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Cancer Research

ADAM17 Regulates Epidermal Growth Factor Receptor Expression through the Activation of Notch1 in Non–Small Cell Lung Cancer

Anja Baumgart[1], Stefan Seidl[2], Petros Vlachou[1], Lars Michel[1], Nadya Mitova[1], Nicole Schatz[1], Katja Specht[2], Ina Koch[2], Tibor Schuster[4], Rebekka Grundler[1], Marcus Kremer[2], Falko Fend[2,5], Jens T. Siveke[3], Christian Peschel[1], Justus Duyster[1], and Tobias Dechow[1]

Abstract

Epidermal growth factor receptor (EGFR) overexpression and activation are hallmarks of non–small cell lung carcinoma (NSCLC). Although EGFR-targeted therapies are used, the prognosis of NSCLC remains poor. ADAM17 induces activation of the EGFR through ligand cleavage. However, we show that inhibition or knockdown of ADAM17 markedly reduces tumorigenesis and survival to a large part independently from EGFR ligand shedding in NSCLC cells. These findings strongly indicate additional oncogenic mechanisms regulated by ADAM17. We identified Notch1 signaling as an ADAM17-controlled pathway and a critical regulator of anchorage-independent growth by using both Notch1 shRNA and ectopic expression of the active intracellular Notch1 fragment. Strikingly, Notch1 knockdown led to a strong reduction of EGFR expression in all analyzed cell lines. Proliferation, survival, and colony formation of Notch1-deficient cells were insensitive to EGF stimulation. Moreover, targeting Notch1 or ADAM17 resulted in substantial cell death, whereas EGFR inhibition predominantly induced cell cycle arrest. Immunohistochemical analysis of primary human tissue revealed a significant correlation between ADAM17, Notch1 signaling, and high EGFR expression levels. In conclusion, this article describes a novel molecular circuitry in NSCLC, incorporating ADAM17 as a regulator of EGFR expression through the activation of Notch1. Due to their central role in tumorigenesis and survival of NSCLC cells, both ADAM17 and Notch1 constitute promising targets for the treatment of NSCLC. *Cancer Res; 70(13); 5368–78. ©2010 AACR.*

Introduction

Non–small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths in the United States and Europe. The prognosis of unresectable, locally advanced, or metastatic disease is poor, and treatment options are limited (1). Both overexpression and constitutive activation of the epidermal growth factor (EGF) receptor (EGFR) are hallmarks of NSCLC. Thus, targeting the EGFR was found to be a promising therapeutic strategy (2–5). However, the majority of patients do not experience a clinical benefit from EGFR-targeted therapies (6, 7).

Authors' Affiliations: Departments of [1]Internal Medicine III, [2]Pathology, and [3]Internal Medicine II; and [4]Institute for Medical Statistics and Epidemiology, Technische Universitat Munchen, Ismaninger Strasse 22, Munich, Germany; and [5]Department of Pathology, University of Tubingen, Liebermeisterstrasse 8, Tubingen, Germany

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Corresponding Author: Tobias Dechow, Department of Internal Medicine III, Technische Universität München, Ismaninger Str. 22, 81675 Munich, Germany. Phone: 49-89-4140-5834; Fax: 49-89-4140-4879; E-mail: t.dechow@lrz.tum.de.

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EGFR ligands are expressed as membrane-anchored precursors. Upon proteolytical cleavage, they are released from the cell surface to activate the EGFR in an autocrine or paracrine manner (8). The key regulators involved in these cleavage processes belong to the a disintegrin and a metalloproteinase family (ADAM), a family of membrane-bound matrix metalloproteinases (MMP; ref. 9). The most critical sheddases in EGFR ligand release are ADAM17 and ADAM10 (10–12). ADAM17-dependent shedding can be activated by several stimuli such as phorbol esters, apoptosis, or activation of G protein–coupled receptors (9, 13–15). It has been shown that ADAM17-mediated EGFR ligand cleavage enhances the proliferation and survival of squamous cell carcinoma cells as well as lung cancer cells (13, 14). In addition to EGFR stimulation, ADAM17 controls the activation of Her3 through cleavage of proheregulin (16).

Besides EGFR ligands and many other, structurally unrelated surface molecules, ADAM17 is involved in the activation of the Notch signaling pathway. Notch receptors are expressed on the cell surface as S1-cleaved heterodimers, consisting of an extracellular domain (N^{EC}) and a transmembrane and intracellular domain (N^{TMIC} or N^{TM}). Upon binding of Notch ligands to Notch receptors, the extracellular S2 site within the N^{TM} domain is proteolytically processed by ADAM17 or ADAM10 to the extracellular truncated Notch

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form (N^{EXT}), followed by the cleavage of the intracellular S3 and S4 sites by the γ-secretase complex. The released intracellular Notch domain (N^{IC}) translocates to the nucleus and induces transcription of several target genes including members of the HES family. N^{IC} is very labile and is rapidly inactivated through lysosomal and proteasomal degradation. In addition, Notch activation is regulated by endocytic trafficking of both the Notch ligands as well as the Notch receptors (17–21). Expression of Notch receptors, Notch ligands, and HES family members has been observed in a large number of solid tumors such as melanoma, cervical carcinoma, lung carcinomas, pancreas carcinomas, renal cell carcinoma, and breast cancer (22–29). Notch signaling is believed to be critical for cell fate decision, stem cell maintenance, and tumorigenesis (30, 31). In addition, Notch can also function as a tumor suppressor as shown by the development of skin tumors in Notch1-deficient mice (32). Our *in vitro* and *in vivo* data show that ADAM17 is required for the tumorigenicity of NSCLC cells through the activation of Notch1 signaling and subsequent regulation of EGFR protein expression. In the era of kinase inhibitors, this study supports the significance that ADAM or Notch inhibition could be an effective strategy in the treatment of NSCLC.

Materials and Methods

Cell lines and reagents

All NSCLC cell lines were obtained from the American Type Culture Collection. EGF and MG132 were from Chemicon. Δ9-Tetrahydrocannabinol was obtained from Sigma. Cetuximab was purchased from Merck. Batimastat was provided by Klaus Maskos (Max-Planck-Institute for Biochemistry, Martinsried, Germany). 2R-2-((3R)-3-amino-3(4-[2-methyl-4-quinolinyl)-methoxy]-phenyl)-2oxopyrrolidinyl)-N-hydroxy-4-methylpentanamide (BMS-561,392) was provided by Bristol-Myers-Squibb. N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester (DAPT) was purchased from Merck.

Western blot

Western blots were performed as previously described (33). Membranes were probed with antibodies to pErk1/2 (Thr-202/Tyr-204), Erk1/2, pAkt, Akt, EGFR, $N1^{IC}$ (cleaved Notch1, Val-1744), cleaved caspase-3 (Cell Signaling), ADAM17 (Chemicon), caspase-3 (H-277), Jagged 1 (C-20), Delta-like (C-20), $N1^{TM}$ and full-length Notch1 (mN1A, Santa Cruz), Notch2 (c651.6DbHN, Developmental Studies Hybridoma Bank), Notch3 (M-134, Santa Cruz), Hes1 (Abcam), phospho-Tyrosine Py20 (BD Pharmingen) and 4G10 (Upstate Biotechnology), β-actin, and flag (Sigma). Horseradish peroxidase–conjugated donkey anti-rabbit and sheep anti-mouse antibodies were purchased from GE Healthcare, and donkey anti-goat was from Santa Cruz.

Stable ADAM17 knockdown

ADAM17 was knocked down with short hairpin RNA (shRNA) technology using the pSuper-retro vector system. Cloning of the plasmid was carried out according to the OligoEngine manual. The sequence used was 5′-GTGCCAGGAGGCGATTAAT-3′. A shRNA construct directed against luciferase RNA was used as a control.

Retroviral and lentiviral expression vectors and infections

The Hoe67-$N1^{IC}$ expression vector containing a flag tag at the 5′ end was kindly provided by Ursula Zimber-Strobl (Helmhotz Center, Munich, Germany). $N1^{IC}$ was cloned into the *EcoR1* and *BamH1* sites of the pLXSN (Clontech). Generation of amphotropic retroviruses has previously been described (34). Notch1 shRNA lentiviral plasmid was obtained from Sigma. For lentiviral supernatants, 4 μg of Vector DNA were transiently introduced into 293T cells with 4 μg of the packaging construct psPAX2 and 4 μg of the envelope plasmid pMD2G using Lipofectamine 2000 (Invitrogen). Infection was performed as described.

Proliferation assay

Cells (1×10^5) were plated in six-well tissue plates. All experiments were performed in duplicates for at least three times in medium containing 0.5% FCS. For analysis, cells were stained with trypan blue and were counted by light microscopy.

Propidium iodide stain

Cells (2×10^6) were plated into 10-cm tissue plates in medium (0.5% FCS). Cells were harvested and fixed in 5 mL of −20°C ethanol (70%). Fixed cells were washed, resuspended in 300 μL DNase-free RNase A (200 μg/mL; Sigma), and stained with propidium iodide (1 mg/mL; Sigma). DNA content was analyzed by flow cytometry on an Epics XL-MCL (Beckman Coulter). The FlowJo software (Tree Star, Inc.) was used to quantify cell cycle distribution.

Soft agar assays

For anchorage-independent growth in soft agar, a bottom layer of 0.75% agar (Difco) in Iscove's modified Dulbecco's medium [Life Technologies (Invitrogen)] was first placed in six-well plates (34). Cells were seeded in duplicates in a final volume of 3 mL containing 0.3% agar. For analysis, colonies were stained with MTT (Sigma). At least three experiments were independently performed.

Xenografts in nude mice

For xenografts 2×10^6 NCI-H292 cells were injected s.c. into the flank of BALB/c *nu/nu* mice 4 weeks of age. Tumor volume was calculated using the formula $V = a \times b \times c \times 0.5$. BMS-561,392 was given orally twice per day in a dosage of 20 mg/kg. All procedures were reviewed and approved by the university's supervisory animal care committee.

Immunhistochemical analysis

For immunhistochemistry of primary human NSCLC samples, serial paraffin sections (4 μm) of selected tumor blocks were cut and deparaffinized. After protease antigen retrieval (6 min), EGFR staining with antibody against EGFR (DAKO, clone E30, dilution 1:20) was carried out on an automated immunostainer (Benchmark, Ventana) with the

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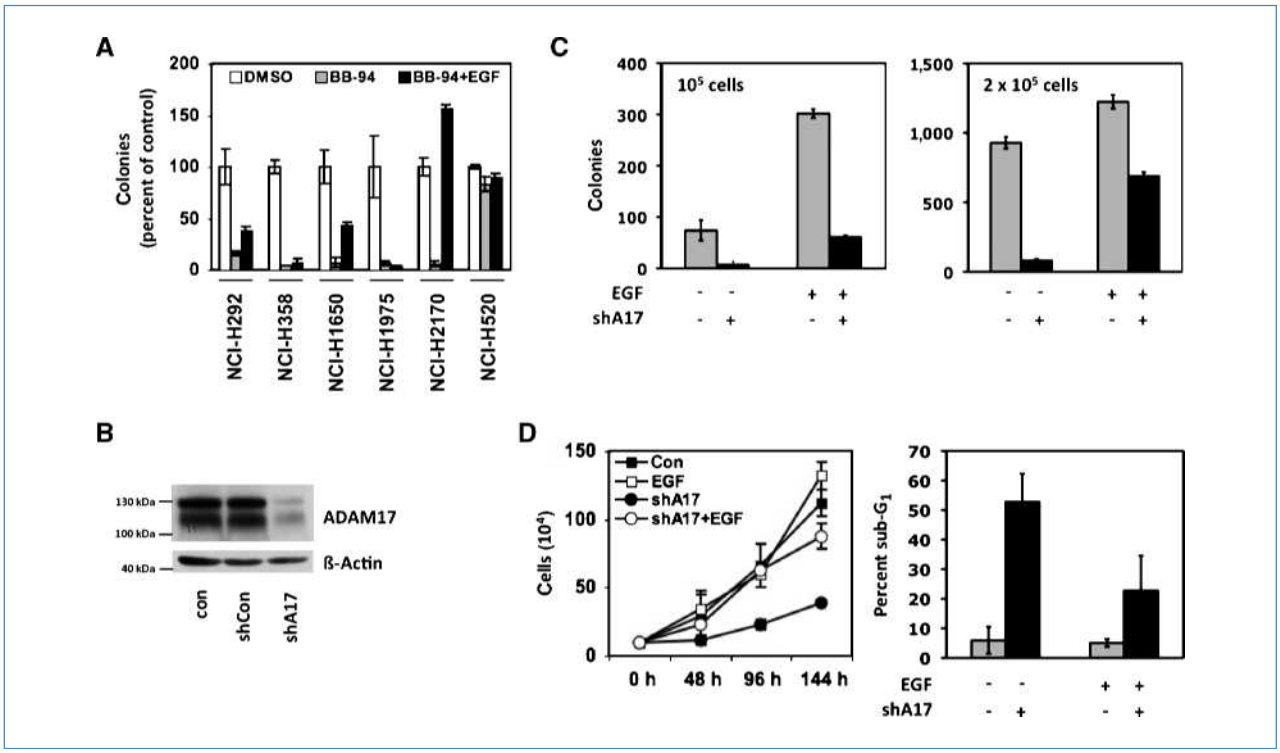

Figure 1. ADAM17 is required for transformation and survival of NSCLC cells through EGFR ligand–dependent and EGFR ligand–independent mechanisms. A, soft agar assay of the indicated cell lines treated with vehicle (DMSO), 10 μmol/L batimastat (BB-94), and 5 ng/mL EGF. B, Western blot analysis of ADAM17 knockdown in NCI-H292 cells using ADAM17 shRNA (shA17) compared with cells infected with empty (con) and control vector (shCon). C, colony formation of ADAM17 knockdown NCI-H292 cells and control-infected cells with 10^5 (left) and 2×10^5 (right) cells. D, proliferation of ADAM17 knockdown NCI-H292 cells (shA17) compared with control-infected cells (con) in the absence or presence of EGF (left). Viability of ADAM17 knockdown cells (shA17) compared with control-infected cells (shCon) with and without EGF as determined by the sub-G_1 fraction using propidium iodide staining (right). Points and columns, mean; bars, 1.96 SD.

recommended reagents, using the avidin-biotin complex method. A manual staining protocol was used for pEGFR (Invitrogen, polyclonal, dilution 1:100), ADAM17 (Calbiochem, clone 807-823, dilution 1:40), Notch1 (Santa Cruz C20, dilution 1:80), and HES1 (rabbit polyclonal, dilution 1:100, generous gift from T. Sudo, Torey Industries Inc., Tokyo, Japan) with heat-induced antigen retrieval in citrate buffer pH 6.0 for 30 minutes. Staining was performed with labelled streptavidin biotin-diaminobenzidin from DAKO. For semiquantitative analysis of the EGFR, pEGFR, and ADAM17 staining, a four-tiered grading system was used as follows: 0 (negative), 1+ (weak positivity), 2+ (intermediate positivity), and 3+ (strong positivity). Two investigators (S.S., M.K.) performed the analysis independently.

Real-time quantitative reverse transcriptase-PCR

Total RNA from cells was isolated using RNeasy kit (Qiagen). cDNA was synthetisized using the RevertAid Premium First Strand cDNA Synthesis kit with oligo(dT) Primers (Fermentas), and gene expression was examined with an ABI Prism using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The data were normalized to glyceraldehyde-3-phosphate dehydrogenase expression. HES1 Primers sense was 5′-TGATTTTGGATGCTCTGAAGAAAGATA-3′, and antisense was 5′-GCTGCAGGTTCCGGAGGT-3′. Tissue preparation, manual macrodissection of pure tumor cell populations, and RNA extraction from formalin-fixed tissues were performed as previously described (35). Details on reverse transcription, PCR conditions, sequences for primers and probes for EGFR and TATA box binding protein as housekeeping gene control, and quantification procedures have been published (35, 36).

Statistical methods

Statistical analyses were performed using the SPSS software (version 16.0, SPSS, Inc.) and Microsoft Excel 2004 (Mac). Data were presented using frequency tables, mean bars ± 1.96 SD, and trend curves of means with 95% confidence intervals. To investigate associations between cell groups and distribution of ordinal-scaled parameters, the generalized Fisher's exact test was used. The Φ coefficient was used to quantify bivariate correlations of dichotomized variables. Φ values of ~0 indicate poor association, whereas an absolute value of Φ = 1 would mean absolute concordance of the binary variables' categories. Comparisons of quantitative measurements between two independent cell groups were performed using the Mann-Whitney *U* test. All statistical comparisons were made in sense of an exploratory data analysis using a two-sided 0.05 level of significance.

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Results

ADAM17 mediates cell growth, survival, and tumorigenicity by EGFR-independent mechanisms

ADAM17 mediates various proteolytical processes implicated in tumorigensis including EGFR ligand cleavage. We first examined the requirement of MMP/ADAM proteolytical activity for anchorage-independent growth of NSCLC cell lines with different *KRAS* and *EGFR* mutational status using the broad spectrum MMP inhibitor batimastat (BB-94; Supplementary Table S1; Fig. 1A). Batimastat considerably reduced colony formation in all but one cell line (NCI-H520). Notably, upon EGF stimulation, BB-94–induced suppression of anchorage-independent growth was only partially attenuated in four cell lines (NCI-H292, NCI-H358, NCI-1650, and NCI-1975). NCI-2170 cells were completely rescued by EGF. However, NCI-H2170 treated with BB-94 and EGF caused markedly fewer colonies compared with control cells stimulated with EGF alone (Supplementary Fig. S1A). To specifically target ADAM17, we significantly reduced ADAM17 expression by shRNA in NCI-H292 cells (Fig. 1B). ADAM17 knockdown led to a marked suppression of both constitutive as well as EGF-induced colony formation, again indicating EGFR ligand–independent functions of ADAM17 (Fig. 1C, left). To increase the concentration of endogenous EGFR ligands, we doubled the number of plated cells per well, which resulted in a higher number of colonies (Fig. 1C, right). The addition of exogenous EGF only had a minor effect on colony formation and failed to completely compensate for ADAM17 deletion. In NCI-H358 cells, ADAM17 shRNA reduced transformation efficiency to a similar extent (Supplementary Fig. S1B and C). In addition, we determined ADAM17-dependent proliferation and survival of NCI-H292 cells. ADAM17 knockdown led to a dramatic cell growth suppression, which was only partially rescued by EGF (Fig. 1D, left). Using propidium iodide staining, ~50% of ADAM17-deficient cells were found

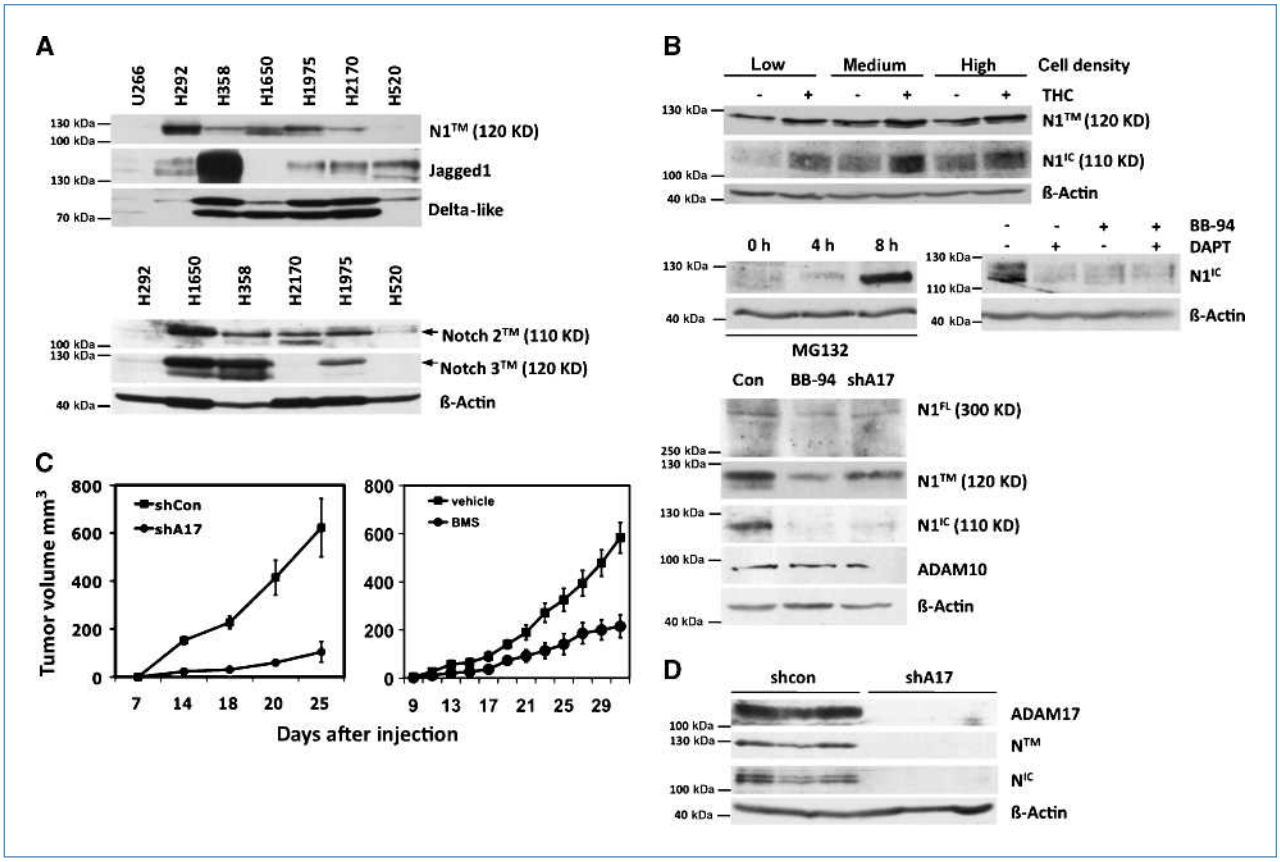

Figure 2. Activation of Notch1 signaling requires ADAM17 *in vitro* and *in vivo*. A, Notch1 ($N1^{TM}$), Jagged1, and Delta-like expression in the indicated NSCLC cell lines. The Notch1-negative myeloma cell line U266 was used as a negative control (top). Notch2 and Notch3 expression in the analyzed panel of NSCLC cell lines (bottom). B, Notch1 activation in NCI-H292 cells plated on 10-cm dishes in low (4×10^6 cells), medium (5×10^6 cells), or high (6×10^6 cells) density stimulated with Δ9-tetrahydrocannabinol (1 μmol/L) or PBS. Lysates were analyzed for S2-cleaved $N1^{TM}$ and intracellular Notch1 ($N1^{IC}$) by Western blot (top). Expression of $N1^{IC}$ in cells treated with MG132 (10 μmol/L) for the indicated time (middle left). $N1^{IC}$ protein levels in THC-stimulated NCI-H292 cells in the presence or absence of 10 μmol/L batimastat (BB-94) and/or 1 μmol/L DAPT (middle right). NCI-H292 control cells treated with BB-94 or cells harboring a stable ADAM17 knockdown (shA17) were analyzed for the indicated Notch1 cleavage forms and ADAM10 (bottom). C, growth of xenograft tumors using 2×10^6 ADAM17 knockdown NCI-H292 cells (shA17) and control cells (shCon; left). NCI-H292 cells (2×10^6) were injected into the flanks of athymic nude mice and treated with BMS-561,392 (BMS) or vehicle. The tumor size was measured every other day (right). Points, mean; bars, 1.96 SD. D, ADAM17, $N1^{TM}$, and $N1^{IC}$ expression in three explanted xenograft tumors of each group as described in C.

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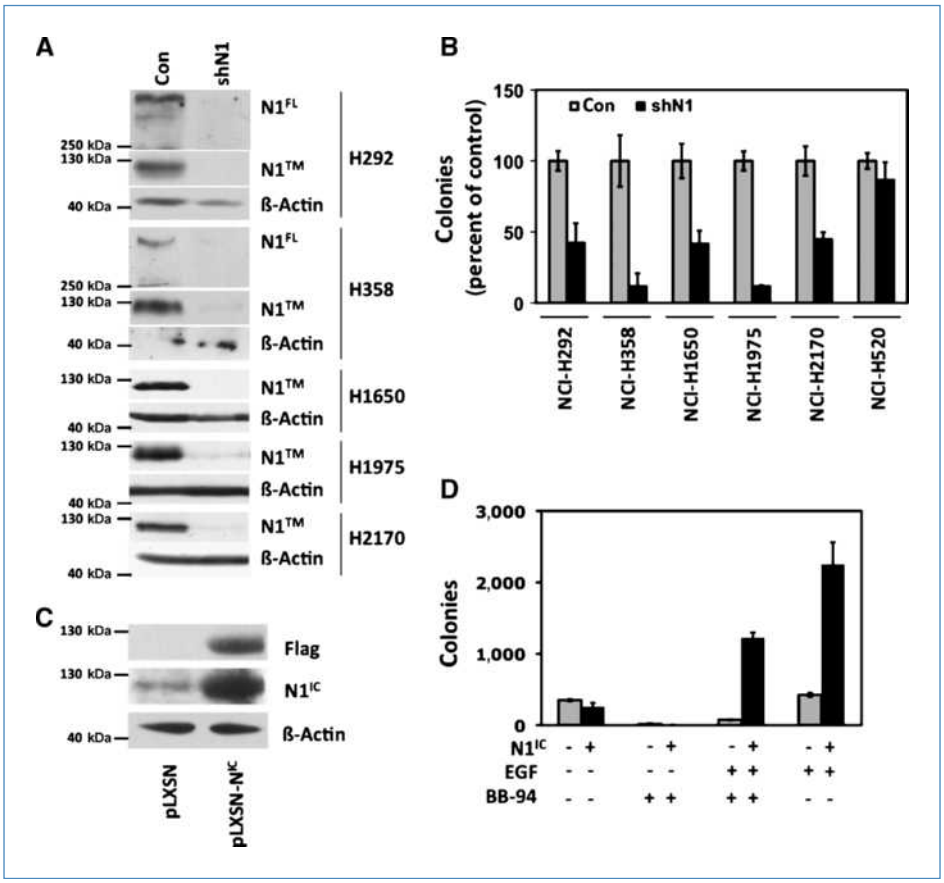

Figure 3. The transformed phenotype of NSCLC cells is dependent on Notch1 signaling. A, Notch1 knockdown using shRNA (shN1) of NSCLC cell lines analyzed by Western blot for $N1^{FL}$ and $N1^{TM}$ as indicated. B, colony formation of NSCLC cell lines infected with Notch1 shRNA. The colony numbers are presented as percent of control. C, ectopic expression of $N1^{IC}$ in NCI-H292 cells detected by anti-flag and anti-$N1^{IC}$ Western blot. D, soft agar colonies of control and $N1^{IC}$-infected NCI-H292 cells treated with 10 μmol/L batimastat (BB-94) and EGF as indicated (right). Columns, mean; bars, 1.96 SD.

within the sub-G_1 fraction compared with <10% of the control cells (Fig. 1D, right). In the presence of EGF, cell death was reduced to ~25%. These findings strongly suggest a critical role of ADAM17 for growth, survival, and transformation of NSCLC cells independently from EGFR ligand shedding.

ADAM17 is required for Notch1 activation *in vitro* and *in vivo*

To determine ADAM17-mediated EGFR stimulation, we induced proteolysis of EGFR ligands by G protein–coupled receptor activation using Δ9-tetrahydrocannabinol (14). Both batimastat and ADAM17 shRNA were used to inhibit ligand cleavage. Western blot analysis revealed a strict requirement of ADAM17 for the activation of the EGFR, Erk1/2, and Akt (Supplementary Fig. S2A and B). Besides EGFR ligands, ADAM17 mediates the shedding of many other soluble factors. However, enhanced cell growth of ADAM17-deficient NCI-H292 cells using conditioned media was dependent on EGFR activation, indicating an ADAM17-cleaved receptor rather than a ligand precursor as a possible candidate (Supplementary Fig. S2C).

Notch signaling is involved in cell-cell communication and tumorigenesis of epithelial tumor cells and requires ADAM17 for its activation as shown in 293T cells (20, 25). We analyzed our panel of NSCLC cell lines for both the expression of Notch receptors and ligands (Fig. 2A). In five NSCLC cell lines, we detected Notch1 as measured by $N1^{TM}$ expression. NCI-H520 cells, which are resistant to MMP/ADAM inhibition, were virtually Notch1-negative. The membrane-tethered Notch ligands Jagged-1 and Delta-like were expressed in five and four cell lines, respectively. Notch2 ($N2^{TM}$) expression was found in four and Notch3 ($N3^{TM}$) expression in three cell lines. Notch1 activation as determined by $N1^{IC}$ using a Val-1744 (neo-epitope)–specific antibody was dependent on cell density and markedly enhanced by Δ9-tetrahydrocannabinol stimulation, particularly in low or medium density (Fig. 2B, top). Because $N1^{IC}$ is very rapidly degraded, we confirmed the expression of $N1^{IC}$ using the proteasomal inhibitor MG132, which led to increased $N1^{IC}$ levels over time (Fig. 2B, middle left). Conversely, batimastat as well as the γ-secretase inhibitor DAPT or the combination of both were able to disrupt the generation of $N1^{IC}$ (Fig. 2B, middle right). More specifically, ADAM17 shRNA reduced $N1^{IC}$ level to the same extent compared with BB-94 (Fig. 2B, bottom). To some degree, $N1^{TM}$ expression seems to be regulated by ADAM17 function, whereas full-length Notch1 ($N1^{FL}$) is not affected by ADAM17 depletion. Expression of ADAM10, which is also a major protease for S2-cleavage, was not influenced by BB-94 or ADAM17 knockdown. These data show that ADAM17 is required for Notch1 receptor proteolysis in NSCLC cells *in vitro*.

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The most stringent assay reflecting oncogenic transformation is tumor growth in nude mice. NCI-H292 cells caused large tumors within <4 weeks, whereas ADAM17 knockdown cells showed a markedly reduced growth (Fig. 2C, left). For pharmacologic ADAM inhibition *in vivo*, we used the orally available ADAM/MMP inhibitor BMS-561,392, which has already been used in clinical trials (37–39). In mice treated with BMS-561,392, the mean volume of tumors reached <50% compared with tumors of nontreated mice (Fig. 2C, right). Western blot analysis of the control tumors revealed activated Notch1 signaling as determined by $N1^{IC}$. In contrast, $N1^{IC}$ were virtually undetectable in ADAM17 knockdown tumors (Fig. 2D). As observed in cell culture experiments, $N1^{TM}$ levels were markedly decreased in ADAM17-deficient tumors, whereas the Notch1 full-length form was similar compared with control tumors (Supplementary Fig. S2D; Fig. 2B, bottom). Taken together, these findings show that ADAM17 functions as a critical regulator of tumor cell growth and Notch1 activation *in vitro* and *in vivo*.

Notch1 signaling is critical for the transformed phenotype of NSCLC cells

To determine the role of Notch signal transduction in lung cancer, we knocked down Notch1 in all Notch1-expressing NSCLC cell lines (Fig. 3A). To avoid off-target effects, we also infected Notch1-negative NCI-H520 cells with Notch1 shRNA (Supplementary Fig. S3A; Fig. 2A). As shown in Fig. 3B, anchorage-independent growth was significantly decreased in all Notch1-deficient cells, indicating the requirement of Notch signaling to maintain the transformed phenotype. In contrast, colony formation of NCI-H520 cells was unchanged by Notch1 shRNA expression.

To determine the effect of constitutively activated Notch signaling in NSCLC, we ectopically expressed $N1^{IC}$ in

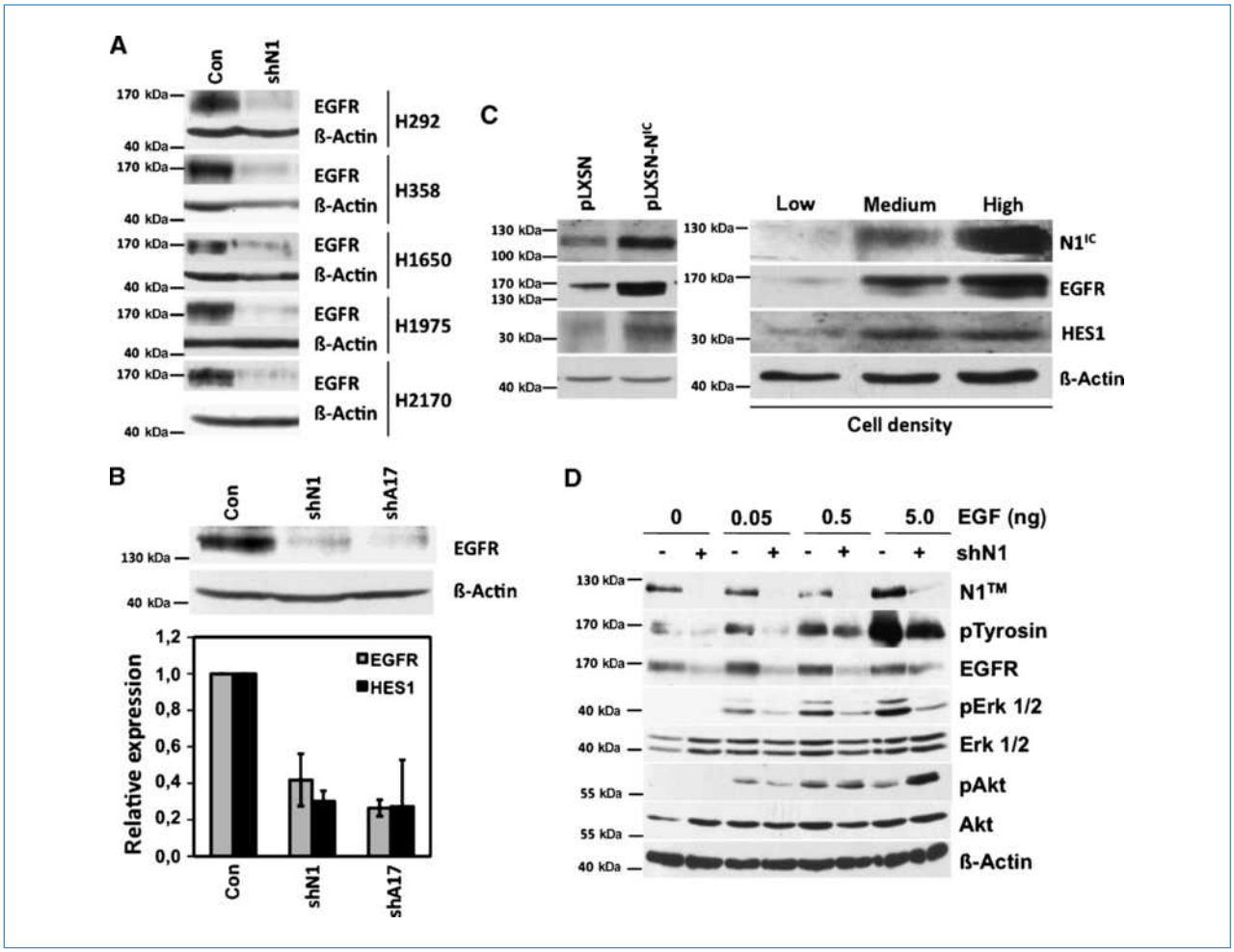

Figure 4. Notch1 regulates EGFR expression. A, Western blot analysis of EGFR expression of the indicated NSCLC cells with (shN1) and without (con) a stable Notch1 knockdown. B, EGFR protein expression of control-infected (con), Notch1 knockdown (shN1), and ADAM17 knockdown (shA17) NCI-H292 cells (top). Relative expression of EGFR and HES1 RNA in NCI-H292 cell lines measured by quantitative real-time PCR. Columns, mean; bars, 1.96 SD (bottom). C, expression of $N1^{IC}$, EGFR, and HES1 in $N1^{IC}$ and control-infected NCI-H292 cells (left) and in parental cells plated in low, medium, and high density (right). D, control-infected and Notch1 knockdown cells were starved and subsequently stimulated with the indicated concentrations of EGF. Lysates were analyzed for the indicated proteins.

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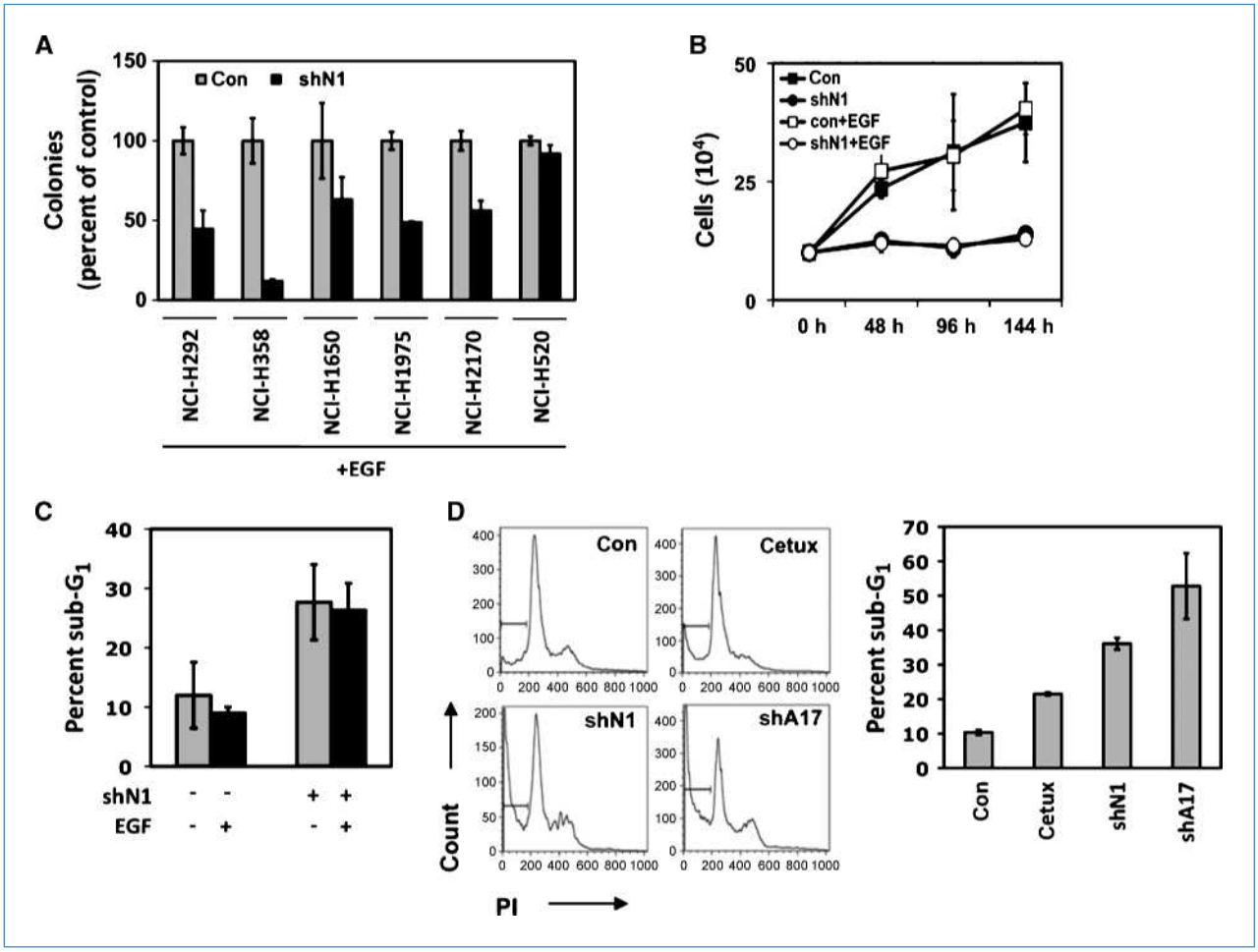

Figure 5. Notch1-deficient cells do not respond to EGF stimulation. A, colony formation in EGF-supplemented soft agar using the indicated NSCLC cell lines infected with Notch1 or control shRNA. B, proliferation assay using Notch1-deficient and control cells in the absence or presence of EGF. C, propidium iodide (PI) stain after 72 h using the same cells and EGF concentration as in E. D, cell cycle analysis (left) and quantification of sub-G_1 fraction (right) of NCI-H292 cells treated with 30 μg/mL cetuximab (cetux) compared with NCI-H292 cells harboring a stable ADAM17 (shA17) and Notch1 (shN1) knockdown. Columns, mean; bars, 1.96 SD.

NCI-H292 cells (Fig. 3C). These cells showed increased proliferation in the presence but not in the absence of exogenous EGF (Supplementary Fig. S3B). $N1^{IC}$ expression considerably enhanced colony formation particularly in the presence of exogenous EGF (Fig. 3D). Similar results were obtained with other NSCLC cells (Supplementary Fig. S3C). Importantly, the combination of $N1^{IC}$ expression and EGF stimulation rendered cells insensitive to batimastat treatment (Fig. 3D). Of note, in the absence of EGF, colony formation of $N1^{IC}$-expressing cells was completely abrogated by batimastat, indicating that EGFR activation is critical for $N1^{IC}$-induced transformation. Analysis of cell viability further supported these findings (Supplementary Fig. S3D). $N1^{IC}$ expression in combination with EGFR activation strongly protected cells from cell death induced by batimastat, whereas constitutive Notch1 signaling alone increased the sensitivity of the cells to the inhibitor. In summary, these findings suggest that Notch1 signaling strongly promotes cellular transformation in the presence of EGFR signaling.

Notch1 signaling regulates EGFR expression

The function of Notch signaling in many epithelial malignancies including NSCLC is largely unclear. Because $N1^{IC}$ expression requires concomitant EGFR signaling to increase anchorage-independent growth and survival, we determined EGFR protein expression in our panel of Notch1-deleted NSCLC cell lines. As shown in Fig. 4A, we observed a substantially reduced EGFR protein expression in all Notch1 knockdown cell lines. In ADAM17 knockdown NCI-H292 cells, EGFR protein levels were reduced to the same extent (Fig. 4B, top). Moreover, quantitative PCR revealed decreased transcript levels of the Notch target gene HES1 and EGFR in Notch1 and ADAM17 knockdown NCI-H292 cells, indicating that Notch1 controls EGFR expression on the transcriptional level (Fig. 4B, bottom). Using the proteasome

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inhibitor MG132, we did not find any evidence for Notch1-regulated proteasomal degradation (Supplementary Fig. S4). To confirm the Notch1-dependent regulation of the EGFR, we analyzed both $N1^{IC}$-expressing NCI-H292 cells as well as cells grown in different densities by EGFR Western blot (Fig. 4C). Ectopic expression of $N1^{IC}$ resulted in substantially elevated EGFR and HES1 protein levels compared with control-infected cells (Fig. 4C, left). In addition, increasing cell densities induced the generation of $N1^{IC}$ as well as enhanced EGFR and HES1 levels (Fig. 4C, right). EGFR protein expression was also upregulated in xenograft tumors (Supplementary Fig. S2D). We next examined EGFR-induced signal transduction in Notch1-deficient NCI-H292 cells. Using increasing concentrations of EGF (0.05-5 ng/mL), phosphorylation of EGFR and Erk1/2 was considerably lower in Notch1 knockdown cells compared with control cells, whereas Akt activation was virtually unchanged (Fig. 4C). These results show that Notch1 signaling is required for EGFR expression and EGFR-dependent Erk but not Akt activation.

Notch1 deficiency renders NSCLC cells insensitive to EGF stimulation

In accordance with our biochemical findings, colony formation of Notch1-deleted NSCLC cells was significantly decreased compared with the respective controls in EGF-supplemented soft agar, indicating that EGF is not able to rescue Notch1 deficiency (Fig. 5A). NCI-H520 again served as Notch1-negative control cells. In addition, exogenous EGF was unable to enhance impaired cell growth and survival of Notch1 knockdown cells, indicating decreased sensitivity to EGF stimulation (Fig. 5B and C). Cleavage of caspase-3 was also independent from EGF stimulation (Supplementary Fig. S5). As recently shown in a prostate cancer cell line, EGFR expression mediates survival to a large part independently from its kinase activity (38). Because induction of cell death is of high clinical relevance, we compared cetuximab treatment with Notch1 and ADAM17 knockdown (Fig. 5D). Inhibition of EGFR activation by cetuximab predominantly led to a G_0-G_1 arrest and to a sub-G1 fraction of only ~20%. In contrast, Notch1 and particularly ADAM17 shRNA strongly induced cell death in more than 35% and 50% of the cells, respectively. Taken together, these *in vitro* results strongly indicate that ADAM17 regulates tumorigenesis of NSCLC cells through the activation of Notch1. Notch1 controls EGFR expression and sensitivity to EGF stimulation. Importantly, abrogation of ADAM17 function and Notch1 signaling results in substantial cell death, whereas EGFR inhibition alone mainly induces cell cycle arrest.

ADAM17 and Notch1 expression correlates with HES1 and EGFR expression in primary lung and NSCLC tissue samples

To find evidence for an association between EGFR, ADAM17, and Notch1 signaling *in vivo*, we analyzed 38 NSCLC specimens (22 adenocarcinomas, 14 squamous cell carcinomas, and 2 large cell carcinomas) and 8 normal lung tissues by immunohistochemistry (IHC; Supplementary Fig. S6A; Table 1). Strikingly, all NSCLC samples revealed a positive staining for ADAM17, whereas all normal tissues were virtually negative (P = 0.012). Phosphorylated EGFR was also only present within the NSCLC group, although not to the level of statistical significance. EGFR expression

Table 1. Correlation of ADAM17, Notch1, HES1, and EGFR in primary tissue samples

A. Associations were assessed using the Fisher's exact test

	IHC	0	1+	2+	3+	*P*
ADAM17	NSCLC % (n = 38)	0	30.6	41.7	27.8	0.012
	Normal % (n = 8)	100	0	0	0	
pEGFR	NSCLC % (n = 38)	61.1	16.7	8.3	13.9	0.259
	Normal % (n = 8)	100	0	0	0	
EGFR	NSCLC % (n = 38)	16.7	36.1	30.6	16.7	0.035
	Normal % (n = 8)	12.5	87.5	0	0	
Notch1	NSCLC % (n = 38)	47.4	44.7	7.9	0	0.710
	Normal % (n = 8)	37.5	37.5	25.0	0	
HES1	NSCLC % (n = 38)	44.7	55.3	0	0	0.015
	Normal % (n = 8)	100	0	0	0	

NOTE: Table showing semiquantified IHC results of normal lung and NSCLC samples.

B. Quantitative EGFR RNA expression of microdissected NSCLC and normal lung tissue

RNA EGFR/TATA box binding protein	Mean	SD	*P*
NSCLC % (n = 38)	1.77	2.19	0.012
Normal % (n = 8)	0.71	0.27	

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was significantly higher in NSCLC compared with normal tissue, although 87.5% of normal lung specimens showed weak (1+) EGFR staining ($P = 0.035$). We also found elevated EGFR RNA levels in microdissected NSCLC tissue ($P = 0.012$). Notch1 showed cytoplasmic staining in >50% of all specimens without a significant difference between NSCLC and normal lung ($P = 0.710$). However, the Notch1 target gene HES1 reflecting Notch activation was expressed in >50% of the NSCLC samples but not in normal lung tissue ($P = 0.015$). In addition, we found a significant correlation between ADAM17 expression and phosphorylated EGFR within all 46 analyzed samples ($\Phi = 0.319$, $P = 0.039$; Supplementary Fig. S6B). Importantly, ADAM17 staining also correlated with high (2+ and 3+) protein levels of EGFR ($\Phi = 0.386$, $P = 0.015$) and HES1 expression ($\Phi = 0.421$, $P = 0.005$). These results suggest that ADAM17-mediated proteolysis is involved in the regulation of the Notch targets HES1 and EGFR in primary lung and NSCLC tissue. To further substantiate our hypothesis, we tested the correlation between Notch1 and its regulated genes *EGFR* and *HES1* (Supplementary Fig. S6C). Within the NSCLC group, Notch1 expression (1+ and 2+) significantly correlated with both high protein levels of EGFR ($\Phi = 0.478$, $P = 0.004$) and HES1 expression ($\Phi = 0.630$, $P < 0.001$). These immunohistochemical results strongly support our *in vitro* and *in vivo* data describing a novel function of ADAM17 and Notch1 in NSCLC.

Discussion

Among the various signaling pathways contributing to the transformation and tumorigenesis of NSCLC, EGFR-dependent signaling has been described most extensively. The requirement of ADAM17-mediated EGFR ligand cleavage for tumor cell growth has been implicated in various epithelial cancer entities including NSCLC (8, 13, 40, 41). This is the first study describing ADAM17 as a critical regulator of tumorigenicity, cell growth, and survival through the activation of Notch1 and subsequent EGFR expression in NSCLC cells (Supplementary Fig. S5).

We found that ADAM17 is required for anchorage-independent growth in five of six NSCLC cell lines independently from the mutational status of *KRAS* and *EGFR*. In addition, we show that ADAM17 knockdown almost completely abrogated tumor growth in nude mice. Although ADAM17 was shown to be required for EGFR ligand cleavage, the addition of exogenous EGF was not sufficient to fully rescue ADAM17 function. These results suggested additional ADAM17 substrates contributing to tumorigenesis. Zhou and colleagues (16) showed that inhibition of ADAM17 restores sensitivity to gefitinib and reduces tumor growth by blocking cleavage of proheregulin. However, in addition to ligand cleavage, our findings suggested an ADAM17 substrate that is involved in cell-cell interactions.

Among the various ADAM17 substrates, we identified Notch1 expression in five of six cell lines. The requirement of ADAM17 for Notch1 cleavage has previously been shown in a 293T cell model (20). In NSCLC cells, we show that Notch signaling was tightly regulated by ADAM17 as shown *in vitro* and *in vivo*. In addition, Notch1 shRNA markedly reduced colony formation, cell growth, and cell viability in all Notch1-expressing cell lines, suggesting that Notch1 mediates cellular transformation in NSCLC cells. Notch1 signaling has been implicated in the pathogenesis of many epithelial cancer types (22, 23, 25, 26, 29, 42, 43). It has been shown that Notch1 and HES1 are necessary for H-RasV12–induced tumorigenesis in fibroblasts (44, 45). In NSCLC cells, Chen and colleagues (46) observed Notch1 expression and sensitivity to γ-secretase inhibition only in low oxygen, whereas the ectopic expression of $N1^{IC}$ led to apoptosis under normal conditions. In our study, $N1^{IC}$ expression abrogated colony formation and induced cell death in the absence of EGFR ligands. In contrast, activation of EGFR and Notch1 signaling supported survival and considerably enhanced transformation efficiency. Other studies have shown that Notch3 is required for the survival of NSCLC cell lines by using a γ-secretase inhibitor (27, 28). We found Notch3 expression in three of six cell lines. However, we focused on the function of Notch1 and used shRNA to specifically disrupt Notch1 signaling without affecting other Notch family members.

The function of Notch1 signaling in oncogenesis is not fully understood. Strikingly, we found a marked reduction of EGFR protein and RNA levels in Notch1 knockdown cells. This is of high biological and clinical significance because elevated levels of the EGFR have been observed in ~60% of the NSCLCs and EGFR overexpression is believed to be an early event in the pathogenesis of NSCLC (47, 48). Moreover, besides EGFR gene amplification, little is known about the regulation of EGFR expression. In glioma cells, Notch1 has been shown to control EGFR expression through p53 (49). However, our panel of NSCLC cells did not reveal a correlation between EGFR and p53 protein expression (data not shown).

Importantly, our *in vitro* model was confirmed by the analysis of human specimens of normal lung and NSCLC by IHC, revealing ADAM17 expression in all tumor samples and not in normal lung tissue as well as a significant association between ADAM17, Notch activation, and high EGFR levels. Using Western blot analysis, Zhou and colleagues (16) showed elevated levels of ADAM17 and activated Her3 in NSCLC. However, this is the first study showing a significant correlation between ADAM17, Notch1 signaling, and EGFR expression in primary NSCLC tissue.

Inhibition of EGFR activation by cetuximab mainly resulted in cell cycle arrest, whereas Notch1 and ADAM17 knockdown led to substantial cell death. In addition, cell death and caspase-3 cleavage were independent from Akt activation. Therefore, this effect might be caused by EGFR kinase–independent mechanisms. In prostate cancer cells, Weihua and colleagues (50) showed that downregulation of the EGFR led to cell death, whereas EGFR kinase inhibition predominantly induced a cell cycle arrest. These results strongly indicate that EGFR downregulation combined with EGFR inhibition by targeting ADAM17 might constitute a highly effective treatment approach.

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In summary, we identified a molecular mechanism in NSCLC with high clinical relevance. EGFR activation and overexpression are frequently found in NSCLC. Using various NSCLC cell lines, we were able to show that targeting ADAM17 or Notch1 markedly suppresses tumorigenicity and induces profound cell death. Based on their ability to regulate both EGFR expression through Notch1 activation and EGFR activation, ADAM17-targeted approaches are particularly attractive for the treatment of NSCLC patients independently from their respective mutational status.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Anja Baumgart, Stefan Seidl, Petros Vlachou, et al.

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