Cross-talk between Tumor and Endothelial Cells Involving the Notch3-Dll4 Interaction Marks Escape from Tumor Dormancy

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Abstract

The Notch ligand Dll4 has a recognized role during both physiologic and tumor angiogenesis, as it contributes to regulate Notch activity in endothelial cells (EC). The effects of Dll4 on Notch signaling in tumor cells expressing Notch receptors remain, however, largely unknown. Here, we report that escape of human T-cell acute lymphoblastic leukemia (T-ALL) cells or colorectal cancer cells from dormancy is associated with Dll4 expression in the tumor microenvironment and increased Notch3 signaling in tumor cells. Dll4 was expressed at early time points during the angiogenic process, and its expression preceded perfusion of the newly established vessels. Treatment of EC with angiogenic factors induced Dll4 expression and increased Notch3 activation in cocultured T-ALL cells. Neutralization of Dll4 greatly reduced EC-mediated activation of Notch 3 signaling in T-ALL cells and blocked tumorigenesis. Moreover, silencing Notch3 by RNA interference had marked antiproliferative and pro-apoptotic effects on T-ALL cells *in vitro* and reduced tumorigenicity *in vivo*. Our results elucidate a novel mechanism by which a direct interplay between endothelial and tumor cells promotes survival and triggers tumor growth. [Cancer Res 2009;69(4):1314–23]

Introduction

The Notch pathway has also been implicated in a variety of human cancers in connection with the genetic alterations and epigenetic events that lead to either constitutive Notch activation or sensitized response to ligand-induced activation (1–4).

Several components of the Notch pathway are prominently expressed in the vasculature. Of note, Dll4 is an endothelium-specific Notch ligand (5–7), which is expressed in large arteries in the developing embryo, whereas in adult mice its expression is limited to small arteries and microvessels (8). Several studies have highlighted the function of Dll4/Notch signaling in angiogenesis and the mechanism underlying the vascular defects resulting from attenuated Dll4/Notch activity (9–16). Moreover, Dll4 is found expressed in tumor vessels, and several groups have recently reported that perturbations of the Dll4/Notch cross-talk might affect tumor angiogenesis and growth (13–15). These studies, however, focused mainly on endothelial cell (EC)–specific Dll4/Notch signaling, and the possible role of Dll4 in supporting Notch signaling in tumor cells has not been established. Here, we investigated this issue by using experimental models of tumor dormancy, a condition defined by the persistence in the host of malignant cells devoid of angiogenic capacity, without tumor formation (17). The availability of angiogenic and nonangiogenic pairs of malignant cells offers a unique opportunity to investigate whether induction of Dll4 expression marks the angiogenic switch and the possible consequences on Notch signaling in cancer cells. We have recently established a model of T-cell acute lymphoblastic leukemia (T-ALL) cell dormancy (18), which we consider relevant to this aim, because T-ALL cells express Notch receptors and the pathogenetic role of Notch deregulation in T-ALL is firmly established. In fact, activating mutations in Notch1 have been found in >50% of human T-ALL (19–22), and overexpression of the Notch3 protein has been reported in virtually all cases of T-ALL, irrespective of any gross abnormalities in the *Notch3* locus (23). The tumorigenic potential of activated Notch in these cells is also indicated by the development of leukemia in mice recipients of bone marrow precursor cells transduced with Notch1 intracellular domain (ICD; ref. 24) or after enforced Dll4 expression (25) or Notch3 signaling in mice (26).

The findings presented here support the possibility that EC embedded in tissues undergoing angiogenesis may communicate activation signals to leukemia cells mediated by the Notch3-Dll4 molecular interaction, which contribute to switching on an aggressive phenotype. These findings that we corroborated in a carcinoma model indicate that Notch3 activity could be regulated through a microenvironmental interaction and suggest a novel function of angiogenesis in tumors.

Materials and Methods

Cell lines and *in vitro* culture. The human T-lymphoblastic cell lines MOLT-3 and Jurkat were purchased from American Type Culture Collection. TALL1 cells were kindly provided by A. Ferrando (Columbia University). T-ALL cell lines were grown in RPMI 1640 supplemented with 10% FCS and 1% L-glutamine (Life Technologies; complete RPMI). MOLT-3–basic fibroblast growth factor (bFGF) cells have been obtained by retroviral vector-mediated transfer of human bFGF cDNA into parental MOLT-3 cells, as detailed elsewhere (18). GT is a cell line derived from growing MOLT-3 tumors obtained by injection of parental MOLT-3 cells s.c. into nonobese diabetic severe combined immunodeficient (NOD/SCID) mice in the presence of exogenous bFGF (18). The MICOL-14 cell line was derived from a metastatic colorectal cancer and was grown in complete RPMI. MICOL14 cells are not tumorigenic in NOD/SCID mice under the experimental conditions described below; a tumorigenic variant of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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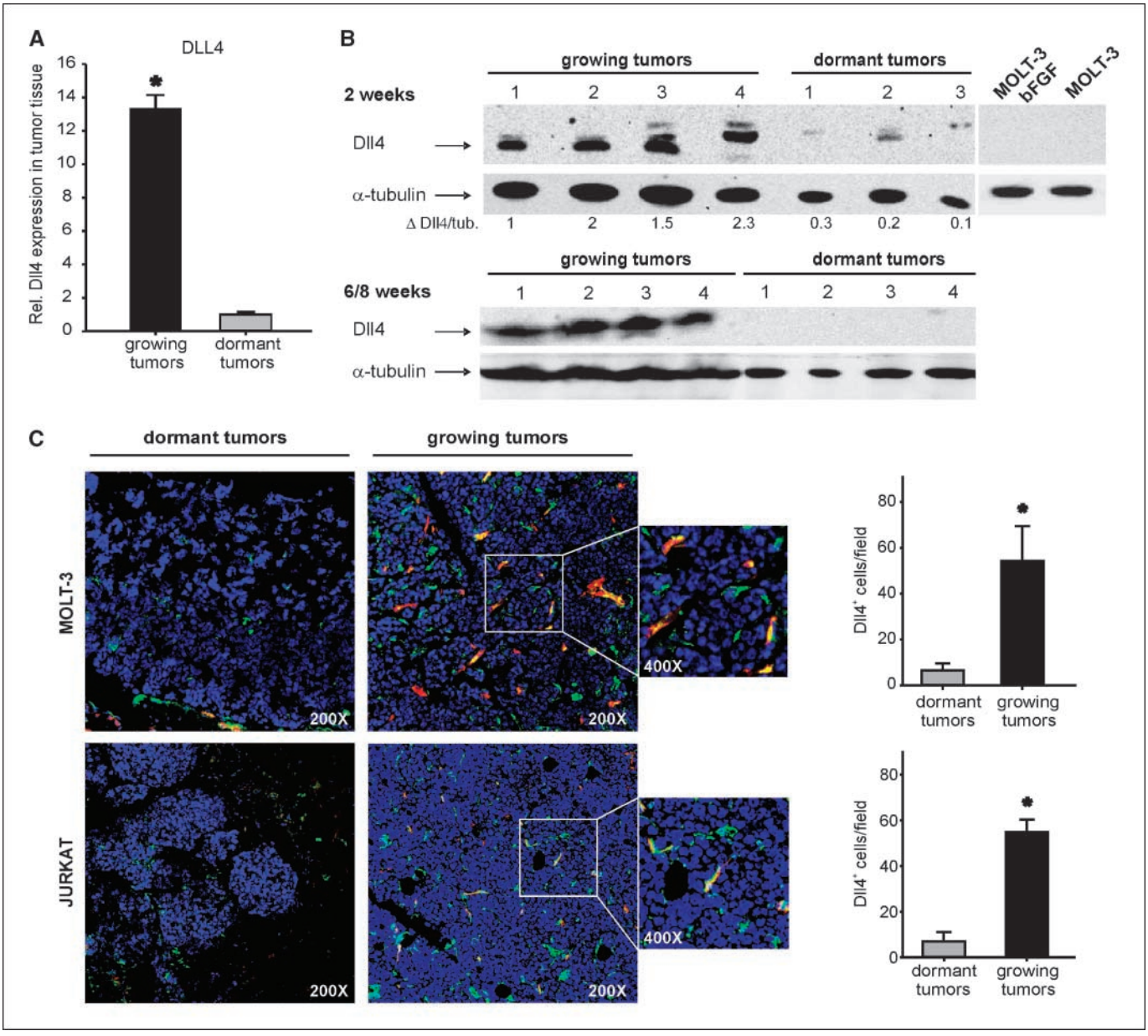

Figure 1. The Notch ligand Dll4 is expressed in growing but not in dormant tumors. *A,* qPCR analysis of murine Dll4 in growing and dormant tumors ($n = 7$ samples per group), 2 wks after inoculation. *Columns,* mean; *bars,* SD. *, $P < 0.001$ compared with dormant tumor value. *B,* Western blot analysis of Dll4 expression in tumors or MOLT-3 and MOLT-3-bFGF cells *in vitro. C,* immunofluorescence analysis of CD31 and Dll4 expression in dormant and growing tumors, 2 wks after inoculation (magnification, 200×; inset, 400×). $Dll4^+$ cells were significantly higher in growing compared with dormant tumors ($n = 4$–8 samples per group). *, $P < 0.05$.

MICOL-14 cells, termed MICOL-14^{tum}, has been obtained from a tumor developed in NOD/SCID mice after s.c. injection of parental MICOL-14 cells in Matrigel plus interleukin-8 (100 ng/mL). The murine dermal microvascular EC line SIEC was obtained from Dr. A. Vecchi (Mario Negri Institute). SIEC cells were cultured in DMEM supplemented with 20% FCS, 1 mmol/L L-glutamine, 1% nonessential amino acids, 1 mmol/L Na pyruvate, EC growth supplement (Sigma-Aldrich), and heparin (100 μg/mL). To show regulation of Notch ligands by angiogenic factors, SIEC cells were first cultured for 48 h in DMEM lacking EC growth supplement and heparin and then for 48 h in DMEM supplemented with vascular endothelial growth factor (VEGF) and bFGF (Peprotech; used at 100 ng/mL). In coculture experiments, 1×10^6 T-ALL cells were layered over subconfluent SIEC cells pretreated for 48 h with angiogenic factors, as detailed above. In some wells, the anti-Dll4 monoclonal antibody (mAb) YM152F was added to the wells (courtesy of Dr. M. Yan, Genentech; final concentration, 2.8 μg/mL; ref. 14). After 48 h coculture, T-ALL cells were recovered and used for further analysis. In a set of experiments, we coated P6 wells with soluble recombinant murine Dll4 (R&D Systems; used at 4 μg/mL) in PBS; 1 d later, the wells were washed with PBS and T-ALL cells were added (1×10^6 per well) in their appropriate medium and cultured for 48 h before RNA extraction and transcriptional analysis.

Tumorigenicity assay. NOD/SCID mice were purchased from Charles River. Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December 1987). For tumor establishment, 7-wk-old to 9-wk-old female mice were injected s.c. with 3.0×10^6 cells in a 300-μL total volume into both dorsolateral flanks, and tumor growth was comparable in both flanks. In experiments aimed at investigating the kinetics of Dll4 expression and perfusion of blood vessels, MOLT-3–bFGF cells were injected in combination with Matrigel

(Becton Dickinson). To test the therapeutic efficacy of Dll4 blockade, the anti-Dll4 mAb YM152F was initially coinjected with tumor cells and subsequently given i.p. every 3 d (10 μg/g). In a set of experiments, mice were injected s.c. in both flanks with 0.5×10^6 MICOL-14 or MICOL-14^{tum} cells. The resulting tumors were inspected twice weekly and measured by caliper; tumor volume was calculated with the following formula: tumor volume (mm^3) = $L \times l^2 \times 0.5$, wherein L is the longest diameter, l is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid.

Optical imaging of tumors. To perform *in vivo* imaging, tumor cells were transduced with a lentiviral vector encoding the *EGFP* reporter gene to obtain an EGFP$^+$ cell population (27). EGFP$^+$ cells were then injected s.c. in anesthetized mice. Immediately after injection and at several time points thereafter, images were acquired using the eXplore Optix System (GE Healthcare) at a fixed integration time (0.3 s) and scan step (1.5 mm); laser power value (μW) was adapted to reach the 70% (photon/s) of maximum laser power. Analysis of images was performed using eXplore Optix OptiView software (GE Healthcare); a region of interest was manually selected around the signal intensity. Fluorescence was calculated as total intensity [normalized counts (NC) × area (mm^2)].

Western blot analysis. Cell lysates were run on 7.5% to 10% polyacrylamide gels; separated proteins were then blotted for 2 h at 400 mA onto a nitrocellulose membrane. Immunoprobing was performed using a rabbit polyclonal antibody against human Notch3 (Santa Cruz Biotechnology), a rabbit monoclonal antibody against human Notch1 (Cell Signaling Technologies), a rabbit polyclonal antibody against Dll4 (Rockland), a rabbit polyclonal anti–β-actin or a mouse polyclonal anti–α-tubulin (both from Sigma-Aldrich), followed by hybridization with a 1:5,000 diluted horseradish peroxidase–conjugated antirabbit or antimouse antibody (Amersham-Pharmacia). Antigens were identified by luminescent visualization using the SuperSignal kit (Pierce). Signal intensity was measured using a Bio-Rad XRS chemiluminescence detection system. The numbers below the bands indicate the ratio between the protein (Notch3 or Dll4) and α-tubulin or β-actin bands normalized by setting at 1 the value obtained from one selected sample.

Evaluation of Dll4 expression, vascularization, and perfusion markers. For immunofluorescence analysis, 5-μm-thick paraffin-embedded or frozen tumor sections were incubated with either rat anti-CD31 (Becton Dickinson) or rabbit anti-Dll4 primary antibody, washed, and incubated with the goat antirat Alexa Fluor 546, antirat Alexa Fluor 488, or antirabbit Alexa Fluor 488 (Invitrogen) secondary antibody.

To measure perfusion, mice were injected with dextran 70-FITC (Invitrogen; 360 μmol/L, 50 μL/mouse) 5 min before sacrifice. Perfused vessels were visualized under a confocal microscope (Zeiss). Nuclei were stained with TO-PRO-3 iodide (Invitrogen). Confocal laser scanning microscopy was carried out with a Zeiss LSM 510 microscope using Argon (488 nm) and helium-neon (543–633 nm) laser sources. The number of fields analyzed varied between 3 and 7 per section, depending on tumor size; at least four sections for each time point were analyzed. Images were collected at magnification of 200× or 400×. Microvessel density (MVD) was quantified by screening the CD31-stained sections for the areas of highest vascularity; 10 representative fields at magnification of 400× for each tumor were counted.

Reverse transcription–PCR and quantitative PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. cDNA was synthesized from 0.5 to 1 μg of total RNA using Superscript II first-strand system for reverse transcription–PCR (Invitrogen). For quantitative PCR (qPCR) analysis, the SYBR Green dye (Invitrogen) and Gene AMP 5700 Sequence Detection System (PE Biosystems) were used. Relative quantification was done using the ΔΔCt method, normalizing to β2-microglobulin mRNA. Primers used for PCR analysis are reported in Supplementary Table S1. Primers for Taqman analysis of Bcl2-A1 and cyclin D1 expression were purchased from Applied Biosystems.

Transduction of cells with lentiviral vectors. Lentiviral vectors encoding short hairpin RNA (shRNA) targeted to human Notch3 or a scrambled shRNA, as a control, were purchased from Sigma-Aldrich. Vector stocks were generated by a transient three-plasmid vector packaging system, as previously described (28). For transduction of MOLT-3 or Jurkat cells, 200 μL of concentrated vector-containing supernatant were layered over target cells that had been seeded into 12-well culture plates at 1×10^6 per well. After 6 to 9 h at 37°C, the supernatant was replaced with 2 mL complete medium. Evaluation of Notch3 silencing was carried out 72 h after transduction. MOLT-3 cells stably expressing the Notch3 ICD were obtained by electroporation of parental MOLT-3 cells with a pcDNA3.1-Notch3ICD plasmid (29), followed by selection in G418-containing medium (500 μg/mL). MOLT-3 cells transfected with a pcDNA3.1-EGFP plasmid were used as a control.

Statistical analysis. Results were expressed as mean value ± SD. Statistical analysis of data was performed using Student's t test. Differences were considered statistically significant when $P < 0.05$.

Details about electrophoretic mobility shift assay, immunoprecipitation of nuclear factor-κB (NF-κB) components and evaluation of apoptosis are presented as supplementary information.

Results

The notch ligand Dll4 is expressed in growing but not in dormant tumors. Our previous study indicated that human T-ALL MOLT-3 cells lack tumorigenic potential and remain dormant in NOD/SCID mice, whereas a derivative of this cell line, termed MOLT-3-bFGF, bearing enforced expression of the angiogenic factor bFGF, forms aggressive tumors (18). Two weeks after inoculation, expression of the Dll4 transcript, a Notch-ligand known to be up-regulated by angiogenic factors on EC (30, 31) and during tumor angiogenesis (8, 11, 31), was increased by 12-fold to 14-fold in MOLT-3–bFGF when compared with MOLT-3 tumors (Fig. 1*A*). Western blotting analysis of tumor lysates confirmed these differences and indicated marked Dll4 protein overexpression in growing compared with dormant tumors even in early stages of tumor development (Fig. 1*B*). Notably, Dll4 was not expressed by MOLT-3 cells *in vitro* nor was it induced by bFGF (Fig. 1*B*), thus indicating that its increased expression in growing tumors could be due to components of the stroma. Indeed, after immunofluorescence analysis of sections from growing tumors, Dll4 was detected mainly in EC, although also some CD31$^-$ cells expressed Dll4 (Fig. 1*C*). In contrast, dormant tumors expressed only marginally Dll4. Similar findings were obtained when Jurkat cells were injected s.c. into NOD/SCID mice with or without bFGF (Fig. 1*C*), thus showing that up-regulation of Dll4 expression during bFGF-induced angiogenesis is not an exclusive feature of MOLT-3 tumors.

To investigate when Dll4 expression begins in respect to the generation of functional blood vessels, we injected MOLT-3-bFGF cells s.c. into NOD/SCID mice in Matrigel plugs and recovered the samples 2, 5, and 14 days thereafter. To evaluate perfusion, the mice were injected with Dextran 70-FITC immediately before sacrifice. The results, shown in Supplementary Fig. S1, indicated that small numbers of Dll4$^+$ cells were found within MOLT-3–bFGF masses already by day 2 after injection, and their number increased later on. Moreover, Dll4 expression invariably preceded perfusion, which was readily detected only in 14-d samples. These findings hint to a possible role of Dll4 during the early phase of tumor formation, before perfusion of the vascular network.

Increased Notch3 activation levels are a distinctive feature of growing MOLT-3 tumors. Next, we evaluated the expression of Notch1 and Notch3 receptors in MOLT-3 cells and in tumors. Notch1 and Notch3 transcripts were expressed by MOLT-3 and MOLT-3-bFGF cells *in vitro* (data not shown), as well as by dormant and aggressive tumors, at comparable levels (Supplementary Fig. S2).

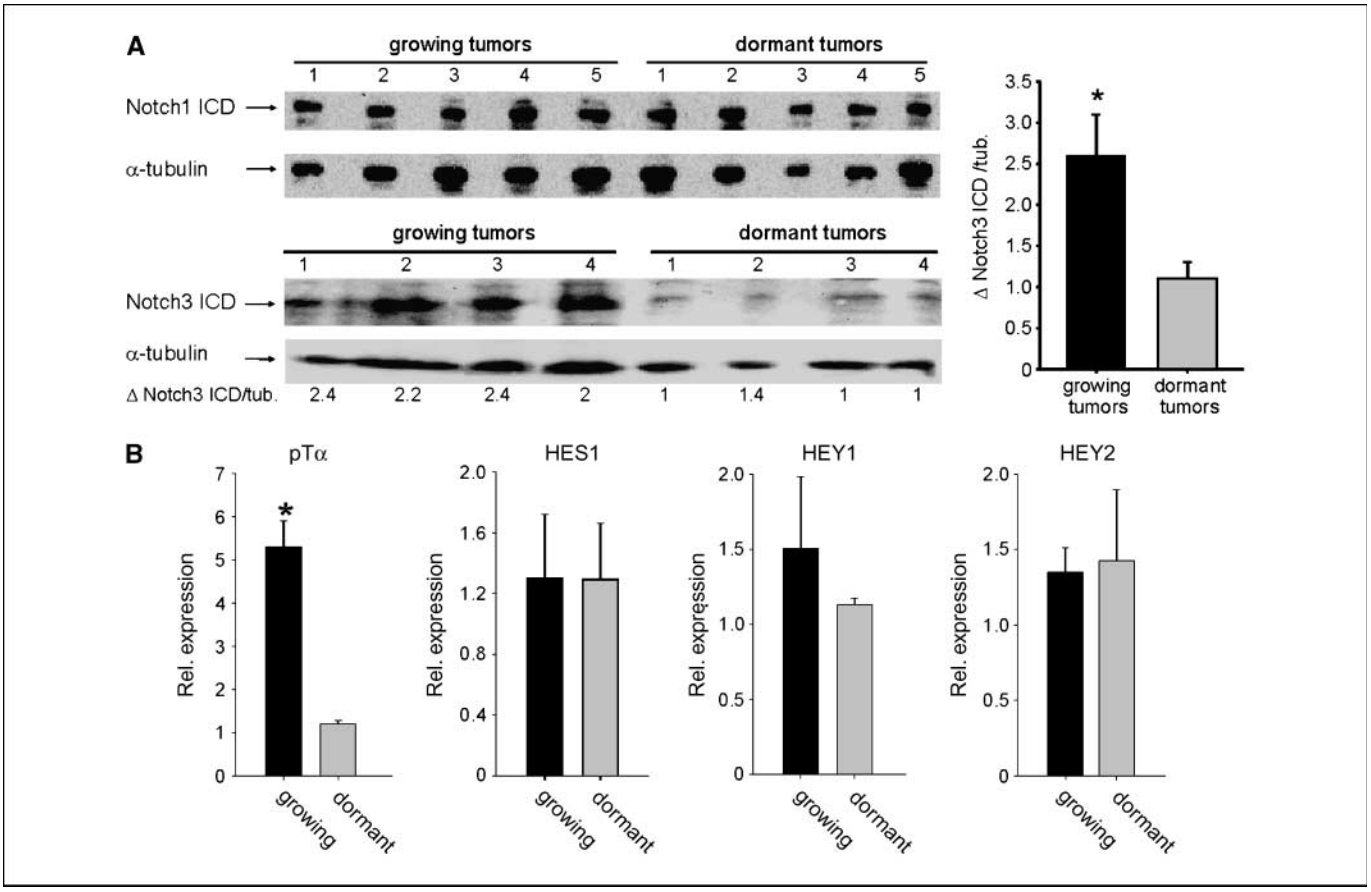

Figure 2. Increased Notch3 activation and pTα expression distinguish growing from dormant tumors. *A,* Western blot analysis of Notch1 and Notch3 ICD levels of tumors removed 6 to 8 wks after tumor cell injection. Five representative samples per group are shown. *Columns,* mean of all samples analyzed; *bars,* SD (n = 10 per group). *, $P < 0.05$ compared with dormant tumor values. *B,* expression of the Notch3 target gene pTα is increased in growing compared with dormant tumors (n = 7 samples per group). Expression of the Notch3-regulated transcripts pTα and HES1 or the Notch1-regulated transcripts HEY1 and HEY2 was determined by qPCR. *Columns,* mean; *bars,* SD. *, $P < 0.001$ compared with dormant tumor values. MOLT-3 cells *in vitro* were used as reference sample.

Analysis of established tumors showed the presence of similar levels of the Notch1 ICD both in dormant and aggressive tumors (Fig. 2*A*); in contrast, Notch3 ICD levels were markedly increased in aggressive compared with dormant tumors (Fig. 2*A*).

Having established that tumors with different growth kinetics show marked differences in expression levels of Notch3 ICD, we extended this analysis to transcripts that are regulated through this pathway. Real-time PCR analysis indicated 5-fold higher levels of the putatively Notch3-specific pTα transcript in MOLT-3–bFGF compared with MOLT-3 tumors (Fig. 2*B*); in contrast, expression levels of HES1, another Notch-regulated gene, and the Notch1 target genes HEY1 and HEY2 were comparable in the two groups of tumors (Fig. 2*B*). These findings indicated that the pTα transcript could represent a valuable specific marker of Notch3 activity in growing versus dormant tumor cells. This is in keeping with previous results, showing that increased pTα expression correlates with the status of active disease, being undetectable in remission stage of T-ALL (23).

As Notch3 signaling may involve activation of the NF-κB pathway through pTα-dependent mechanisms (32), the expression of some components of this pathway was also investigated. As shown in Supplementary Fig. S3, NF-κB DNA-binding activity, as well as the generation of p50/phosphorylated p65 heterodimer, was significantly increased in growing compared with dormant tumors. Moreover, the expression levels of genes known to be responsive to NF-κB transcriptional regulation, including cyclin D1 (32) and Bcl2-A1 (33, 34), were increased in aggressive compared with dormant tumors (Supplementary Fig. S3).

Dll4 expression correlates with increased Notch3 cleavage in a colorectal carcinoma tumor model. To extend these results, we analyzed Dll4 expression in a model of tumor dormancy that we established for this purpose using colorectal carcinoma cells. MICOL-14 cells remained viable, albeit non-tumorigenic in NOD/SCID mice, as shown by optical imaging techniques (Fig. 3*A*) and by measurement of tumor size (Supplementary Fig. S4), whereas their tumorigenic variant, termed MICOL-14^{tum}, formed aggressive tumors. Expression of Dll4 and CD31 was barely detectable in dormant MICOL-14 tumors, whereas it was clearly found in EC in 5-week-old tumors formed by MICOL-14^{tum} cells (Fig. 3*B*); in addition, several CD31^{-} cells were also labeled by the anti-Dll4 antibody. When levels of cleaved Notch3 were measured, we found that they were invariably higher in growing tumors compared with the dormant tumors (Fig. 3*C*); in contrast, Notch1 cleavage was apparently reduced in growing compared with dormant tumors. Overall, these results confirmed that Dll4 expression is a feature of angiogenic tumors and that it correlates with increased levels of Notch3 activation in the tumor cells.

Coculture of T-ALL cells with EC triggers Notch3 signaling. Because Dll4 expression was predominantly found in tumor-associated EC, we speculated that a possible interplay between EC and tumor cells could represent the key event involved in conferring a tumorigenic phenotype to otherwise dormant tumor cells. To establish whether this heterotypic interaction might modulate Notch signaling, we set up a coculture system using the T-ALL cells. *In vitro* treatment of murine EC (SIEC) with bFGF and VEGF readily induced Dll4 expression (Supplementary Fig. S5). Other components of the Notch pathway were also expressed by SIEC cells, including Jagged1, Jagged2, and Dll1, as previously reported (31); however, except for Dll1, they were not induced by bFGF/VEGF (Supplementary Fig. S5). Moreover, Notch1 and Notch3 were also expressed by SIEC cells, but their expression was not detectably increased by the angiogenic factors (Supplementary Fig. S5). Cocultivation of leukemia cells with SIEC increased by ~5-fold the expression of the Notch-regulated pTα transcript in MOLT-3 cells and it also up-modulated pTα transcript levels in other T-ALL cell lines cocultured with EC, including Jurkat and T-ALL1 cells, with heterogeneous increase in HES1 levels (Fig. 4*A*). In contrast, HEY1 and HEY2 levels remained unchanged in MOLT-3 cells after coculture (not shown). Notch3 ICD levels in MOLT-3 and Jurkat cells were also increased after coculture (Fig. 4*B*), whereas Notch1 ICD levels did not substantially change (Fig. 4*B*). Neutralization of Dll4 using the anti-Dll4 antibody YW152F reduced Notch3 ICD levels in cocultured MOLT-3 and Jurkat cells (Fig. 4*B*), and it almost abolished pTα overexpression in Jurkat cells (Fig. 4*B*), thus showing that Dll4 was substantially contributing to Notch3 activation in this cocolture system. Furthermore, cultivation of T-ALL cells on plastic wells coated with sDll4 for 48 h resulted in marked up-regulation of pTα levels and heterogeneous increase of HES1 levels (Fig. 4*C*), thus reinforcing the evidence that Dll4 can trigger Notch signaling in these cells.

Finally, we investigated the consequences of coculture on leukemia cell survival. By using prolonged serum starvation to

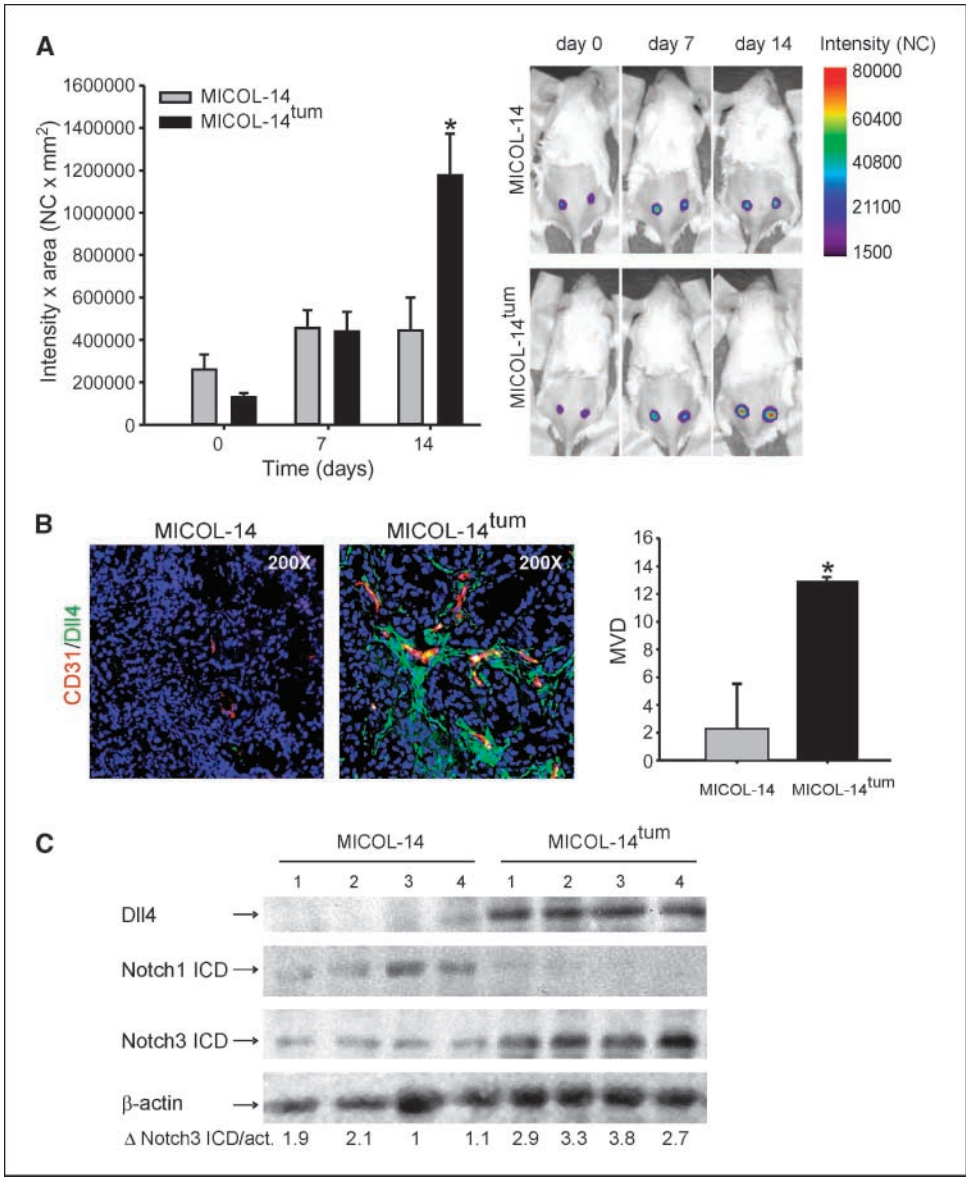

Figure 3. Dll4 and Notch3 ICD expression levels in dormant and growing tumors formed by colorectal cancer cells. *A,* optical imaging of EGFP expressing MICOL-14 and MICOL-14^{tum} tumors. Representative fluorescence intensity images of tumor cells immediately after injection (day 0), 7 and 14 d later. *B,* immunofluorescence analysis of Dll4 and CD31 expression in growing (MICOL-14^{tum}) compared with dormant (MICOL-14) tumors 5 wks after tumor cells injection. The diagram reports MVD values.*, $P < 0.05$. *C,* expression of Dll4, Notch1, and Notch3 ICD in four growing compared with dormant MICOL-14 tumors by Western blot analysis.

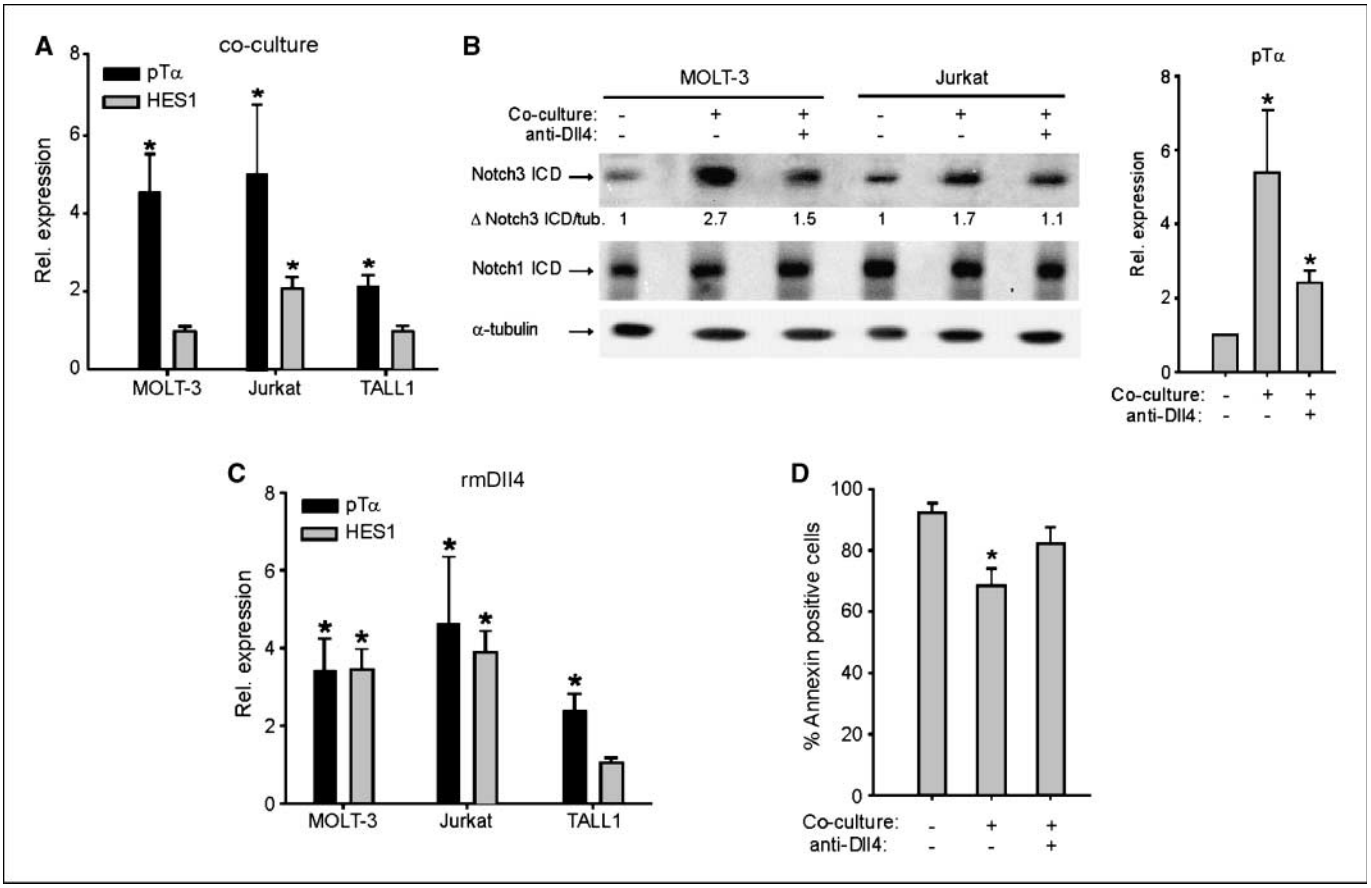

Figure 4. Triggering of Notch3 signaling in T-ALL cells after coculture with EC. *A,* Notch3 is activated in T-ALL cells after coculture with EC for 48 h. Expression of the Notch3 target genes pTα and HES1 by qPCR. *Columns,* mean values of three experiments; *bars,* SD. Relative expression was calculated using leukemia cells not cocultured as reference sample. *, $P < 0.05$. *B,* analysis of Notch activation in MOLT-3 cells after coculture with EC for 48 h. *Left,* Western blot analysis of Notch1 and Notch3 ICD levels of cocultured or not MOLT-3 and Jurkat cells with or without a Dll4-neutralizing mAb (2.8 μg/mL). *Right,* expression of pTα in Jurkat cells by qPCR. Jurkat cells were cocultured or not in the presence or absence of anti-Dll4 mAb. *Columns,* mean values of three experiments; *bars,* SD. Relative expression was calculated using not cocultured Jurkat cells as reference sample. *, $P < 0.05$. *C,* qPCR analysis of pTα and HES1 in T-ALL cells grown for 48 h in wells precoated with rmDLL4 (4 μg/mL) or PBS. *Columns,* mean values of five experiments; *bars,* SD. *, $P < 0.05$ compared with untreated cells. *D,* coculture with EC protects T-ALL cells from apoptosis. Jurkat cells were serum-starved for 48 h. Then, cells were cultured either alone or in the presence of SIEC for further 48 h in the absence of serum. Apoptosis levels were measured by flow cytometric analysis after Annexin V staining on CD5+ cells (CD5 is a Jurkat cell marker). *Columns,* mean values of four experiments; *bars,* SD. *, $P < 0.05$ compared with untreated or cocultured Jurkat cells in the presence of anti-Dll4 mAb.

induce cell death, we observed that control Jurkat cells underwent massive apoptosis, as evaluated by Annexin V staining (Fig. 4*D*); apoptosis levels, however, were reduced by 30% ($P < 0.05$) when serum-starved Jurkat cells were cocultured with SIEC for the same time, and this protective effect of SIEC was almost abolished after addition of the anti-Dll4 mAb to the cultures (Fig. 4*D*). Similar results were obtained with MOLT-3 cells, and altogether, they indicated that SIEC cells can provide a survival signal involving the Notch-Dll4 interaction to T-ALL cells under stress conditions.

Dll4 neutralization impairs tumor growth and reduces Notch3 activation. The above studies pointed to Dll4 as a Notch ligand implicated in triggering Notch3 activation in T-ALL cells. To investigate the role of Dll4 in tumors, we injected NOD/SCID mice bearing MOLT-3–bFGF tumors with the anti-Dll4 mAb YW152F (14). This resulted in significant reduction of tumor size and weight (Fig. 5*A*); these effects were also confirmed by digital imaging of the tumors after injection of EGFP-labeled tumor cells (Supplementary Fig. S6). Anti-Dll4 treatment was associated with a significant increase in the MVD of the tumors (41.0 ± 6.7 versus 16.3 ± 1.3 in anti-Dll4-treated and control tumors, respectively; $P < 0.001$; Fig. 5*B*), in agreement with previous studies (13–15). Importantly, anti-Dll4-treated tumors had 65% reduced levels of cleaved Notch3 compared with controls ($P < 0.05$; Fig. 5*C*), and this was associated with a significant reduction in pTα expression levels (Fig. 5*C*). Altogether, these results showed that Dll4 neutralization impairs Notch3 signaling and tumor growth in this model.

Modulation of Notch3 levels affects T-ALL cell survival and tumorigenicity. To further investigate the role of Notch3 signaling in this model, MOLT-3 cells were transduced by lentiviral vectors encoding specific shRNA, which reduced Notch3 expression levels specifically by 70% to 80% compared with control cells (not shown) and also markedly attenuated Notch3 ICD protein levels (Fig. 6*A*).

Silencing Notch3 expression had dramatic effects on the viability of the leukemia cells *in vitro*, which seemed 7 to 10 days after gene silencing. The proapoptotic effect of Notch silencing was initially shown by analysis of poly(ADP-ribose) polymerase (PARP) cleavage in cell lysates; reduction of Notch3 ICD protein levels was associated with increased levels of cleaved PARP (not shown). The percentage of Annexin V^+ cells increased from 10% to 16% in control to 40% to 50% in shNotch3 MOLT-3 cells (Fig. 6*A*). Similar findings were obtained after silencing of Notch3 in Jurkat cells and GT cells, a derivative of MOLT-3 cells bearing high levels of Notch3

activation (Fig. 6*A*). Proliferation of shNotch3 MOLT-3 cells was reduced by >80% in an MTS assay compared with the control (not shown). In line with these *in vitro* findings, silencing of Notch3 in GT cells dramatically impaired tumor engraftment (Fig. 6*A*), further indicating that the Notch3 pathway is required for tumor formation. On the other hand, injection of parental MOLT-3 cells stably transfected with a Notch3 ICD-encoding plasmid, which showed increased levels of cleaved Notch3 protein and pTα expression (Fig. 6*B*), conferred tumorigenic properties to these otherwise dormant cells (Fig. 6*B*). In conclusion, these findings indicated that modulation of the Notch3 pathway plays a pivotal role in determining the tumorigenic phenotype of MOLT-3 cells.

Discussion

Recent studies highlighted the role of Dll4 during tumor angiogenesis and showed the therapeutic potential of Dll4 blockade (11, 13, 14). Here, we show, for the first time, involvement

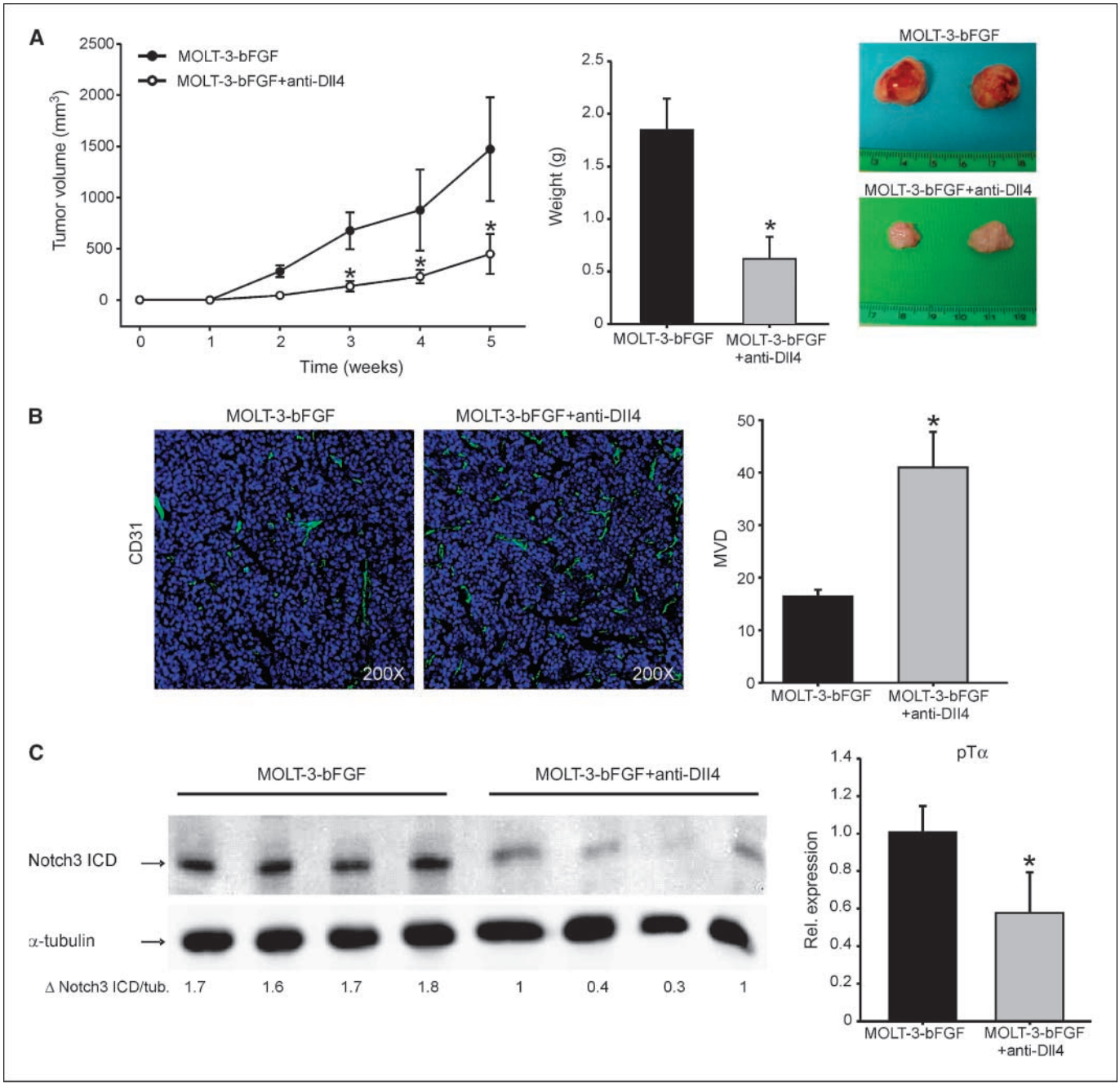

Figure 5. Dll4 blockade impairs tumor growth and Notch3 signaling in tumors. Mice bearing s.c. MOLT-3-bFGF tumors on both flanks were injected biweekly with the neutralizing anti-Dll4 YW152F mAb (10 μg/g) for 3 wks and sacrificed 2 wks later (n = 6 mice per group). *A,* anti-Dll4 treatment impairs tumor growth (*left*) and weight (*right*). Mean ± SD of 8 to 12 different samples. The macroscopic appearance of some representative tumors is also shown. *B,* Dll4 neutralization increased MVD in MOLT-3-bFGF tumors. *Left,* representative CD31 staining patterns (magnification, 200×); *right,* quantification of the MVD. *, $P < 0.05$. *C,* Dll4 neutralization reduces Notch3 signaling. *Left,* reduced Notch3 ICD levels were found in treated tumors by Western blot analysis. *Right,* pTα expression was measured by qPCR analysis in n = 8 samples; *, $P < 0.05$.

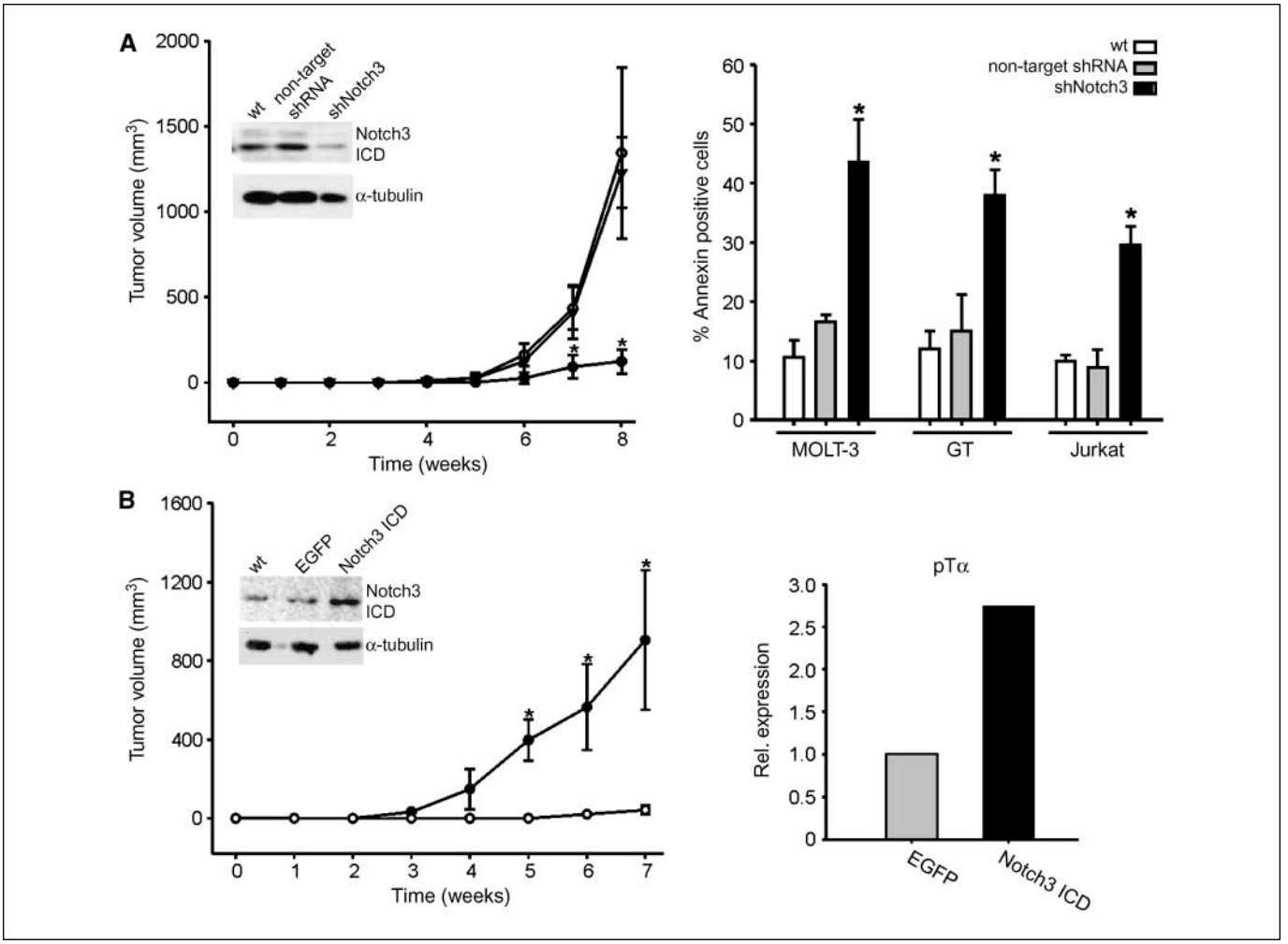

Figure 6. Effects of modulation of Notch3 levels on apoptosis and tumorigenicity of T-ALL cells. *A, left,* tumor growth curves of xenotransplants in NOD/SCID mice. Parental GT cells (▼), a tumorigenic variant of MOLT-3 expressing high levels of Notch3 ICD (see Materials and Methods), GT cells transduced by the shNotch3 (●) or the control vector (○; 3×10^6 cells, $n = 6$ mice/group) were injected s.c. into NOD/SCID mice 72 h after transduction. In all cases, cell viability at the beginning of the experiment was >95%. The tumor volume was plotted as a function of time. *, $P < 0.05$ compared with GT control tumors. *Inset,* Western blot analysis of Notch3 ICD expression in parental (*wt*), control, and Notch3-silenced MOLT-3 cells. *Right,* flow cytometric analysis of apoptosis in T-ALL cells transduced by the same vectors. Annexin V staining was performed 1 wk after gene transfer. *Columns,* mean of three experiments; *bars,* SD. *, $P < 0.05$ compared with parental (*wt*) and nontarget shRNA-transduced MOLT-3 cells. *B, left,* tumor growth curves of MOLT-3 cells overexpressing Notch3 ICD in NOD/SCID mice. MOLT-3 cells stably transfected with a Notch3 ICD-encoding plasmid (●) or an EGFP-encoding control plasmid (○) were injected s.c. in NOD/SCID mice (3×10^6 cells, $n = 4$ mice per group). The tumor volume was plotted as a function of time. *, $P < 0.05$ compared with control tumors. Western blot analysis of Notch3-ICD transfected and control MOLT-3 cells (insertion). *Right,* expression of pTα in MOLT-3 Notch3 ICD cells by qPCR.

of Dll4 in the regulation of tumor dormancy. We have previously found that T-ALL cells are poorly angiogenic and acquire a dormant phenotype in NOD/SCID mice and that this condition can be interrupted by an angiogenic switch delivered to the tumor microenvironment (18). More recently, we observed that poorly angiogenic CRC cells also establish dormant tumors in NOD/SCID mice and have isolated angiogenic variants of these cells with increased tumorigenic potential. Intriguingly, these CRC cells express Notch receptors, and were therefore useful to further investigate the effects of angiogenesis on Notch signaling. By using these experimental models, we report that quiescent tumors lack Dll4 expression, whereas aggressive tumors show intense Dll4 expression; in agreement with previous studies (8, 11, 31), Dll4 was found expressed mainly in ECs, although some $CD31^-$ cells were unexpectedly labeled by the anti-Dll4 antibody. As T-ALL cells did not express Dll4, it is likely that some nonendothelial stromal cells also express this Notch ligand. In this regard, some $Dll4^+$ cells were stained by the macrophage marker F4/80 (not shown), thus confirming recent data which report Dll4 expression in macrophages exposed to proinflammatory stimuli (35). A further interesting result of our studies concerns the kinetics of Dll4 expression during tumor angiogenesis, an aspect which has not been carefully analyzed before. The observation that Dll4 expression *in vivo* was detected soon after tumor cell injection, when a vascular network capable of providing adequate oxygen and nutrient supply was not yet established, supports a role for Dll4-mediated Notch signaling during the early steps of tumor formation.

The main finding of this study is the demonstration that expression of the Notch ligand Dll4 in the angiogenic tumor microenvironment contributes to regulate Notch3 signaling in tumor cells. The striking association between Dll4 expression and levels of Notch3 active forms in tumors and, more importantly, the finding that Dll4 neutralization *in vivo* markedly impairs Notch

signaling in the tumor cells support this hypothesis. Moreover, Notch3 activation in T-ALL cells was triggered by coculture on EC or immobilized recombinant Dll4, and this was blocked by an anti-Dll4 mAb. Although we stress the role of Dll4 in the regulation of Notch3 activity, it is known that several Notch receptors and ligands are expressed on EC surface, enabling interactions between adjacent cells upon receptor-ligand binding (36). Therefore, other Notch ligands, in addition to Dll4, could possibly contribute to activate the Notch pathway in the T-ALL cells, and this could explain why Dll4 neutralization did not abrogate Notch3 activation both *in vitro* and *in vivo*.

Intriguingly, while activated forms of both Notch1 and Notch3 were expressed in leukemia and colorectal cancer cells, as well as in tumors, acquisition of the tumorigenic phenotype in both models was associated with selective up-modulation of Notch3 signaling. Given the recognized cross-talk between these two Notch members (37), it is possible that in dormant tumors basal Notch3 expression may be sustained by Notch1. Notch1 activity seems to be required for T-ALL cell survival, as shown by the detection of Notch1 ICD and expression of Notch1 target genes HEY1-2 in tumors and by the negative consequences of Notch1 silencing on T-ALL cell viability (not shown) but yet insufficient to confer an aggressive phenotype, which rather needs combined Notch1 and Notch3 signaling. Moreover, the finding that Notch1 expression was down-regulated in aggressive tumors formed by colorectal cancer cells, for unknown reasons, may suggest that Notch1 plays a different role during tumorigenesis in the two models analyzed. In patients with T-ALL, Notch3 expression is extremely common and it has been observed in virtually 100% of these tumors, including their major molecular and immunophenotypic subtypes (23). Hypothetically, Notch3 activation in T-ALL could be driven by expression of Dll4 or other Notch ligands in the bone marrow microenvironment of T-ALL patients; in this regard, it is important to note that we found Dll4 expressed in the microvasculature of three T lymphoblastic lymphoma samples, a rare type of lymphoma which represents the counterpart of T-ALL outside bone marrow (Supplementary Fig. S7).

Triggering the Notch3 receptor seems to deliver a survival signal to T-ALL cells. In support of this hypothesis, we found increased activation of the NF-κB pathway, which has an established role in protection from apoptosis (38), in aggressive tumors compared with dormant ones. Increased NF-κB levels may contribute to explain the reduced numbers of apoptotic cells detected in growing compared with dormant tumors in our previous study (18). The protective role of Notch3 signaling from apoptosis was also noted in coculture experiments with serum-starved T-ALL cells and by the consequences of silencing the *Notch3* gene in T-ALL cells. In any case, Notch3 expression seemed necessary to maintain T-ALL cell viability. This latter finding is in line with a previous report, showing that inhibition of Notch signaling in T-ALL cell lines induces cell cycle arrest and apoptosis (39). The importance of Notch3 activation *in vivo* was further shown by acquirement of tumorigenic capacity by MOLT-3 cells transfected with the full active Notch3 ICD form and by the finding that silencing Notch3 expression in MOLT-3 cells by shRNA techniques greatly impaired tumor growth.

Could these findings be generalized? Notch receptors have been found in several solid tumors, and in some cases, they are expressed by cancer stem cells (2). Recently, *Notch3* gene has been detected amplified or overexpressed in lung, pancreatic, and ovarian cancer, although its contribution to the pathogenesis of these tumors remains unknown (40–42). Although carcinoma cells are usually capable of inducing the angiogenic switch, the interaction of Notch with its ligands on EC may contribute to the regulation of tumor cell behavior by the microenvironment, as also remarked by our findings with colorectal cancer cells. The finding that Notch3 could be regulated through a tumor-EC interaction suggests a novel function of angiogenesis in cancer, uncoupled from perfusion. Finally, these data may have implications for our understanding of the molecular interactions occurring in the so-called "vascular niche" around cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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