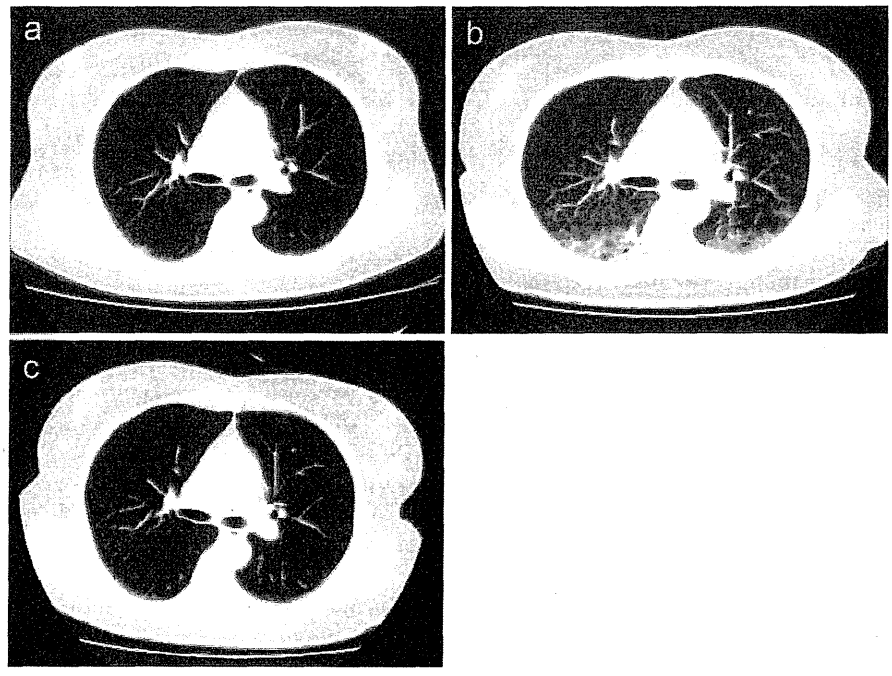

Fig. 1 **a** Chest computed tomography (CT) scan before PLD therapy showed clear lung field. **b** Twenty-six days after second administration of PLD, CT revealed bilateral interstitial infiltrates and ground-glass opacities. **c** Two months after steroid therapy, CT showed significant improvement

factor, which could induce interstitial pneumonitis. In addition to PLD, she received ascorbic acid, pyridoxal phosphate hydrate, rebamipide, and brotizolam. As they were all unlikely to induce interstitial pneumonitis, administration of these drugs except PLD was continued. The patient was treated with intravenous methylprednisolone 500 mg/day for 3 days. Azithromycin 1,000 mg per os and intravenous cefepime 4 g/day were administrated until all examinations of infection proved to be negative, including blood culture, β-D-glucan, influenza antigen detection, urinary pneumococcal antigen test, Chlamydia IgA/IgG, candida antibody assays, and galactomannan antigen of aspergillosis.

After the steroid pulse therapy, symptoms resolved promptly and lung function tests improved remarkably. Two months after the diagnosis of interstitial pneumonitis, a chest CT scan showed significant improvement (Fig. 1c). PLD was discontinued and her chemotherapy regimen was changed to docetaxel (70 mg/m^2). She has not shown any respiratory symptoms after cessation of PLD. Currently, she is alive with disease 24 months after the surgery and undergoing fifth-line chemotherapy.

Discussion

Pegylated liposomal doxorubicin is a reformulated version of doxorubicin, which takes the active agent doxorubicin and places it into a phospholipid bilayer called a liposome and another outer layer of methoxypolyethylene glycol. This coating allows PLD to evade detection and destruction by the immune system and to remain longer in the blood circulation.

PLD has a different toxicity profile compared with free doxorubicin. Though cumulative cardiac toxicities are unique to free doxorubicin, cardiac toxicities associated with PLD are rarely reported. Toxicities relatively unique to PLD are hand-foot syndrome or plantar palmar erythema (PPE), which are rarely reported with free doxorubicin.

It is reported that lung toxicity induced by doxorubicin is rare. Several cases of interstitial pneumonitis associated with doxorubicin or PLD have been described [6, 7]. It was unclear whether the lung toxicities were directly attributable to doxorubicin in published case reports, because all patients were concurrently receiving other agents, mostly antineoplastic drugs, which were also implicated in causing lung toxicity.

In our case, though the symptoms were initially severe, discontinuation of PLD and steroid therapy immediately resolved them. Serum KL-6 levels have been reported to correlate with grade of interstitial lung disease [8]. Normal serum KL-6 level in this case might associate with her excellent clinical course.

Two possible mechanisms of drug-induced interstitial pneumonitis have been described, one of which is the direct toxicity of the drug to the pulmonary organ and the other is immunological mechanism, although the etiology of PLD-induced interstitial pneumonitis is unclear.

Drug-induced pulmonary toxicity in Japan got a great deal of attention because of pulmonary toxicity induced by molecular-targeted chemotherapeutic drugs, gefitinib and an antirheumatic drug, leflunomide. It is reported that the

rates of interstitial lung disease associated with gefitinib and leflunomide are 2 and 1.1% in Japan and 0.3 and 0.02% in the United States, respectively. These data indicate that chemotherapeutic-drug-induced pulmonary toxicity is more frequent in Japan than in other nations [9, 10]. Fatal pneumonitis induced by gefitinib or leflunomide is less frequent in other Asian countries than in Japan. It may be that such drugs including PLD cause fatal pneumonitis predominantly in Japanese. The differences of genetic background or lifestyle between Japanese and non-Japanese might be involved in this event.

Drug-induced interstitial pneumonitis should be taken into consideration in the differential diagnosis of otherwise unexplained ground-glass lung lesions. Pulmonary toxicity induced by PLD is rare, but awareness of this toxicity is important, since it could be lethal. Additional investigation is required to elucidate how PLD induces interstitial pneumonitis or whether PLD-induced interstitial pneumonitis is more frequent in Japanese.

Conflict of interest No author has any conflict of interest.

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LETTER TO THE EDITOR

Second-line chemotherapy with docetaxel and carboplatin in paclitaxel and platinum-pretreated ovarian, fallopian tube, and peritoneal cancer

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Abstract We retrospectively evaluated the efficacy and toxicity of docetaxel and carboplatin in patients with platinum and paclitaxel-pretreated recurrent ovarian, fallopian tube, and peritoneal cancer. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four patients had measurable disease. The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, 34 weeks, and the median overall survival time was 94, 224, 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes (8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months.

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Keywords Docetaxel · Carboplatin · Chemotherapy · Early progression · Recurrent ovarian cancer

The standard regimen as second-line chemotherapy in recurrent ovarian cancer has not been established, especially in the patients with a short progression-free interval from the previous treatment. Docetaxel is an active drug as second-line chemotherapy for recurrent ovarian cancer as well as pegylated liposomal doxorubicin, irinotecan, topotecan, gemcitabine, and etoposide [1].

The purpose of this study was to evaluate activity and toxicity of the combination of docetaxel and carboplatin retrospectively in patients with paclitaxel and platinum resistant (progression-free interval less than 6 months) and partially resistant (progression-free interval of 6–12 months) ovarian, fallopian tube, and peritoneal cancers. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four (81%) patients had measurable disease. Twenty-six (62%) patients had experienced progression of disease within less than 6 months of their last treatment, whereas 16 patients (38%) within 6–12 months. The median number of courses of treatment per patient was 4.5 (range: 1–8 courses). The median follow-up period was 107 weeks (range: 9–373 weeks). The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, and 34 weeks, and the median overall survival time was 94, 224, and 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes

(8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. On the other hand, grade 2/3 neuropathy was observed only in two (4.8%) patients.

Several chemotherapeutic agents such as pegylated liposomal doxorubicin, topotecan, irinotecan, gemcitabine, and etoposide have been used in the treatment of platinum-resistant disease with response rates in the range 10–15% [2–5]. The results from our study about overall response rate are in line with other chemotherapeutic agents. Notably, our data about median time to tumor progression and overall survival are longer than the previously reported data of other regimens.

The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months. However, chemotherapy with docetaxel and carboplatin may improve time to tumor progression and overall survival time in these cases; this regimen can be an alternative in patients whose hematological toxicity is relatively weak at their previous treatment.

Conflict of interest None.

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第３次対がん総合戦略研究事業

ヒトパピローマウイルスを標的とする発がん予防の研究

平成２２年度～２５年度　総合研究報告書

Ⅲ　研究成果の刊行物・別冊
その４

研究代表者　温川　恭至

平成２６（２０１４）年　５月

Ⅲ　研究成果の刊行物・別冊

その４
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Noncanonical NOTCH Signaling Limits Self-Renewal of Human Epithelial and Induced Pluripotent Stem Cells through ROCK Activation

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NOTCH plays essential roles in cell fate specification during embryonic development and in adult tissue maintenance. In keratinocytes, it is a key inducer of differentiation. ROCK, an effector of the small GTPase Rho, is also implicated in keratinocyte differentiation, and its inhibition efficiently potentiates immortalization of human keratinocytes and greatly improves survival of dissociated human pluripotent stem cells. However, the molecular basis for ROCK activation is not fully established in these contexts. Here we provide evidence that intracellular forms of NOTCH1 trigger the immediate activation of ROCK1 independent of its transcriptional activity, promoting differentiation and resulting in decreased clonogenicity of normal human keratinocytes. Knockdown of NOTCH1 abrogated ROCK1 activation and conferred sustained clonogenicity upon differentiation stimuli. Treatment with a ROCK inhibitor, Y-27632, or ROCK1 silencing substantially rescued the growth defect induced by activated NOTCH1. Furthermore, we revealed that impaired self-renewal of human induced pluripotent stem cells upon dissociation is, at least in part, attributable to NOTCH-dependent ROCK activation. Thus, the present study unveils a novel NOTCH-ROCK pathway critical for cellular differentiation and loss of self-renewal capacity in a subset of immature cells.

Notch is an evolutionarily conserved cell surface receptor that plays essential roles in cell fate decisions as well as maintenance of self-renewing tissue organization (1–3). Notch proteins are expressed in most adult tissues, and the biological consequence of Notch activation is critically dependent on the cell type and the cellular context (4–7). In keratinocytes, Notch1 has been shown to be a key inducer of differentiation (8–11). Keratinocyte-specific conditional deletion of the *Notch1* gene results in epidermal hyperproliferation and tumor formation in mice, thus indicating a tumor-suppressive role of Notch1 in mammalian postnatal epidermis (12). The Notch receptor is generally activated by interaction with its ligands displayed on the neighboring cell surface. Cell-cell contact is a strong inducer of keratinocyte differentiation in culture, where Notch1 acts as a critical determinant in the transition from proliferation to differentiation (13, 14). Due to *cis* inhibition of Notch by its ligand when these are expressed on the same cell surface (15, 16), the relative increase in expression levels of the Notch receptor over its ligand is also shown to be a pivotal cue to activate Notch signaling and generate distinct cell fates among neighboring cells (17). We previously demonstrated that p53 and TAp63 transactivate *Notch1* gene expression and induce keratinocyte differentiation, while ΔNp63 is a transcriptional repressor of the *Notch1* gene and inhibits keratinocyte differentiation (14, 18). p63, especially ΔNp63α, is a master regulator of development and maintenance of stratified epithelia (19, 20). ΔNp63α expresses predominantly in the basal proliferating compartment, where Notch1 signaling is suppressed (21). In suprabasal layers, downregulation of ΔNp63α by miR-203 or another factor(s) (22–24) evokes activation of Notch1 signaling, which in turn further downmodulates ΔNp63α expression so as to induce differentiation (9, 21). The Notch1 precursor (~300 kDa) is processed by furin protease in the Golgi apparatus and transported to the cell surface as a mature heterodimeric complex (~120/~180 kDa) that is held by Ca^{2+}-dependent noncovalent interaction (25). Ligand binding dissociates the Notch1 extracellular domain (~180 kDa) by *trans* endocytosis. The residual transmembrane domain (~120 kDa) is sequentially cleaved by tumor necrosis factor alpha-converting enzyme/metalloprotease (TACE) and γ-secretase, resulting in release of the Notch1 intracellular domain (~110 kDa) into the cytosol (3). EDTA is reported to activate Notch signaling through disruption of the heterodimeric complex of Notch1 (25) and thus used as a tool to study Notch1 signaling (26–28). In canonical Notch1 signaling, the liberated Notch1 intracellular domain (~110 kDa) translocates into the nucleus to activate Notch-responsive genes, such as Hes1, by making a complex with CSL family members {CBF1 and RBP-Jκ in mammals, Suppressor of hairless [Su(H)] in *Drosophila*, and Lag1 in *Caenorhabditis elegans*} and its transcriptional coactivator Mastermind (MAM). Besides this canonical pathway, accumulating evidence suggests noncanonical cytoplasmic Notch functions (29–31).

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Rho-associated coiled-coil protein kinases (ROCKs) (also known as Rho kinases [ROKs]) are effectors of the small GTPase Rho and belong to a family of protein serine/threonine kinases (32–34). Activated ROCK proteins regulate actomyosin cytoskeletal dynamics and contractility through phosphorylation of multiple downstream targets, such as myosin phosphatase (MYPT1), to drive cell motility. In keratinocytes, ROCK proteins play a role in differentiation (35, 36), and their selective inhibitor, Y-27632, completely inhibits differentiation as well as stratification of keratinocytes in organotypic raft culture (37). Y-27632 also enables efficient immortalization of not only human primary keratinocytes but also several other primary human epithelial cells in the presence of fibroblast feeders (37, 38), although molecular details supporting immortalization remain elusive.

In addition, Y-27632 has been shown to increase the survival rate and cloning efficiency of human embryonic stem cells (hESCs) dissociated with EDTA (39) through blocking the Rho-ROCK-myosin light chain signaling cascade (40, 41). However, the precise mechanisms by which EDTA activates ROCK have not been elucidated (41, 42).

These results let us hypothesize a possible link between NOTCH1 and ROCK activation. Here we show a novel function of NOTCH1 as a critical upstream regulator of ROCK1 and its relevance to loss of self-renewal capacity in human keratinocytes as well as human induced pluripotent stem (hiPS) cells.

MATERIALS AND METHODS

Cell culture. Normal human cervical keratinocytes (HCKs) were obtained with written consent from a patient who underwent abdominal surgery for a gynecological disease other than cervical cancer and were retrovirally transduced with the catalytic subunit of human telomerase reverse transcriptase (hTERT) for immortalization (HCK1Ts) (14). HCK1Ts were cultured in serum-free keratinocyte-SF medium supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 μg/ml of bovine pituitary extract (Invitrogen, Life Technologies, Saint Aubin, France). Primary human dermal keratinocytes (HDKs) were purchased from Cell Applications Inc. (San Diego, CA). Primary human foreskin keratinocytes (HFKs) were obtained from Denise A. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA). HDKs and HFKs were cultured in serum-free keratinocyte-SF medium supplemented with 5 ng/ml EGF and 50 μg/ml of bovine pituitary extract (Invitrogen, Life Technologies). Human endometrium cells were collected by scraping tissues from surgical specimens, with signed informed consent and with ethical approval of the Institutional Review Board of the National Institute for Child Health and Development, Japan. All experiments involving human cells and tissues were performed in line with Tenets of the Declaration of Helsinki. Human iPS cell lines, MRC-hiPSCs and UtE-hiPSCs, were established from MRC-5 fetal lung fibroblasts (43) and UtE1104 endometrium-derived cells (44), respectively, via procedures described by Takahashi et al. (45) with slight modification (46, 47). Human iPS cells were maintained in iPSellon medium (Cardio Incorporated, Osaka, Japan) supplemented with 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in the presence of irradiated mouse embryonic fibroblast (MEF) feeders.

Retroviral vector construction and transduction. Retroviral vector plasmids were constructed using the Gateway system according to the manufacturer's instructions (Invitrogen). Segments of the intracellular domain of human NOTCH1 (ICN1), a truncated form of MAML1 corresponding to amino acids 13 to 74 fused to N-terminal hemagglutinin (HA) tag (MAML61-3HA), and c-MYC were cloned and recombined into retroviral expression vectors to generate pCLXSN-ICN1 (14), pCLXSN-MAML61-3HA (48, 49), and pCMSCVpuro-c-MYC (50). Human ROCK1, ROCK1ΔC241, ROCK1-D1113A, ROCK1-K105A, ICN1-ERT, ICN2-ERT2, RhoA, and enhanced green fluorescent protein (EGFP) were cloned into a lentiviral vector, CSII-TRE-Tight-RfA, in which the elongation factor promoter in CSII-EF-RfA (a gift from Hiroyuki Miyoshi, RIKEN, BioResource Center) was replaced with the tetracycline-responsive promoter from pTRE-Tight (Clontech, Mountain View, CA). The Notch1 short hairpin RNA (shRNA) vectors were described previously (14, 18). To generate ROCK1- or ROCK2-specific shRNA expression vectors pCL-SI-MSCVpuro-ROCK1Ri-1,-2,-3 and pCL-SI-MSCVpuro-ROCK2Ri-1,-2,-3, the following sequences were chosen as the targeted sites: 5′-GTACTTGTATGAAGATGA-3′ (51), 5′-GGTATATGCTATGAAGCTT-3′, and 5′-GCGAAATGGTGTAGAAGAA-3′ for ROCK1 and 5′-GAAACTAATAGGACACTAAC-3′ (52), 5′-GGTTTATGCTATGAAGCTT-3′, and 5′-GGATAAACATGGACATCTA-3′ for ROCK2. The retroviral vector and packaging constructs pCL-GagPol and pEF6/env (10A1) or the lentiviral vector and packaging constructs pCAG-HIVgp and pCMV-VSV-G-RSV-Rev were cotransfected into 293FT cells (Invitrogen) using TransIT-293 (Mirus Co., Madison, WI) according to the manufacturer's instructions, and the culture fluid was harvested at 60 to 72 h posttransfection. Titers of the recombinant viruses were determined by drug resistance with HeLa cells or a real-time PCR method (TaKaRa, Otsu, Japan) to detect the viral RNA genome, yielding titers equivalent to greater than 1×10^6 CFU/ml. Following addition of the recombinant viral fluid to cells in the presence of 4 μg/ml Polybrene, infected cells were selected in the presence of 0.5 μg/ml puromycin or 50 μg/ml G418, and promptly after drug selection, pooled cell populations were used for most subsequent experiments.

Tet-On keratinocytes. HCK1T cells were stably transduced with Tet-On ADV and tTS expression vectors, encoding the rtTA-Advanced transactivator and transcriptional silencer, respectively (Clontech). The resultant HCK1T Tet-On cells were then introduced with CSII-TRE-Tight-ROCK1, ROCK1ΔC241, ROCK1-D1113A, ROCK1-K105A, ICN1-ERT, ICN2-ERT2, RhoA (constitutive active and dominant negative forms), and EGFP by retroviral gene transfer. Induction of these transgenes was routinely achieved by treatment with 1 μg/ml doxycycline (DOX) for 72 h.

Inhibitors. The following pharmacological inhibitors were used: cycloheximide (CHX) (239764; Calbiochem, Darmstadt, Germany), z-VAD-fmk (caspase inhibitor IV) (219007; Calbiochem), γ-secretase inhibitor IX (DAPT) (565784; Calbiochem), Y-27632 (08945-84; Nacalai Tesque, Kyoto, Japan), C3 ADP-ribosyltransferase (Rho inhibitor) (CT04; Cytoskeleton, Inc., Denver, CO), and blebbistatin (sc-203532; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were pretreated with inhibitors for 2.5 h. For DAPT, in addition to pretreatment, cells were incubated with this inhibitor during and after exposure to EDTA or differentiation stimuli for up to 48 h.

Induction of keratinocyte differentiation. At 48 h after plating, HCK1T cells were treated with 2.5 mM EDTA in phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)] for 10 min or exposed to 0.7% and 5% bovine serum albumin (BSA) or 10% serum-containing medium in the presence of 10 μg/ml of bovine pituitary extract. To induce ligand-dependent NOTCH activation, HCK1T cells were harvested in subconfluent and 7-day-postconfluent states. HCK1T cells were also introduced with ICN1 by retroviral gene transfer to induce differentiation.

Dissociation of human iPS cells. First, hiPSC colonies were treated with collagenase IV solution at 37°C for 10 min. The detached hiPSC clumps were recovered, incubated with 0.005% trypsin–2.5 mM EDTA solution at 37°C for 5 min, and dissociated into single cells by pipetting. The dissociated cells were counted with Vi-CELL (Beckman Coulter, Brea, CA) and seeded onto MEF feeders.

Immunoblotting. Whole-cell protein extracts were used for analysis, and immunoblotting was conducted as described previously (14). Primary antibodies against Notch1 (sc-6014; Santa Cruz Biotechnology), activated Notch1 (cleaved Notch1 Val1744 2421; Cell Signaling Technology, Danvers, MA), Notch2 (clone C651.6DbHN; Developmental Studies Hybridoma Bank, University of Iowa), Hes1 (Toray Industries, Inc., To-

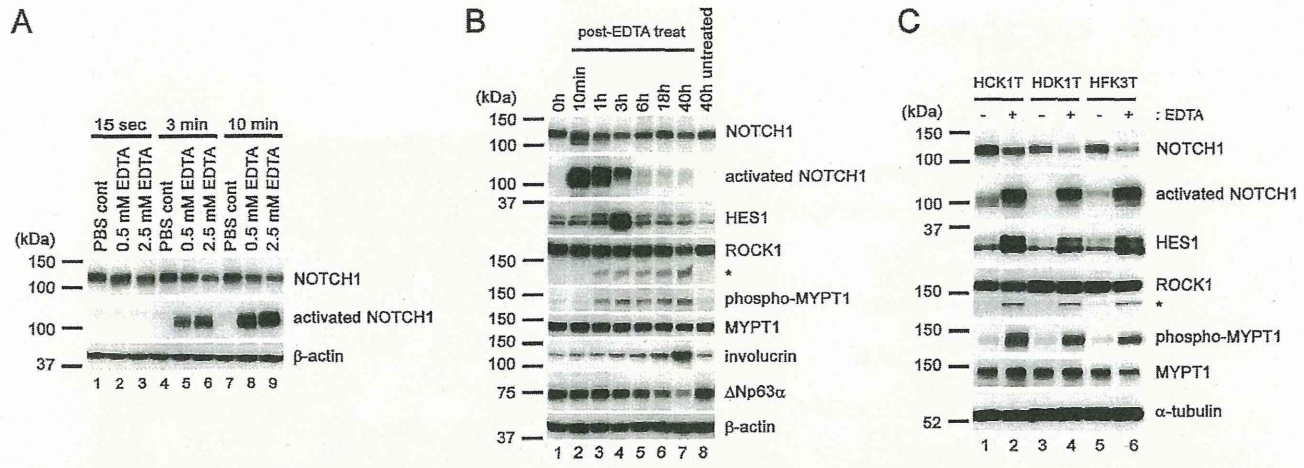

FIG 1 Immediate activation of ROCK1 following release of the NOTCH1 intracellular fragment in normal human keratinocytes. (A) HCK1T cells were either left untreated (PBS cont) or treated with 0.5 or 2.5 mM EDTA in PBS(−) for indicated time points. Cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. (B) HCK1T cells were either left untreated or treated with 2.5 mM EDTA in PBS(−) for 10 min at 37°C. After washing twice with PBS(−), cells were incubated with keratinocyte-SF medium. Cell lysates were prepared at the indicated time points after EDTA treatment. Extracts were analyzed by immunoblotting with the indicated antibodies. The band corresponding to the furin-processed transmembrane domain of NOTCH1 with a molecular mass of 120 kDa is shown as NOTCH1 here. An asterisk indicates a smaller fragment of ROCK1 protein with a molecular mass of ~130 kDa. (C) Keratinocytes from cervix (HCK1T), dermis (HDK1T), and foreskin (HFK1T) were either left untreated or treated with 2.5 mM EDTA for 10 min. After washing twice with PBS(−), cells were incubated with keratinocyte-SF medium for 3 h. Extracts were analyzed by immunoblotting with the indicated antibodies.

kyo, Japan), Hey1 (sc-16424; Santa Cruz Biotechnology), involucrin (clone SY5; Sigma, Saint-Quentin Fallavier, France), loricrin (AF 62; Covance, Princeton, NJ), Rock1 (sc-5560; Santa Cruz Biotechnology), phospho-MYPT1 (07-251; Merck-Millipore, Billerica, MA), MYPT1 (07-672; Merck-Millipore), Rock2 (sc-5561; Santa Cruz Biotechnology), p63 (clone 4A4; Santa Cruz Biotechnology), caspase-3 (9662; Cell Signaling Technology), poly(ADP-ribose) polymerase (PARP) (9542; Cell Signaling Technology), OCT3/4 (sc-5279; Santa Cruz Biotechnology), HA tag

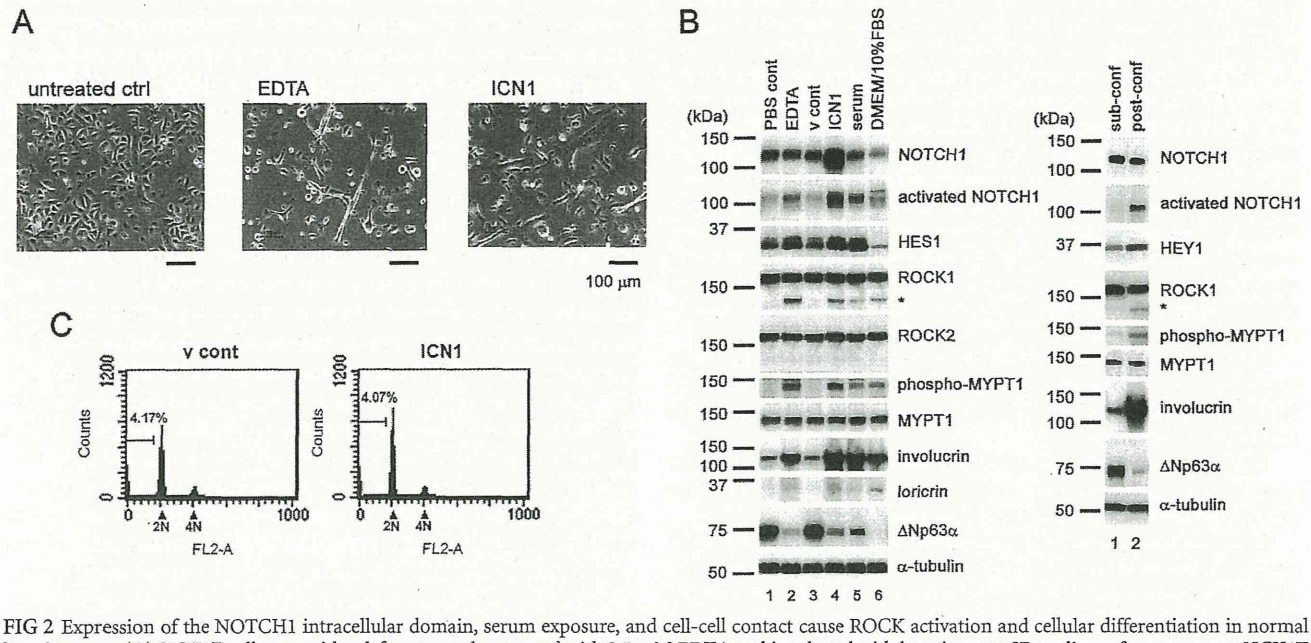

FIG 2 Expression of the NOTCH1 intracellular domain, serum exposure, and cell-cell contact cause ROCK activation and cellular differentiation in normal keratinocytes. (A) HCK1T cells were either left untreated or treated with 2.5 mM EDTA and incubated with keratinocyte-SF medium after treatment. HCK1T cells were transduced with the constitutively active form of NOTCH1 (ICN1). Typical areas were photographed at 3 days posttreatment or posttransduction. Scale bars represent 100 μm. (B) HCK1T cells were either left untreated or treated with 2.5 mM EDTA and incubated with keratinocyte-SF medium after treatment. HCK1T cells were transduced with the constitutively active form of NOTCH1 (ICN1) or control (v cont). HCK1T cells were exposed to serum-containing keratinocyte-SF medium (serum) or Dulbecco modified Eagle medium with 10% fetal bovine serum (DMEM–10% FBS). Cell lysates were harvested at 3 days posttreatment or posttransduction. HCK1T cells were also harvested in subconfluent and 7-day-postconfluent states. Extracts were analyzed by immunoblotting with the indicated antibodies. (C) HCK1T cells were transduced with the constitutively active form of NOTCH1 (ICN1) or control (v cont). At 3 days posttransduction, cells were collected and DNA content was analyzed by flow cytometry. The percentage of apoptotic cells displaying a sub-G_1 DNA content is shown between markers.