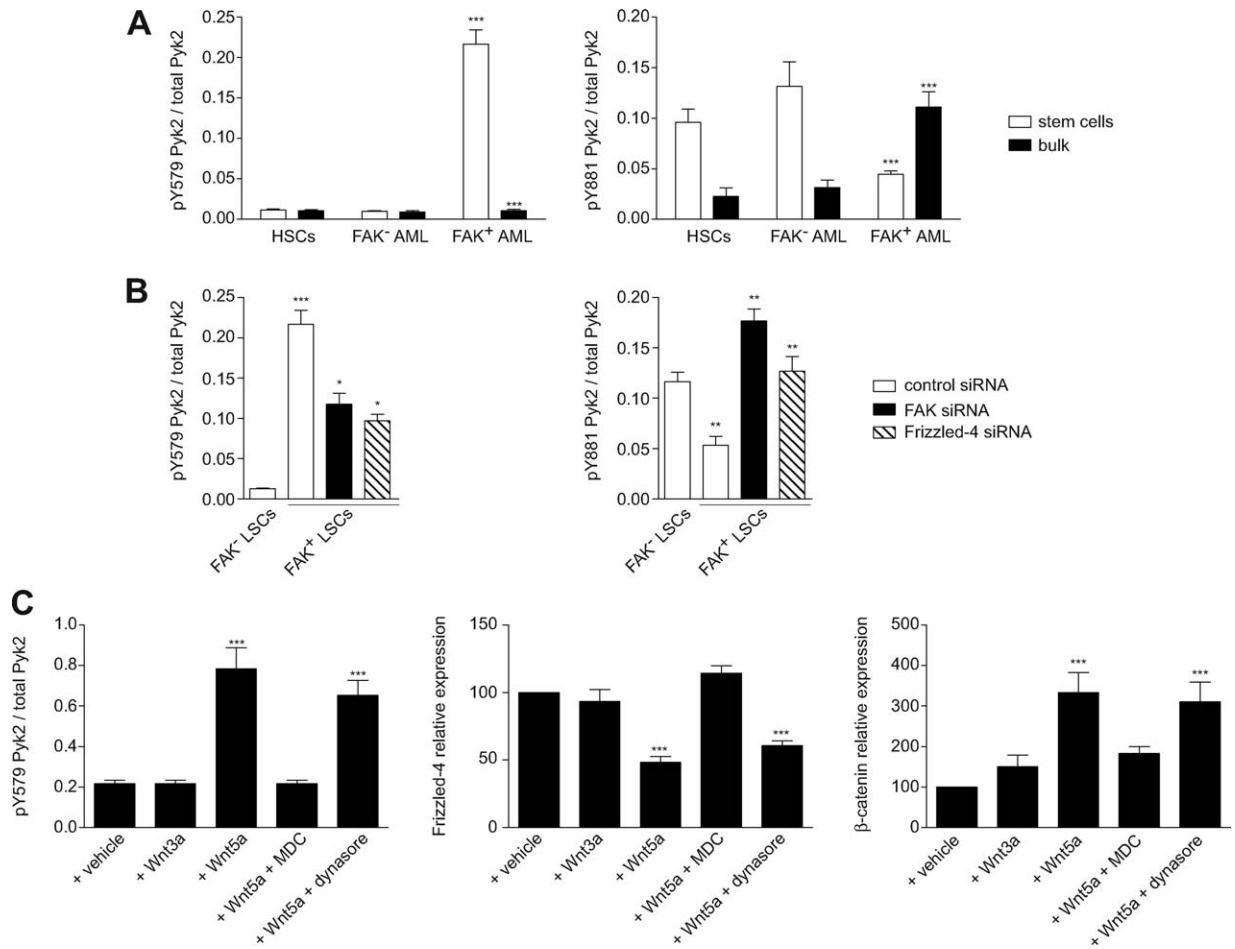

Figure 3. FAK-induced phosphorylation of Pyk2 on Y579 is involved in endocytosis and canonical signaling of Wnt5a/Frizzled-4. **(A):** Pyk2 is specifically phosphorylated on Y579 in FAK^+ LSCs. Average phosphorylation of Pyk2 on residues Y579 and Y881 (ratio of phospho-Pyk2 mean fluorescence intensity [MFI]/total Pyk2 MFI) in HSCs, and in FAK^- and FAK^+ LSCs/bulk. **(B):** FAK and Frizzled-4 are necessary for Y579 Pyk2 phosphorylation. Average phosphorylation of Pyk2 on residues Y579 and Y881 (expressed as MFI of pPyk2 normalized by MFI of total Pyk2) in control, FAK, or Frizzled-4 siRNA transfected FAK^+ LSCs and in FAK^- LSCs. **(C):** pY579 Pyk2 is correlated with Wnt5a-mediated canonical signaling. Average quantification of pY579 Pyk2 (ratio of pY579 Pyk2 MFI/total Pyk2 MFI, left), average quantification of Frizzled-4 extracellular expression (center), and average β-catenin expression (right) in FAK^+ LSCs treated by Wnt3a or Wnt5a and clathrin endocytosis inhibitor (MDC) or caveolae endocytosis inhibitor (dynasore). Data are expressed as mean ± SEM of three different primary samples. Statistical comparison to HSCs or to stem cell subpopulation (A), to FAK^- LSCs or to control siRNA (B), to vehicle (C): *, $p < .05$; **, $p < .01$; ***, $p < .005$. Abbreviations: AML, acute myeloid leukemia; FAK, focal adhesion kinase; HSC, hematopoietic stem cell; LSC, leukemic stem cell; MDC, monodansylcadaverin; siRNA, small interfering RNA.

detected by flow cytometry (Fig. 3C; Supporting Information Fig. S3D). Note that monodansylcadaverin, an inhibitor of the clathrin-mediated endocytosis pathway, but not dynasore (caveolin inhibitor), blocked endocytosis of the Frizzled-4/pY579 Pyk2 complex, significantly decreased Wnt5a-dependent phosphorylation of Pyk2, and abolished β-catenin accumulation in the nucleus (Fig. 3C; Supporting Information Fig. S3C). Together, these data suggest that FAK induces the specific phosphorylation of Pyk2 on Y579, which in turn allows it to interact with the Wnt5a/Frizzled-4/LRP complex and facilitate its endocytosis in a clathrin-dependent fashion. Endocytosis of this complex appears to be essential to the downstream activation of β-catenin.

Enforced Expression of FAK Variants Induces a Leukemic Phenotype in HSCs

We then investigated the consequences of overexpressing different FAK variants in normal $CD34^+$ cells on their proliferation/differentiation behavior and Wnt/β-catenin signaling. For this purpose, $CD34^+$ cells from normal human bone marrow samples were transduced with a pTRIP-IRES-GFP lentiviral vector [26] encoding either the FAK^0or FAK^6 cDNA [7] (Fig. 4A). Transduction efficiencies of more than 75% were determined from flow cytometric measurements of GFP- and FAK-positive cells a few days later (Fig. 4A). The normal human bone marrow $CD34^+$ cells that had been transduced with either FAK^0 or FAK^6 showed increased proliferation (Fig. 4B) and clonogenic activity (Fig. 4C) as compared to cells transduced with the control vector. Moreover, assessment of the type of colonies obtained from the FAK^0 or FAK^6-transduced cells showed that a much higher proportion of these were granulopoietic with a marked decrease in colonies derived from erythroid burst-forming unit (Fig. 4D). Importantly, FAK-transduced $CD34^{hi}CD38^{-/lo}$ cells displayed increased expression of both β-catenin and Frizzled-4 and a switch from the Wnt3a- to Wnt5a-controlled canonical pathway (Fig. 4E, 4F). *c-MYC* and *WNT5a* expression were

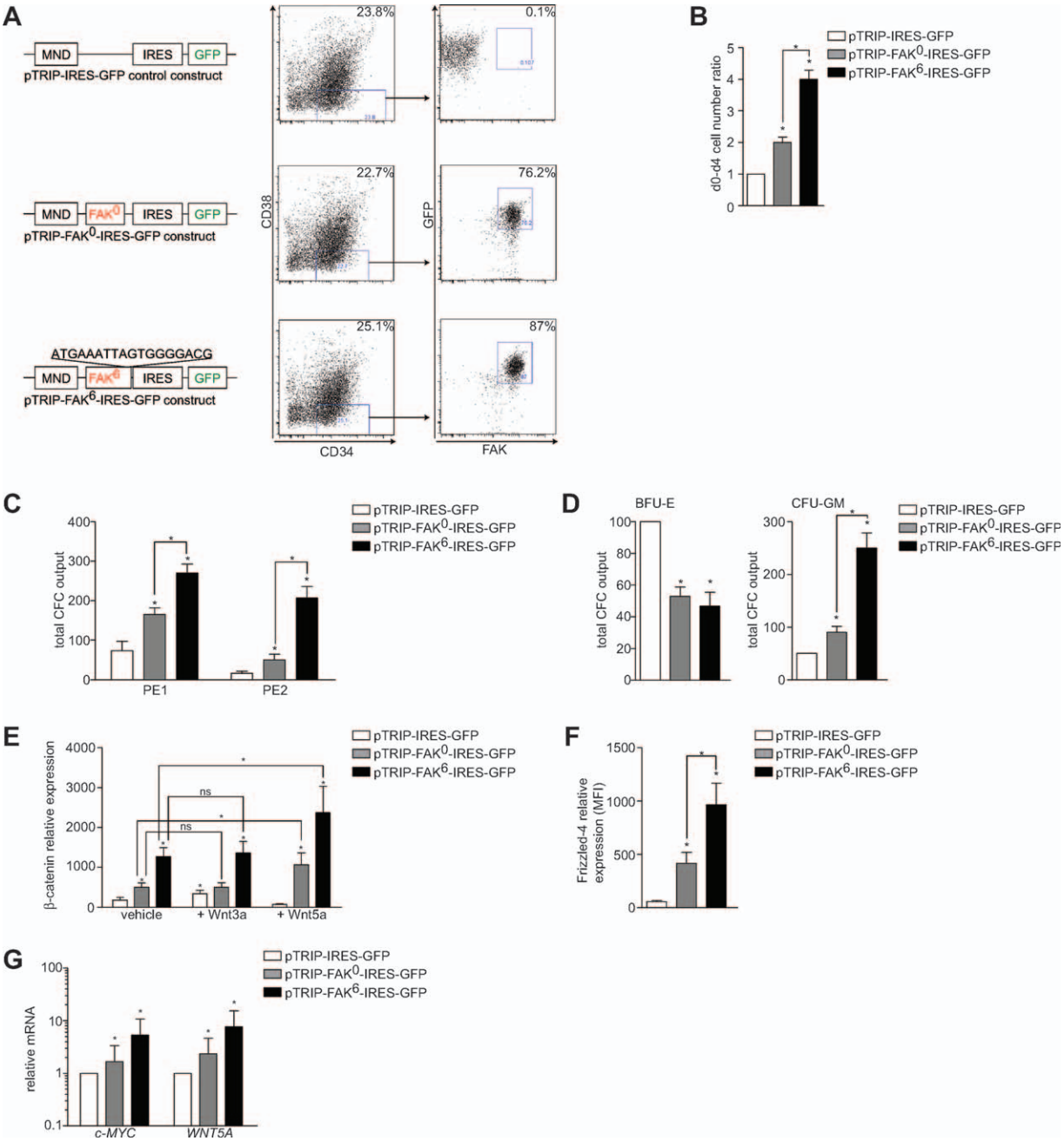

Figure 4. Overexpression of FAK variants in normal CD34$^+$ cells induces features of leukemic cells in vitro. CD34$^+$ normal bone marrow cells were, respectively, transduced by pTRIP-IRES-GFP (control), pTRIP-FAK^0-IRES-GFP, or pTRIP-FAK^6-IRES-GFP lentiviral constructs and their clonogenic/differentiation potential and Wnt/β-catenin signaling pathway were analyzed. **(A):** FAK variants lentiviral constructs and transduction efficacy of CD45^{hi}CD34^{hi}CD38$^{-/lo}$ cells. **(B):** FAK variants induce increased proliferation of CD34$^+$ cells. **(C):** FAK$^+$ CD34$^+$ cells have increased clonogenic activity. **(D):** FAK$^+$ CD34$^+$ cells display abnormal differentiation. **(E):** FAK$^+$ CD45^{hi}CD34^{hi}CD38$^{-/lo}$ cells switch to Wnt5a-controlled canonical pathway. **(F):** FAK$^+$ CD45^{hi}CD34^{hi}CD38$^{-/lo}$ cells overexpress Frizzled-4. **(G):** FAK$^+$ CD34$^+$ cells overexpress *c-MYC* and *WNT5a* mRNA. Data are expressed as mean ± SEM of three independent experiments. Statistical comparison to control vector or as indicated: ns, non significant; *, $p < .05$. Abbreviations: BFU-E, erythroid burst-forming unit; CFC, colony-forming cells; CFU-GM, colony-forming unit-granulocyte-macrophage; FAK, focal adhesion kinase; GFP, green fluorescent protein; IRES, internal ribosome entry site; MFI, mean fluorescence intensity.

increased in FAK-transduced normal CD34$^+$ cells as shown in Figure 4G. Overall, enforced expression of FAK variants in HSCs recapitulated the Wnt-deregulated phenotype seen in FAK$^+$ primitive AML cells, the FAK^6 isoform being the most efficient.

FAK Expression and β-Catenin Activation Are Essential to the Maintenance of Primitive AML Cells

To further address the biological importance of FAK-dependent activation of β-catenin in FAK$^+$ primitive AML cells, we

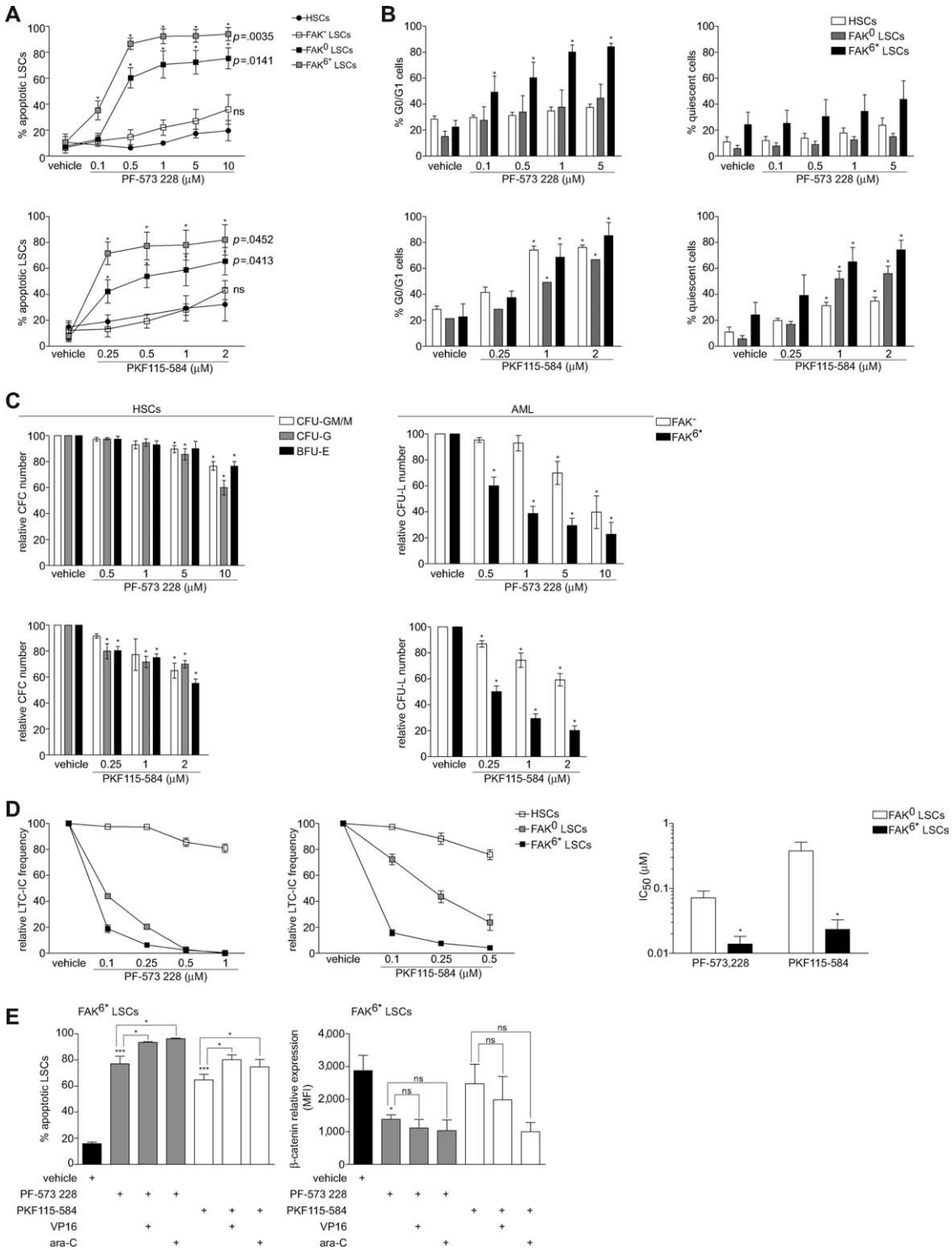

Figure 5. FAK and β-catenin are essential to the stem cell phenotype of FAK$^+$ AML LSCs. Whole primary samples were treated with FAK inhibitor (PF-573228) or β-catenin inhibitor (PKF 115-584) at different concentrations. Data are expressed as mean ± SEM of six different primary samples (statistical comparison to vehicle *, $p < .05$ or to HSCs as indicated). **(A):** FAK and β-catenin inhibition lead to apoptosis of FAK$^+$ LSCs. The average percent of apoptotic cells (calculated as the percent of Annexin V-fluorescein isothiocyanate (FITC)$^+$, 7-aminoactinomycin D (7-AAD)$^-$ LSCs) treated by FAK or β-catenin inhibitors is represented. **(B):** FAK and β-catenin both regulate proliferation of FAK$^+$ LSCs. The average percent of nonproliferating cells (calculated as the percent of cells in G_0/G_1 phase from 5-ethynyl-2 deoxyuridine/4′-6-diamidino-2-phenylnidole (EdU/DAPI) staining), and the average percent of quiescent cells (calculated as the percent of G_0 phase from Hoechst/pyronin-Y staining) treated by FAK or β-catenin inhibitors is represented. **(C):** FAK and β-catenin inhibition leads to reduced clonogenic activity in FAK$^+$ AML samples. The relative number of colonies (calculated as the percent of colonies in the vehicle assay) formed by normal or AML samples treated by FAK or β-catenin inhibitors is shown. **(D):** FAK and β-catenin inhibitors suppress CFC output from FAK$^+$ AML LTC-ICs. Sorted LSCs were treated with FAK or β-catenin inhibitors and then seeded in limiting dilution assay on MS5 feeder cells. Results are expressed as the LTC-IC frequency relatively to untreated condition and average IC_{50} calculated as the dose responsible for a 50% decrease of LTC-IC frequency from three different AML samples. Statistical comparison to FAK^0 LSCs: *, $p < .05$. **(E):** Characterization of FAK$^+$ LSCs surviving to FAK or β-catenin inhibition. FAK$^+$ AML samples were treated with FAK or β-catenin inhibitors (0.25 μM) for 24 hours and further incubated with VP16 or ara-C (1 μM, 24 hours). LSCs were analyzed for apoptosis and β-catenin expression after each treatment. Data are expressed from three different AML samples. Statistical comparison to vehicle or as indicated: ns non significant; *, $p < .05$; **, $p < .01$; ***, $p < .005$. Abbreviations: AML, acute myeloid leukemia; BFU-E, erythroid burst-forming unit; CFC, colony-forming cells; CFU, colony-forming unit; FAK, focal adhesion kinase; HSC, hematopoietic stem cell; LSC, leukemic stem cell; LTC-IC, long-term culture-initiating cell; MFI, mean fluorescence intensity.

tested the effect of treating FAK^+ LSCs with pharmacological agents that specifically inhibit the kinase activity of FAK (PF-573228) [44], or that block the ability of β-catenin to interact with TCF (PKF 115-584) [45]. The efficacy of these two inhibitors was confirmed by testing the tyrosine phosphorylation of paxillin, which depends on FAK ([46]; Supporting Information Fig. S4A), and the coimmunoprecipitation of β-catenin and TCF (Supporting Information Fig. S4B) in model cells. A brief exposure of FAK^+ LSCs to low doses of the same inhibitors (for 4 hours) led to a massive induction of cell death whereas both HSCs and FAK^- LSCs were spared when similarly treated (Fig. 5A). The FAK inhibitor also specifically blocked the proliferation of FAK^{6*}, but not FAK^0 LSC proliferation, whereas the β-catenin inhibitor also blocked the proliferation of FAK^0 LSCs (Fig. 5B). This indicates a biological difference in the FAK requirement for β-catenin activation in primitive AML cells that show differences in FAK variant expression.

We also examined the effects of these inhibitors on specific subsets of functionally defined cells within the FAK^+ primitive AML cells. The same brief treatment protocol followed by assessment of surviving AML CFCs showed these were highly sensitive to both inhibitors (Fig. 5C). Assessment of effects of these inhibitors on more primitive, functionally defined, AML cells was performed by measuring the post-treament output of AML CFCs in a 6-week LTC-IC assay. Both FAK and β-catenin inhibitors strongly decreased this AML LTC-IC activity (Fig. 5D) providing further support to the idea that FAK/β-catenin signaling is essential for the survival of the most primitive leukemic subsets. Interestingly, and as predicted by previous studies of the kinase activity of FAK variants, FAK^6 cells were again more sensitive to FAK and β-catenin inhibition.

Notably, inhibition of β-catenin, but not of FAK, also appeared to promote LSC quiescence (Fig. 5B). Characterization of the LSCs surviving treatment with either the FAK or β-catenin inhibitor at concentrations close to their IC_{50}s (Fig. 5E) showed that the LSCs surviving β-catenin inhibition were less sensitive to VP16 or ara-C treatment and were killed with a reduced efficacy (80%/75% apoptotic LSCs with β-catenin inhibition plus VP16/ara-C treatment vs. 90%/95% with FAK inhibition plus VP16/ara-C treatment). Moreover, the apoptosis induced by FAK inhibition followed by drug treatment was specifically accompanied by a strong decrease of β-catenin expression. Overall these data show that the FAK/β-catenin pathway may represent an interesting target to eradicate FAK^+ primitive AML cells.

Discussion

The Importance of FAK Variant Expression in Human Primitive AML Cells

AMLs comprise a genetically and biologically heterogeneous group of malignant hematopoietic diseases. Much evidence suggests that the malignant clone originates from the initial transformation of a primitive hematopoietic cell although a fully leukemic phenotype may occur in cells with properties of late "progenitors" that acquire stem cell properties. Thus, the property of indefinite and deregulated growth appears to remain vested in a rare subset of phenotypically distinct LSCs. These LSCs have attracted much attention as the cells that need to be eliminated to achieve cures.

Given our previous evidence that deregulated FAK expression is associated with a poor outcome in AML, we designed the present studies with the following dual objectives. The first was to test the hypothesis that abnormal FAK expression contributes to the abnormal growth and/or survival properties of primitive AML cells. The second was to obtain a further understanding of the pathways that might be deregulated with the expectation that such knowledge would facilitate the design of therapeutic strategies that could be more effective against LSCs than conventional chemotherapy. From a broad survey of primary AML samples, we confirmed and extended our previous observations to obtain evidence of upregulated expression of variant isoforms of FAK as a new feature of poor prognosis AML patients. In addition, we show that the kinase activity of abnormally expressed FAK isoforms in primitive AML cells is crucial for their functional integrity in vitro. Specifically, we found that a FAK inhibitor that displays a 50–250-fold selectivity for FAK over other protein kinases, including Pyk2, and efficiently targets FAK kinase activity, prevented the hyperactivation of canonical Wnt signaling. FAK^6, the most active FAK isoform, was also the most sensitive isoform to this inhibitor. Thus FAK appears to be a key determinant of FAK^+ primitive AML cells maintenance, in line with what has been reported for breast cancer stem cells [47]. Furthermore, it is likely that this role of FAK is enhanced with increasing expression of variant isoforms leading to a constantly activated form of FAK [9].

The Dependence of Primitive AML Cells Function on FAK Variant Expression Involves a Switch in Wnt Signaling and Activation of β-Catenin

FAK activation in other systems has most often been described as an event that occurs downstream of the activation of the noncanonical Wnt pathway leading to activation of PI3K/Akt. However, we did not find evidence of a crosstalk between the PI3K/AKT and Wnt pathways in FAK^+ primitive AML cells, possibly due to differences in the pool of GSK3β present [48]. What we found was a higher expression of β-catenin and its greater activity in unstimulated FAK^+, as compared to FAK^- LSCs (or normal HSCs, which are also FAK^-). We also found that FAK^+ LSCs have a unique ability to activate a Wnt5a-mediated canonical signaling pathway (Fig. 6). It has been suggested that Frizzled-4 and LRP5 are key determinants of Wnt5a-induced canonical signaling [38], and Frizzled-4 overexpression has been previously reported in FLT3-ITD and MLL AML [39, 20]. In FLT3-ITD and MLL AML, the overexpression of Frizzled-4 has been correlated with higher β-catenin activity. Here, we show that expression of both Frizzled-4 and LRP5 as well as Wnt5a are all increased in FAK^+ LSCs. We also show that, forced expression of FAK variants in normal $CD34^+$ bone marrow cells induces an upregulation of Frizzled-4 expression and a switch to a Wnt5a-controlled canonical response as well as an increased clonogenic ability and an abnormal differentiation behavior.

An essential role of Wnt5a in promoting quiescence and HSC maintenance has been proposed in steady-state adult hematopoiesis [49, 50]. However, Wnt5a and Frizzled-4 are also highly expressed during fetal life when the entire HSC pool is rapidly proliferating [51, 52] suggesting that Wnt5a and Frizzled-4 can have opposite effects in different cellular contexts and recreate a similar fetal-like process in AML LSCs [53]. Interestingly, the microenvironmental entity of FAK^+ AML may be characterized by a pro-Wnt5a and proangiogenic pattern [54]. Recently, FAK has been shown to control both the Wnt3a expression and Wnt-β-catenin signaling in the developing neural plate, and this FAK-Wnt3a linkage was also found in human breast cancer cells [55]. Together with our results, these data suggest that FAK activity could widely control the transcription of Wnt-related genes through its cytoplasmic activity regulating directly or not β-catenin,

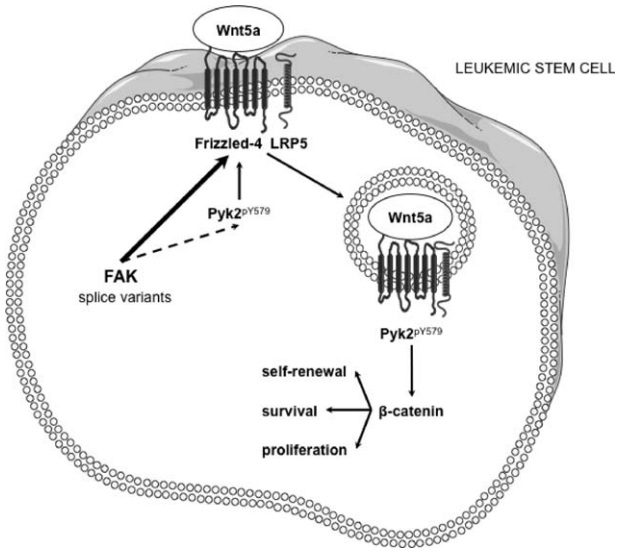

Figure 6. Schematic representation of the Wnt5a-mediated canonical signaling pathway in FAK$^+$ acute myeloid leukemia leukemic stem cells. The potential implication of pY579 Pyk2 in Wnt receptors endocytosis is shown. Abbreviation: FAK, focal adhesion kinase.

and/or its nuclear activity as a modulator of chromatin and transcriptional factors [46].

Our analyses of primitive AML cells also support a role of endocytosis of the Wnt5a/Frizzled-4/LRP5 complex in the enhanced activation of β-catenin. We obtained evidence that endocytosis of this complex in AML LSCs is mediated by a clathrin-dependent mechanism that involves the recruitment of pY579 Pyk2 by Frizzled-4 to the plasma membrane, under Wnt5a stimulation. To date Pyk2 has only been linked to endocytosis in mature cells executing phagocytosis or pathogen internalization but, in this setting, does so through effects on the cytoskeleton [56]. Therefore, we would propose that in AML LSCs pY579 Pyk2 may act downstream of Frizzled-4 to participate in cytoskeleton remodeling and thereby facilitate endocytosis and subsequent activation of canonical signaling (activation of β-catenin) from endocytic vesicles. Importantly, in the absence of FAK, Pyk2 did not display a compensatory role on Wnt deregulation and LSCs maintenance oppositely to FAK-null mammary cancer stem cells where it has a protumorigenic role [47].

Implications for Therapy

This study highlights the dependence of the primitive AML cells from poor prognosis AML on the FAK isoforms they could express and delineate a new pathway that mediates this dependence that culminates in constitutively activated β-catenin. Here, we show that neutralization of either FAK or β-catenin activity strongly decreased LSC survival. However, inhibition of β-catenin also caused the surviving FAK$^+$ LSCs to arrest in G0/G1 and become less sensitive to standard chemotherapeutic drugs. Thus, despite an increasing interest in targeting β-catenin in drug-resistant AML [20], our data suggest that targeting FAK may be more efficacious for killing FAK$^+$ LSCs. Additional agents that would interfere with other critical elements in this mechanism might also be useful. For this, a better understanding of the mechanisms that deregulate FAK expression in primitive AML cells will be useful. NFκB overactivation [57] and, in some cases, p53 inactivation [58], both potential regulators of FAK transcription [59, 60], and known to be features of some AML clones, might explain why FAK is expressed in some LSCs. Understanding the mechanisms of abnormal splicing of FAK, and investigating FAK variant expression in other subsets of AML cells will also be of great interest. Abnormal splicing of Pyk2 has been described in chronic myeloid leukemia (CML), due to alterations of the splicing machinery [61]. It is interesting that in AML (this study) and CML [61], splicing deregulation leads to the expression in hematopoietic cells of FAK and Pyk2 isoforms normally expressed in neurons. We suggest that abnormal expression of FAK splice variants may be a significant factor of poor prognosis in other types of cancers.

Conclusion

This study reports the first evidence that FAK variants are abnormally expressed in AML stem cells and delineates why this could be an important new therapeutic target for poor prognosis patients. The FAK variants deregulate β-catenin signaling through previously undescribed molecular mechanisms, offering a therapeutic target unique to AML stem cells.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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