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Differential Regulation of *MMP-9* and *TIMP-2* Expression in Malignant Melanoma Developed in *Metallothionein*/*RET* Transgenic Mice

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We recently established a *metallothionein-I(MT)*/*RET* transgenic mouse line in which skin melanosis, benign melanocytic tumor and malignant melanoma develop stepwise. Malignant melanoma cells but not benign melanocytic tumor cells had metastatic ability in transgenic mice. In the present study, we investigated the expression of several matrix metalloproteinases (*MMP*s) and tissue inhibitors of matrix metalloproteinases (*TIMP*s), including *MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MT1-MMP, TIMP-1* and *TIMP-2,* in these tumors. Western and northern blot analyses revealed that malignant transformation of melanocytic tumors developed in *MT*/*RET* transgenic mice accompanied with upregulation of *MMP-9* and downregulation of *TIMP-2*. Expression of other *MMP* and *TIMP* genes examined was very low or undetectable in both benign and malignant tumors. Since activation of MMP-9 in malignant tumors was detected by gelatin zymography, these results suggest that imbalance of expression of the *MMP-9* and *TIMP-2* genes might be associated with metastatic ability of melanoma cells developed in *MT*/*RET* transgenic mice.

Key words: *RET* — Transgenic mice — Malignant melanoma — *MMP-9* — *TIMP-2*

By introducing the *RET* recombinant oncogene (*RFP*/*RET*) fused to the mouse *metallothionein-I* (*MT*) promoter-enhancer, we previously established three transgenic mouse lines (designated lines 192, 304 and 319) in which skin melanosis and benign melanocytic tumors develop stepwise.[1–3] In these lines, benign melanocytic tumors grew slowly and did not metastasize. In addition, development of malignant melanoma was very rare in *MT*/*RET* transgenic mice.

We have recently generated a new transgenic mouse subline by crossing line 304 with C57BL/6 mice (designated line 304/B6) in which benign melanocytic tumors frequently progress to malignant melanoma.[4] We observed that benign melanocytic tumors, which grew very slowly for several months, suddenly began to grow rapidly in more than 60% of 304/B6 mice, showing the histological appearance of malignant melanoma. The malignant tumors metastasized to lymph nodes, brain, lung, kidney, liver and spleen.[4] The malignant transformation of tumor cells was accompanied with increased expression of the *RFP*/*RET* transgene, mitogen-activated protein kinases (MAPKs) and *c-Jun*.

In the present study, we further investigated the expression of several matrix metalloproteinases (*MMP*s) and tissue inhibitors of matrix metalloproteinases (*TIMP*s) in benign melanocytic tumors and malignant melanomas developed in 304/B6 mice. We found that, in addition to upregulation of *MMP-9* expression, the *TIMP-2* expression is drastically decreased in malignant melanoma cells.

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MATERIALS AND METHODS

***Metallothionein*/*RET* transgenic mouse** Two *MT*/*RET* transgenic mouse lines (designated lines 192 and 304) that develop skin melanosis and benign melanocytic tumors were previously described.[1] By crossing line 304 with C57BL/6 mice, a new subline (designated line 304/B6) in which benign melanocytic tumors frequently progress to malignant melanoma was established.[4]

cDNA cDNAs for *MMP-1*, *MMP-2*, *MMP-3*, *MMP-7*, *membrane-type (MT)1-MMP*, *TIMP-1* and *TIMP-2* genes were kindly provided by Dr. Shimizu (Aichi Cancer Center Research Institute, Nagoya). *E1AF* cDNA was provided by Dr. Fujinaga (Sapporo Medical School, Sapporo).

RNA probes for northern blot analysis and *in situ* hybridization The sense- and antisense-cDNAs were inserted into the pGEM plasmids and linearized by enzyme digestion. Digoxigenin (DIG)-labelled riboprobes were generated using the SP6 and T7 promoters according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany).

Western blot analysis Total cell lysates were prepared from tumor tissues as described previously.[5] The lysates were subjected to sodium dodecyl sulfate (SDS)/8% polyacrylamide gel electrophoresis and transferred to polyvi-

nylidene difluoride membranes (Nihon Millipore Kogyo KK, Tokyo). The membranes were reacted with anti-Ret antibody,[5] anti-phosphotyrosine antibody (Zymed Laboratories, South San Francisco, CA), anti-MMP-9 monoclonal antibody[6] or anti-TIMP-2 antibody (Chemicon International Inc., Temecula, CA), and the reaction was examined by enhanced chemiluminescence (Amersham Corp., Buckinghamshire, United Kingdom).

***In situ* hybridization** Cryostat sections 10–15 μm thick were placed on 3-aminopropyltriethoxysilane (APES)-coated slides and fixed with 4% paraformaldehyde for 10 min at room temperature. The sections were washed twice in phosphate-buffered saline (PBS) and acetylated in 0.1 *M* triethanolamine containing 0.25% acetic anhydride for 15 min. After having been washed twice in 4× SSC (0.6 *M* NaCl, 0.06 *M* sodium citrate) for 10 min, the sections were allowed to air-dry for 1 h at room temperature. Then the sections were prehybridized with 50% deionized formamide, 2× SSC for 1–2 h at 42°C and hybridized with the DIG-labelled riboprobe in hybridization mixture overnight at 42°C. The hybridization mixture contained 2× SSC, 50% deionized formamide, 1 mg/ml tRNA, 1 mg/ml sonicated salmon sperm DNA, 1 mg/ml bovine serum albumin (BSA) and 10% dextran sulfate. After hybridization, the sections were washed with 2× SSC, 50% formamide at 42°C for 20 min three times. Then they were incubated with 20 mg/ml RNase A in TE buffer (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA) containing 0.5 *M* NaCl at 37°C for 30 min. The sections were washed with buffer 1 (100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl) and treated with 1% blocking reagent (Boehringer Mannheim) in buffer 1 for 30 min at room temperature. The sections were reacted with 1:500 dilution of anti-DIG Fab fragments conjugated with alkaline phosphatase in buffer 1 for 1 h and washed twice with buffer 1 and then with buffer 2 (100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl and 50 m*M* $MgCl_2$). The enzyme reaction was detected by incubation with 13.5 μl of 4-nitro blue tetrazolium chloride (NBT) (Boehringer Mannheim) and 10.5 μl of X-phosphate/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Boehringer Mannheim) in 3 ml of buffer 2. The sections were washed in 100 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA, then air-dried and mounted in 90% glycerol.

Northern blot analysis Total RNA was isolated by the guanidinium thiocyanate/phenol-chloroform extraction method. Total RNA (10 μg) was electrophoresed in formaldehyde agarose gels (1% wt./vol.) and transferred to nitrocellulose membranes. The membranes were air-dried, cross-linked by exposure to UV light for 2 min, prehybridized and hybridized in 5× SSC containing 50% formamide, 10 m*M* sodium phosphate (pH 6.5), 0.5% SDS, 10× Denhardt's solution, 0.1 mg/ml salmon sperm DNA and 2% blocking reagent at 65°C in the presence of DIG-labelled riboprobe for 18 h. The membranes were washed twice with 2× SSC, 0.1% SDS at room temperature and twice with 0.2× SSC, 0.1% SDS at 65°C for 30 min. They were incubated with buffer A (100 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl) for 5 min, and with 2% blocking reagent in buffer A for 1 h and then reacted with a 1:10,000 dilution of anti-DIG-Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim). After the reaction, the membranes were washed twice with buffer A for 15 min and treated with buffer B (50 m*M* Tris-HCl, pH 9.5, 50 m*M* NaCl and 50 m*M* $MgCl_2$) for 5 min. The immunoreactive bands were visualized using the CDP star system (New England Biolab, Beverly, MA).

Reverse-transcription polymerase chain reaction (RT-PCR) Total RNAs isolated from tumor tissues were reverse-transcribed into cDNA. The oligonucleotides used were as follows; forward primer for *MMP-2* (CTGGGTCTATTCTGCCAGCACTCCTG), reverse primer for *MMP-2* (AGCCAGTCTGATTTGATGCTTCCAA), forward primer for *MT1-MMP* (ATCAACACTGCCTACGAGAGGAAGG), reverse primer for *MT1-MMP* (AGCCAGTCTGATTTGATGCTTCCAA). PCR mixture and the respective primers were amplified for 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. *β-Actin* primers were used as the positive control. Each PCR reaction mixture was subjected to 2% agarose gel electrophoresis.

Zymographic analysis Zymograms demonstrating gelatinolytic activity in unreduced samples were prepared by incorporating 2 mg/ml gelatin (Difco Laboratories, Detroit, MI) into 8% polyacrylamide gels. Prior to electrophoresis, samples (50 μg) were incubated with nonreducing sample buffer (50 m*M* Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 25 μg/ml bromophenol blue). Electrophoresis was carried out in a cold room and the gels were washed with 50 m*M* Tris-HCl (pH 7.5) containing 2.5% Triton X-100 for 30 min at room temperature twice and with water for 5 min three times to remove SDS. Then the gels were incubated overnight in 50 m*M* Tris-HCl (pH 7.5), 10 m*M* $CaCl_2$ at 37°C. Following staining with Coomassie blue, regions of proteolytic activity were visualized as clear zones against a blue background.

RESULTS

We previously established transgenic mouse lines that develop melanocytic tumors by introducing the *RFP*/*RET* fusion gene linked to the mouse metallothionein-I promoter-enhancer (*MT*/*RET*).[1–4] Tumor tissues were obtained from two *MT*/*RET* transgenic mouse lines (lines 192 and 304/B6). Transgenic mice of line 192 develop only benign melanocytic tumors (Fig. 1a), whereas benign tumors in mice of line 304/B6 (Fig. 1b) frequently progress to malignant melanoma with metastatic ability (Fig. 1, c and d). Total cell lysates for western blotting and RNAs for northern blotting were prepared from

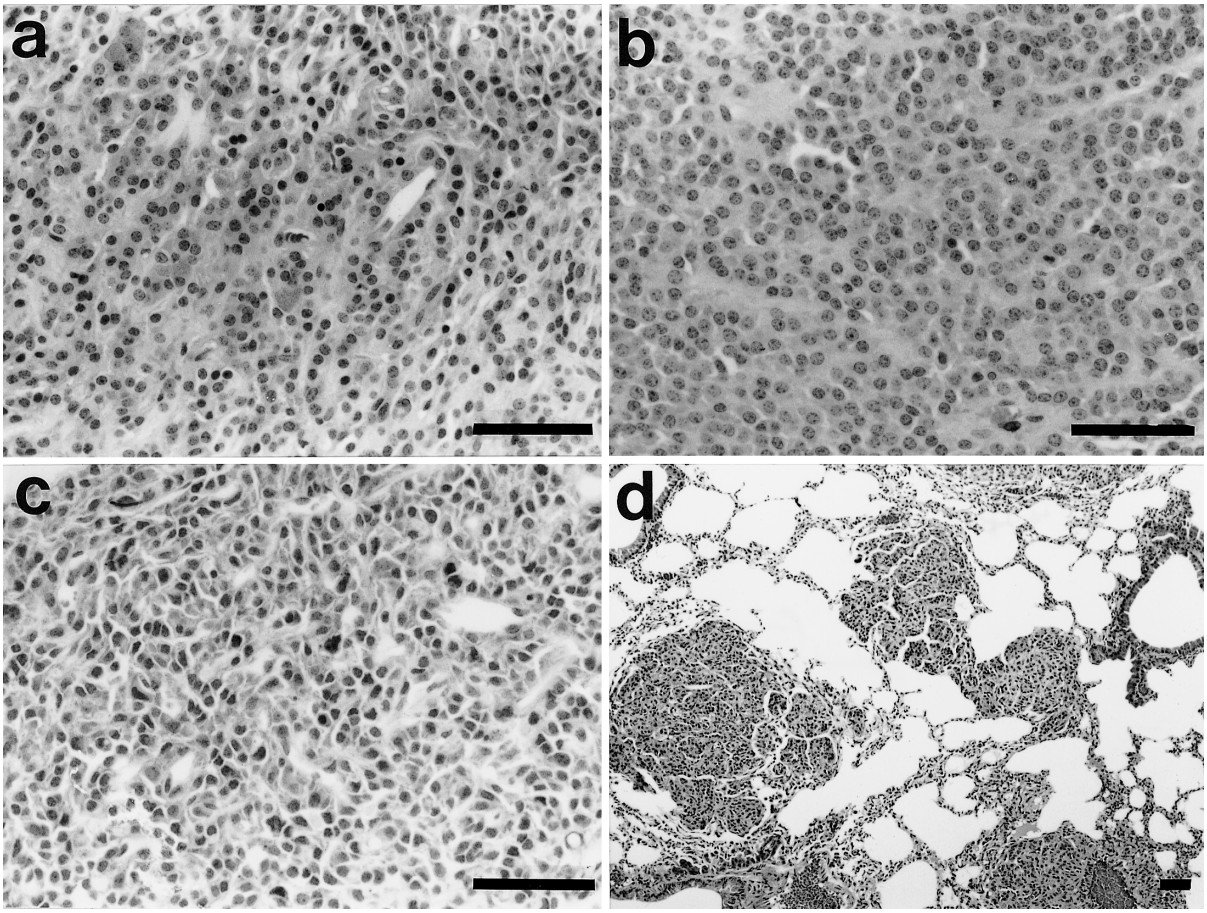

Fig. 1. Histopathology of tumors developed in *MT*/*RET* transgenic mice. (a) A benign melanocytic tumor in the skin of a line 192 mouse. (b) A benign melanocytic tumor in the skin of a line 304 mouse. (c) Malignant melanoma in the skin of a line 304/B6 mouse. (d) Lung metastasis of melanoma cells in a line 304/B6 mouse. Bars represent 70 μm.

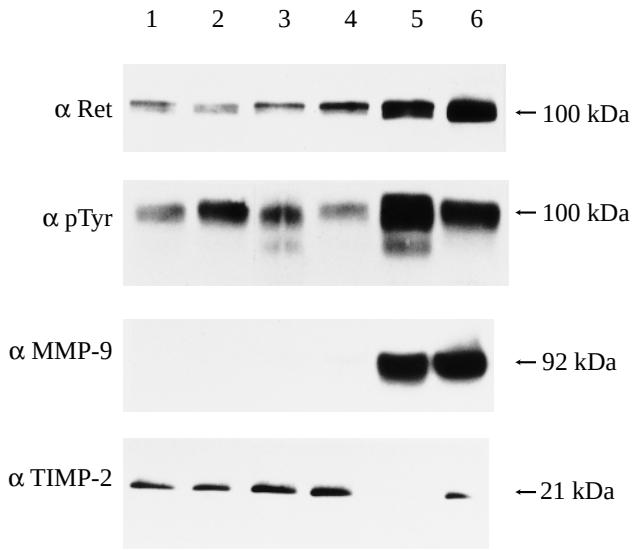

Fig. 2. Expression of Ret, MMP-9 and TIMP-2 in melanocytic tumors of *MT*/*RET* transgenic mice. Total cell lysates (50 μg) from two benign melanocytic tumors of line 192 mice (lanes 1 and 2), two benign melanocytic tumors of line 304/B6 mice (lanes 3 and 4) and two malignant tumors of line 304/B6 mice (lanes 5 and 6) were analyzed by western blotting with anti-Ret antibody, anti-phosphotyrosine (pTyr) antibody, anti-MMP-9 antibody or anti-TIMP-2 antibody. 100 kDa Rfp/Ret, 92 kDa MMP-9 and 21 kDa TIMP-2 proteins are indicated.

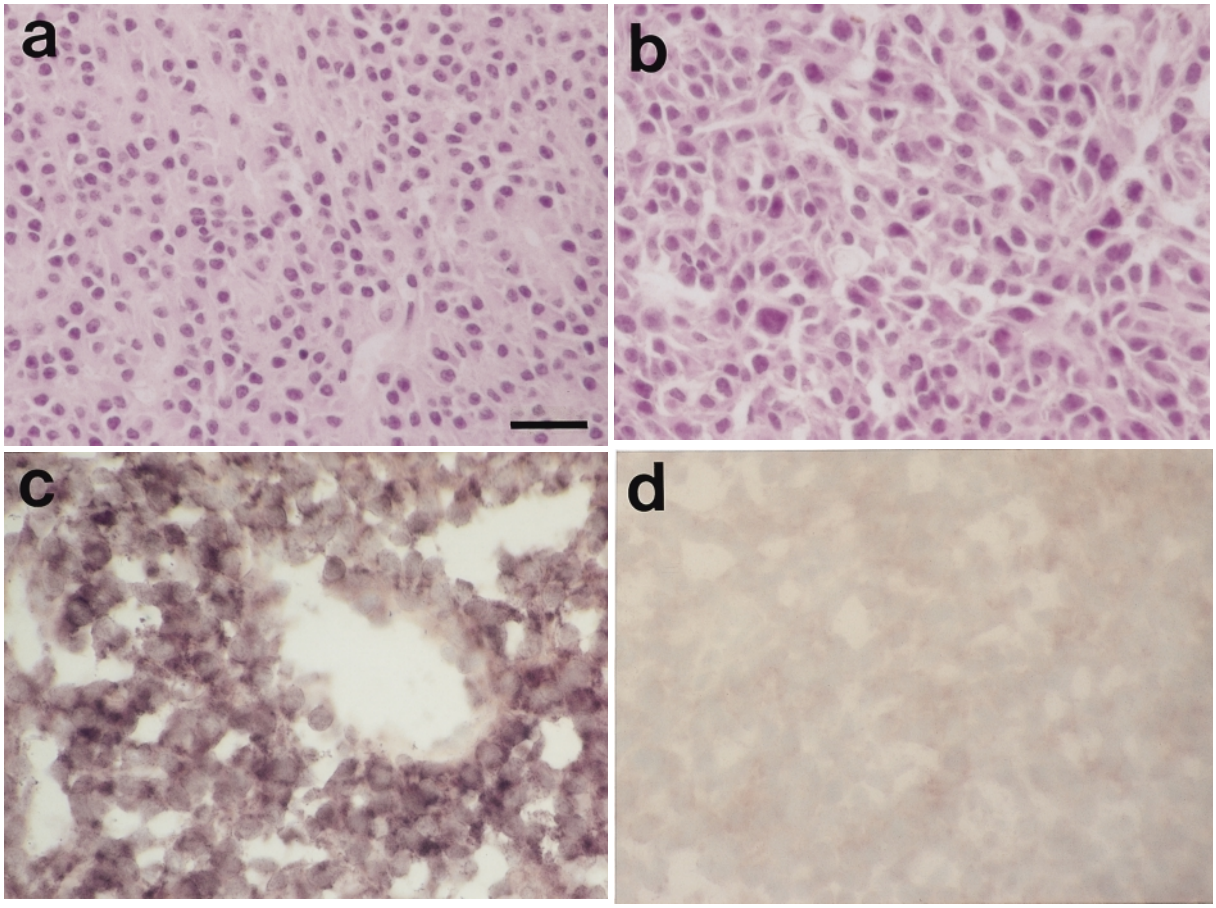

Fig. 3. *In situ* hybridization of the *TIMP-2* gene. (a and b) Hematoxylin and eosin staining of benign (a) and malignant (b) tumors of line 304/B6 mice. (c and d) Frozen sections of benign (c) and malignant (d) tumors were hybridized with a digoxigenin (DIG)-labelled antisense *TIMP-2* probe. Strong signals were detected in a benign tumor (c). A bar represents 30 μm.

benign tumors of line 192 mice and from both benign and malignant tumors of line 304/B6 mice. The sizes of benign and malignant tumors were 50–100 mm^3 and >50,000 mm^3, respectively.

As we have recently reported,[4] expression of the Rfp/Ret protein and its tyrosine phosphorylation were significantly increased in malignant melanomas from line 304/B6 mice compared with those in benign tumors from lines 192 and 304/B6 mice (Fig. 2). Interestingly, when expression of MMP-9 and TIMP-2 was investigated by western blotting, marked increase of MMP-9 expression and decrease of TIMP-2 expression were observed in malignant melanomas. Decrease of the *TIMP-2* expression in a malignant tumor was confirmed by *in situ* hybridization, whereas a benign tumor in a 304/B6 mouse showed a high level of *TIMP-2* expression (Fig. 3).

We further investigated the expression of several *MMPs* and *TIMPs* including *MMP-1*, *MMP-2*, *MMP-3*, *MMP-7*, *MT1-MMP*, *TIMP-1* and *TIMP-2* by northern blotting. The expression of these genes, except *TIMP-2*, was undetectable in both benign and malignant tumors by northern blotting (Fig. 4a and data not shown), although the transcripts of the *MMP-2* and *MT1-MMP* genes were detected in both by RT-PCR (Fig. 4b). In addition, since it was reported that the *Ets*-related *E1AF* gene regulates the expression of the *MMP* genes,[7] its expression was examined in each tumor by northern blotting. As shown in Fig. 4a, *E1AF* was expressed at similar levels in both benign and malignant tumors.

Finally, activation of MMP-2 and MMP-9 was analyzed by gelatin zymography. Upregulation of enzymatic activity of MMP-9, but not that of MMP-2, was clearly detected in malignant tumors and their culture medium (Fig. 5, lanes 3 and 6), although the activity of MMP-2 was observed in serum from transgenic mice with either benign or malignant tumors (Fig. 5, lanes 7 to 9).

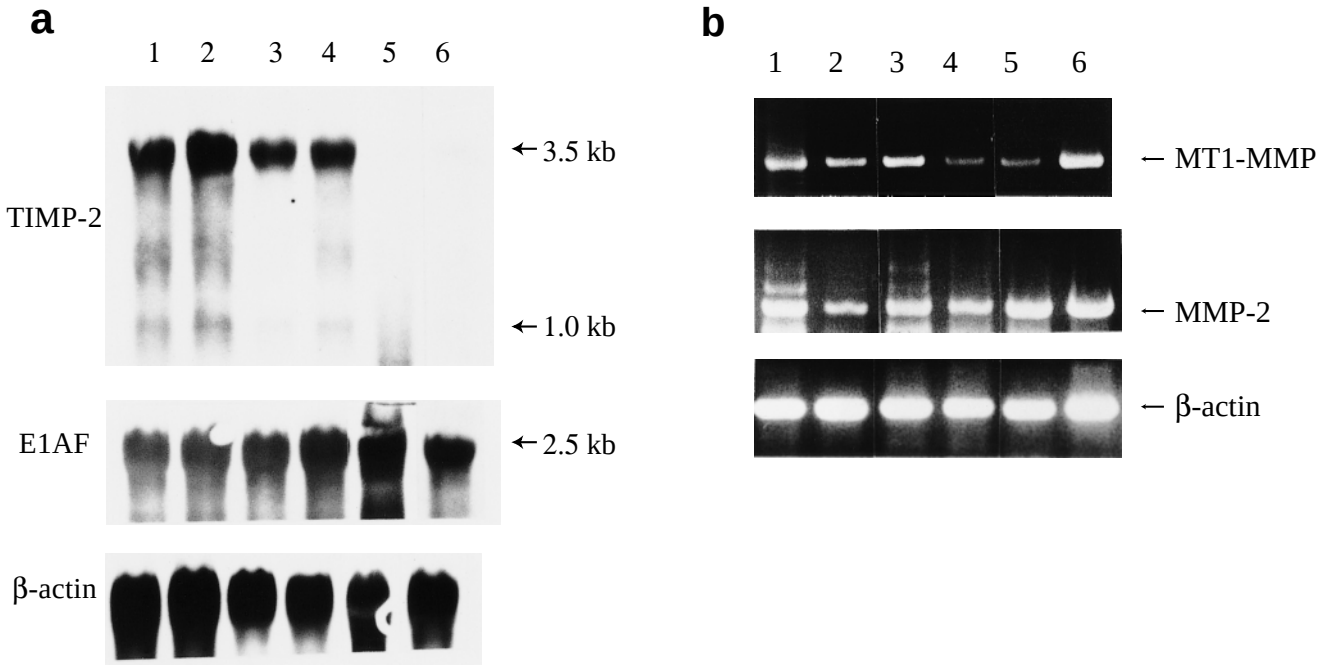

Fig. 4. Analyses of expression of *MMP*s and *TIMP*s by northern blotting and RT-PCR. (a) Total RNAs (10 μg) isolated from two benign melanocytic tumors of line 192 mice (lanes 1 and 2), two benign melanocytic tumors of line 304/B6 mice (lanes 3 and 4) and two malignant tumors of line 304/B6 mice (lanes 5 and 6) were analyzed by northern blotting with *TIMP-2*, *E1AF* and *β-actin* probes. The 3.5 kb and 1.0 kb transcripts of the *TIMP-2* gene and 2.5 kb transcripts of the *E1AF* gene are indicated. (b) Expression of the *MT1-MMP*, *MMP-2* and *β-actin* genes was analyzed by RT-PCR.

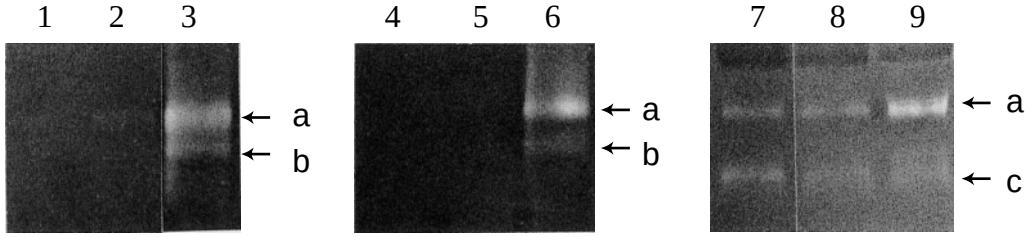

Fig. 5. MMP-9 activity in tumors of *MT*/*RET* transgenic mice. The MMP-9 and MMP-2 activities in tumor tissues (lanes 1 to 3), their culture media (lanes 4 to 6) and serum (lanes 7 to 9) were analyzed by gelatin zymography. A benign melanocytic tumor of a line 192 mouse (lanes 1, 4 and 7), a benign melanocytic tumor of a line 304/B6 mouse (lanes 2, 5 and 8) and a malignant tumor of a line 304/B6 mouse (lanes 3, 6 and 9) were used in this assay. Proenzyme (a) and activated form (b) of MMP-9 and proenzyme (c) of MMP-2 are indicated.

DISCUSSION

In the present study, we investigated the expression of *MMP*s and *TIMP*s in melanocytic tumors developed in two *MT*/*RET* transgenic mouse lines (lines 192 and 304/B6). Line 192 mice develop only benign melanocytic tumors whereas line 304/B6 mice frequently develop malignant melanoma by progression from benign tumors.[4] We found that malignant transformation of melanocytic tumors in 304/B6 mice was accompanied with upregulation of *MMP-9* and downregulation of *TIMP-2*, among various *MMP*s and *TIMP*s examined. Activation of MMP-9 was confirmed by gelatin zymography as previously described.[4] Since many studies suggesting a correlation between malignant progression or metastatic potential of tumor cells and overexpression of *MMP-9* have been

reported,[8–14] it is possible that upregulation of *MMP-9* is associated with the metastatic ability of malignant tumors of 304/B6 mice.

It is noteworthy that expression of the *TIMP-2* gene was drastically downregulated in malignant melanoma of 304/B6 transgenic mice. TIMP-2 is known to regulate the function of MT1-MMP, that can activate MMP-2.[15–18] Thus, we examined the expression of *MT1-MMP* and *MMP-2* in tumors of *MT*/*RET* transgenic mice. Analyses by northern blotting and RT-PCR revealed that expression of these two genes was low in both benign and malignant tumors. Although we have recently reported the activation of MMP-2 in malignant tumors of *MT*/*RET* transgenic mice by gelatin zymography,[4] its activation was unclear in the tumors as well as in their culture medium in the present study, suggesting that it may not be crucial for the metastatic ability of malignant melanoma cells in our transgenic mice. Rather, the expression and activation level of MMP-2 could depend on the individual tumors examined and downregulation of TIMP-2 may play a role in the activation of MMP-9.

At present, we do not know how *MMP-9* and *TIMP-2* gene expression is regulated in tumors of transgenic mice. Although we investigated the expression of the *E1AF* gene, that was reported to regulate *MMP* expression,[7] it was expressed at similar levels in both benign and malignant tumors. Thus, it seems unlikely that the *E1AF* gene is involved in the regulation of *MMP-9* and *TIMP-2* expression in malignant transformation of tumor cells in our transgenic mice. Since the Rfp/Ret, MAPK and c-Jun expression was markedly upregulated in malignant tumors of 304/B6 mice,[4] this suggests that the intracellular signalling through Rfp/Ret may be important for upregulation of *MMP-9* and downregulation of *TIMP-2*. Further investigation will be required for elucidation of the mechanisms of the aberrant expression of *MMP-9* and *TIMP-2* in melanoma cells.

Our transgenic mice are interesting model animals in which benign melanocytic tumors spontaneously progress to malignant melanoma. This transgenic line should provide a useful system to study the mechanisms of malignant transformation and metastasis of tumor cells *in vivo*.

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