Phyllodes tumors (PTs) are rare fibroepithelial tumors of the breast with epithelial and stromal components, and surgical resection is the standard and only available treatment for malignant PTs. To provide a better understanding of these tumors, we developed mouse models that recapitulate the pathological and clinical properties of human malignant PTs. Mouse undifferentiated mammary gland cells were infected with a retrovirus encoding the human oncoprotein H-Ras\(^{G12V}\), and the infected cells were transplanted orthotopically into the mammary fat pads of syngeneic mice. The transplanted cells showed a high tumorigenic activity, with the resulting tumors manifesting pathological characteristics including stromal overgrowth similar to those of human malignant PTs. The tumors also showed high rates of both local recurrence and lung metastasis. Our models may prove useful for studies of the pathophysiology of malignant PTs as well as facilitate the development of new treatments.

**Introduction**

Phyllodes tumors (PTs) are fibroepithelial tumors of the breast with epithelial and stromal components (Zurrida et al. 1992; Kim et al. 2013; Spitaleri et al. 2013; Borhani-Khomani et al. 2016; Tan et al. 2016). They are characterized by stromal overgrowth and a leaf-like architecture resulting from an exaggerated intracanalicular pattern, and they are graded as benign, borderline or malignant on the basis of stromal cellularity, atypia and mitotic activity as well as the nature of the tumor margin (Zurrida et al. 1992; Kim et al. 2013; Spitaleri et al. 2013; Borhani-Khomani et al. 2016; Tan et al. 2016). PTs account for <1% of all breast neoplasms, with local recurrences developing in 8%–50% of patients depending on the grade of the primary tumor and distant metastases having been detected in up to 47% of malignant cases (Zurrida et al. 1992; Chen et al. 2005; Taira et al. 2007; Kim et al. 2013; Borhani-Khomani et al. 2016; Tan et al. 2016). Surgery remains the standard and only treatment for PTs. The development of new therapeutic approaches for refractory cases would be facilitated by the establishment of mouse models that recapitulate the biological and pathological characteristics of human PTs.

Comparative genomic hybridization has showed copy number changes in malignant PTs, with inactivation of the tumor suppressor p16\(^{INK4a}\) appearing to be important for progression to malignancy (Jones et al. 2008). Many molecules have also been found to affect the biological behavior of malignant PTs (Karim et al. 2013). Exome sequencing has identified highly recurrent MED12 and RARA somatic mutations in breast fibroepithelial tumors (Lim et al. 2014; Tan et al. 2015), with PTs also manifesting mutations in FLNA, SETD2, KMT2D, BCOR and MAP3K1 (Tan et al. 2015).

Ras is a small GTPase that functions as a molecular switch in the activation of multiple downstream effectors (Downward 2003; Eckert et al. 2004; McLaughlin et al. 2013). It thus activates the mitogen-activated protein kinase (MAPK) signaling pathway composed...
of Raf, MEK and ERK, which regulates gene expression as well as cell proliferation, differentiation and survival (Dhillon et al. 2007; Young et al. 2013). It also activates the PI3K–Akt–mTOR pathway, which plays a key role in the regulation of glucose metabolism as well as cell proliferation, migration and apoptosis (Eckert et al. 2004; Castellano & Downward 2011). The activation state of Ras is controlled at the level of its cycling between GTP-bound (active) and GDP-bound (inactive) forms (Milburn et al. 1990; Downward 2003; Karnoub & Weinberg 2008). Ras GTPase-activating proteins (RasGAPs), such as p120GAP and NF1, negatively regulate Ras signaling by promoting the conversion of Ras-GTP to Ras-GDP (Milburn et al. 1990; Downward 2003; Karnoub & Weinberg 2008). Ras GTPase-activating proteins (RasGEFs) positively regulate the conversion of Ras-GTP to Ras-GDP (Milburn et al. 1990; Downward 2003; Karnoub & Weinberg 2008). Mis-sense gain-of-function mutations in the RAS genes (HRAS, NRAS, KRAS) have been detected in 27% of all human cancers, with 98% of the mutations occurring at three mutational hotspots: amino acids Gly12, Gly13 and Gln61 (Karnoub & Weinberg 2008), whereas Ras guanine nucleotide exchange factors (RasGEFs) positively regulate such signaling by promoting the conversion of Ras-GDP to Ras–GTP (Milburn et al. 1990; Downward 2003). Mis-sense gain-of-function mutations in RAS genes (HRAS, NRAS, KRAS) have been detected in 27% of all human cancers, with 98% of the mutations occurring at three mutational hotspots: amino acids Gly12, Gly13 and Gln61 (Karnoub & Weinberg 2008; Baines et al. 2011; Hobbs et al. 2016). Stabilization of Ras proteins in their active state results in malignant transformation and contributes to tumor growth in cancers bearing such Ras mutations (Karnoub & Weinberg 2008; Baines et al. 2011; Hobbs et al. 2016).

In an attempt to develop a mouse model of human PTs, we infected normal undifferentiated mammary gland cells derived from C57BL/6 mice with a retrovirus encoding H-RasG12V. The oncogene-expressing cells were then injected into mammary fat pads of syngeneic mice. The tumors that formed from the injected cells were found to be similar to human malignant PTs in terms of their pathological and clinical characteristics.

Results and discussion

Establishment of mouse undifferentiated mammary gland cells that express H-RasG12V

Although xenograft models based on injection of established cell lines into immune-deficient mice are often used in preclinical studies, such models alone are insufficient because they do not fully recapitulate the heterogeneous nature of native tumors that results from various microenvironmental stimuli and variability in the differentiation ability of cancer cells. We previously showed that H-RasG12V transforms Ink4a/Arf knockout C57BL/6 mouse mammary gland cells and thereby established a syngeneic mouse model of triple-negative breast cancer (TNBC) (Kai et al. 2014). In the present study, we set out to establish malignant mesenchymal tumor models through the expression of H-RasG12V in wild-type mouse mammary gland cells. Normal mammary glands of C57BL/6 mice were digested with collagenase, and the isolated cells were maintained in culture as mammospheres. The mammospheres were subsequently dissociated and cultured on Matrigel for the establishment of mammosphere-derived cells (MDCs) (Fig. 1A). In addition, we isolated CD24medCD29high cells (defined as basal stem cells, or BSCs) (Stingl et al. 2006; Lim et al. 2010; dos Santos et al. 2013) from normal mouse mammary gland cells by fluorescence-activated cell sorting (FACS) (Fig. 1B), and cells were subsequently subjected to culture on Matrigel. Flow cytometric analysis for these stromal markers showed that both cultured MDCs and BSCs were predominantly CD24lowCD29high (Fig. 1C), indicating that the isolated BSCs changed from CD24medCD29high to CD24lowCD29high during the course of in vitro culture.

We next infected the cultured MDCs and BSCs either with a retrovirus encoding both oncogenic H-RasG12V and green fluorescent protein (GFP) or with a control virus encoding GFP alone. The infected cells were isolated by FACS on the basis of their expression of GFP. Expression of H-RasG12V induced a change in the morphology of both MDCs and BSCs from a spindle shape to a more spherical shape as well as triggered the formation of focal cell aggregates (Fig. 1D). It also induced phosphorylation of the kinases Akt and ERK (Fig. 1E), indicating that both the PI3K–Akt and MEK–ERK pathways were activated as downstream effectors of mutant H-Ras signaling. Furthermore, both H-RasG12V/MDCs and H-RasG12V/BSCs showed a marked growth advantage in comparison with parental MDCs and BSCs or corresponding cells infected with the control retrovirus (Fig. 1F).

H-RasG12V-induced mammary tumors are pathologically similar to human malignant PTs

To examine the tumorigenic potential of the mammary cells expressing H-RasG12V, in vivo, we transplanted these cells into mammary fat pads of syngeneic mice. Both H-RasG12V/MDCs and H-RasG12V/BSCs formed tumors in the recipient mice at rates of 97.9% (47/48) and 100% (20/20), respectively (Fig. 2A).
Both types of tumors manifested marked stromal cellularity and atypia, a high mitotic rate, and pronounced stromal overgrowth (Fig. 2B). They were thus pathologically similar to human malignant PTs, which are characterized by stromal overgrowth and a leaf-like architecture resulting from an exaggerated intracanalicular pattern (Tan et al. 2016). Although the expression of hormone receptors in PTs has been examined in previous studies, the relevance of such expression is unclear (Tse et al. 2002; Tan et al. 2016). Immunohistochemical analysis showed that the tumors formed by H-RasG12V/MDCs or H-RasG12V/BSCs in mice did not express estrogen receptor α (ER) or the progesterone receptor (PR) at detectable levels (Fig. 2C). It has been reported that H-Ras genetically engineered mice developed lung bronchiolo-alveolar adenomas, lung bronchiolo-alveolar adenocarcinomas, splenic hemangiosarcomas, cutaneous squamous cell papillomas, Harderian gland adenoma and hepatocellular adenomas (Nambiar et al. 2012; Paranipe et al. 2012).
Gene expression signature of H-RasG12V-induced mammary tumors

Triple-negative breast cancer is a type of human breast cancer that is characterized by a lack of ER, PR and human epidermal growth factor receptor 2 (HER2) expression. TNBC tumors have been classified into six subtypes—basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and luminal androgen receptor (LAR)—on the basis of the activity of canonical signaling pathways and differential gene expression (Chen et al. 2012). We carried out cDNA microarray analysis with total RNA isolated from H-RasG12V-induced mouse mammary tumors as well as from similar tumors formed by MDCs infected with a retrovirus encoding the R1275Q oncogenic mutant of human anaplastic lymphoma kinase (ALK) (Chen et al. 2008; Mosse et al. 2008; Azarova et al. 2011), which were also negative for ER and PR expressions by immunohistochemical analysis (Fig. S1 in Supporting Information).

To classify our tumor models, we converted the mouse cDNA data to the equivalent for human genes and then uploaded the results to ‘TNBCtype’ <http://cbc.mc.vanderbilt.edu/tnbc>, a subtyping tool for TNBC. This tool showed that none of the tumors formed by H-RasG12V/MDCs, H-RasG12V/BSCs or ALKR1275Q/MDCs could be assigned to a TNBC subtype (Fig. 2D). We then compared the patterns of gene expression between each pair of the three mouse tumor types. The patterns for H-RasG12V/MDC tumors and H-RasG12V/BSC tumors were more similar to each other than were those for H-RasG12V/BSC tumors and ALKR1275Q/MDC tumors or for H-RasG12V/MDC tumors and ALKR1275Q/MDC tumors (Fig. 2E), suggesting that the gene expression signature is largely influenced by the type of oncogene.

H-RasG12V-induced mammary tumors are clinically similar to human malignant PTs

Complete surgical excision is the standard treatment for PTs. However, the local recurrence rate for malignant PTs is 23%–30% even in patients who experience successful tumor resection (Zurrida et al. 1992; Kim et al. 2013; Tan et al. 2016). We investigated local recurrence and metastasis to the lungs after surgical resection of tumors in our mouse models. The pathology of local recurrences and lung metastases resembled that of the primary tumors for mice injected with H-RasG12V/MDCs or H-RasG12V/BSCs (Fig. 3A). H-RasG12V/MDCs formed tumors in all 10 injected mice within nine days, with local recurrences and lung metastases being observed in seven and two mice, respectively, after surgical resection of primary tumors. H-RasG12V/BSCs formed tumors in all 10 injected mice within 10 days, with local recurrences and lung metastases being detected in nine and six mice, respectively (Fig. 3B). The rate of lung metastasis was thus greater for H-RasG12V/BSCs than for H-RasG12V/MDCs. These observations suggested that the H-RasG12V-induced mammary tumors are highly malignant and are clinically similar to human malignant PTs.

In conclusion, we have established mouse models of human malignant PTs through injection of H-RasG12V-expressing mouse CD24lowCD29high undifferentiated mammary gland cells (MDCs or BSCs) into syngeneic recipients. The clinico-pathologic features of the mouse tumors are highly similar to those of human malignant PTs. Our models may therefore provide new insight into the pathogenesis of PTs as well as assist in the development of new treatments for these tumors.

Experimental procedures

Isolation of mammary gland cells

Mammary glands of six-week-old female C57BL/6J mice were minced and then digested for 3–4 h at 37 °C with collagenase type II (1 mg/mL; Sigma, St Louis, MO, USA) in Dulbecco’s modified Eagles’ medium (DMEM)–F12 (Sigma) supplemented with 21.4 mM NaHCO₃, 25 μM HEPES and bovine serum albumin (20 mg/mL; Sigma). The digested tissue was then treated with DNase I (0.1 mg/mL; Sigma) before isolation of single cells by passage through a 100-μm cell strainer (BD Biosciences, San Jose, CA, USA). Red blood cells were lysed by the addition of NH₄Cl to a final concentration of 150 mM.

Preparation of MDCs

Primary mammary gland cells were maintained as mammospheres under 5% CO₂ at 37 °C in DMEM–F12 supplemented with recombinant human epidermal growth factor (20 ng/mL; PeproTech, Rocky Hill, NJ, USA), recombinant human basic fibroblast growth factor (20 ng/mL; PeproTech), B27 supplement without vitamin A (Invitrogen, Carlsbad, CA, USA), heparin sulfate (200 ng/mL), penicillin
(100 U/mL) and streptomycin (100 ng/mL) (Nacalai Tesque, Kyoto, Japan). The mammospheres were subsequently dissociated and cultured in a Matrigel adhesion dish for the preparation of MDCs.

Isolation of BSCs

A single-cell suspension of primary mammary gland cells in phosphate-buffered saline was labeled with allophycocyanin-conjugated rat monoclonal antibodies to mouse CD24 (BioLegend, San Diego, CA, USA) and phycoerythrin-conjugated hamster monoclonal antibodies to mouse/rat CD29 (eBioscience, San Diego, CA, USA) for FACS with a MoFlo XDP Cell Sorter (Beckman Coulter, Brea, CA, USA). The CD24<sup>med</sup>CD29<sup>high</sup> fraction was isolated as BSCs. Cultured MDCs and BSCs were also analyzed for CD24 and CD29 expressions by flow cytometry with a Gallios Flow Cytometer (Beckman Coulter) and FLOWJO software.

Maintenance of MDCs and BSCs

Mammosphere-derived cells and BSCs were maintained in DMEM–F12 supplemented with recombinant human epidermal growth factor (20 ng/mL; PeproTech), recombinant human basic fibroblast growth factor (20 ng/mL; PeproTech), B27 supplement without vitamin A (Invitrogen), heparin sulfate (200 ng/mL), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Nacalai Tesque) on a Matrigel adhesion dish coated with a combination of poly-L-ornithine hydrobromide (100 μg/mL; Sigma) and EZ cell matrix (10 μg/mL; Asahi Glass, Tokyo, Japan).

Retrovirus infection

Plat-E packaging cells were transfected with pMXs-HRAS (G12V)-IRES-GFP (Kitamura et al. 2003), pMXs-IRES-GFP (control plasmid), pGCDN-ALK(R1275Q)-IRES-Kusabira Orange (KuO) (Tamase et al. 2009) or pGCDN-IRES-KuO (control plasmid) with the use of the FugeneHD reagent (Roche Diagnostics, Mannheim, Germany). Culture supernatants were collected, passed through a 0.45-μm cellulose acetate filter (Iwaki, Kyoto, Japan) and centrifuged at 17 970 g for 4 h at 4 °C. The virus pellets were resuspended in culture medium and added to MDCs or BSCs. Infected cells were sorted by FACS on a Vantage SE instrument (BD, Franklin Lakes, NJ, USA) on the basis of GFP or KuO fluorescence.

Figure 2 Formation of PT-like tumors by H-Ras<sup>G12V</sup>-expressing mouse mammary stromal cells in vivo. (A) Tumorigenesis rate for H-Ras<sup>G12V</sup>-MDCs and H-Ras<sup>G12V</sup>-BSCs injected into mammary fat pads of syngeneic mice. (B) Hematoxylin–eosin staining of tumors formed by H-Ras<sup>G12V</sup>-MDCs or H-Ras<sup>G12V</sup>-BSCs. Arrows indicate a leaf-like architecture. Scale bars, 500 μm (upper panels) or 100 μm (lower panels). (C) Immunohistochemical staining of GFP, ER, and PR in tumors formed by H-Ras<sup>G12V</sup>-MDCs or H-Ras<sup>G12V</sup>-BSCs. Scale bars, 100 μm. (D) TNBC subtype analysis carried out on the basis of gene expression profile with the TNBCtype tool for tumors formed by the indicated cells. UNS, unstable; NA, not available. (E) MA (log intensity ratio versus average log intensity) plots for pairwise comparisons of the distribution of gene expression among tumors formed by H-Ras<sup>G12V</sup>-BSCs, H-Ras<sup>G12V</sup>-MDCs or ALK<sup>R1275Q</sup>-MDCs.

Cell viability assay

Cells (3 × 10<sup>5</sup> per well) were seeded in 96-well plates, cultured for 72 h and assayed for viability with the use of a Cell TiterGlo assay (Promega, Madison, WI, USA).

Immunoblot analysis

Cells were lysed with SDS sample buffer [2% SDS, 10% glycerol, 50 mM Tris–HCl (pH 6.8)] containing 1 mM dithiothreitol for fractionation by SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to a polyvinylidene difluoride membrane and exposed to primary antibodies including those to H-Ras (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), to Akt (#9272; Cell Signaling Technology, Danvers, MA, USA), to Ser<sup>473</sup>–phosphorylated Akt (#4060; Cell Signaling Technology), to ERK1/2 (#9102; Cell Signaling Technology), to Thr<sup>202</sup>/ Tyr<sup>204</sup>–phosphorylated ERK1/2 (#4370; Cell Signaling Technology) and to α-tubulin (Sigma). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Chemi-Lumi One L; Nacalai Tesque).

Injection of cells into mammary fat pads of mice

All mouse experiments were approved by the animal ethics committee of Keio University School of Medicine. Female C57BL/6j mice aged 6–8 weeks were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Retrovirus-infected MDCs or BSCs [1 × 10<sup>6</sup> in 20 μL of a 1 : 1 (v/v) mixture of DMEM–F12 and EZ cell matrix] were injected into the no. 4 mammary fat pads of the anesthetized mice.

Immunohistochemistry

Tumor tissue was fixed with 4% paraformaldehyde, embedded in paraffin and sectioned, after which the sections were dehydrated in paraffin and stained with rabbit polyclonal antibodies to ER, to PR or to GFP (Santa Cruz Biotechnology). Immune complexes were detected with biotinylated secondary antibodies and a Vectastain avidin and biotinylated horseradish peroxidase macromolecular complex reagent and 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA).
Mouse model of malignant phyllodes tumor

| Cells     | Introduced gene          | Tumorigenesis rate |
|-----------|--------------------------|--------------------|
| MDCs      | H-Ras<sup>G12V</sup>-GFP | 97.9% (47/48)      |
|           | Control vector (GFP)     | 0% (0/5)           |
| BSCs      | H-Ras<sup>G12V</sup>-GFP | 100% (20/20)       |
|           | Control vector (GFP)     | 0% (0/5)           |

(B) Lower magnification

Higher magnification

(C) H-Ras<sup>G12V</sup>/MDCs

H-Ras<sup>G12V</sup>/BSCs

GFP

ER

PR

(D) Tumor Subtype Correlation P value

| Tumor       | Subtype | Correlation | P value |
|-------------|---------|-------------|---------|
| ALK<sup>R1275Q</sup>/MDC | UNS     | NA          | NA      |
| H-Ras<sup>G12V</sup>/MDC   | UNS     | NA          | NA      |
| H-Ras<sup>G12V</sup>/BSC   | UNS     | NA          | NA      |

(E) H-Ras<sup>G12V</sup>/BSCs versus H-Ras<sup>G12V</sup>/MDCs

H-Ras<sup>G12V</sup>/BSCs versus ALK<sup>R1275Q</sup>/MDCs

H-Ras<sup>G12V</sup>/MDCs versus ALK<sup>R1275Q</sup>/MDCs
Figure 3  H-RasG12V-induced mammary tumors are clinically similar to human malignant PTs. (A) Hematoxylin–eosin staining of primary tumors, local recurrences and lung metastases formed in mice injected with H-RasG12V/MDCs or H-RasG12V/BSCs. (B) Clinical course with the evaluation of local recurrence and lung metastasis in mice injected with H-RasG12V/MDCs or H-RasG12V/BSCs on day 1 and subjected to surgical excision of the primary tumor on the indicated days. Data are shown for each of the no. 4 mammary fat pads injected in each of 10 mice. NT, not tested.
Microarray analysis

Total RNA was isolated from tumors formed by H-RasG12V/MDCs, H-RasG12V/BCSs or ALK1275Q/MDCs with the use of Trizol (Thermo Fisher Scientific K.K., Yokohama, Kanagawa, Japan) and RNeasy Kit (Qiagen, Tokyo, Japan). The RNA was then converted to cRNA for the analysis of gene expression with a cDNA array (Agilent Gene Expression DNA Microarray; Agilent Technologies, Santa Clara, CA, USA).

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Y Takamoto et al.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1ALKR1275Q–induced mammary tumors.