Priority Report

Cancer Research

A Novel Mouse Model for *De novo* Melanoma

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Abstract

Nevus-associated melanomas arise from pre-existing benign lesions, but *de novo* melanomas can also develop in the absence of such lesions. Few studies have addressed the latter phenomenon because no animal models have been described in which melanomas clearly develop in a *de novo* manner. In this study, we have address this need in defining RFP-RET-transgenic mice (RET mice) as a mouse model for multi-step melanomagenesis that proceeds via tumor-free, benign, premalignant, and malignant stages. Melanomas from RET mice exhibited decreased expression levels of endothelin receptor B (Ednrb) compared with benign tumors. In RET mice that were heterozygous for Ednrb (Ednrb+/−;RET mice), >80% of the arising primary tumors were malignant. Life span after tumor development in the mice was significantly shorter than in RET mice. Lung metastasis after tumor development was significantly higher than in RET mice. The observed process of melanomagenesis in Ednrb+/−;RET mice, which proceeded without a pre-existing benign lesion, along with the emergent characteristics in the model after tumor development corresponded well with the formation of *de novo* melanoma in humans. Our findings define a novel transgenic mouse model for *de novo* melanoma and suggest that reduced expression of Ednrb might facilitate the development of *de novo* melanoma in humans. *Cancer Res; 70(1); 24–9. ©2010 AACR.*

Introduction

It has been shown histopathologically that there are two kinds of carcinogenesis in human cancers. One is multistep carcinogenesis that arises from a pre-existing benign lesion, and the other is *de novo* carcinogenesis that arises without a pre-existing lesion (1–4). At present, however, mechanisms for multistep carcinogenesis and *de novo* carcinogenesis are still largely unclear. To our knowledge, there are no melanoma animal models in which tumors have been clearly shown to be *de novo* melanoma.

Human melanomas develop from pre-existing benign lesions (multistep melanomagenesis) and in the absence of benign lesions (*de novo* melanomagenesis; refs. 5–7). However, there is very limited information about the biochemical mechanisms underlying multistep melanomagenesis and *de novo* melanomagenesis. This is because observation of the entire process for melanomagenesis in humans is impossible. Establishment of animal models that can be used for the study of both multistep melanomagenesis and *de novo* melanomagenesis may contribute to the elucidation of their pathogenetic differences.

We previously established metallothionein-I/RFP-RET-transgenic mice of line 304/B6 (RET mice) that spontaneously develop systemic skin melanosis, benign melanocytic tumors, and melanoma metastasizing to distant organs stepwise (Fig. 1*A*; ref. 8). In this study, we introduce RET mice with heterozygously deleted Ednrb [Ednrb(+/−);RET mice] as a novel mouse model for *de novo* melanoma.

Materials and Methods

Mice. RET mice (Fig. 3*A*), in which solitary or multiple primary dome-shaped tumors macroscopically develop on the skin (head, neck, trunk, limbs, and tail) and eyes (9, 10), were used in this study. Endothelin receptor B (Ednrb)-heterozygously deleted RET mice [Ednrb(+/−);RET mice] were newly generated in this study by crossing RET mice and Ednrb-deficient mice (11). Location and shape of tumors in Ednrb (+/−);RET mice were similar to those in RET mice (Fig. 3*A*). We calculated tumor volumes by the integral method based on their radius and height with calculation of hemisphere volume after choosing the largest primary tumors in the case of mice with multiple tumors. Ednrb-homozygously deleted RET mice [Ednrb (−/−);RET mice] died of Hirschsprung disease within a month. This study was approved by the Animal

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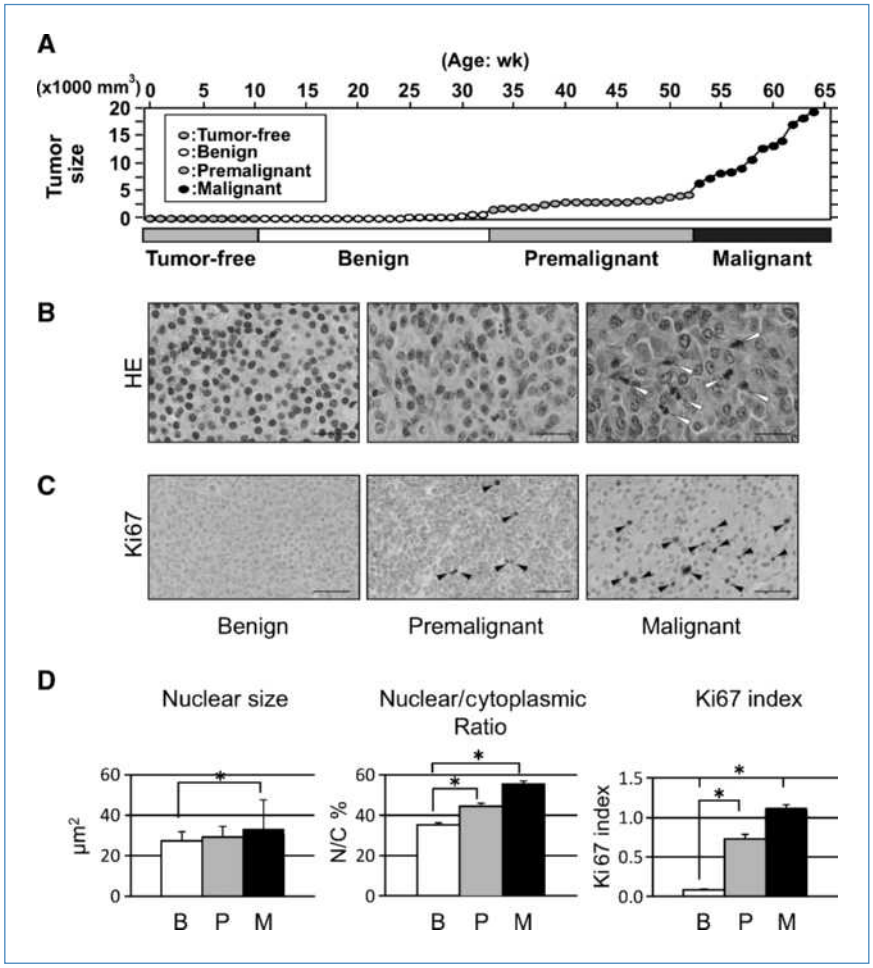

Figure 1. Stage-dependent histopathologic characterization of primary tumors from RET mice. *A* to *C,* representative time course (*A*) and microscopic appearances (*B,* HE staining; *C,* immunohistochemistry with anti-Ki67 antibody) in tumor-free (*border*), benign (*white*), premalignant (*gray*), and malignant (*black*) stages in primary tumors from RET mice. *White arrowheads,* mitotic cells (*B*). *Black arrowheads,* Ki67-positive cells (*C*). *Bars,* 20 μm. *D, columns,* mean values of nuclear size, nuclear-cytoplasmic ratio (N/C ratio) and Ki67 index in benign (*B, white*), premalignant (*P, gray*), and malignant (*M, black*) tumors; *bars,* SEM. *, $P < 0.01$, significantly different from benign tumors by Kruskal-Wallis test.

Care and Use Committee (approval no.18001) and Recombination DNA Advisory Committee (approval no. 06-01) in Chubu University, Japan.

Real-time PCR, immunohistochemical, and Western blot analyses. We biochemically and histopathologically analyzed primary cutaneous tumors and metastatic tumors from RET mice and Ednrb(+/−);RET mice by real-time PCR, immunohistochemical, and Western blot analyses. Real-time PCR was performed by the method previously described (12). More than 10 tumors in each tumor stage were analyzed. The immunohistochemistry with anti–Ki67 (Novocastra) and anti-Ednrb (Chemicon) antibodies was performed using a Vectastain ABC Kit (Vector) and Vector VIP (Vector) for colorization. Results from 90 cells from six different samples (nuclear size and N/C ratio) and 5,000 cells from six different samples (Ki67 index) in each stage were analyzed. Western blot analysis was performed using anti-Ednrb (Abcam) and anti–glyceraldehyde-3-phosphate dehydrogenase (Epitomics) antibodies. Analysis of HE staining and immunohistochemistry and Western blot was performed in six tumors in each stage, and representative results are presented.

Results

Histopathologic definitions of stages in the process of melanomagenesis in RET mice. Tumors in the benign stage (benign melanocytic tumors) in RET mice were histopathologically composed of round cells with regular round nuclei without mitotic activity (Fig. 1*B*). Tumors in the malignant stage (melanoma) were histopathologically composed of cells with nuclei of various sizes (Fig. 1*B*). The tumors also had a high level of mitotic activity (Fig. 1*B*) and Ki67 proliferation index (percentage of Ki67-positive cells; Fig. 1*C*). Microscopic characteristics of tumors in the newly defined premalignant stage (premalignant tumors) were intermediate between benign and malignancy (Fig. 1*B–D*). No lung metastases were detected in the mice with premalignant tumors ($n = 18$), although lung metastasis was found in 43.8% of the RET mice with malignant tumors ($n = 16$). Tumor size–oriented histopathologic analysis in RET mice (Fig. 4*A, top*) revealed that >80% (18 of 22) of tumors with sizes of <500 mm^3 were benign. All (12 of 12) tumors with sizes of >4,000 mm^3 were malignant, whereas >80% (11 of 13) of tumors with sizes of 500 to 4,000 mm^3 were premalignant (Fig. 4*A, top*). The entire process of

melanoma development via tumor-free, benign, premalignant, and malignant stages in RET mice (Figs. 1 and 4*A*) corresponded to multistep melanomagenesis in humans.

Biochemical characterization for various stages of tumors from RET mice. To further analyze tumor stage–oriented biochemical characters in RET mice, we selected four melanoma-related molecules [melanoma cell adhesion molecule (MCAM), E-cadherin, N-cadherin, and membrane type-1 matrix metalloproteinase (MT1-MMP)] as progression markers (Fig. 2*A*; refs. 13–16).

Transcript expression levels of MCAM, N-cadherin, and MT1-MMP in malignant tumors were significantly higher ($P < 0.01$) than those in benign tumors, whereas E-cadherin expression level in malignant tumors was significantly lower ($P < 0.01$) than that in benign tumors in RET mice (Fig. 2*A*). These results corresponded well with results in human melanoma (15). Transcript expression levels of MCAM, N-cadherin, and MT1-MMP in premalignant tumors were similar to those in benign tumors, whereas E-cadherin expression level in premalignant tumors was comparable with that in malignant tumors in RET mice (Fig. 2*A*). Taken together with the histopathologic results shown in Fig. 1*B* to *D*, these results suggest that the characteristics of premalignant tumors are intermediate between benign and malignancy.

To investigate the correlation between Ednrb and melanomagenesis in RET mice, we next examined transcript expression levels of Ednrb in tumors of various stages. Ednrb transcript expression levels in malignant tumors were significantly ($P < 0.01$) decreased compared with those in benign and premalignant tumors (Fig. 2*A*). Both immunohistochemical (Fig. 2*B*) and Western blotting (Fig. 2*C*) analyses also showed reduction of Ednrb protein expressions in malignant tumors compared with those in benign tumors. These results suggest that the reduction

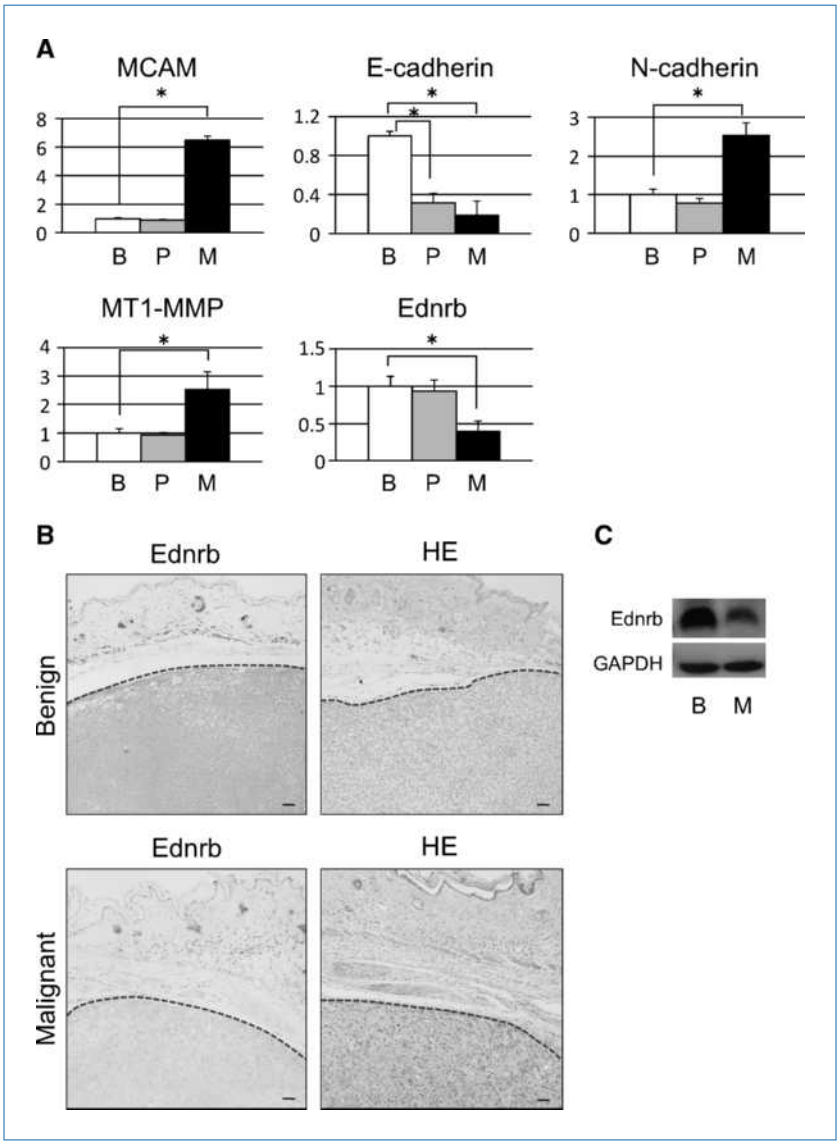

Figure 2. Stage-dependent biochemical characterization of primary tumors from RET mice. *A,* transcript expression levels (*columns,* mean; *bars,* SEM) of MCAM, E-cadherin, N-cadherin, MT1-MMP, and Ednrb in benign (*B, white*), premalignant (*P, gray*), and malignant (*M, black*) tumors from RET mice examined by real-time PCR. *, $P < 0.01$, significantly different from benign tumors by Kruskal-Wallis test. *B* and *C,* microscopic appearance (*B*) and Ednrb protein expression (*B* and *C*) in benign and malignant tumors from RET mice examined by immunohistochemistry with anti-Ednrb antibody, HE staining (*B*), and Western blot (*C*). Tumors were located under the dotted lines (*B; bars,* 50 μm).

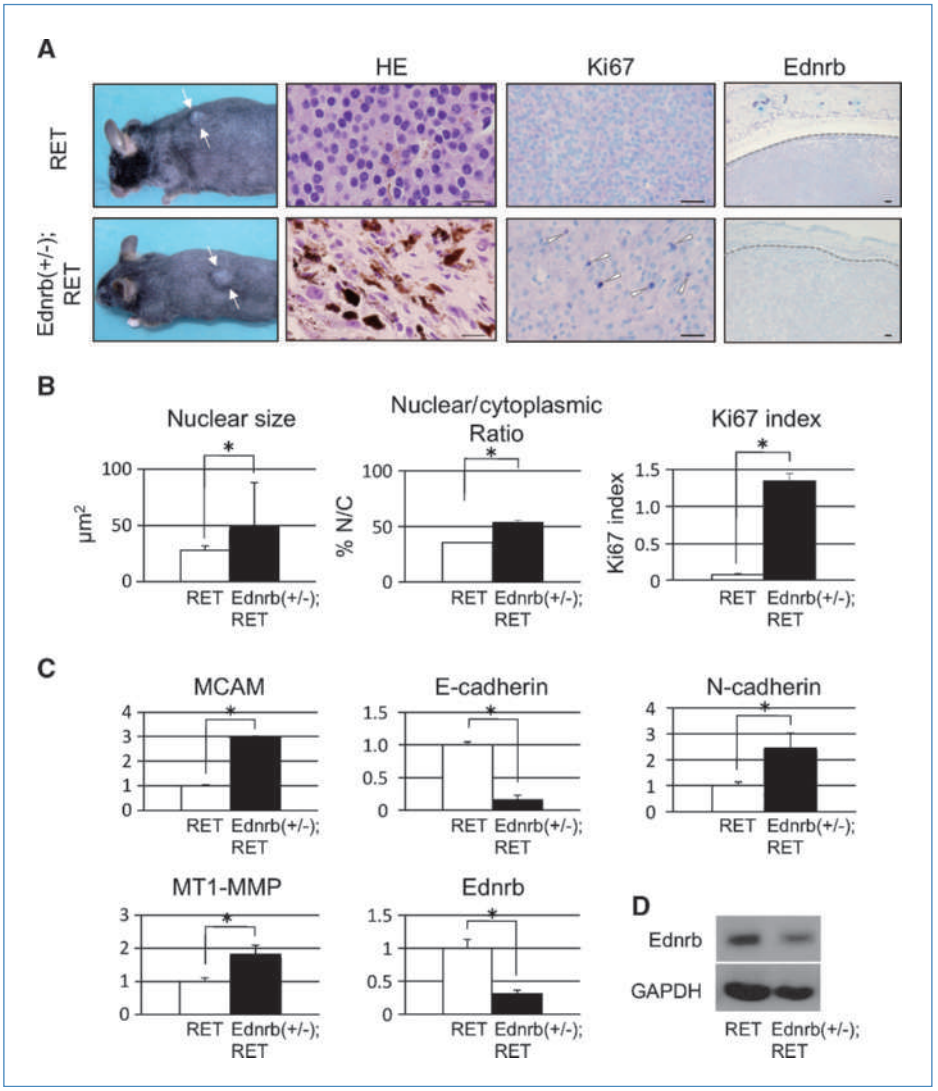

Figure 3. Characterization of small tumors (<500 mm^3) from RET mice and Ednrb(+/–);RET mice. *A,* macroscopic and microscopic appearances of <500 mm^3 primary tumors from RET mice and Ednrb(+/–);RET mice examined by HE staining and immunohistochemistry with anti-Ki67 and anti-Ednrb antibodies. *Arrows,* melanocytic tumors from RET mice (*top*) and Ednrb(+/–);RET mice (*bottom*) after shaving. *Arrowheads,* Ki67-positive cells. Ednrb protein was detected (*purple*) and nuclei were counterstained (*green*). Tumors were located under the dotted lines. *Bars,* 50 μm. *B, columns,* mean of nuclear size, nuclear-cytoplasmic ratio (N/C ratio), and Ki67 index in small tumors from RET mice (*white*) and Ednrb(+/–);RET mice (*black*) are presented; *bars,* SEM. *C,* transcript expression levels (*columns,* mean; *bars,* SEM) of MCAM, E-cadherin, N-cadherin, MT1-MMP, and Ednrb in tumors from RET mice and Ednrb(+/–);RET mice examined by real-time PCR. *, $P < 0.01$, significantly different from RET tumors by Mann-Whitney test. *D,* protein expression levels of Ednrb in <500 mm^3 primary tumors from RET mice and Ednrb(+/–);RET mice determined by Western blot.

of Ednrb expression in tumors occurs with progression of tumor stage in RET mice.

Characterization of tumors from RET mice and Ednrb-heterozygously deleted RET mice [Ednrb(+/–);RET mice]. To address the biological significance of reduced Ednrb expression levels in melanoma, we next produced Ednrb-heterozygously deleted RET mice [Ednrb(+/–);RET mice]. There were no macroscopic differences in tumors between RET mice and Ednrb(+/–);RET mice (Fig. 3*A*). Melanocytic tumors (Fig. 3*A*), which were microscopically composed of Dct- and S100-positive cells (Supplementary Fig. S1), deve-loped in Ednrb(+/–);RET mice. Both Ednrb transcript (Fig. 3*C*) and protein (Fig. 3*A* and *D*) expressions in <500 mm^3 tumors from Ednrb(+/–);RET mice were decreased compared with those in tumors of equivalent size from RET mice. Both Ednrb transcript (Supplementary Fig. S2*A*) and protein (Supplementary Fig. S2*B*) expressions in tumors from Ednrb(+/–); RET mice were further decreased compared with those in >4,000 mm^3 malignant tumors from RET mice.

We next histopathologically compared nuclear size, N/C ratio, and Ki67 index in tumors with sizes of <500 mm^3 from RET mice and Ednrb(+/–);RET mice (Fig. 3*A* and *B*). Interestingly, 68.8% (11 of 16) of <500 mm^3 tumors from Ednrb(+/–);RET mice were histopathologically malignant, and the rest (5 of 16 = 31.2%) were premalignant. In contrast, 82.6% (19 of 23) of <500 mm^3 tumors from RET mice were benign and 17.4% (4 of 23) were premalignant. Biochemical analysis in <500 mm^3 tumors from Ednrb(+/–);RET mice and RET mice suggests malignant characteristics in tumors in Ednrb(+/–);RET mice compared with those in tumors in RET mice (Fig. 3*C*).

We further statistically analyzed all tumors from RET mice and Ednrb(+/–);RET mice (Fig. 4*A*). Different from the above-described results of tumor size–oriented histopathologic analysis in tumors from RET mice (Fig. 4*A, top*), most (21 of 26 = 80.8%) tumors from Ednrb(+/–); RET mice were malignant and the rest were premalignant tumors (5 of 26 = 19.2%). Although the formation of the

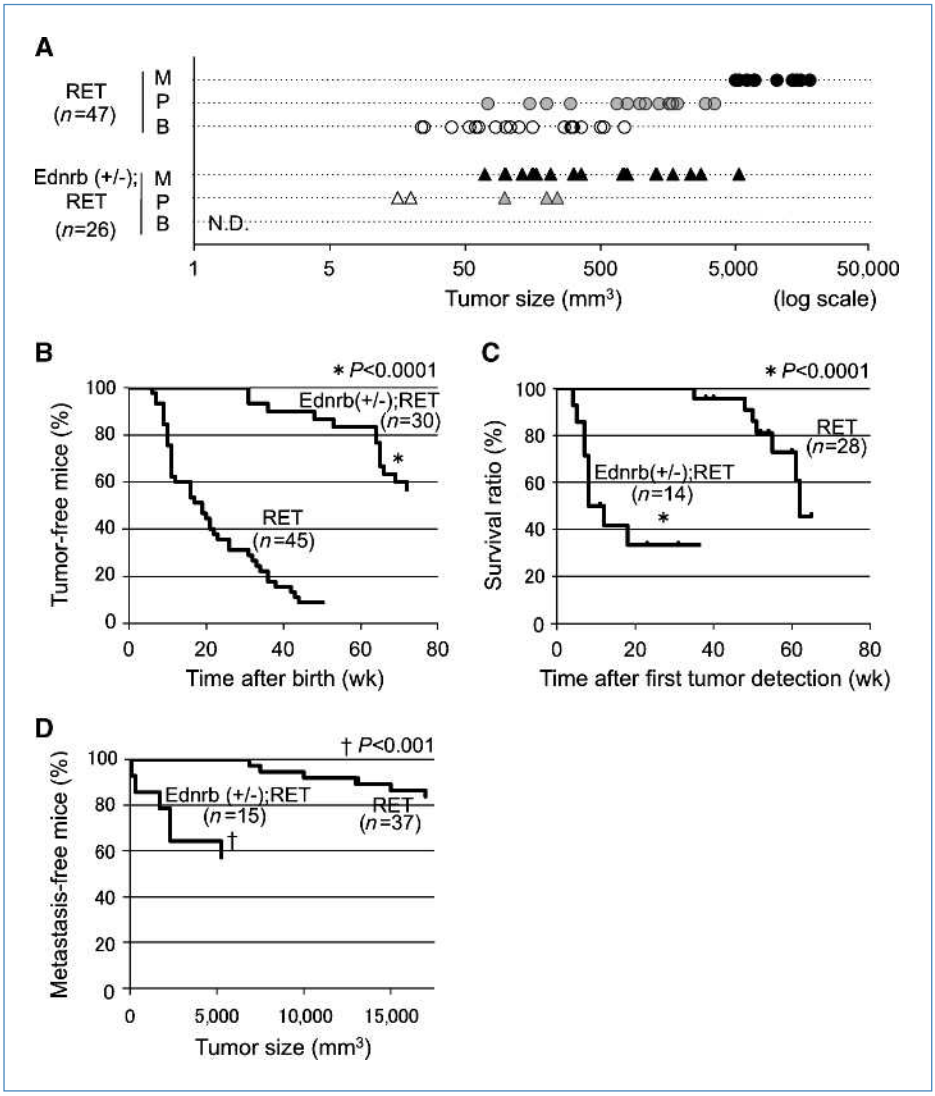

Figure 4. Comparative study between RET mice and Ednrb(+/–);RET mice. *A,* correlation between stage (*B,* benign; *P,* premalignant; *M,* malignant) and size in tumors from RET mice (*circles*) and Ednrb (+/–);RET mice (*triangles*). Volumes of 47 tumors from RET mice and 26 tumors from Ednrb(+/–);RET mice. *N.D.,* no detection. *B,* percentage of tumor-free RET mice and Ednrb(+/–);RET mice after birth. *C* and *D,* survival ratio (*C*) and percentage of lung metastasis–free mice (*D*) after detection of the first tumors in RET mice and Ednrb(+/–);RET mice. *, $P < 0.0001$ and †, $P < 0.001$, significantly different between RET mice and Ednrb(+/–)RET mice by log rank test (*B–D*).

first tumor after birth in Ednrb(+/–);RET mice was significantly ($P < 0.0001$) later than that in RET mice (Fig. 4*B*), life span after macroscopic detection of the first tumor in Ednrb(+/–);RET mice was significantly ($P < 0.0001$) shorter than that in RET mice (Fig. 4*C*). The percentage of lung metastasis, which was confirmed by immunohistochemical analysis with anti-Dct and anti-S100 antibodies (Supplementary Fig. S3), after macroscopic detection of the first tumor in Ednrb(+/–);RET mice was significantly ($P < 0.001$) higher than that in RET mice (Fig. 4*D*). Thus, we showed different processes of melanomagenesis in RET mice and Ednrb(+/–);RET mice.

Discussion

Previous studies have shown that 20% to 25% of melanomas developed from benign melanocytic lesions with multistep progression (multistep melanoma) and that 75% to 80% of melanomas arose without pre-existing benign lesions (*de novo* melanoma; Supplementary Table S1; refs. 5–7). The percentage of metastases in patients with *de novo* melanoma was shown to be higher than that in patients with multistep melanoma, whereas age at onset of *de novo* melanoma was later than that of multistep melanoma in humans (Supplementary Table S1). Prognosis in the patients with *de novo* melanoma was worse than that in the patients with multistep melanoma (Supplementary Table S1). Because the entire process of melanomagenesis in RET mice via tumor-free, benign, premalignant, and malignant stages corresponds well with multistep melanomagenesis in humans, RET mice could be an animal model for multistep melanomagenesis. On the other hand, not only were >80% of the tumors in Ednrb(+/–);RET mice malignant but also none of the tumors in Ednrb(+/–);RET mice were benign (Fig. 4*A*). The characteristics of late-onset, high percentage of metastasis, and poor prognosis after tumor development in Ednrb (+/–);RET mice are compatible with those in *de novo* melanoma in humans. Ednrb(+/–);RET mice may be the first

animal model in which tumors have clearly developed in a *de novo* manner.

Recently, it has been reported that melanoma risk in humans was significantly increased in patients with loss of function-related mutation of EDNRB (17). In this study, we showed reduction of Ednrb expression in malignant tumors compared with that in benign tumors in RET mice (Fig. 3*A, C*, and *D*). Reduction of EDNRB activity and expression by treatment with an inhibitor of EDNRB (BQ788) greatly increased vascular endothelial growth factor expression and decreased the angiogenic suppressor gravin (18). Reduced EDNRB expression might enhance metastatic ability in tumors from Ednrb(+/−);RET mice through modulation of vascular endothelial growth factor–related and gravin-related angiogenesis, resulting in their poor prognosis after development of tumors. High metastatic ability in the mice might be correlated with poor prognosis in patients with *de novo* melanoma compared with that in patients with multistep melanoma. These enhancements of metastatic ability by reduced Ednrb activity and expression suggest that Ednrb has a tumor-suppressing effect. In contrast, the tumor-free stage in Ednrb(+/−);RET mice was prolonged compared with that in RET mice in this study (Fig. 4*B*). Lahav and colleagues also revealed that reduction of EDNRB expression by BQ788 suppressed the growth of melanoma via the promotion of apoptosis (18, 19). These suppressions of tumor growth activity by reduced Ednrb activity and expression suggest that Ednrb has a tumor-promoting effect. Thus, Ednrb might have bidirectional effects on tumor growth and metastasis.

In summary, we have developed a novel animal model for *de novo* melanoma in addition to the previously reported animal model for multistep melanoma. We have also shown that alteration in the expression level of one molecule (Ednrb) may have a crucial effect on the process of melanoma development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Chiang JM, Wu Chou YH, Ma SC, Chen JR. Influence of age on adenomatous polyposis coli and p53 mutation frequency in sporadic colorectal cancer-rarity of co-occurrence of mutations in APC, K-ras, and p53 genes. Virchows Arch 2004;445:465–71.
2. Olschwang S, Hamelin R, Laurent-Puig P, et al. Alternative genetic pathways in colorectal carcinogenesis. Proc Natl Acad Sci U S A 1997;94:12122–7.
3. Hede SM, Hansson I, Afink GB, et al. GFAP promoter driven transgenic expression of PDGFB in the mouse brain leads to glioblastoma in a Trp53 null background. Glia 2008;57:1143–53.
4. Zhu H, Acquaviva J, Ramachandran P, et al. Oncogenic EGFR signaling cooperates with loss of tumor suppressor gene functions in gliomagenesis. Proc Natl Acad Sci U S A 2009;106:2712–6.
5. Kaddu S, Smolle J, Zenahlik P, Hofmann-Wellenhof R, Kerl H. Melanoma with benign melanocytic naevus components: reappraisal of clinicopathological features and prognosis. Melanoma Res 2002;12:271–8.
6. Friedman RJ, Rigel DS, Kopf AW, et al. Favorable prognosis for malignant melanomas associated with acquired melanocytic nevi. Arch Dermatol 1983;119:455–62.
7. Weatherhead SC, Haniffa M, Lawrence CM. Melanomas arising from naevi and *de novo* melanomas—does origin matter? Br J Dermatol 2007;156:72–6.
8. Kato M, Takahashi M, Akhand AA, et al. Transgenic mouse model for skin malignant melanoma. Oncogene 1998;17:1885–8.
9. Kato M, Takeda K, Kawamoto Y, et al. c-Kit-targeting immunotherapy for hereditary melanoma in a mouse model. Cancer Res 2004;64:801–6.
10. Kato M, Liu W, Yi H, et al. The herbal medicine Sho-saiko-to inhibits growth and metastasis of malignant melanoma primarily developed in ret-transgenic mice. J Invest Dermatol 1998;111:640–4.
11. Hosoda K, Hammer RE, Richardson JA, et al. Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell 1994;79:1267–76.
12. Yajima I, Kumasaka M, Thang ND, et al. Zinc finger protein 28 as a novel melanoma-related molecule. J Dermatol Sci 2009;55:68–70.
13. Genis L, Galvez BG, Gonzalo P, Arroyo AG. MT1-MMP: universal or particular player in angiogenesis? Cancer Metastasis Rev 2006;25:77–86.
14. Watson-Hurst K, Becker D. The role of N-cadherin, MCAM and β3 integrin in melanoma progression, proliferation, migration and invasion. Cancer Biol Ther 2006;5:1375–82.
15. Perlis C, Herlyn M. Recent advances in melanoma biology. Oncologist 2004;9:182–7.
16. Mills L, Tellez C, Huang S, et al. Fully human antibodies to MCAM/MUC18 inhibit tumor growth and metastasis of human melanoma. Cancer Res 2002;62:5106–14.
17. Soufir N, Meziani R, Lacapere JJ, et al. Association between endothelin receptor B nonsynonymous variants and melanoma risk. J Natl Cancer Inst 2005;97:1297–301.
18. Lahav R, Suva ML, Rimoldi D, Patterson PH, Stamenkovic I. Endothelin receptor B inhibition triggers apoptosis and enhances angiogenesis in melanomas. Cancer Res 2004;64:8945–53.
19. Lahav R, Heffner G, Patterson PH. An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells *in vitro* and *in vivo*. Proc Natl Acad Sci U S A 1999;96:11496–500.

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