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The Simultaneous Induction of Tumorigenesis and Cre-*loxP* Recombination in Mice

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To investigate the role of Rac1 for tumorigenesis, we generated inducible transgenic (Tg) mice that simultaneously express polyomavirus middle T antigen (mT) and Cre recombinase under the control of mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter (MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg). MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice formed tumors in the subcutaneous tissue and developed lung metastasis. We examined tumor latency and types in *rac1* deficient (*rac1*$^{flox/-}$) and control (*rac1*$^{+/+}$, *rac1*$^{+/-}$ or *rac1*$^{flox/+}$) MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice and found that formation of cutaneous appendage tumor was suppressed although tumor latency in these mice was not affected by loss of Rac1. These results suggested that Rac1 may play a pivotal role in induction and growth of the mT-mediated epithelial tumors. MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice would provide a versatile animal model to investigate genetic interaction in the tumorigenesis.

There are several reports describing transgenic mouse models in which tumorigenesis can be induced by expression of mT (15). Of these, the MMTV-LTR-*mT* Tg mice expressing mT under the control of MMTV-LTR promoter induce breast cancer, and develop lung metastasis (6). This mouse model has been used for elucidating the molecular mechanisms in tumorigenesis as well as metastatic progression of tumor. The metastatic progression of tumor is a pleiotropic process requiring the disregulation of numerous cellular signaling pathways such as cell adhesion, migration, and invasion. In this study, we combined the mT transgenic system with Cre-*loxP* system to generate a versatile model animal which simultaneously induces mT-mediated tumorigenesis and knockout of a specific gene. We applied this new model to elucidate the role of a Rho family GTPase Rac1 in tumorigenesis.

Rac1 is a ubiquitously expressed member of Rac GTPases and activates lamellipodia formation and inhibits stress fiber formation by regulation of actin cytoskeleton (11). Aberrant Rac activation is associated with invasive behavior or malignant transformation of various types of tumors, including breast carcinoma, glioblastoma, hepatocarcinoma, and

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melanoma (1, 2, 3, 9). However, elucidation of role of Rac1 in tumorigenesis and metastasis *in vivo* has been hampered by lack of good animal models.

Therefore, we established new transgenic mice, MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg, with inducible and reversible expression of both mT and Cre in the same cells. Using Tet-off system, the Tg mice carrying the *mT* oncogene developed epithelial tumor such as cutaneous appendage tumor and mesenchymal tumors including hemangioma and lymphangioma. Furthermore, MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice developed lung metastasis. We examined the effect of Rac1 deletion on latency and number of tumors induced in the Tg mice and showed that our model mice provide a new method for analysis of genetic interaction in the mT-mediated tumorigenesis.

MATERIALS AND METHODS

Transgene constructs

A MMTV-LTR-*tTA* transgene was composed of *tTA* cDNA fragment from pTet-Off vector (Clontech, Palo Alto, CA, USA), MMTV-LTR sequence from pMSG vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and pBst-N vector containing donor and acceptor splice sites derived from the rabbit β-globin gene and polyadenylation sites derived from the rabbit β-globin and the SV40 early genes. To create the MMTV-LTR-*tTA* transgene, the 1.0 kb *tTA* cDNA fragment was introduced into pBst-N vector at *Eco*RI site located between the acceptor splice site and the polyadenylation site from the rabbit β-globin gene. And then, the 1.5 kb *Hind*III-*Sma*I MMTV-LTR fragment was inserted into the *Bam*HI site, which was located upstream of *tTA*.

A *mT*-TRE-*cre* transgene was composed of cDNA encoding the *mT* from pPyMT1 vector (15), *cre* sequence (13), bidirectional tetracycline responsive element (TRE) sequence from pBI Tet vector (Clontech), splice sites and polyadenylation site derived from pBst-N vector and splice sites and polyadenylation site derived from pCI vector (Promega, Madison, WI, USA). To create the *mT*-TRE-*cre* transgene, the 1.0 kb *cre* fragment was introduced into pCI vector at *Xho*I-*Not*I site located between the acceptor splice site and the polyadenylation site (pCI-*cre*). The 1.3 kb mT cDNA was inserted into pBst-N vector at *Eco*RI site located between the acceptor splice site and the polyadenylation site. TRE sequence was inserted into pBst-N vector at *Not*I-*Bam*HI site, which located downstream of mT cDNA. Finally, the 1.6 kb fragment containing *cre* sequence and polyadenylation site from pCI-*cre* was inserted into pBst-N vector at *Not*I site, which located downstream of TRE sequence.

Generation of transgenic mice

This study was approved by the Institutional Animal Care and Use Committee and carried out according to the guidelines for the Kobe University Animal Experimentation Regulations.

MMTV-LTR-*tTA* and *mT*-TRE-*cre* DNA fragments were co-injected into the fertilized eggs of C57BL/6N mice. For genotyping by Southern blot analysis, tail DNAs were digested with *Eco*RI, separated on a 1% agarose gel and transferred to Biodyne PLUS membrane (Pall Corporation, Pensacola, FL, USA). The 1.0 kb DNA fragment of *tTA* or the 1.3 kb DNA fragment of *mT* was used as hybridization probes. Hybridized bands were visualized by using Bio-Imaging Analyzer BAS-2500 (Fuji Photo Film, Tokyo, Japan).

MMTV-LTR-*tTA*/*mT*-TRE-*cre*/CAG-CAT-Z triple Tg mice were obtained by crossing MMTV-LTR-*tTA*/*mT*-TRE-*cre* double Tg mice with CAG-CAT-Z Tg mice (12).

MMTV-LTR-*tTA*/*mT*-TRE-*cre* double Tg mice were crossed to $rac1^{+/-}$ mice (14) to generate $rac1^{+/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* double Tg mice. And then, $rac1^{+/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* double Tg mice were crossed with $rac1^{flox/flox}$ mice (7) to

generate *rac1*$^{flox/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* double Tg mice. Genetic background of these mice was a hybrid of C57BL/6N and 129/Ola. Only female mice were used for this study.

RNA isolation and reverse transcription-PCR analysis

Total RNA from tumor or non-tumor tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA (500 ng) was reverse-transcribed into cDNA with TaKaRa RNA PCR Kit (AMV) Ver.3.0 using oligo-dT primers (Takara, Shiga, Japan). PCR was performed with cDNA equivalent to 25 ng of total RNA, 10 μM each forward and reverse primer in a total volume of 20 μl.

The primer pairs used were as follows:
mT forward, 5'-TGGAAGCCATGCCTTAATGC-3',
and reverse, 5'-AGTCATCGTGTAGTGGACTG-3';
cre forward, 5'-GCATACCTGGAAAATGCTTC-3',
and reverse, 5'-CCAGTGAAACAGCATTGCTG-3';
tTA forward, 5'-AAGTGATTAACAGCGCATTAG-3',
and reverse, 5'-ATCGCGATGACTTAGTAAAGC-3';
β-actin forward, 5'-CTACAATGAGCTGCGTGTGG-3',
and reverse, 5'-CCATCATGAAGTGTGACGTT-3'.

β-galactosidase staining

Skin or tumor tissues from MMTV-LTR-*tTA*/*mT*-TRE-*cre*/CAG-CAT-Z triple Tg mice were washed three times with 0.01% deoxycholate, 0.02% NP-40 and 2 mM $MgCl_2$ in phosphate-buffered saline (PBS) for 30 min at 4°C, and then washed in PBS for 15 min at 4°C. After washing, tissues were incubated in a staining solution containing 0.1% X-gal, 5 mM $K_4[Fe(CN)_6]$, 5 mM $K_3[Fe(CN)_6]$ and 2 mM $MgCl_2$ in PBS overnight at 37°C, and then washed in PBS for 30 min at 4°C. After X-gal staining, tissues were fixed in 3.7% formalin in PBS, embedded in paraffin, sectioned on a sliding microtome at 6 μm thickness and counterstained with hematoxylin-eosin or nuclear fast red.

Histological and immunohistochemical analysis

Tissues were fixed in 3.7% formalin in PBS, embedded in paraffin, and sectioned on a sliding microtome at 3 μm thickness. Hematoxylin-eosin staining was carried out according to standard protocols.

Immunohistochemistry was carried out using the streptavidin-biotin peroxidase method with a LSAB2 kit (Dako, Glostrup, Denmark). For immunohistochemical analysis, paraffin-embedded sections were mounted on MAS-coated glass slides (Matsunami glass, Osaka, Japan), deparaffinized and incubated with 3% hydrogen peroxide for 15 min at room temperature to quench endogenous peroxidase, and blocked with 10% normal goat serum (Dako) for 30 min at room temperature. The sections were incubated overnight at 4°C with an antibody to keratin 14 (2 μg/ml, Covance, Berkeley, CA, USA) or normal rabbit immunoglobulin fraction (2 μg/ml, Dako) for negative control. The sections were then washed with TBS (50 mM Tris-HCl and 150 mM NaCl, pH 7.6) and incubated with biotinylated antibody to rabbit immunoglobulin (2.5 μg/ml, Dako) for 1 hr at room temperature. Streptavidin-biotinylated peroxidase complex was applied, and then the sections were incubated with diaminobenzidine. The sections were counterstained with hematoxylin to enhance nuclear detection.

Statistical analysis

Transgenic mice were monitored for the appearance of tumors by weekly physical palpation. The percentage of tumor-free mice in each genotype was analyzed by using a Kaplan-Meier analysis, and was compared by the log-rank test.

RESULTS

Generation of MMTV-LTR-*tTA*/*mT*-TRE-*cre* double transgenic mice

To induce the tumorigenesis and Cre-*loxP* recombination in the same cell, we generated double Tg mice carrying MMTV-LTR-*tTA* and *mT*-TRE-*cre* transgenes. We utilized tetracycline-controlled gene expression system for inducible and simultaneous expression of the two genes, *mT* and *cre* (Figure 1A). In this system, two components are essential. One is the tTA, a fusion protein that contains *tet* repressor (TetR) and transcription activation domain of protein 16 of the herpes simplex virus, VP16 (5). The other is the bidirectional TRE, which consists of heptamerized *tet* operator (*tet*O) sequence, the binding sites of TetR, flanked by identical two minimal cytomegalovirus promoters. In this study, a transgene named MMTV-LTR-*tTA* expressed tTA under the control of MMTV-LTR promoter (Figures 1A and 1B) and another transgene named *mT*-TRE-*cre* expressed mT and Cre proteins under the control of TRE sequence without doxycycline administration (Figure 1A). Therefore, expression of mT and Cre through tTA binding to the TRE was expected to induce tumorigenesis and Cre-*loxP* recombination simultaneously.

After microinjection of DNA fragments containing MMTV-LTR-*tTA* and *mT*-TRE-*cre* into the fertilized eggs of C57BL/6N mice, offspring were screened for presence of the transgenes by Southern blot analysis (Figure 1B) and PCR analysis (data not shown) of tail DNAs. We obtained four Tg founder mice, and one Tg founder mouse (line 6) transmitted the transgene to progeny over many generations.

To examine the Cre expression, MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice were crossed with CAG-CAT-Z reporter mice (12). In CAG-CAT-Z reporter mice carrying CAG promoter-*loxP*-CAT-*loxP*-*lacZ* transgene expression of *lacZ* gene depends on Cre-*loxP* recombination. In MMTV-LTR-*tTA*/*mT*-TRE-*cre*/CAG-CAT-Z triple Tg mice, Cre expression, as monitored by *lacZ*-encoded β-galactosidase activity, was observed in sebaceous glands in the MMTV-LTR-*tTA*/*mT*-TRE-*cre*/CAG-CAT-Z triple Tg mice (blue staining in Figures 1C and 1D). These results indicated that MMTV-LTR-*tTA* and *mT*-TRE-*cre* transgenes induced Cre-*loxP* recombination in the sebaceous glands in the Tg mice without doxycycline administration.

MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg induced tumorigenesis and Cre-*loxP* recombination in sebaceous glands

To investigate the tumorigenesis in MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, the MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice were maintained without doxycycline administration. All MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice produced tumors by 20 months of age. These tumors could be classified into three types, cutaneous appendage tumor (51.2%), hemangioma (39.0%) and lymphangioma (9.8%) (Table). Cutaneous appendage tumor showed glandular structure and keratinization that are typically observed in the tumors derived from epithelial cells. Hemangioma originated from endothelial cell was characterized by increase vessels and filled with blood. Lymphangioma was a tumor of lymphatic analogue of the hemangioma. These tumors were mainly observed in subcutaneous tissue of buttock, flank and base of extremities in the MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice (Figure 2A and data not shown).

To confirm the transgene expression in the tumor tissue of MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, we carried out RT-PCR analyses of RNAs from tumor tissue or non-tumor tissue. *mT*, *cre* and *tTA* mRNAs were detected from the tumor tissue, but not from the non-tumor tissue in the MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice (Figure 2B).

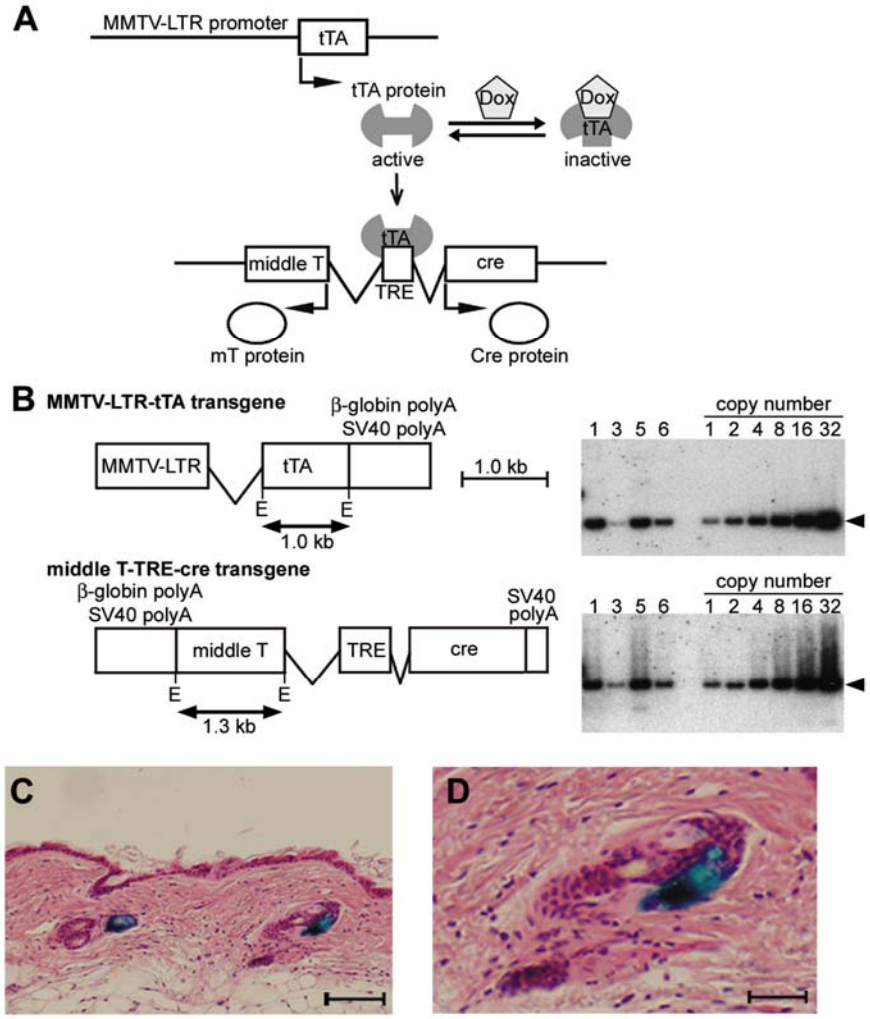

Figure 1 Generation of MMTV-LTR-*tTA*/*mT*-TRE-*cre* double Tg mice. (A) Schematic diagram of mT and Cre expression by the tetracycline-responsive gene system. In MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, mT and Cre expression depends on two transgenes in the absence of doxycycline (Dox) administration. Tetracycline-controlled transactivator (tTA) binds to tetracycline responsive element (TRE) sequence and activates transcription of both *mT* and *cre*. (B) Structures of MMTV-LTR-*tTA* transgene (top) and *mT*-TRE-*cre* transgene (bottom) and Southern blot analysis of MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice. The 1.0 kb-tTA fragment or 1.3 kb-mT fragment was used as probes. The *tTA* and *mT* transgenes were detected by the presence of 1.0 kb band (arrowhead; top) and 1.3 kb band (arrowhead; bottom), respectively. Among four lines of MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg founder mice obtained, line 6 was used in this study. E: *Eco*RI. (C and D) To detect the Cre-*loxP* recombination, skin of MMTV-LTR-*tTA*/*mT*-TRE-*cre*/CAG-CAT-Z triple Tg mice was stained with X-gal and hematoxylin-eosin. The blue signals indicated Cre-*loxP* recombination occurred in sebaceous glands. Scale bars: 100 μm (C), 50 μm (D).

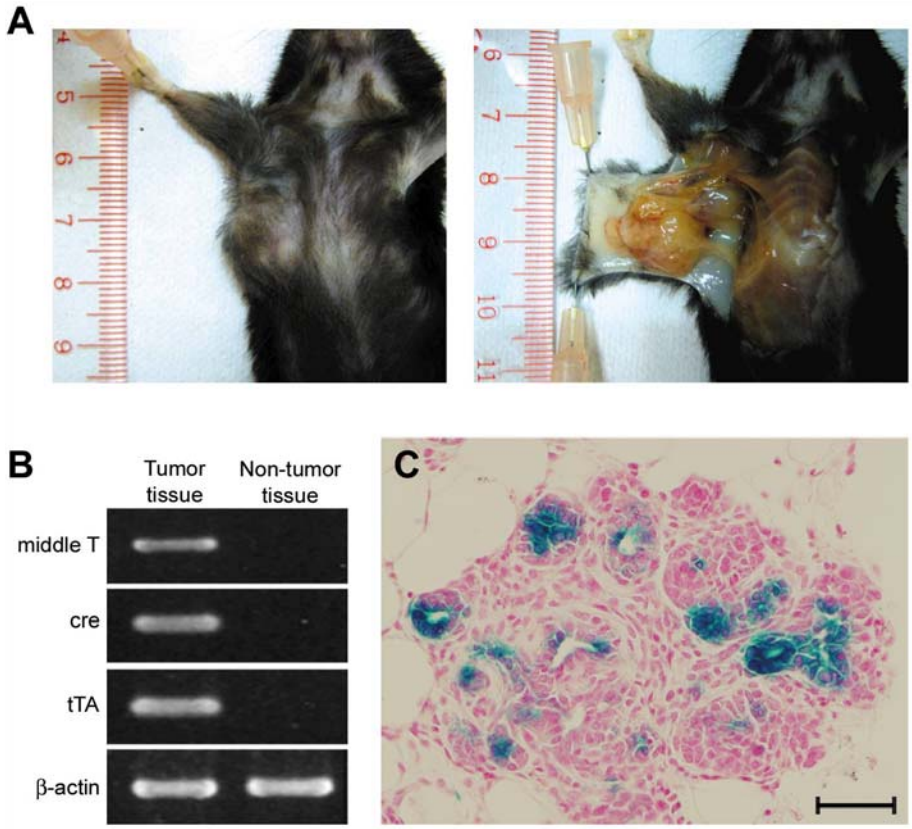

Figure 2 Induction of tumor formation by mT in sebaceous glands. (A) A representative cutaneous appendage tumor of an MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mouse. In this mouse, the tumor appeared 14 months after birth. (B) RT-PCR analyses of RNAs extracted from tumor or non-tumor tissues of the MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice. *mT*, *cre* and *tTA* mRNAs were detected in tumor tissues, but not in non-tumor tissues. β-actin was used as a control. (C) Cre-*loxP* recombination (blue) was detected in tumor tissue from an MMTV-LTR-*tTA*/*mT*-TRE-*cre*/CAG-CAT-Z triple Tg mouse. Nuclei were counterstained with nuclear fast red. Scale bar: 25 μm (C).

Cre-*loxP* recombination in tumor tissues of MMTV-LTR-*tTA*/*mT*-TRE-*cre*/CAG-CAT-Z triple Tg mice was confirmed by X-gal staining. Cre-*loxP* recombination was observed in a portion of cutaneous appendage tumor (Figure 2C) and hemangioma (data not shown). These observations confirmed that both tumorigenesis and Cre-*loxP* recombination was induced in MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice.

MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice induced lung metastasis

Previous reports showed that MMTV-LTR-*mT* Tg mice induced mammary tumor and multiple metastases in the lungs (6). Thus, we examined the lung metastasis in our MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice. Fourteen out of 19 MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice (C57BL/6N background) induced cutaneous appendage tumors (Figure 3A) and all of them showed aggressive tumor behavior. Two of these mice induced lung tumors (Figure

	rac1$^{+/+}$; Tg mice	*rac1*$^{+/-}$; Tg mice	*rac1*$^{flox/+}$; Tg mice	*rac1*$^{flox/-}$; Tg mice
Cutaneous appendage tumor	51.2% (21/41)	51.7% (15/29)	51.2% (21/41)	23.8% (5/21)
Hemangioma	39.0% (16/41)	41.4% (12/29)	41.5% (17/41)	71.4% (15/21)
Lymphangioma	9.8% (4/41)	6.9% (2/29)	7.3% (3/41)	4.8% (1/21)

Table Comparison of tumor types in *rac1*$^{+/+}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, *rac1*$^{+/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, *rac1*$^{flox/+}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice and *rac1*$^{flox/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice. All tumors in Tg mice could be classified into three types, cutaneous appendage tumor, hemangioma, or lymphangioma. Loss of Rac1 in the tumor tissue resulted in decrease in the ratio of cutaneous appendage tumor and increase in the ratio of hemangioma.

3B). To investigate whether these lung tumors originated from cutaneous appendage tumor, we performed RT-PCR (Figure 3C) and histological analyses (Figure 4) of these lung tumors. RT-PCR analysis showed that these lung tumor expressed *mT*, *cre* and *tTA* mRNAs which were detected in the primary tumor (i.e., cutaneous appendage tumor) tissues (Figure 3C). Furthermore, the histological analysis showed that lung tumors and cutaneous appendage tumor shared similar histological characteristics of epithelial cell-derived tumors such as glandular structures (Figure 4, top panels). Supporting these pathological similarities, the

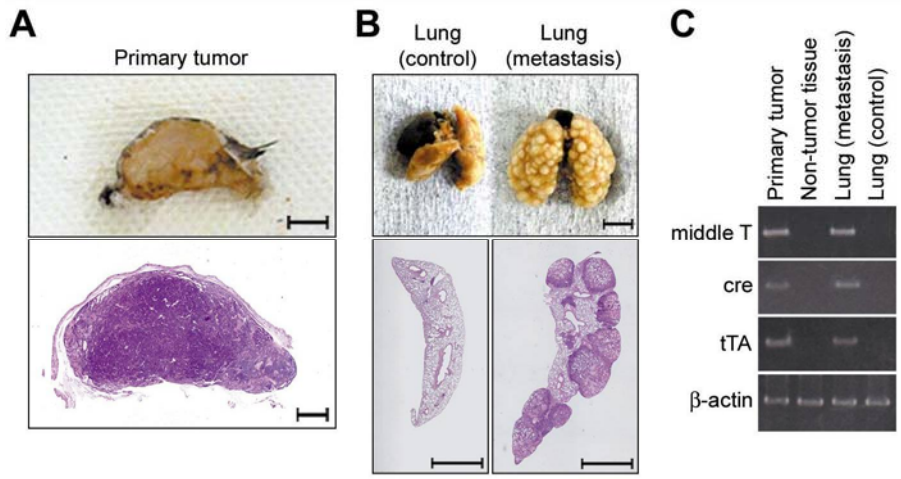

Figure 3 Lung metastasis of cutaneous appendage tumor in an MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mouse. (A) Gross and histological analyses of cutaneous appendage tumor (primary tumor) of the MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mouse. (B) Gross and histological analyses of lungs in control and MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice. Cutaneous appendage tumor metastasized to the lung was observed in the MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mouse. (C) Transcription products of *mT*, *cre* and *tTA* were detected in primary tumor and the lung tumor of the MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mouse, but not in the non-tumor tissue and the lung of the control mouse. β-actin was used as a control. Scale bars: 5 mm (A and B; top), 2 mm (A and B; bottom).

expression of keratin 14, a marker of epithelial basal cells, was observed in primary tumor and lung tumor, but not in the lung tissue of control mice (Figure 4, middle panels). These results suggest that the tumorigenesis in the sebaceous glands by mT leads to the development of lung metastasis.

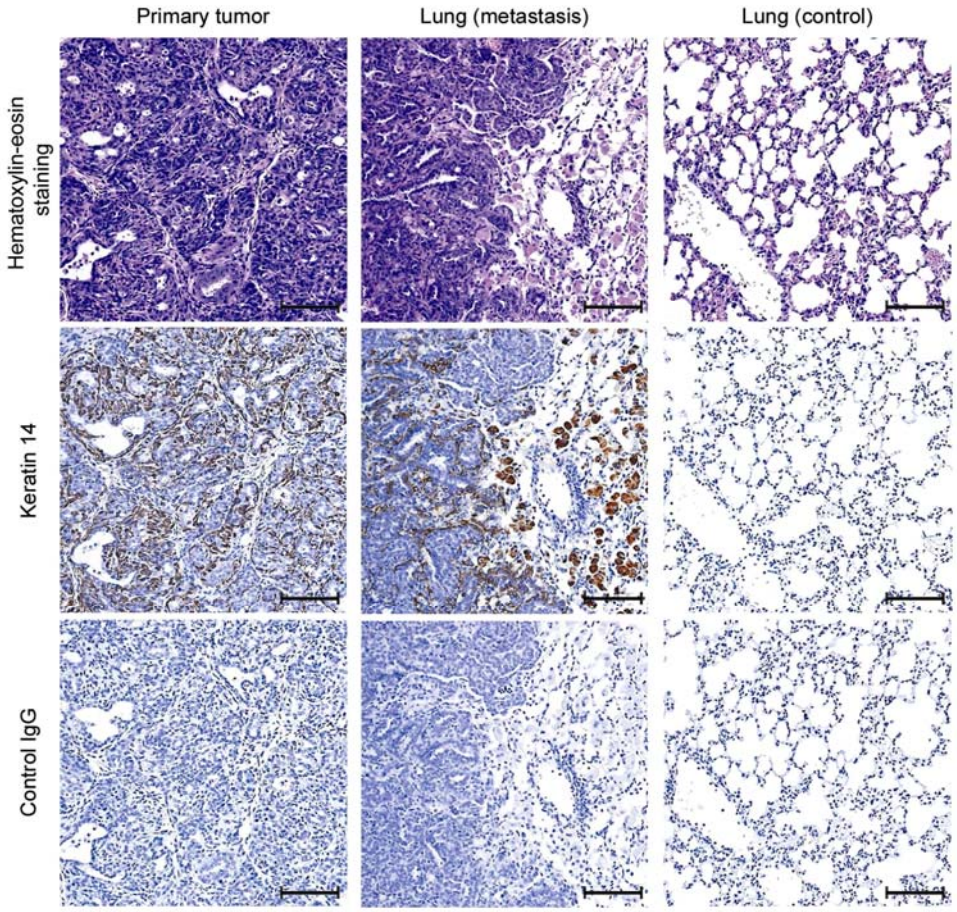

Figure 4 Expression of keratin 14 in lung tumor from a MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mouse. Consecutive paraffin sections were stained with hematoxylin-eosin (top) or immunostained with anti-keratin 14 antibody (middle) as a marker of epithelial basal cells. The keratin 14 expression was observed in primary tumor tissue (cutaneous appendage tumor) and lung tumor from MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mouse, but not in the lung tissue of a control mouse. The sections incubated with control IgG for rabbit were shown as negative controls (bottom). Scale bars: 100 μm.

Loss of Rac1 in MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice resulted in decrease of cutaneous appendage tumor

To assess whether Rac1 is involved in tumorigenesis induced by mT in MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, we examined tumor latency and types in *rac1* deficient ($rac1^{flox/-}$) and control ($rac1^{+/+}$, $rac1^{+/-}$ or $rac1^{flox/+}$) MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice (Figure 5 and Table). The deletion of *rac1* gene was confirmed by PCR analysis for *rac1* allele in the tumor tissue from $rac1^{flox/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice (data

not shown). The mean latency for tumor development exhibited no significant difference between *rac1* deficient (*rac1*$^{flox/-}$) and control (*rac1*$^{+/-}$ or *rac1*$^{flox/+}$) MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice (Figure 5; *rac1*$^{flox/-}$; Tg vs *rac1*$^{+/-}$; Tg mice, P = 0.919; *rac1*$^{flox/-}$; Tg vs *rac1*$^{flox/+}$; Tg mice, P = 0.499, Kaplan-Meier analyses and log-rank test). Although Rac1 deficiency did not affect the latency for tumor development, the number of cutaneous appendage tumor significantly decreased in MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice (Table). In the control MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, more than 50% of tumor were cutaneous appendage tumor, and around 40% of tumor were hemangioma. In contrast, the incidence of cutaneous appendage tumor in *rac1* deficient MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice was 23.8% (Table). Lung metastasis was not observed in these MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice with the hybrid genetic background of C57BL/6N and 129/Ola. Taken together, these data provide the possibility that Rac1 plays an important role in tumorigenesis of cutaneous appendage tumor.

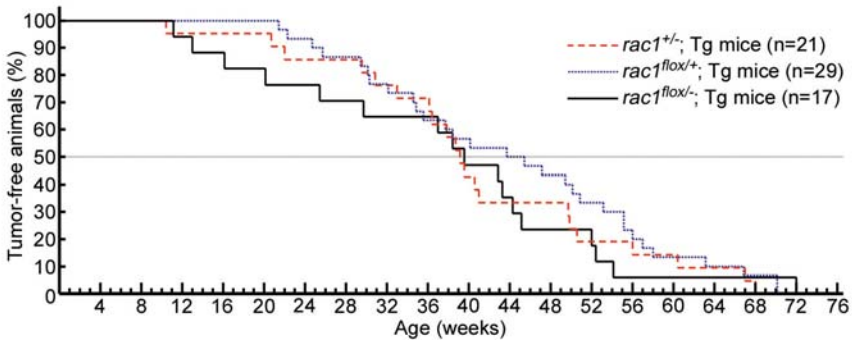

Figure 5 Tumorigenesis in the *rac1*$^{flox/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice. Kaplan-Meier analyses for the age at which the first palpable tumor was detected. The median latency for tumor development was 274 days for *rac1*$^{+/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, 318 days for *rac1*$^{flox/+}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice and 277 days for *rac1*$^{flox/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice.

DISCUSSION

We established a novel transgenic line, MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg, which induces tumorigenesis and Cre-*loxP* recombination in subcutaneous tissue and develop lung metastasis. We examined the role of Rac1 in mT-mediated tumorigenesis by examining the latency of tumor development and tumor types in *rac1*$^{flox/-}$; MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice. By Rac1 deletion, formation of cutaneous appendage tumor was suppressed although tumor latency in these mice was not affected. Blockade of Rac1 signaling by dominant negative Rac1 resulted in inhibition of *in vivo* growth of malignant keratinocytes which were subcutaneously injected into the flanks of SCID mice (8). Taking together with our results, Rac1 may play a pivotal role in tumor induction and growth of epithelial tumors. Rac1 was also reported to be essential for transformation of primary cultured mouse brain endothelial cells via PI3-kinase signaling pathway (4). However, in our Tg mice, number of hemangioma derived from endothelial cells was not affected by Rac1 deletion in spite of regression of epithelial tumors. mT-mediated hemangioma formation in our mice might have genetic events or activation of signaling pathway other than Rac1-PI3-kinase signaling.

Cre-*loxP* recombination was detected in the limited number of cells in each tumor of MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice (Figure 2C). Based on the hypothesis that tumor cells were derived from monoclonal origin, all cells in each tumor were expected to express transgene-encoded tTA, mT and Cre. These results suggested the two possibilities. One possibility is that each tumor in MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice was not derived from monoclonal origin. mT expression in sebaceous glands was required for initiation, but other cells without mT expression also participate in outgrowth of tumor. Another possibility is that variation of Cre expression level in each cells results in mosaic pattern for Cre-*loxP* recombination events in same tumors. A transgene expression depends on the genomic structures which might be influenced by the expression of the genes surrounding the transgene.

MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg induced tumorigenesis in sebaceous gland, but not in mammary gland. Previous reports showed that the MMTV-LTR promoter drove target genes efficiently in mammary glands. MMTV-LTR-*mT* Tg induced mammary tumors and showed high incidents of lung metastasis. The tTA-TRE-mediated Tet expression system often drives target genes more weakly than promoters by themselves. To overcome this problem in Tet systems, we might utilize tTA whose sequences were modified according to mammalian codon usage to achieve higher expression of target genes.

The MMTV-LTR-*mT* Tg mice cause development of aggressive tumor with an incidence of lung metastasis at high frequency (6). In case of our MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice, only 2 of 14 MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice in the C57BL/6N background with cutaneous appendage tumor induced lung metastasis. Furthermore, the MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice in the hybrid genetic background of C57BL/6N and 129/Ola did not induce lung metastasis (n = 14). The lung metastasis rate in the MMTV-LTR-*mT* Tg mouse model is known to depend on genetic background (10). To induce lung metastasis of our mice, MMTV-LTR-*tTA*/*mT*-TRE-*cre* Tg mice should be bred to inbred strains such as AKR/J strain that is assumed to possess the genetic modifiers accelerating metastasis of mT-mediated tumors.

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