Epidermal Growth Factor Receptor and Notch Pathways Participate in the Tumor Suppressor Function of γ-Secretase*

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γ-Secretase, a unique aspartyl protease, is required for the regulated intramembrane proteolysis of Notch and APP, pathways that are implicated, respectively, in the pathogenesis of cancer and Alzheimer disease. However, the mechanism whereby reduction of γ-secretase causes tumors such as squamous cell carcinoma (SCC) remains poorly understood. Here, we demonstrate that γ-secretase functions in epithelia as a tumor suppressor in an enzyme activity-dependent manner. Notch signaling is down-regulated and epidermal growth factor receptor (EGFR) is activated in SCC caused by genetic reduction of γ-secretase. Moreover, the level of EGFR is inversely correlated with the level of γ-secretase in fibroblasts, suggesting that the up-regulation of EGFR stimulates hyperproliferation in epithelia of mice with genetic reduction of γ-secretase. Supporting this notion is our finding that the proliferative response of fibroblasts lacking γ-secretase activity is more sensitive when challenged by either EGF or an inhibitor of EGFR as compared with wild type cells. Interestingly, the up-regulation of EGFR is independent of Notch signaling, suggesting that the EGFR pathway functions in parallel with Notch in the tumorigenesis of SCC. Collectively, our results establish a novel mechanism linking the EGFR pathway to the tumor suppressor role of γ-secretase and that mice with genetic reduction of γ-secretase represent an excellent rodent model for clarifying pathogenesis of SCC and for testing therapeutic strategy to ameliorate this type of human cancer.

γ-Secretase was initially recognized as an activity catalyzing the intramembranous cleavage of the amyloid-β precursor protein (APP)3 C-terminal fragment to generate amyloid-β (Aβ) peptides, aberrant accumulation of which is central to the pathogenesis of Alzheimer disease (1). Substantial evidence supports the view that presenilins (PS), which when mutated cause autosomal dominant AD (2), form high molecular weight complexes with several other transmembrane proteins critical for γ-secretase activity, including Nicastrin (Nct), APH-1, and PEN-2 (3). Recent studies demonstrate that all four components are required for the assembly of the active enzyme complex of γ-secretase. Other than APP family of proteins, γ-secretase is also critical for the regulated intramembranous proteolysis of a growing list of type I transmembrane proteins, including the Notch family of proteins (4, 5).

Previous studies of PS1 transgene rescued-PS1 null mice (6) indicated that PS1/γ-secretase functions as a tumor suppressor in epithelia. To explain the tumorigenesis occurring in this mouse model (6), it was proposed that PS1 suppresses the β-catenin signaling pathway through interactions with the large hydrophilic loop domain of PS1 (6, 7), such that the lack of PS1 in the skin results in increased stability of β-catenin. However, contrary to this idea is the finding (8) that mice with a homozygous deletion of exon 10 encoding part of the hydrophilic loop domain of PS1 (a mutation that effectively abolished interactions between PS1 and β-catenin but retains γ-secretase activity) do not exhibit overt abnormalities, such as spontaneous skin cancers. Because deletion of PS1 leads to reduction of γ-secretase activity, other pathways directly regulated by γ-secretase are likely to participate in the tumorigenesis of γ-secretase-deficient mice.

One likely downstream pathway regulated by γ-secretase that may participate in the tumorigenesis of epithelia is Notch, a well known developmental signaling pathway critical for binary cell fate determination involving differentiation, proliferation, and survival (9). Supporting this notion are two recent reports (10, 11) showing that notch serves as a tumor suppressor in the skin. However, it remains to be determined whether the Notch pathway is solely responsible for tumorigenesis in mice with reduced γ-secretase activity because considerable pathological differences exist between mice with reduced γ-secretase activity and Notch1 conditional knockout mice (10).

To clarify the mechanism whereby reduction of γ-secretase leads to increased risk of squamous cell carcinoma (SCC), we analyzed mice with genetic reduction of γ-secretase, including animals with deletion of one allele of Nct (Nct+/− mice). We also demonstrate that pharmacological inhibition of enzymatic

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3 The abbreviations used are: APP, amyloid-β precursor protein; MAPK, mitogen-activated protein kinase; PS, presenilins; Nct, nicastrin; SCC, squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; EGFP, enhanced green fluorescence protein; RT, reverse transcriptase.

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activity of γ-secretase in mice led to hyperplastic epidermis that resembles skin lesions observed in Nct<sup>+/−</sup> mice at a pre-tumor stage, indicating that tumor suppressor function of γ-secretase is dependent on its enzymatic activity. Because the skin tumors in Nct<sup>+/−</sup> mice resemble human head and neck squamous cell carcinoma (HNSCC), we examined pathways that are known to be activated in human HNSCC, including epidermal growth factor receptor (EGFR) pathways. We show that whereas Notch signaling is reduced, EGFR is activated in skin lesions of carcinoma (HNSCC), we examined pathways that are known to be regulated by γ-secretase that functions in parallel with Notch in SCC tumorigenesis. Together, these results demonstrate that γ-secretase activity functions to prevent tumor formation in the skin, and identify a novel mechanism linking both EGFR and Notch signaling to the tumor suppressor functions of γ-secretase.

**EXPERIMENTAL PROCEDURES**

**Animals and Cell Lines—**Nct<sup>+/−</sup> mice were generated as described previously (12). The congenic Nct<sup>+/−</sup> mice were generated by cross-breeding hybrid (C57BL/6;129SvJ) Nct<sup>+/−</sup> mice with C57BL/6 mice for 10 generations. Notch<sup>+/−</sup> and Tg2576 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All procedures involving mice were performed under the guidelines of JHMI Institutional Animal Care and Use Committee. Nct<sup>+/−</sup>, Nct<sup>−/−</sup>, Aph-1a<sup>−/−</sup>, Aph-1a<sup>/−</sup>, Nct<sup>+/−</sup>;PS1<sup>+/−</sup>, Notch1<sup>−/−</sup>, Notch1<sup>−/−</sup>, and wild type fibroblasts were generated as described previously (12, 13). All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplied with 10% fetal bovine serum with antibiotic and l-glutamine (Invitrogen).

**Immunoblot and Antibodies—**Immediately after euthanasia, mouse tissues (brain, skin) were dissected and proteins were extracted with TEPER buffer containing complete protease inhibitor mixture (Roche). The protein concentrations in the supernatants were determined by the BCA method (Pierce) and inhibited mixture (Roche). The protein concentrations in the extracts were determined with TEPER buffer containing complete protease inhibitor mixture (Roche).

**Inhibitors—**Immediately after euthanasia, mouse tissues (brain, skin) were dissected and proteins were extracted with TEPER buffer containing complete protease inhibitor mixture (Roche). The protein concentrations in the supernatants were determined by the BCA method (Pierce) and equal amounts of protein lysates resolved on 4–20% Tris glycerine SDS-PAGE gels, then transferred to polyvinylidene difluoride (PVDF) membranes, and probed with the following antibodies: anti-Nicastrin (12), antisera specific for PS1 (14) (PS1-NTF, 1:5000; PS1-loop, 1:2500), and anti-PEN-2 (1:2000) antibodies: anti-Nicastrin (12), antisera specific for PS1 (14) (PS1-NTF, 1:5000; PS1-loop, 1:2500), and anti-PEN-2 (1:2000). Immunoblotting of protein extracts from fibroblasts blotted by antisera against EGFR were measured by ImageJ software from the National Institutes of Health.

**Quantitative RT-PCR—**Quantitative RT-PCR was performed as previously described (17), with all reactions normalized to actin or glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems). Assays-on-Demand TaqMan probes (Applied Biosystems) were used to measure murine Hes1, Hes5, and EGFR mRNAs expression. All samples were analyzed in triplicate.

**Treatment of HEK293 Cell or Mice with γ-Secretase Inhibitors—**HEK293 cells were plated on 6-well plates the day before treatment. When the cells were ~50% confluent on the plates, the standard medium was replaced with medium containing different concentrations (0.5–4 μM) of γ-secretase inhibitor JC-22 (18). JC-22 was a gift from Dr. Yueming Li (Memorial Sloan-Kettering Cancer Center). The medium was changed every 24 h, and the cells were collected for analysis 48 h post-treatment.

**The γ-secretase inhibitor LY-411,575** was synthesized at the Mayo Clinic Chemistry Core. Purified LY-411,575 at concentrations of 0.01375 or 0.02756 g/kg was homogenously incorporated into Harlan Teklad 7012 chow by Research Diets, Inc. (New Brunswick, NJ). Based upon dietary consumption at this age, these diets were designed to deliver 2.5 or 5 mg of LY-411,575 on average per day. Tg2576 mice (7-month-old, females) were then fed LY-411,575 formulated in chow at various dosing regimes (from 2 weeks up to 6 weeks). Diet consumption, general health, and body weight were monitored on a weekly basis.

**Proliferative Assays of Cells Treated with AG1478 and EGF—**Nct<sup>+/−</sup> and Nct<sup>−/−</sup> cells were plated in 96-well plates at a density of 2.5 × 10<sup>3</sup> cells/well and allowed to adhere overnight. For AG1478 (Invitrogen) treatments, the cells were incubated with medium containing 1 or 10 μM of AG1478 for 48 h. For EGF (Sigma) or the combination of EGF and AG1478 treatments, the cells were plated in serum-free medium for 12 h prior to the treatment. After 0 and 48 h, viable cell number was determined by using the CellTiter 96<sup>Aqueous</sup> Non-Radioactive Cell Proliferation Assay (MTS assay, Promega), and the percentage proliferation was calculated by the following formula: [A<sub>490</sub> (48 h) of treated cells – A<sub>490</sub> (0 h)]/[A<sub>490</sub> (48 h) of control cells – A<sub>490</sub> (0 h)] × 100.

**Treatment of Nct<sup>−/−</sup> Cell with Adenovirus—**Nct<sup>−/−</sup> cells were plated on 6-well plates the day before treatment. When the cells were ~50% confluent on the plates, the standard medium was replaced with medium containing different doses of adenovirus (1–5 × 10<sup>6</sup> plaque forming units/ml) expressing...
Notch1-ICD or Notch2-ICD as described previously (19). The cells were collected for analysis 48 h post-treatment.

RESULTS

Congenic Nct+/− Mice on C57BL/6 Background Develop Spontaneous Skin Tumors—We recently demonstrated that genetic reduction of Nct/γ-secretase in mice on hybrid C57BL/6:129svj background increased the risk of developing squamous cell carcinoma, indicating that Nct/γ-secretase can serve as a tumor suppressor in epithelia (20). To investigate whether the tumor suppressor role of Nct/γ-secretase is dependent on the genetic background in mice, we generated Nct+/− congenic mice on a C57BL/6 background. As we have observed in Nct+/− mice with a hybrid strain background, congenic C57BL/6 Nct+/− mice start to develop skin lesions as early as 3 months of age (Fig. 1A). The initial skin lesions in Nct+/− mice were usually at the face and around the tail (Fig. 1B), and some lesions gradually progressed to invasive skin tumors. The median age at which tumors appear in congenic Nct+/− mice (n = 74) was ~57 weeks (Fig. 1A), similar to that we have observed in Nct+/− mice with the hybrid strain (~60 weeks, n = 89). Although a small percentage of wild type C57BL/6 mice developed various tumors at older ages, none of the wild type mice developed skin tumors.

The early skin lesions (nodules <5 mm diameter, >10 nodules per mouse) observed in Nct+/− mice were characterized by follicular or cystic hyperkeratosis (Fig. 1C). These skin abnormalities were most severe on the muzzle (nose and mouth), in the inguinal-perineal areas, the meibomian (sebaceous) glands of the eyelids, and auditory sebaceous (Zymbal) gland. Proliferative changes with squamous metaplasia were present in all nodules, and some more advanced lesions also contained areas of dysplasia with proliferation of atypical cells. Inflammatory changes and ulceration were also occasionally present. Some lesions gradually progressed to invasive SCC (Fig. 1, C and D). Interestingly, these SCCs of the head and neck areas in Nct+/− mice resembled HNSCC of human. Because HNSCC is a very common and clinically aggressive tumor for which no transgenic animal model is currently available, Nct+/− mice serve as a valuable animal model for understanding the pathogenesis of HNSCC and evaluating therapies for this human cancer.

Mice Treated with a γ-Secretase Inhibitor Develop Skin Lesions—Whereas we demonstrated that Nct/γ-secretase serves as a tumor suppressor in mice, it is not known whether the tumor suppressor role of γ-secretase is regulated by one of the components of γ-secretase complex or by its enzymatic activity. To clarify this issue, we treated mice with a γ-secretase inhibitor, LY-411,575 (~2.5 mg/kg/day, greater than ED50). This dose results in an inhibition of γ-secretase activity, as measured by alterations in steady state levels of Aβ; >90% and >70% reduction in levels of plasma Aβ and brain Aβ, respectively, although having no effect on the levels of γ-secretase complex (data not shown). However, animals cannot endure long-term treatment of the drug at doses higher the ED50, with most mice not surviving treatment of LY-411,575 at ~2.5 mg/kg/day for more than 6 weeks. When treated with a higher dose of LY-411,575 (~5 mg/kg/day), the mice do not survive more than 4 weeks. Although mice treated with the LY-411,575 for such a short period of time fail to develop spontaneous skin tumors, they did develop severe skin abnormalities, such as ulcers, dermatitis, nodules, and thickening of skin (Fig. 2A), similar to the lesions commonly observed in Nct+/− mice at pre-tumor stages. Histological analysis of mice treated with 2.5 mg/kg/day dose of LY-411,575 for 6 weeks revealed hyperproliferative skin lesions characterized by epidermal hyperplasia, as well as follicular and epidermal inclusion cysts (Fig. 2D), features resembling those observed in the pre-tumor stage of Nct−/− mice (Fig. 2C), but not found in wild type mice (>100 mice analyzed). These results indicate that suppression of γ-secretase activity causes hyperproliferation in squamous epithelia, and that the tumor suppressor function of γ-secretase is dependent on its enzymatic activity.

Down-regulation of Notch Signaling and Elevation of Cytosolic β-Catenin in Skin Lesions of Nct+/− Mice—Previous studies have shown that Notch signaling prevents proliferation and induces early differentiation events in the epidermis, and that Notch functions as a tumor suppressor in the skin (10, 11).
Because Notch is a well recognized substrate of γ-secretase (4, 21), we determined whether reduction of γ-secretase activity resulted in altered Notch signaling in the epidermis of Nct+/− animals by measuring the level of the transcription factor Hes-1, a direct target gene up-regulated by Notch signaling. Hes-1 protein level was significantly reduced in Nct+/− skin, especially in skin with neoplastic lesions (Fig. 3A). Consistent with this finding are our analysis of mRNA levels of Hes1 and Hes5 using quantitative RT-PCR (Fig. 3B), as the level of Hes1 (Fig. 3B) and Hes5 (data not shown) mRNA in SCC of Nct+/− mice was 3-fold lower (p = 0.001; Student’s t test) than that of the skin of wild type siblings. Levels of Hes1 and Hes5 mRNA from normal skin of Nct+/− mice were also reduced, but to a lesser extent as compared with that of SCC in Nct+/− mice. Taken together, these results indicate that Notch signaling is reduced in the skin of Nct+/− mice. Consistent with previous studies showing that levels of p21 are decreased in the epidermis of the Notch1 conditional mutant and in notch1−/− primary keratinocytes (10), the levels of CDK2 were increased in skin tumors of Nct+/− mice (Fig. 3A), suggesting that its inhibition by p21 is decreased. However, another cyclin-dependent kinase inhibitor, p27, was slightly increased and its target gene, CDK4, was not changed in tumor tissues of Nct+/− mice (Fig. 3A), suggesting that p21/CDK2 is specifically affected in tumors of Nct+/− mice. Collectively, our data suggest that attenuation of Notch signaling plays an important role in tumorigenesis of Nct+/− mice. The activation of β-catenin signaling correlating with the development of skin tumors has been previously documented in three different mouse models (6, 10, 11). Whereas abnormal
stabilization and accumulation of β-catenin has been implicated in various neoplasms, including some types of skin cancer such as pilomatrixomas (22) and basal cell carcinoma (23), no such activation of β-catenin signaling has been directly linked to development of SCC (24). To determine whether β-catenin signaling is altered in the skin of Nct+/− mice, we examined levels of total and Thr41/Ser45 phosphorylated β-catenin (the activated form of the protein). Although they were modestly increased in hyperplastic skins of Nct+/− mice as compared with that of wild type mice (Fig. 3A), a marked increase of both total and phosphorylated β-catenin were observed in neoplastic skin lesions and correlated with the malignancy of lesions (Fig. 3, A and D).

Because β-catenin also participates in complexes with cadherin cell adhesion molecules in epithelia, it is possible that the increased level of β-catenin in skin lesions are due to either activation of β-catenin signaling or to an increase of adhesion complexes in skin lesions. To clarify this issue, we first examined β-catenin levels in fibroblasts derived from embryos of Nct+/− and Nct+/−;PS1+/− mice. Surprisingly, the β-catenin levels were unchanged in Nct−/− and Nct−/−;PS1−/− fibroblasts as compared with wild type cells (Fig. 3C), suggesting that β-catenin is not stabilized simply by reduction of γ-secretase. Interestingly, immunocytochemical analysis revealed that β-catenin was only detected in epidermis and near the plasma membrane, with no obvious nuclear localization in normal skin of Nct+/+ and Nct−/− mice or skin with hyperplastic lesions in Nct+/− mice (Fig. 3D, middle panel), indicating that β-catenin levels in hyperproliferative skin is correlated with the increased levels of adherens junction complexes. In contrast, β-catenin was readily detectable in the nucleus in regions displaying evidence of neoplasia (Fig. 3D, inset of bottom panel stained with β-catenin). The malignancy of skin lesions and alteration in β-catenin signaling were also correlated with cellular proliferation. The most intensive Ki-67 staining (Fig. 3D, lane 3) was correlated with invasive foci and coupled with an increase of β-catenin translocation into the nucleus. These data suggest that partial reduction of γ-secretase and Notch signaling is not sufficient to directly facilitate the activation of β-catenin. However, at advanced stages in the development of neoplastic lesions in Nct+/− mice, β-catenin signaling was up-regulated, supporting the idea that β-catenin signaling is not an initial response to the reduction of γ-secretase, but a later event correlated with the progression of tumors. Consistent with this idea is the observation that mice with a homozygous deletion of exon 10 of PS1, a mutation that effectively abolished interaction between PS1 and β-catenin and deregulated β-catenin signaling, do not develop spontaneous skin cancers (8).

EGFR Signaling Participates in the Tumorigenesis of Nct+/− Mice—Because there are obvious significant differences between the phenotypes observed in Nct+/− mice and Notch1 conditional knock-out mice (10), we attempted to identify additional molecular mechanisms critical for tumorigenesis occurring in Nct+/− mice. The fact that the skin tumors developed in Nct+/− mice are strikingly similar to HNSCC encouraged us to examine pathways that are known to be involved in the human disease. Because EGFR is overexpressed in at least 90% of HNSCC cases (25), we asked whether EGFR signaling and its downstream pathways are altered in our Nct+/− mice. EGFR is normally tightly controlled and expressed at low levels on the surface of most normal cells, except for those of hematopoietic origin (26). Consistent with this view, we detected low levels of EGFR in the skin of Nct−/− mice (Fig. 4, A and B). In contrast, EGFR was overexpressed in hyperplastic and neoplastic skin lesions of Nct−/− mice (Fig. 4A). Because Akt kinase is activated by the EGFR pathway (27, 28), we examined whether Akt signaling was altered in the skin of Nct−/− mice. Both total Akt and the Ser473 phosphorylated active form of Akt were increased in skin of Nct+/− mice, especially in the neoplastic regions (Fig. 4A). Furthermore, in skin tumors, we also detected an increase in the levels of phosphorylated p70S6K, a downstream target of Akt activation (Fig. 4A). Taken together, these data suggested that the EGFR pathway is activated in skin lesions of Nct+/− mice.

Although up-regulation of EGFR is associated with hyperplastic and neoplastic skin lesions in Nct+/− mice, it is not established as to whether activation of EGFR is initiated by reduction of γ-secretase activity, or is simply a downstream effect of tumorigenesis in Nct+/− mice. To address this issue, we assessed EGFR levels in normal skin tissues of Nct−/− mice. Interestingly, protein levels of EGFR in the skin of Nct+/− mice were higher as compared with that of wild type mice (Fig. 4B), suggesting that up-regulation of EGFR occurs prior to the tumorigenesis observed in Nct+/− mice.

To further test whether the EGFR is regulated by one of the components or enzymatic activity of γ-secretase, we treated HEK293 cells with various concentrations of a γ-secretase inhibitor, IC-22 (18), and examined the levels of EGFR by protein blot analysis. As expected, this compound effectively inhibited γ-secretase activity (IC50 ≈ 1 μM, data not shown), but did not impact on the level of γ-secretase complex as judged by the level of Nct (Fig. 4C). Interestingly, the levels of EGFR in these treated cells correlated with the concentration of the γ-secretase inhibitor in the medium (Fig. 4C). These results strongly support the notion that the up-regulation of levels of EGFR in cells are dependent on the levels of γ-secretase activity.

To corroborate these inhibitor studies, we examined levels of EGFR in fibroblasts derived from Nct+/+, Nct+/−, Nct−/−;PS1+/−, PS1−/−, and Nct−/− embryos, in which the γ-secretase complex is abolished in Nct−/− cells, reduced to ~30% in Nct+/−;PS1−/− cells, and reduced to ~50% in Nct−/− cells as compared with Nct+/+ cells (Fig. 4D). Whereas EGFR was detected at low levels in Nct+/+ cells as judged by antisera specific for EGFR, EGFR expression was up-regulated in Nct−/− cells and was further increased in Nct+/−;PS1−/− and Nct−/− cells as compared with Nct+/− cells (Fig. 4D). These findings corroborate outcomes from γ-secretase inhibitor studies and strongly support the view that reduction of γ-secretase leads to up-regulation of EGFR in fibroblasts. Interestingly, the accumulated levels of EGFR appear to be inversely related to levels of γ-secretase in these cell lines (Fig. 4E). To validate this inverse relationship between levels of γ-secretase and EGFR, we examined the expression of EGFR in Aph-1a+/− cells (which harbor ~65% of normal level of γ-secretase) and Aph-1a−/− cells (which harbor ~30% of normal level of γ-secretase) (13) (Fig. 4D). Consistent with our expectation, the expression level of EGFR in Aph-1a−/− cells was markedly reduced compared with that in Aph-1a+/− cells.
EGFR in *Aph-1a*+/− fibroblasts, SCC is not observed in *Aph-1a*+/− mice (even at two years of age). Taken together, these findings establish that the risk and severity of developing skin tumors are correlated with reduced levels of γ-secretase and parallels the magnitude of EGFR up-regulation in fibroblasts.

To investigate the effects of EGFR signaling on proliferation, we treated *Nct*+/+ and *Nct*−/− cells with an EGFR inhibitor, AG1478. Whereas the growth rate of *Nct*+/+ cells was slightly reduced, the proliferation of *Nct*−/− cells was significantly inhibited by treatment with AG1478 (Fig. 5A), confirming that the up-regulation of EGFR signaling is responsible for the altered proliferative potential of *Nct*−/− cells. This proliferative response to AG1478 is specific to EGFR signaling because exposure of fibroblasts to EGFR dramatically stimulated proliferation of *Nct*−/− cells, but not *Nct*+/+ cells (Fig. 5B), and this proliferation effect can be blocked by the presence of an EGFR inhibitor (Fig. 5B). Collectively, these data are consistent with the view that up-regulation of EGFR stimulates proliferation of *Nct*−/− cells and leads to hyperproliferation in epithelia of mice with reduced γ-secretase activity.

**Up-regulation of EGFR in the *Nct*−/− Cells** is independent of Alteration of Notch Signaling—Although the exact molecular mechanism whereby EGFR is up-regulated remains to be defined, the observation that LIN-12/Notch signaling antagonizes the EGFR-MAPK pathway in *Caenorhabditis elegans* (29, 30) suggests that up-regulation of EGFR may result from down-regulation of Notch signaling in *Nct*−/− mice. To examine the relationship between Notch1 signaling and EGFR, we first assessed the levels of EGFR in fibroblasts derived from *Notch1*+/− and *Notch1*−/− embryos. Interestingly, loss of function of Notch1 in *Notch1*+/− and *Notch1*−/− cells did not lead to up-regulation of EGFR as we observed in *Nct*−/− cells (Fig. 6A). The β-catenin levels were also unchanged in *Notch1*+/− and *Notch1*−/− fibroblasts as compared with that of wild type cells (Fig. 5A), findings that are consistent with our view that β-catenin is not directly regulated

1a−/− cells was significantly higher than that of *Nct*+/− cells (−50% of normal level of γ-secretase) (12), and similar to that of *Nct*+/+;PS1+/− cells (−35% of normal level of γ-secretase) but lower than that observed in *Nct*−/− cells (Fig. 4, D and E). However, we failed to detect increased levels of EGFR in *Aph-1a*+/− cells (−65% of normal level of γ-secretase). Interestingly, consistent with our observation that there is no up-regulation of EGFR in fibroblasts derived from *Notch1*+/− and *Notch1*−/− embryos.
Regulation of EGFR Pathway by γ-Secretase

by γ-secretase. To test whether Notch signaling can suppress the level of EGFR, we infected Nct⁻/⁻ cells with different doses of adenovirus expressing Notch1-ICD and Notch2-ICD, the constitutive active forms of Notch signaling. Interestingly, the up-regulation of EGFR in Nct⁻/⁻ cells was not suppressed by Notch signaling (Fig. 6B). These results indicate that the up-regulation of EGFR in Nct⁻/⁻ cells is not due to reduction of the Notch pathway, and strongly support the view that EGFR and Notch pathways are both regulated independently by γ-secretase and function in parallel in the tumorigenesis occurring in Nct⁺/⁺ mice.

Microarray Analysis of Nct⁺/⁺ and Nct⁻/⁻ Fibroblasts—We have provided substantial evidence that up-regulation of EGFR is related to the tumorigenesis occurring in Nct⁺/⁺ mice. However, the mechanism of up-regulation of EGFR remains unclear. We first asked whether EGFR is regulated at the transcriptional level. RT-PCR analysis of mRNA extracted from Nct⁺/⁺ and Nct⁻/⁻ cells showed that the mRNA level of EGFR is increased in Nct⁺/⁺ cells (-fold change = 0.55 ± 0.14, n = 4, p < 0.001) and Nct⁻/⁻ cells (-fold change = 1.04 ± 0.21, n = 4, p < 0.001) as compared with wild type cells, suggesting that reduction of γ-secretase increases the rate of transcription of EGFR. However, it is difficult to reconcile the less than 2-fold increase of the mRNA level of EGFR with the dramatic elevation in the level of EGFR proteins in these cells. It is plausible that other pathways also contribute to regulation of EGFR or participate in tumor development. To address these questions, we examined the differential expression of genes in fibroblasts with reduced levels of γ-secretase activity using a microarray approach. Because we have demonstrated that the risk of developing skin tumor in mice is correlated with the levels of γ-secretase, we focused on genes that were consistently changed in Nct⁺/⁺ and Nct⁻/⁻ cells, and ones whose expression was correlated with levels of γ-secretase activity. We found about 92 and 112 genes that were, respectively, up-regulated or down-regulated at least 2-fold in Nct⁺/⁺ and Nct⁻/⁻ fibroblasts as compared with wild type cells.

Whereas many of the differentially expressed genes are structural proteins, or are involved in general metabolism, some are related to cell proliferation, differentiation, and apoptosis (Table 1). Interestingly, expression of several genes that are related to Notch, Wnt/β-catenin, and EGFR pathways are significantly changed in Nct⁺/⁺ and Nct⁻/⁻ fibroblasts. For example, consistent with the notion that Notch signaling is regulated by γ-secretase, Jagged1 (a ligand of Notch receptor) was decreased in Nct⁺/⁺ and Nct⁻/⁻ cells as compared with wild type cells. Interestingly, whereas the mRNA level of β-catenin was not changed in these cells, mRNA levels of lymphoid enhancer binding factor 1 (Lef1), a downstream factor of the β-catenin pathway, were decreased in Nct⁺/⁺ and Nct⁻/⁻ fibroblasts as compared with wild type cells, supporting the idea that the β-catenin pathway is not activated when the levels of γ-secretase are diminished in fibroblasts. Importantly, consistent with our in vivo and in vitro findings, the mRNA levels of EGFR were increased in Nct⁺/⁺ cells (-fold change = 0.24 ± 0.06) and Nct⁻/⁻ cells (-fold change = 0.62 ± 0.18). Interestingly, several genes that are directly related to the EGFR signaling pathway are also altered. For example, ERBB receptor feedback inhibitor 1 (Erff1; also known as mitogen-inducible gene 6, Mig6; RALT or Gene 33), a negative feedback regulator of receptor tyrosine kinases, was significantly down-regulated in Nct⁺/⁺ and Nct⁻/⁻ cells (Table 1). Consistent with these findings is the recent report documenting that deletion of the mouse gene encoding Mig6 caused hyperactivation of endogenous EGFR and resulted in hyperproliferation and impaired differentiation of epidermal keratinocytes (31), phenotypes that resemble those in Nct⁺/⁺ mice. It is conceivable that down-regulation of Mig6 leads to activation of the EGFR pathway in mice with reduced γ-secretase activity. Further sup-

![FIGURE 5. Effects of AG1478 and EGF on proliferation of Nct⁺/⁺ and Nct⁻/⁻ fibroblasts. A, Nct⁺/⁺ and Nct⁻/⁻ fibroblasts were treated in vitro with AG1478 (1 and 10 μM). B, Nct⁺/⁺ and Nct⁻/⁻ fibroblasts were treated in vitro with EGF (10 ng) or a combination of EGF (10 ng) and AG1478 (5 μM). Data were expressed as average percentage ± S.E. of cell growth of three independent experiments.](image)

![FIGURE 6. EGFR pathway is independent of Notch signaling in fibroblasts. A, protein extracts (40 μg each) from Notch1⁺/⁺, Notch1⁻/⁻, and wild type fibroblasts were immunoblotted with antisera against Nct, PS1-loop, EGFR, and β-catenin. The same blot was stripped and reprobed with antisera specific to mouse actin as loading control. B, protein extracts (40 μg each) from Nct⁻/⁻ cells that were infected with adenovirus expressing Notch1-ICD (5–20 μl), Notch2-ICD (5–20 μl), and green fluorescent protein (GFP) (10 μl) were immunoblotted with antisera against EGFR and GFP. The same blot was stripped and reprobed with antisera specific to mouse actin as loading control.](image)
Regulation of EGFR Pathway by γ-Secrease

A partial list of genes that are differentially expressed in Nct+/− and Nct−/− fibroblasts as compared to Nct+/+ cells (+/+) (log 2 base).

| Probe       | Symbol | Description                      | GenBank     | UniGene     | +/+ vs. +/+ Fold change | −/+ vs. +/+ Fold change |
|-------------|--------|----------------------------------|-------------|-------------|-------------------------|-------------------------|
| Nct pathway | Jag1   | Jagged 1                         | AV359819    | Mm.2239     | −1.36 ± 0.06a           | −1.85 ± 0.17a           |
| Wnt/β-catenin pathway | Lef1 | Lymphoid enhancer binding factor 1 | NM_010703   | Mm.255219   | −1.08                   | −1.71                   |
| EGFR pathway | Errfl | ERBB receptor feedback inhibitor 1 | NM_133753   | Mm.318841   | −1.44 ± 0.13a           | −1.13 ± 0.09a           |
|            | Eps8   | Epidermal growth factor receptor pathway substrate 8 | NM_007945   | Mm.235346   | 1.38 ± 0.12a            | 2.25 ± 0.04a            |
|            | Ereg   | Epiregulin                        | NM_007950   | Mm.4791, Mm.381463 | 1.05                   | 2.89                   |
| Cell cycle | Ccnd2  | Cyclin D2                         | NM_009829   | Mm.333406   | 1.87 ± 0.1a             | 2.66 ± 0.17a            |
|            | Maf    | Avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog | AV284857    | Mm.275549   | 1.15                    | 3.59                    |
|            | Gas1   | Growth arrest specific 1          | BB550400    | Mm.22701   | 1.86 ± 0.18a            | 1.33 ± 0.18a            |
| Growth factors | Bmp4 | Bone morphogenetic protein 4     | NM_007554   | Mm.6813     | 1.85                    | 2.34                    |
|             | Grem1  | Gremlin                           | BC015293    | Mm.166318   | −1.01                   | −2.93                   |
|             | Grem2  | Gremlin 2 homolog, cysteine knot superfamily (Xenopus laevis) | BC018360    | Mm.25760    | −1.1                    | −1.18                   |
|             | Igfl   | Insulin-like growth factor 1      | BG075165    | Mm.268521   | 1.82 ± 0.11a            | 2.58 ± 0.09a            |
|             | Igfbp4 | Insulin-like growth factor binding protein 4 | BC019836    | Mm.233799   | 1.18                    | 3.07                    |
|             | Hbegf  | Heparin-binding EGFR-like growth factor | L07264     | Mm.289681   | −1.22 ± 0.18a           | −1.02 ± 0.01a           |
|             | Cgfl   | Connective tissue growth factor   | NM_010217   | Mm.1810     | −1.46                   | −2.06                   |
|             | Pdgfc  | Platelet-derived growth factor, C polypeptide | NM_019971   | Mm.331089   | −2.32 ± 0.13a           | −1.82 ± 0.10a           |
|             | Ndn    | Nedarin                           | AW743020    | Mm.250919   | −1.07 ± 0.03a           | −2.44 ± 0.18a           |
|             | Ngfb   | Nerve growth factor β             | NM_013609   | Mm.1259     | −1.78                   | −1.43                   |
|             | Gdnf   | Glial cell line derived neurotrophic factor | NM_010275   | Mm.4679     | −2.69                   | −3.47                   |
|             | Artn   | Artemin                           | AK015393    | Mm.56897    | −1.18                   | −1.36                   |

a The -fold change values indicated are averages for all the probes listed for each gene.

Port of the view that EGFR is activated by reduction of γ-secretase activity came from our observations that epiregulin, an autocrine growth factor for human keratinocytes (32) and EPS8 (epidermal growth factor receptor pathway substrate 8), an EGFR-activated protein (33), were both up-regulated in Nct+/− and Nct−/− cells. Collectively, these findings confirm our view that activation of the EGFR pathway in the skin plays a central role in tumorigenesis of Nct+/− mice exhibiting reduced levels of γ-secretase activity.

Apart from EGFR and Notch pathways, expression of several cell signaling genes were also altered, such as IGF1, BMP4, and some neurotrophic factors (Table 1). In this regard, we observed that cyclin D2 is significantly up-regulated in Nct+/− and Nct−/− fibroblasts as compared with wild type cells. D-type cyclins (D1, D2, and D3) are expressed in the G1 phase of the cell cycle and form complexes with and activate CDK4 and CDK6. Overexpression of cyclin D2 has been reported in several human cancers (34–36). Interestingly, mRNA levels of MAF (avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog), an oncogene that transactivates cyclin D2 and enhances myeloma proliferation (37), were increased in Nct+/− and Nct−/− cells, suggesting that up-regulation of cyclin D2 may contributes to the tumorigenesis in skin of Nct+/− mice.

DISCUSSION

γ-Secrease has been viewed as a potential target for mechanism-based anti-amyloid therapy for AD, however, the full function of this intriguing enzyme remains incompletely understood. Recently, it has been speculated that individual components of γ-secretase complex may function independent of γ-secretase complex or activity. For example, it has been proposed that PS1 serves as a tumor suppressor through the β-catenin pathway. However, our demonstration that reduction of Nct, another critical component of the γ-secretase complex, also leads to tumorigenesis in mice strongly favor the idea that the tumor suppressor function of γ-secretase is dependent on its enzymatic activity. This view is supported by our findings that pharmacological inhibition of γ-secretase in mice induced hyperproliferation in epithelia.
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This notion also led us to examine pathways regulated by γ-secretase activity that may account for increased risk of age-dependent skin cancer in \textit{Nct}^−/− mice. Consistent with this view is our observation that Notch signaling is altered in \textit{Nct}^+/− mice, and, given that Notch is a tumor suppressor (10), it is likely that Notch plays a critical role in the tumorigenesis of SCC in \textit{Nct}^+/− mice. However, there are obvious differences between the phenotypes observed in \textit{Nct}^+/− mice and \textit{Notch1} conditional knock-out mice (10). First, the frequency of spontaneous skin tumors in \textit{Nct}^+/− mice is clearly much greater than that observed in the \textit{Notch1} conditional knock-out mice; spontaneous skin tumors occur at high frequency in \textit{Nct}^+/− mice, whereas \textit{Notch1} conditional knock-out mice require chemical carcinogens to induce skin tumors (10). Moreover, the tumor types are different in these mouse models; the tumor type observed in \textit{Nct}^+/− mice is mainly SCC with occasional basal cell hyperplasia, whereas BCC is found in \textit{Notch1} conditional knock-out mice (10), suggesting that other pathways may also participate in the tumorigenesis of \textit{Nct}^+/− mice.

Because there are four Notch genes in mice, it is possible that weak tumorigenesis observed in \textit{Notch1} conditional knock-out mice is due to compensatory mechanisms and genetic redundancy. Because γ-secretase regulates all members of the Notch family, reduction of ∼50% of γ-secretase activity in \textit{Nct}^+/− mice may have a more profound affect on Notch signaling as compared with a \textit{Notch1} null. Supporting this notion is the finding that pan-Notch inhibitor (DNMAML1) conditional transgenic mice also develop SCC (11). However, it remains to be determined whether DNMAML1 affects other pathways in addition to Notch signaling. In contrast to the low frequency of tumors occurring in Notch1 conditional knock-out mice, our findings that there is a minimum threshold level of γ-secretase required to prevent SCC and that the risk of tumorigenesis is dependent on the magnitude of γ-secretase in mice (20) would strongly support that idea that pathways other than Notch also participate in the tumorigenesis of SCC observed in \textit{Nct}^+/− mice. Consistent with this view, our demonstration that EGFR is activated in skin lesions of \textit{Nct}^+/− mice, and levels of EGFR is inversely correlated with levels of γ-secretase \textit{in vivo} and \textit{in vitro} and with the risk of developing SCC in mice with genetic reduction of γ-secretase, establish EGFR as a critical participant in the tumorigenesis of \textit{Nct}^+/− mice. Taken together with our observation that activation of EGFR is independent of Notch signaling, our findings strongly support the view that activation of EGFR, in parallel with altered Notch signaling, plays a critical role in tumor suppressor function of γ-secretase in epithelia of mice.

Interestingly, another group has independently also demonstrated an inverse relationship between the levels of PS and EGFR in skin tumors of \textit{PS1}^+/−:\textit{PS2}^−/− mice and in brains of \textit{PS1}^+/−:\textit{PS2}^−/− mice (38). Furthermore, consistent with our observation that reduction in γ-secretase activity leads to tumorigenesis in \textit{Nct}^+/− mice, they showed that the PS/γ-secretase activity-dependent pathways mediate transcriptional regulation of EGFR (38). Our observation of modest up-regulation of EGFR mRNA in \textit{Nct}^+/− and \textit{Nct}^−/− fibroblasts is consistent with their findings. However, our microarray analysis suggests that EGFR signaling may also be regulated by other mechanisms, such as by Errf1, a negative feedback regulator of EGFR. It has been reported that deletion of mouse Errf1 leads to hyperactivation of endogenous EGFR and results in hyperproliferation of epidermal keratinocytes in mice (31), a phenotype that resembles that of \textit{Nct}^+/− mice. In addition, our observation that other pathways are also altered in \textit{Nct}^+/− and \textit{Nct}^−/− cells, such as cyclin D2 and Maf, overexpression of which have been reported in several cancers (34–37), raises the possibility that these pathways may contribute to tumorigenesis in \textit{Nct}^+/− mice. However, because our microarray analysis is based on an expression profile of fibroblasts, it will be critical to validate these results in the future using our \textit{in vivo} animal models.

EGFR is an oncogene that is involved in a variety of human cancers, including human HNSCC (25). Our observation that tumors developed in the head and neck of \textit{Nct}^+/− mice histopathologically and molecularly resemble that of human HNSCC supports the idea that \textit{Nct}^+/− mice serve as a useful model system to study HNSCC, a common and clinically aggressive tumor for which no transgenic animal model currently exists. Future efforts to clarify regulatory pathways that control tumor development should provide new insights toward our understanding of cancer biology as well as identification of a novel mechanism-based therapy for the treatment and prevention of human skin cancer.

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