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A facile one-step strategy for the generation of conditional knockout mice to explore the role of Notch1 in oroesophageal tumorigenesis

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ABSTRACT

NOTCH1 plays an important role in epithelial differentiation and carcinogenesis. To investigate the impact of *Notch1* inactivation in oroesophageal epithelium, we generated conditional knockout (cKO) mice, using a combined construct which induces the expression of single guide RNA targeting *Notch1* and *Cas9* by the *KRT14* promoter. The cKO mice exhibited patchy hair loss and multiple NOTCH1-negative areas in the tongue epithelium, indicative of heterogeneous knockout. The cKO mice showed susceptibility to esophageal tumorigenesis, underscoring *Notch1* as a tumor suppressor. Our one-step strategy for generation of cKO mice provides a versatile method to examine a gene function *in vivo*.

1. Introduction

The Notch signaling pathway is a fundamental intercellular signaling system that regulates cell fate decisions in terms of proliferation and differentiation in multiple tissues [1]. Notch proteins are cell surface transmembrane receptors that transmit the signal from ligands on the surfaces of neighboring cells through direct contact. Interaction with the ligands induces proteolytic release of the Notch intracellular domain. The intracellular domain translocates to the nucleus where it participates in transcriptional regulation of downstream target genes [2]. In mammals, there are four Notch paralogues (NOTCH 1, 2, 3, and 4), each of which have unique and/or redundant influences on diverse tissues, conferring complexity on this signaling system.

In squamous epithelium, three Notch paralogues (NOTCH1, 2, 3) are expressed [3]. Among them, NOTCH1 seems to play a dominant role in regulation of epithelial differentiation. Since conventional knockout of *Notch1* resulted in embryonic lethality [4], conditional gene inactivation using cre-*loxP* technology has been applied in the generation of keratinocyte-specific *Notch1* null mice. These mice exhibited corneal and epidermal hyperplasia [5,6]. A more severe consequence of *Notch1* inactivation was the increased incidence of skin tumor, suggesting the role of *Notch1* as a tumor suppressor [6,7]. We previously demonstrated that reduced expression of NOTCH1 is associated with oral and esophageal tumors [3]. In line with this, recent next generation sequencing studies uncovered *NOTCH1* as one of the most frequently affected genes in head and neck cancer [8–11]. Intriguingly, mutations of the *NOTCH1* gene are not frequent in other types of solid tumors [8], suggesting that *NOTCH1* may be a tumor suppressor gene specific for oral cancer, although it remains to be clarified whether all those *NOTCH1* mutations are pathogenic.

Our goal was to elucidate the role of NOTCH1 in the pathogenesis of oral and esophageal cancers. Despite the putative contribution to human oral and esophageal cancers, alterations in oroesophageal mucosa in the *Notch1* knockout mice have not been described. To investigate the impact of *Notch1* inactivation in oroesophageal epithelium, we were determined to generate conditional knockout mice.

Here, we report the generation of conditional *Notch1* knockout mice by utilizing a combined transgene which elicits tissue-specific expression of *Cas9* and the ubiquitous expression of a single guide RNA (*sgRNA*) targeting *Notch1*. We found that *Notch1* was inactivated in keratinocytes and hair follicle cells in a heterogeneous

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manner, showing patches of NOTCH1-negative areas. The novel strategy we employed in this study will be beneficial to examine the roles of tumor suppressor genes in an *in vivo* setting.

2. Experimental procedures

Animals-The Animal Care and Use Committees at Tokyo Medical and Dental University reviewed and approved all experimental procedures. Mice were sacrificed by cervical dislocation. The tissues were dissected, fixed in 10% neutral buffered formalin for 24–48 h and embedded in paraffin wax. E18 embryos were decalcified in 5% formic acid for 24 h after fixation.

Immunohistochemistry-Formalin-fixed-paraffin-embedded tissue blocks were cut into sections of 4 μm thickness. Antigen retrieval was performed by heating the sections at 120 °C for 15 min or at 80 °C for 60 min in alkaline buffer (10 mM Tris; pH = 9.0, 1 mM EDTA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min. The primary antibodies used in this study were anti-NOTCH1 (EP1238Y, Abcam, CA, USA), Keratin (KRT) 13 (AE8, Abcam), KRT14 (LL002, Thermo Fisher Scientific, Waltham, MA, USA), KRT15 (EP14, Abcam), and PCNA (PC10, Dako, Glostrup, Denmark). The antibodies were used at 1:500 dilution. Secondary antibody used was EnVision™ (Dako).

Construction of the transgene-Plasmid *pSpCas9(BB)-2A-GFP* (*PX458*) was purchased from Addgene (plasmid #48138) [12]. The target genomic sequence of mouse *Notch1* was determined using the online CRISPR Design Tool (http://tools.genome-engineering.org). We tried 8 sets of complementary oligonucleotides for the generation of knockout constructs. Only 1 set (5′-AAACCTTGGCTGGGAGCATCTCAAC-3′ and 5′-CACCGTTGAGATGCTCCCAGCCAAG-3′) successfully induced a mutation in the experiment described below. The oligonucleotides were annealed and cloned into *PX458*. The resulting plasmid *pSN1pCas9* was confirmed by sequencing. *pSN1pCas9* was transfected to NIH/3T3 cells by using Polyethylenimine Max (Polysciences, PA, USA). To enrich the transfected cells, GFP-positive cells were sorted 72 h after transfection by FACSAria™ II (BD Biosciences, CA, USA). DNA was extracted from the sorted cells and analyzed by PCR-direct sequencing of the corresponding genomic region of *Notch1*. The PCR primer sequences were, forward: 5′-GGTAAGTGTCCCAGAACGAA-3′ and reverse: 5′-CACCTTAATCCCCTCCCACT-3’. PCR was performed using PrimeSTAR® GXL DNA Polymerase (Takara, Shiga, Japan) and DNA sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). In order to induce squamous epithelium-specific *Notch1* ablation, the *CBh* promoter (hybrid form of the chicken beta actin promoter) of *pSN1pCas9* was replaced with human *KRT14* promoter. The *KRT14* promoter was excised from *pG3Z-K14* (ATCC® MBA-124™) [13] by double digestion with Sac1 and BamH1, ligated into *pAcGFP1-N1* (Takara) as a shuttle vector, and then excised from *pAcGFP1-N1* by double digestion with Age1 and Kpn1 to ligate into *pSN1pCas9*. The resulting plasmid *pSN1pK14Cas9* was confirmed by sequencing. *pSN1pK14Cas9* was linearized by double digestion with Pci1 and Not1.

Generation of transgenic mice—The linearized *pSN1pK14Cas9* without the vector backbone was injected into zygotes of CD-1 mice. Zygotes were transplanted to pseudopregnant CD-1 mice. The tails of three week old infants were cut and genomic DNA was extracted as previously described [14]. PCR of 35 cycles of denaturation at 98 °C for 30 s, annealing 58 °C for 30 s, and extension at 72 °C for 30 s was performed using the primers specific for *Cas9* (forward: 5′-GGTTCTTGTCGCTTCTGGTC-3′, reverse: 5′-TCCTGCAGACAGTGAAGGTG-3′). Hair genomic DNA was extracted according to the method described previously [15] and used for PCR-direct sequencing to examine *Notch1* mutations. RNA was extracted from biopsied dorsal skin using NucleoSpin® RNA (Takara). RNA was transcribed into cDNA using oligo (dT) primers.

Scanning electron microscopy—Hairs were plucked, pasted to carbon tape on an aluminum mount, coated with platinum, and examined using scanning electron microscope (Hitachi S-4500; Hitachi, Tokyo, Japan) at 15 kV.

Chemically induced oroesophageal carcinogenesis—4-Nitroquinoline-1-oxide (4-NQO) was added at a concentration of 100 μg/mL to drinking water and administered to mice for 16 weeks, *ad libitum*. 4-NQO-containing drinking water was prepared fresh weekly. After the administration period, mice were sacrificed and the tongue and esophagus were dissected for histological analysis.

3. Results

To introduce an epithelial-specific expression of Cas9, we used the 2.0 kb fragment of human *KRT14* promoter. To foresee the tissues that would be affected by *KRT14*-mediated gene ablation of *Notch1*, we first investigated the expression of NOTCH1 and KRT14 in fetuses (E18) and adult mice. Immunohistochemical examination revealed that NOTCH1 was expressed in epithelial basal cells of the skin including the outer root sheath of hair follicles (Fig. 1A), in the oral mucosa (Fig. 1B), the esophagus (Fig. 1C), and in the urothelial epithelium of the bladder (data not shown) in both fetuses and adults. KRT14 expression was observed in the basal layer of skin including hair follicles (Fig. 1D), in the oral mucosa (Fig. 1E), and the esophagus (Fig. 1F), which completely overlapped to NOTCH1, suggesting that the influence of *KRT14*-dependent inactivation of *Notch1* would appear mainly in these tissues.

The design of the transgenic construct is shown in Fig. 2A. Transcription of the *sgRNA* targeting *Notch1* is induced by the human *U6* promoter and transcription of *Cas9* is induced by the human *KRT14* promoter (Fig. 2A). We first selected eight putative target sequences in exon 1, 2 and 3 of *Notch1* and introduced each into *PX458*, in which the *CBh* promoter induces universal *Cas9* expression. The resulting plasmid is referred to as *pSN1pCas9*. To check the Cas9 effect aided by each *sgRNA*, NIH/3T3 cells were transfected with each *pSN1pCas9* by lipofection and the targeted *Notch1* loci were examined by PCR-direct sequencing. Among 8 constructs, one construct that targeted exon 2 yielded an electropherogram indicative of a single base deletion mutation (Fig. 2B). Next, we replaced the *CBh* promoter in this *pSN1pCas9* with human *KRT14* promoter. The resulting plasmid is referred to as *pSN1pK14Cas9*. After confirming the expression of Cas9 in HeLa cells, the linearized *pSN1pK14Cas9* was used for the generation of transgenic mice.

Two out of 57 infants were positive for the transgene (Fig. 2C). They initially had no apparent phenotype in gross appearance. However, on week 8, one mouse (N1cKO#1-F0; male) manifested hairless patches in the dorsal skin followed by another mouse (N1cKO#2-F0; female) showing similar patches on week 10. N1cKO#2-F0 exhibited a more severe phenotype (Fig. 2D).

To see whether the hair loss was caused by *Notch1* mutation, DNA was obtained from remaining hairs that grew in the hairless areas. PCR-direct sequencing revealed deletion mutations in exon 2 of *Notch1* in both N1cKO#1-F0 and N1cKO#2-F0. Both of these deletions were predicted to cause frameshift mutations that render a premature stop codon (Fig. 2D). No mutation was detected in hairs taken from a normal looking skin (data not shown), suggesting that the hair loss was caused by *Notch1* mutations that were induced heterogeneously in limited numbers of cells.

We expanded the transgenic strains by mating them to wild type mice. Since all offspring of N1cKO#1-F0 had the wild type genotype, we concentrated our analysis on the N1cKO#2 strain