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Original Article

Correlated expression levels of endothelin receptor B and Plexin C1 in melanoma

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Abstract: Discussion concerning the effect of endothelin receptor B (Ednrb) on melanoma continues because Ednrb has been reported to have both tumor promoting and suppressive effects for melanoma. In order to examine Ednrb-related signaling in melanomagenesis, DNA microarray analysis for a melanoma from a *RFP/RET*-transgenic mouse (RET-mouse) and a melanoma from an *Ednrb*-heterozygously deleted RET-mouse [Ednrb(+/-);RET-mouse], in both of which melanoma spontaneously develops, was performed in this study. We found that the expression level of *Plexin C1* (*PlxnC1*), a suppressor for melanoma, in a melanoma from an Ednrb(+/-);RET-mouse was drastically decreased compared to that in a melanoma from a RET-mouse. Therefore, we further examined the correlation between *Ednrb* and *PlxnC1* expression levels in melanomas. *PlxnC1* transcript expression levels in melanomas from Ednrb(+/-);RET-mice were lower than those in melanomas from RET-mice. A strong correlation between *Ednrb* and *PlxnC1* transcript expression levels (R = 0.78, $p < 0.01$) was also found in melanomas from both RET-mice and Ednrb(+/-);RET-mice. Correspondingly, there was a significant correlation between transcript (R = 0.80; $p < 0.01$) and protein (R = 0.60; $p < 0.01$) expression levels of EDNRB and PLXNC1 in human primary melanomas. Together with our results showing that the expression level of *PLXNC1* transcript was reduced in *EDNRB*-depleted human melanoma cells, our results showing positively correlated expression levels of Ednrb/EDNRB and PlxnC1/PLXNC1 in melanoma suggest that PlxnC1/PLXNC1 is involved in the Ednrb/EDNRB-mediated suppressive effect on melanoma.

Keywords: Ednrb, Plexin C1, melanoma

Introduction

Endothelin receptor B (Ednrb), a receptor for endothelins, enhances migration and proliferation of early melanocyte precursors [1]. Previous studies showed that the specific EDNRB antagonist BQ788 suppressed growth and induced cell death of transplanted melanoma in nude mice [2, 3] and decreased expression of a survival factor, B-cell leukemia/lymphoma 2 (BCL-2) [3]. These results suggest a therapeutic effect for melanoma through decreased activity and expression of Ednrb. In contrast, previous studies showed that reduction of the activity and expression of EDNRB by treatment with BQ788 increased expressions of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 alpha subunit (HIF-1α) and decreased expression of the angiogenic suppressor GRAVIN [3] and that melanoma risk in humans was significantly increased in patients with loss of function-associated mutation of *EDNRB* [4]. These results suggest a risk of melanoma progression through decreased activity and expression of Ednrb. Thus, it remains unknown whether Ednrb promotes or suppresses melanomagenesis because the associated signaling is not sufficiently understood.

Both Plexin C1 (PlxnC1) and β1-integrin are receptors for Semaphorin 7a [5]. PlxnC1 has opposing actions of β1-integrin, which promotes cell adhesion and invasion [5]. An increased level of PlxnC1 expression has an inhibitory

Table 1. A list of gene expression levels in melanoma developed in a RET-mouse and an Ednrb(+/-);RET-mouse

Gene	Expression values in melanoma		Ednrb(+/-); RET/RET ratio
	RET	Ednrb-(+/-);RET	
Vascular endothelial growth factor A (Vegf)	228.1	573.7	2.5
Hypoxia inducible factor-1 alpha subunit (Hif-1α)	4320.6	5859.9	1.4
Endothelin receptor type B (Ednrb)	958.8	1848.2	0.5
A kinase (PRKA) anchor protein 12 (Gravin)	63.3	131.1	0.5
B-cell leukemia/lymphoma 2 (Bcl-2)	1801.1	993.6	0.6
Plexin A1	4020.7	1613.8	0.4
Plexin A2	209.5	479.5	2.3
Plexin A3	384.5	282.3	0.7
Plexin B1	1344.5	1230.9	0.9
Plexin B2	1777.5	3277.4	1.8
Plexin B3	363.4	1325.3	3.7
Plexin C1	3502.7	472.1	0.1
Plexin D1	764.0	1614.1	2.1

Five *Ednrb* signaling-associated genes and eight plexin family genes are shown as a result of DNA microarray (GeneChip Mouse Genome 430 2.0).

effect on Semaphorin 7a-mediated spreading of melanocytes [5] and on melanoma progression [6]. Therefore, PlxnC1 might be a useful tool for evaluating the pathogenesis of melanoma.

We noted that PlxnC1 expression level in melanomas from Ednrb(+/-);RET-mice was definitely decreased compared to that in melanomas from RET-mice in our DNA microarray analysis. Therefore, in this study, we further analyzed the correlation between Ednrb/EDNRB and PlxnC1/PLXNC1 expression levels in primary melanomas of mice and humans to evaluate the effect of Ednrb/EDNRB on melanomas by PlxnC1/PLXNC1, a tumor suppressor.

Materials and methods

Mice

RET-transgenic mice of line 304/B6 carrying *RFP/RET* oncogene (RET-mice) and Ednrb-heterozygously deleted RET-mice [Ednrb(+/-);RET-mice] were used in this study.

Cell line and culture conditions

Human SK-Mel28 melanoma cells (Riken Bioresource Center Cell Bank) were cultured in RPMI1640 supplemented with 10% fetal bovine serum.

Quantitative PCR analysis

Real-time PCR analysis was performed by the method previously described [7]. The primers of GCTTAATCCCTTTCAGAAAACAGCC and GGCAAGCAGAAGTAGAAACTGAAC and ATAGTTCCTGCCACCACCTG and ACATTTGCGTTTCCCTTCAG were used for murine *Ednrb* and *PlxnC1*, respectively. *Hprt* was used as a reference gene for normalization, and its primer sequences were described previously [7]. The primers of ATCTGCGAATCTGCTTGCTT and TCCCGTCTCTGCTTTAGGTG and TGCCTCCTTCCTAACCATTG and GTTCAGAGTCGTCACCAGCA were used for human *EDNRB* and *PLXNC1*, respectively. *TBP* was used as a reference gene for normalization, and its primer sequences were described previously [7].

Immunohistochemistry

Immunohistochemical analysis for primary human melanoma in a tissue microarray (US Biomax, Inc.) was performed using a VECTASTAIN® ABC Kit (VECTOR) and VECTOR NovaRED (VECTOR) according to the protocol previously described [8]. Rabbit polyclonal anti-EDNRB IgG (CHEMICON) and goat polyclonal anti-PLXNC1 IgG (Santa Cruz) were used as primary antibodies. Counterstaining was performed by hematoxylin (MUTO PURE CHEMICALS). Intensities of EDNRB and PLXNC1 in melanoma were digitalized by WinROOF (MITANI Corporation) according to the method previously described [9].

Depletion of EDNRB

Human SK-Mel28 melanoma cells were transiently transfected with pRNAT-U6.1 siRNA expression plasmid (GeneScript Corporation)

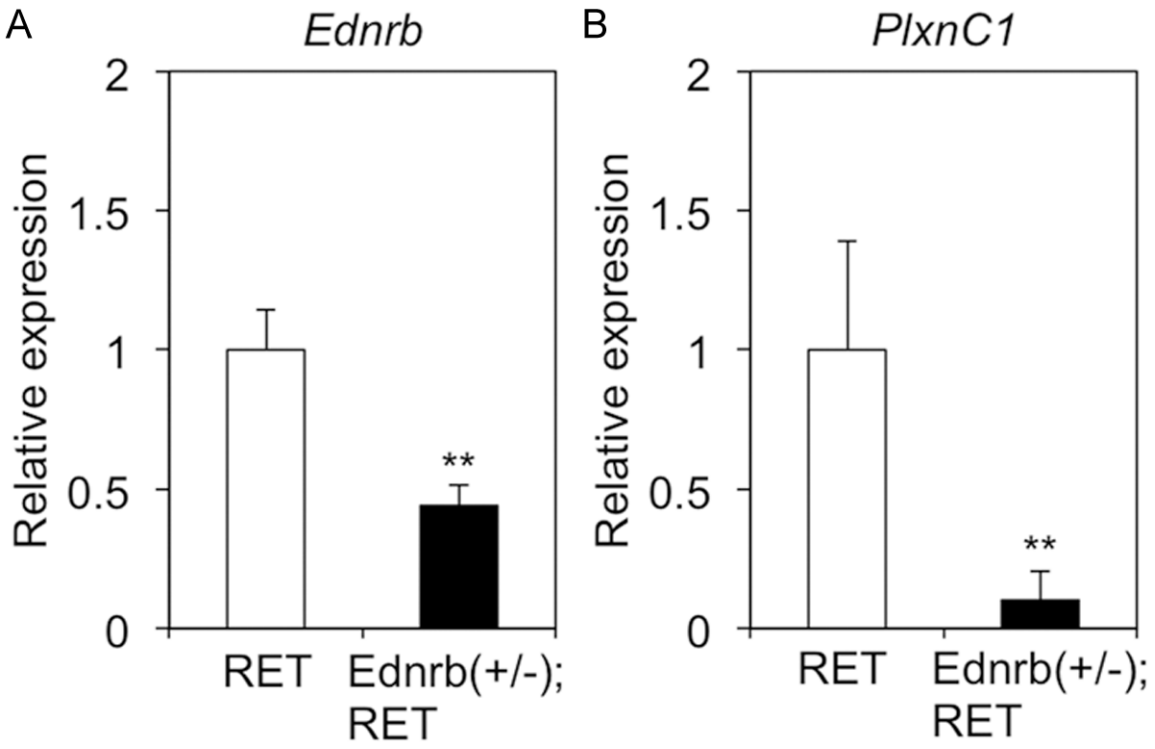

Figure 1. *Ednrb* and *PlxnC1* transcript expression levels in murine melanomas. (A, B) Ratios (mean ± SD) of *Ednrb* (A) and *PlxnC1* (B) transcript expression levels in melanomas from Ednrb(+/-);RET-mice (n = 6) relative to those in melanomas from RET-mice (n = 6) evaluated by quantitative PCR are presented. **Significantly different ($p < 0.01$) from the melanomas developing in RET-mice by Student's t-test.

containing target sequence for EDNRB or control sequence using Lipofectamine 2000 (Invitrogen Life Tech) according to the manufacturer's protocol. The target sequence for human EDNRB siRNA was 5'-CTGTTGGTATTGGACTATA-3' and control siRNA was 5'-CAAGTGGAATAA-CTCCGAGTGCTCT-3'.

Statistical analysis

Statistical analysis in this study was performed according to the method previously described [10]. The JMP Pro10 software package (SAS Institute Inc.) was used for statistical analyses.

Results

DNA microarray analysis

In order to determine the role of Ednrb in melanomagenesis, DNA microarray analysis of melanomas from a RET-mouse and an Ednrb(+/-); RET-mouse was performed. The expression level of *Ednrb* in a melanoma from an Ednrb(+/-); RET-mouse was about half of that in a melanoma from a RET-mouse in our DNA microarray analysis (**Table 1**), in good agreement with our previous results [8]. Moreover, expression levels of angiogenesis-related molecules including *vascular endothelial growth factor A* (*Vegf*) and *hypoxia inducible factor-1 alpha subunit* (*Hif-1α*) in a melanoma from an Ednrb(+/-);RET-mouse were higher than those in a melanoma from a RET-mouse (**Table 1**). In contrast, expression levels of an angiogenic suppressor gene, *A kinase* (*PPKA*) *anchor protein 12* (*Gravin*), and an anti-apoptotic gene, *B-cell leukemia/lymphoma 2* (*Bcl-2*), in a melanoma from an Ednrb(+/-);RET-mouse were lower than those in a melanoma from a RET-mouse (**Table 1**). These results partially correspond to the results of a previous study showing that Ednrb inhibition triggers apoptosis and enhances angiogenesis in melanomas [3].

Correlation between expression levels of Ednrb and PlxnC1 in murine melanomas

We found that the *PlxnC1* expression level in a melanoma from Ednrb(+/-);RET-mouse was only

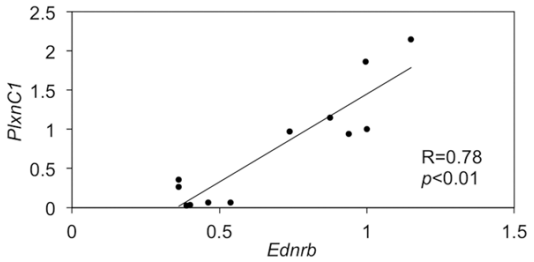

Figure 2. Correlation between transcript expression levels of *Ednrb* and *PlxnC1* in murine melanomas. Correlation between relative expression levels of *Ednrb* and *PlxnC1* transcripts in melanomas from RET-mice (n = 6) and Ednrb(+/-);RET-mice (n = 6) is presented. The correlation (R = 0.78; $p < 0.01$; n = 12) was examined by Spearman's correlation analysis.

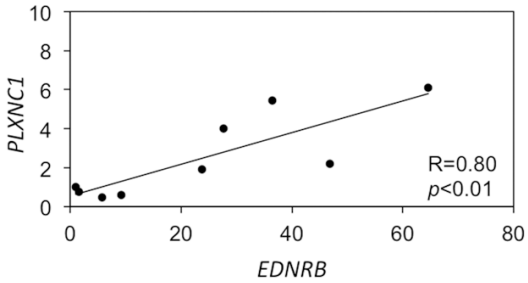

Figure 3. *EDNRB* and *PLXNC1* transcript expression levels in human melanomas. Correlation between relative expression levels of *EDNRB* and *PLXNC1* transcripts in human melanomas (n = 9) evaluated by quantitative PCR is presented.

13.4% of that in a melanoma from RET-mouse, whereas there was no drastic difference between a melanoma from RET-mouse and a melanoma from Ednrb(+/-);RET-mouse in the expression levels of Plexin family molecules except for *PlxnC1* (**Table 1**). Therefore, we further analyzed expression levels of *Ednrb* and *PlxnC1* in melanomas from RET-mice and Ednrb(+/-);RET-mice by real-time PCR (**Figure 1**). Our real-time PCR analysis showed that the expression level of *Ednrb* transcripts in melanomas from Ednrb(+/-);RET-mice was 44.1% of that in melanomas from RET-mice (**Figure 1A**). The expression level of *PlxnC1* transcripts in melanomas from Ednrb(+/-);RET-mice was only 10.2% of that in melanomas from RET-mice (**Figure 1B**). We then found a strong correlation (R = 0.78, $p < 0.01$) between transcript expression levels of *Ednrb* and *PlxnC1* in melanomas from these mice (**Figure 2**).

Correlation between expression levels of EDNRB and PLXNC1 in human melanomas

We next examined the expression levels of *EDNRB* and *PLXNC1* transcripts (**Figure 3**) and proteins (**Figure 4**) in primary melanomas in humans. Levels of *EDNRB* transcripts were strongly (R = 0.80; $p < 0.01$) correlated with those of *PLXNC1* transcripts (**Figure 3**). In immunohistochemical analysis, a high expression level of EDNRB protein was accompanied by a high expression level of PLXNC1 protein (**Figure 4A** and **4C**), while a low expression level of EDNRB protein was accompanied by a low expression level of PLXNC1 protein in human primary melanomas (**Figure 4B** and **4D**). More importantly, we could find a significant correlation (R = 0.60; $p < 0.01$) between EDNRB and PLXNC1 protein expression levels (**Figure 4E**).

Discussion

Multistep melanomagenesis and de novo melanomagenesis in RET-mice and Ednrb(+/-);RET-mice, respectively, found in our previous study [8] suggest a melanoma progressive effect of decreased Ednrb expression via promotion of transformation from a benign melanocytic tumor to a melanoma. In contrast, the less than half tumor incidence in Ednrb(+/-);RET-mice than that in RET-mice found in that study [8] suggests a melanoma suppressive effect of decreased Ednrb expression. Not only our previous results [8] but also results of various studies worldwide [2-4] showed bidirectional effects of Ednrb/EDNRB on melanomagenesis. On the other hand, EDNRB is considered to be a candidate for molecular-targeted therapy for melanoma in humans as shown in phase II trials of bosentan, an EDNRA/EDNRB antagonist, for patients with melanoma [11, 12]. Therefore, the bidirectional effects of Ednrb/EDNRB on melanomagenesis must be clarified.

Identification of Ednrb/EDNRB-related signaling might contribute to clarification of the role of Ednrb/EDNRB in melanoma. In this study, we tried to characterize Ednrb/EDNRB in primary melanoma by PlxnC1/PLXNC1. We found a significant correlation between transcript and protein expression levels of Ednrb/EDNRB and PlxnC1/PLXNC1 in murine and/or human melanomas. Moreover, we showed that depleted *EDNRB* expression decreased *PLXNC1* expres-

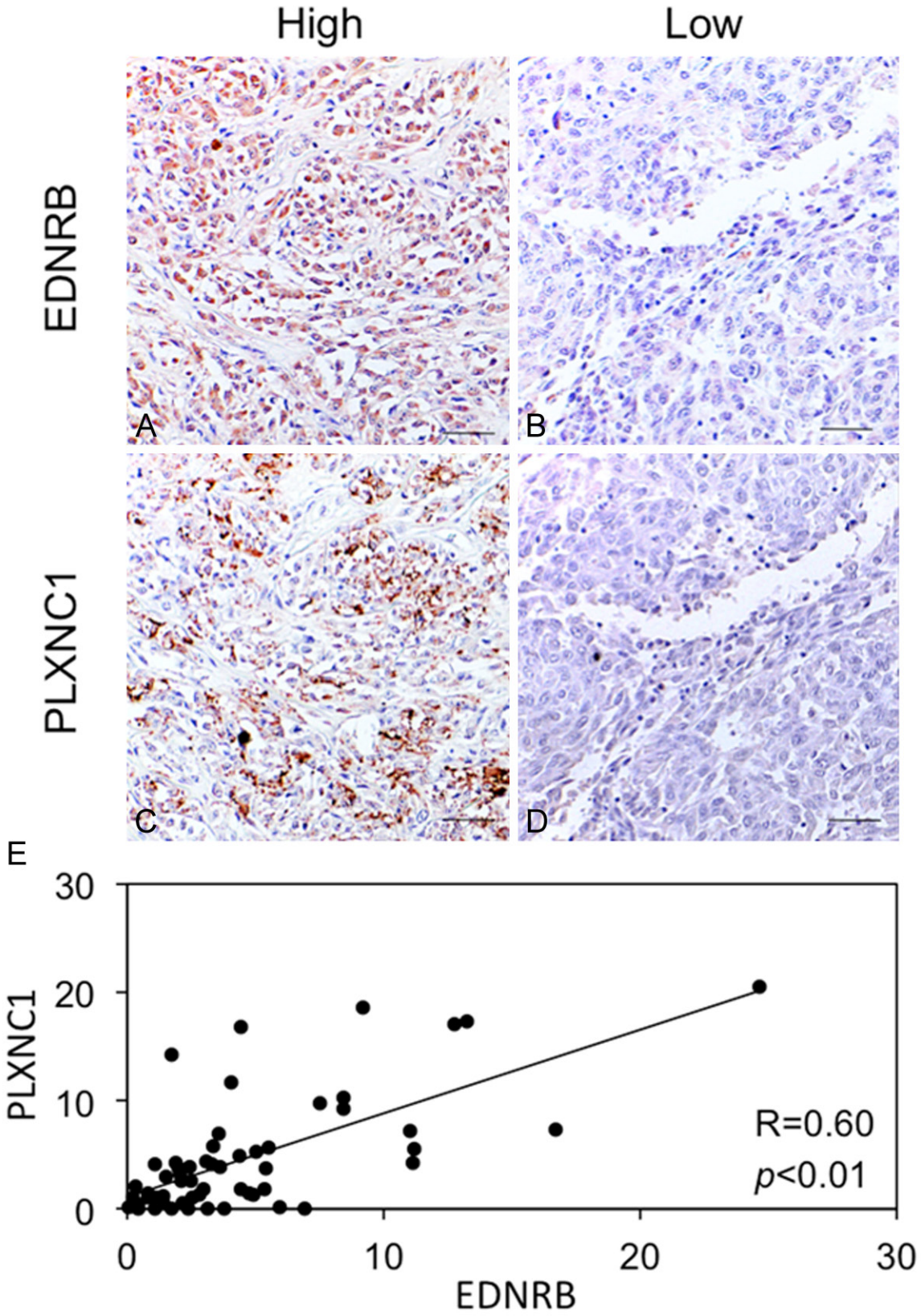

Figure 4. EDNRB and PLXNC1 protein expression levels in human melanomas. (A-D) Immunohistochemical analysis for primary human melanomas (n = 56) in a tissue microarray was performed. Representative photographs with high (A, C) and low (B, D) intensities of EDNRB (A, B) and PLXNC (C, D) in human melanomas are presented. (E) Correlation between relative expression levels of EDNRB and PLXNC1 protein in melanomas is presented. The correlation (R = 0.60; $p < 0.01$; n = 56) was examined by Spearman's correlation analysis. Scale bars: 100 μm.

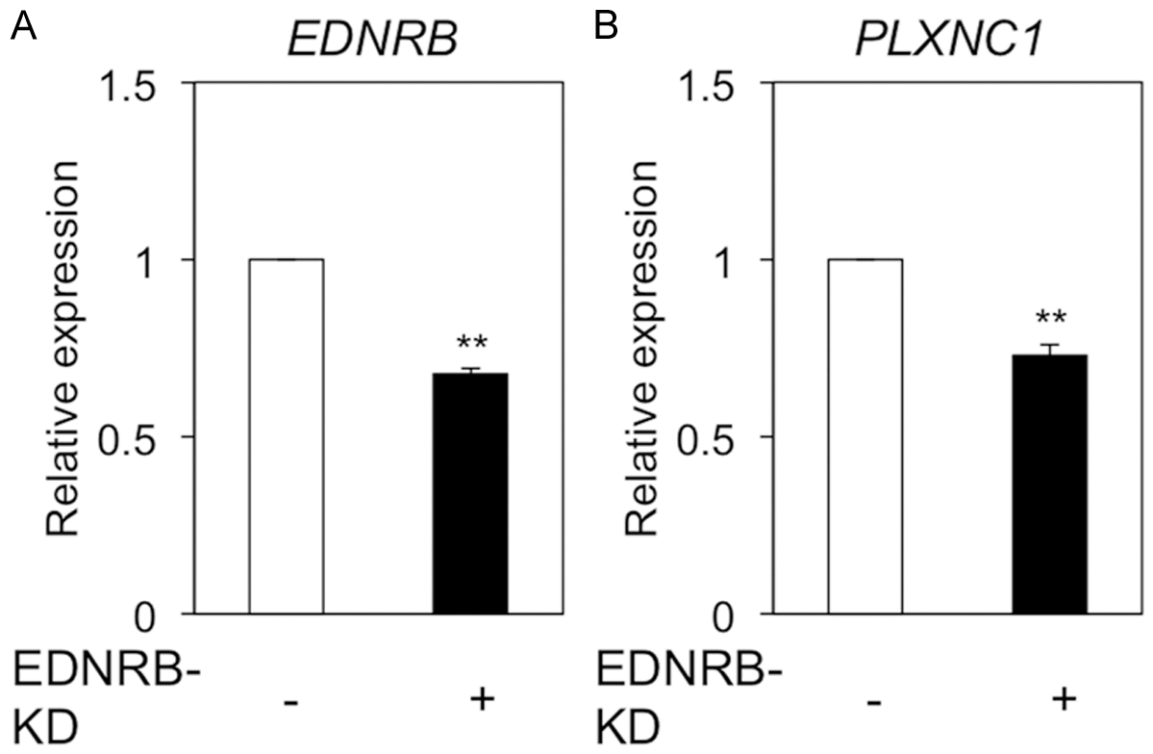

Figure 5. Effect of *EDNRB* expression on *PLXNC1* expression. Human SK-Mel28 melanoma cells were transiently transfected with *EDNRB* shRNA or control shRNA. The cells were incubated in RPMI1640 with 10% FBS for 48 hrs and transcript expression levels of *EDNRB* (A) and *PLXNC1* (B) were analyzed by real-time PCR. Ratios (mean ± SD) of *EDNRB* (A) and *PLXNC1* (B) transcript expression levels in *EDNRB* knockdown (EDNRB-KD) cells relative to those in control shRNA-transfected cells are presented. **Significantly different ($p < 0.01$) from control cells by Student's t-test.

sion level in human SK-Mel28 melanoma cells (**Figure 5**). Our results suggest that decrease in the level of *PlxnC1* expression occurs in accordance with decrease in the level of *Ednrb* expression in melanoma cells. Thus, PlxnC1/PLXNC1 may be downstream of Ednrb/EDNRB signaling and may be associated with an Ednrb/EDNRB-mediated signaling for tumor suppression. Further study is needed to clarify the biological significance of and mechanism for the positive correlation between *Ednrb* and *PlxnC1* expression levels.

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Disclosure of conflict of interest

None.

Abbreviations

RET-mice, *RFP-RET*-transgenic mice of line 304/B6; MT-I, metallothionein-I; Hprt, hypoxanthine guanine phosphoribosyl transferase; TBP, TATA-box-binding protein.

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