様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）悪性黒色腫の顎骨浸潤プロセスにおけるBMPシグナルの役割

研究課題名（英文）The role of BMP signaling in bone metastasis by malignant melanoma

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研究成果の概要（和文）：悪性黒色腫は口腔粘膜にも発生する悪性度の高い悪性腫瘍であり、増殖能が高く容易に肺などに転移するために予後が悪い。データベース解析からヒトの健常皮膚、良性母斑、悪性黒色腫への変化に伴い転写コファクターであるTLE3の発現が上昇した。TLE3を過剰発現したB16細胞は増殖能が亢進し、CyclinD1などの細胞周期関連遺伝子の発現量が上昇した。この細胞を12週齢雄BALB/cA Jcl-nu/nuマウス背部皮下に接種するとコントロール細胞に比べて大きな腫瘍を形成した。一方、TLE3を恒常的にノックダウンしたB16細胞をマウス皮下に摂取するとコントロール細胞に比べて小さな腫瘍を形成した。

研究成果の学術的意義や社会的意義
近年、抗PD-1抗体nivolumabなどの免疫チェックポイント阻害剤の登場により悪性黒色腫の治療成績が著しく向上してきたが、これら薬剤は間質性肺炎や大腸炎など様々な臓器に対して免疫関連有害事象をもたらすことも明らかとなってきた。そのため、悪性黒色腫の病態を正確に理解し、新たな治療法を確立する必要性は依然として残っている。ヒストン脱アセチル化酵素（HDAC）阻害剤は悪性黒色腫の新規治療薬として注目されている。本研究はTLE3の悪性黒色腫細胞の増殖促進作用にHDACが関与することを明らかにしたものであり、HDAC阻害剤を利用した悪性黒色腫の治療法に新たなエビデンスを提唱することができたと考える。

研究成果の概要（英文）：Melanoma, one of the most aggressive neoplasms, is characterized by rapid cell proliferation. Transducin-like Enhancer of Split (TLE) is an important regulator of cell proliferation via Histone deacetylase (HDAC) recruitment. Given that HDAC activity is associated with melanoma progression, we examined the relationship between TLE3, a TLE family member, and melanoma. TLE3 expression was increased during the progression of human patient melanoma. Overexpression of Tle3 in B16 murine melanoma cells led to an increase in cell proliferation as well as the number of cyclinD1-positive cells. in vivo injection of mice with B16 cells overexpressing Tle3 resulted in larger tumor formation than in mice injected with control cells. In contrast, siRNA-mediated knockdown of Tle3 in B16 cells or TLE3 in HMV-II human melanoma cells decreased proliferation. Treatment of B16 cells with trichostatin A, a class I and II HDAC inhibitor, prevented the effect s of Tle3 on proliferation.

研究分野：歯科麻酔学、悪性腫瘍、分子生物学

キーワード：悪性黒色腫　BMP　TLE3　HDAC

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９、ＣＫ－１９（共通）

１．研究開始当初の背景

悪性黒色腫は口腔粘膜にも発生する悪性度の高い悪性腫瘍であり、増殖能が高く容易に肺などに転移するために予後が悪い。近年、抗 PD-1 抗体 nivolumab などの免疫チェックポイント阻害剤の登場により悪性黒色腫の治療成績が著しく向上してきたが、これら薬剤は間質性肺炎や大腸炎など様々な臓器に対して免疫関連有害事象をもたらすことも明らかとなってきた。そのため、悪性黒色腫の病態を正確に理解し、新たな治療法を確立する必要性は依然として残っている。

２．研究の目的

マウス悪性黒色腫株 B16 細胞を骨形成タンパク質 BMP で処理をすると形態変化をしたため、BMP シグナルと悪性黒色腫の悪性度の関係を明らかにすることを目的に研究を開始した。しかしながら、研究を遂行する中で、転写コファクターの TLE3 が悪性黒色腫の増殖に深く関わっている可能性に気づき、研究の若干の方向修正を行った。
そこで､本研究では悪性黒色腫の増殖における TLE3 の役割を明らかにすることを目的とした。

３．研究の方法

①マイクロアレイ解析

To examine TLE3 expression in patient melanoma samples, dataset GSE3189 (Talantov D et al., 2005) was downloaded from the NCBI Gene Expression Omnibus (GEO) and analyzed.

②動物

12 week-old male C57BL/6 and 12 week-old male BALB/cA Jcl-nu/nu mice were purchased from CLEA Japan Inc. (Tokyo, Japan). All mice were used in accordance with guidelines from the Kyushu Dental University Animal Care and Use Committee. All experiments were carried out with the approval of the Animal Use and Care Committee of the Kyushu Dental University (Approval number #17-23).

③細胞、遺伝子導入、細胞の選択

B16 cells (RCB1283) (MTA; RM87746), HMV-II cells (RCB0777) (MTA; RM87747), or COLO679 cells (RCB0989) (MTA; RM87746) were purchased from RIKEN BRC (RIKEN, Ibaragi, Japan). B16 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM L-Glutamine (Douglas RG et al., 2018). HMV-II cells were maintained in DMEM/Ham's F-12 supplemented with10% FBS. COLO679 were maintained in RPMI Medium1640 supplemented with 20% FBS. B16 cells were transfected with Myc-tagged murine Tle3 (1-782), Myc-taged murine Tle3 (1- 140) (Kokabu S et al., 2017), Myc-pcDEF3 empty vector (Control), shRNA against murine TLE3 (#1792), or shRNA against LacZ (Control) (Baron R and Kneissel M, 2013). Cells transfected with Myc-pcDEF3 or Myc-pcDEF3 empty vector were treated with G418 (Roche, Basel, Switzerland) for 2 weeks until G418-resistant clones emerged. Cells transfected with shRNA against murine Tle3 (#1792) or LacZ (control), were treated with blasticidin (Wako, Osaka, Japan) to obtain blasticidin resistant clones.

④HDAC 阻害剤

B16 cells, HMV-II cells, or COLO678 cells were treated with trichostatin A (TSA) (Sigma Aldrich Chemicals, St. Louis, MO), Apicidin (BioVison, Milpitas, CA), M344 (BioVison, Milpitas, CA), Sodium 4-Phenylbutyrate (BioVison, Milpitas, CA), Splitomicin (BioVison, Milpitas, CA), or Valproic Acid (VPA) (BioVison, Milpitas, CA) at the indicated concentrations for 12 hours.

⑤B16 悪性黒色腫モデルマウス

Mice were injected subcutaneously with 1 x 105 B16 cells in a 100 μL volume (Zhu ML et al., 2013). Tumor diameters were measured with calipers. Body weight and physiologic status were monitored daily.

⑥RNA の抽出と qPCR

Total RNA was isolated from cells using FastGeneTM RNA Basic Kit (Nippon Genetics, Tokyo, Japan) and then reverse-transcribed into cDNA using ReverTra Ace (Toyobo, Osaka, Japan). The cDNA was amplified by PCR using specific primers for murine Tle3 (primer sequences: forward, agtctcgcctccattcctg; reverse, catctgcccatcagcactc), murine Cyclin A2 (primer sequences: forward, cttggctgcaccaacagtaa ; reverse, caaactcagttctcccaaaaaca), murine Cyclin D1 (primer sequences: forward, tttctttccagagtcatcaagtgt; reverse, tgactccagaagggcttcaa), murine Cyclin D2 (primer sequences: forward, tcccgactcctaagacccatc; reverse, taccagttcccactccagca), and β-actin (forward, aaggccaaccgtgaaaagat; reverse, gtggtacgaccagaggcatac). SYBR green-based quantitative real-time PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA) with QuantStudio 3 system (Thermo Fisher Scientific). Values were normalized to □-actin using the 2-□□Ct method (Kokabu et al., 2014).

⑦免疫組織化学

Freshly isolated skin from C57BL/6 mice were immediately fixed in 4% paraformaldehyde

in PBS and subsequently embedded in paraffin. Vertical sections, 6 to 8 μm thick, were deparaffinized in xylene and rehydrated with a graded series of ethanol concentrations. Sections were incubated at 4 ° C overnight with polyclonal anti-TLE3 antibody (Proteintech, Chicago, IL), or normal Rabbit IgG (MBL). After washing, sections were incubated for 1h with peroxidase-labeled secondary antibodies (Histofine Simple Stain)(Nichirei Biosciences, Tokyo, Japan). Diaminobenzidine (Histofine DAB-3S kit)(Nichirei Biosciences) served as the peroxidase substrate. ABZ-9000 (Keyence, Tokyo, Japan) microscope was used for these analyses.

⑧免疫細胞化学

B16 cells were incubated with primary antibodies at 4□C overnight following blocking/permeabilization with PBS containing 0.3% Triton X100 and 5% goat serum for 20 minutes at room temperature. The following antibodies were used for immunocytochemistry: polyclonal anti-TLE3 antibody (Proteintech), and CyclinD1 mouse monoclonal antibody (72-13G)(Santa Cruz, Santa Cruz, CA). anti-Ki67 rabbit monoclonal antibody (ab92742, Abcam, Cambridge, UK). The target proteins were visualized using an Alexa 488- or Alexa 594-conjugated secondary antibody (Invitrogen, Carlsbad, CA). ABZ-9000 (Keyence) microscope was used for these analyses. To visualize the cell nuclei, the cells were mounted with Hard Set Mounting Medium with DAPI (Vector laboratories, Burlingame, CA) and to visualize the cellular skeleton, the cells were stained with Rhodamine Phalloidin (Thermo Fisher Scientific).

⑨Western blot 法

The following antibodies were used for Western blot analysis: anti-TLE3 antibody (Proteintech), anti-cyclinD1 mouse monoclonal antibody (72-13G)(Santa Cruz), anti-Myc-tag polyclonal antibody (MLB), CyclinA2 rabbit polyclonal antibody (GTX103042, GeneTex, Irvine, CA), and anti-β-actin mouse monoclonal antibody (Sigma Aldrich Chemicals, St. Louis, MO). The target proteins were detected using an anti-mouse or anti-rabbit IgG antibody conjugated with horseradish peroxidase (Cell signaling, Beverly, MA) and visualized by ImmunoStar LD (Wako).

⑩発現ベクター、shRNA、siRNA

Myc-tagged murine TLE3 expression plasmid (Kokabu S et al., 2017), C-terminally truncated forms of myc-tagged TLE3 plasmids (Kokabu S et al., 2017), murine Wnt3a (Chien AJ et al., 2009) and constitutively active form of murine β-catenin (Kokabu S et al., 2014) were previously described. Super TOP flash luciferase reporter vector (Fukuda T at al., 2010) was kindly provided by Dr. Randall Moon. Short hairpin RNA against murine Tle3 constructs (shTLE3) was designed using BLOCK-IT RNAi designer tool (Invitrogen), sense and antisense oligos were annealed and cloned into pcDNA 6.2-GW/miR (Kokabu S et al., 2013). The following shRNA oligos were used: control shRNA, TGCTGAAATCGCTGATTTGTGTAGTCGTTTTGGCCACTGACTGACGACTACACATCAGCGATTT; shRNA against Tle3, TGCTGTGCTGAGGCTGTCTTTCTCTT GTTTTGGCCACT GACTGACAAGAGAAACAGCCTCAGCA. Only sense strands are shown (Kokabu S et al., 2013). siRNA-1 against murine Tle3 (Stealth siRNA, MSS238514)(Thermo Fisher Scientific), siRNA-2 against murine Tle3 (Stealth siRNA, MSS2385136), siRNA-1 against human TLE3 (Stealth siRNA, HSS186348), or siRNA-2 against human TLE3 (Stealth siRNA, HSS110791) were transfected into B16 cells or HMV-II cells using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's Protocol.

⑪細胞増殖試験

The proliferation of B16 cells was assessed using a Cell Counting kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer ' s Protocol (Tada Y et al., 2014).

⑫レポーターアッセイ

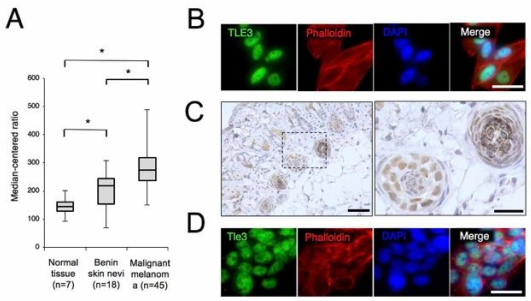
Luciferase assays were performed using Super TOPflash-luciferase reporter vector or phRL-SV40 (Promega, Madison, WI) with the Dual-Glo Luciferase Assay System (Promega).

⑬統計処理

Comparisons were made using an unpaired ANOVA with Tukey-Kramer post-hoc test and Wilcoxon's signed rank test. The results are shown as the mean ± S.D. The statistical significance is indicated as follows: **, $p < 0.01$ and *, $p < 0.05$.

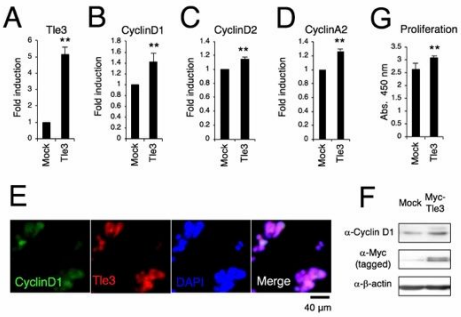
４．研究成果

図１：ヒト悪性黒色腫で TLE3 の発現量が上昇する。



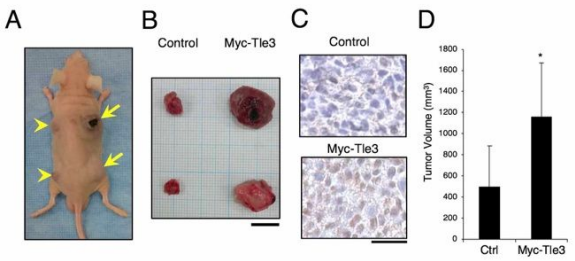
The expression of TLE3 in normal skin, benign nevi and malignant melanoma of patients (GSE3189 dataset). Expression levels of TLE3 are presented as boxplots and means were compared using unpaired ANOVA with Tukey-Kramer post-hoc test and Wilcoxon's signed rank test (A). HMV-II cells were stained with TLE3 antibody, rhodamine phalloidin (phalloidin), or DAPI (B). Skin from 12-week-old C57BL/6J male mice was immunostained with anti-Tle3 antibody. The boxed areas in the left panel are shown as magnified images of hair follicles in the right panel. Scale bars indicate 500 μm (left panel) and 100 μm (right panel) respectively. Representative images of several sections are shown (C). B16 cells were stained with Tle3 antibody, phalloidin, or DAPI (D). Representative images are several experimental repeats shown. Scale bar corresponds to 100 μm (B and D).

図 2: TLE3 の過剰発現は B16 細胞の増殖を増加させる。



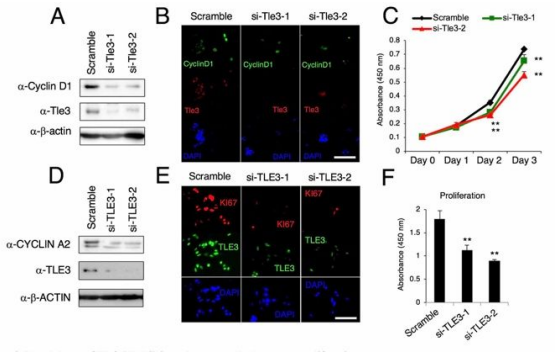
(A-F) B16 cells stably expressing Myc-tagged Tle3 or empty vector were generated after positive selection with G418. The messenger RNA levels of Tle3 (A), CyclinD1 (B), CyclinD2 (C), or CyclinA2 (D) were determined by qPCR on day 2. B16 cells with high expression of Tle3 co-expressed CyclinD1 in the nuclei. Scale bar corresponds to 40 μm (E). Protein levels of CyclinD1, Myc-tagged Tle3, or β-actin were determined by western-blot analysis on day 2 (F). Overexpression of Tle3 increased the proliferation of B16 cells assessed by water-soluble tetrazolium salt (WST) -8 assay. Proliferation was quantified on day 2 by spectrophotometric absorbance measurement at 450 nm (G). Data are expressed as the mean ± SD (n = 3). **, $p < 0.01$ versus control (A-D, G). Representative images were shown (E and F).

図３：TLE3 を過剰発現した B16 細胞をマウス皮下に注射すると大きな腫瘍を形成する。



BALB/cA Jcl-nu/nu mice (n=5) were injected subcutaneously with 1 x 105 control B16 cells (left side; arrow heads) or cells stably expressed Myc-tagged Tle3 (right side; arrows). Representative photograph of a mouse (A) and resected tumors (B) 3 weeks after injection of with B16 cells. Scale bar corresponds to 10 mm (B). Resected tumors were immunostained with anti-Tle3 antibody. Scale bars indicate 25 μm (C). Representative images of several sections are shown. The volume of resected tumors was quantified. The data are expressed as the mean ± SD (n = 10). *, $p < 0.05$ versus control (D).

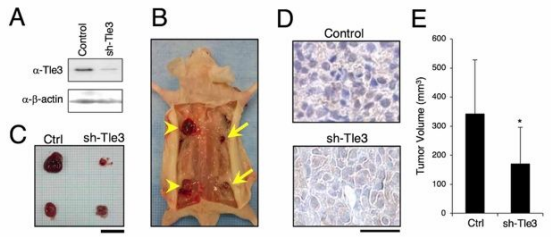
図４: TLE3 のノックダウンでメラノーマの増殖が低下する



(A - C) B16 cells were transfected with scramble siRNA, or siRNA against murine Tle3 (siTle3-1, siTle3-2). Protein levels of Tle3, cyclinD1, or β-actin were assessed by western blotting analysis (A). The numbers of cyclinD1 positive cells were decreased in the Tle3 knockdown B16 cells (B). In cells Tle3 knockdown cells,

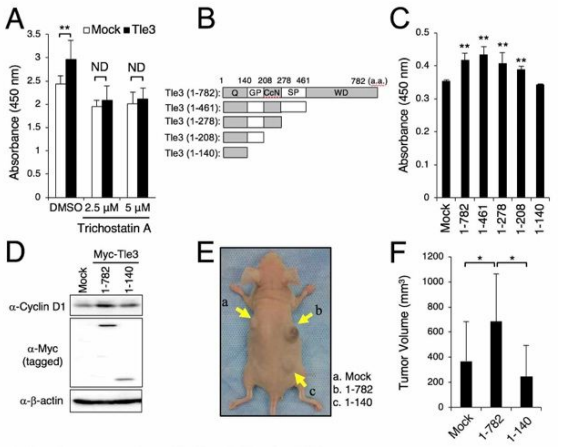
proliferation ability on day 2 and day 3 was decreased in comparison to scramble siRNA cells (C). (D - F) HMV-II cells were transfected with scrambled siRNA or siRNA against human TLE3 (siTLE3-1, siTLE3-2). Protein levels of TLE3, CYCLIN A2, or β-ACTIN were assessed by western blotting analysis (D). The numbers of KI67 positive cells were decreased in the TLE3 knockdown HMV-II cells (E). In TLE3 knockdown HMV-II cells, proliferation on day 4 was decreased in comparison to scrambled siRNA cells (F). Scale bar corresponds to 100 mm (B and E). **, $p < 0.01$ versus scramble (C and F).

図５：TLE3 をノックダウンした B16 細胞をマウス皮下に注射すると小さな腫瘍を形成する。



B16 cells, stably expressing control shRNA (Control) or shRNA against Tle3 (sh-Tle3) were generated by blasticidin selection. Protein levels of Tle3 were decreased in cells stably expressing sh-Tle3 (A). BALB/cA Jcl-nu/nu mice (n=5) were injected subcutaneously with 1 x 105 control cells (left side; arrow heads) or sh-Tle3 (right side; arrows). Representative pictures of subcutaneous tumors (B) and resected tumors (C) 3 weeks after injection of B16 cells are shown. Scale bar corresponds to 10 mm (C). Resected tumors were immunostained with anti -Tle3 antibody. Scale bars indicate 25 μm. Representative images of several sections are shown (D). The volume of resected tumors were quantified (E). The data are expressed as the mean ± SD (n = 10). *, $p < 0.05$ versus control (E).

図６：TLE3 によるメラノーマの増殖抑制効果は HDAC を介する。



(A-C) B16 cells were transiently transfected with empty vector (Mock) or Myc-tagged Tle3 and then treated with DMSO, or the indicated concentration of trichostatin A. Cell proliferation was evaluated on day 2 by water-soluble tetrazolium salt (WST) assay and absorbance measurement at 450 nm (A). Schematic of the C-terminally truncated forms of the Myc-tagged Tle3 plasmids used in these experiments. Q; glutamine rich domain, GP; glycine/proline rich domain, CcN; CcN domain, SP; serine/proline rich domain, WD, tryptophan/aspartic acid repeat domain (B). C-terminally truncated forms of Tle3 were transfected in B16 cells and proliferation ability measured on day 2 by WST assay (C). The data are expressed as the mean ± SD (n = 3). **, $p < 0.01$ versus Mock transfection (A and C). B16 cells were transfected with empty vector (Mock), Myc-tagged Tle3 (1-782), or Myc-tagged Tle3 (1-140). Protein levels of cyclinD1, Myc (tagged) or β-actin were assessed by western blotting analysis on day 2 (D). (E and F) B16 cells stably expressing Myc-tagged Tle3 (1-782), Myc-tagged Tle3 (1-140), or empty vector were generated after positive selection with G418. BALB/cA Jcl-nu/nu mice (n=5) were injected subcutaneously with 1 x 105 mock B16 cells (a), cells stably expressing Myc-tagged Tle3 (1-782) (b), or cells stably expressing Myc-tagged Tle3 (1-140) (c). Representative photograph of a mouse 3 weeks after injection with B16 cells (E). The volume of resected tumors was quantified. The data are expressed as the mean ± SD (n = 5). *, $p < 0.05$ (G).

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〔図書〕（計　0　件）

〔産業財産権〕
○出願状況（計　0　件）

○取得状況（計　0　件）

〔その他〕
ホームページ等

６．研究組織

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ローマ字氏名：OGAWA masahiro