の発現レベルを調節するなど遺伝子発現のグルーバルな変化をもたらすことが判ってきているが、神経芽腫についてはまだ十分なデータが揃っていない miRNA の遺伝子発現レベルについて昨年度より解析を開始し、これまでに得られた遺伝子発現プロファイルや幹細胞性維持に関与する遺伝子群との関連性について検討を行った。神経芽腫予後良好例 19 例と予後不良例 13 例について約 700 の miRNA の発現レベルを解析した。両群の間で Fold Change>2.0、かつ t-test で有意差($p<0.05$)を示す miRNA を 70 種類ピックアップし、TargetScan データベースに公開されている各 miRNA の予想ターゲット遺伝子のリストと、進行神経芽腫における遺伝子発現レベルとの相関を検討したところ、mRNA 発現レベルが有意に予後と相関する遺伝子群が複数含まれていた。

さらに今年度は上記 I-type 幹細胞様神経芽腫細胞株と N-type 細胞株との miRNA 発現プロファイルの比較も進めた。I-type 細胞はこれまでに取得してきた予後不良プライマリー腫瘍における miRNA プロファイルとは異なる傾向を示していたことから、さらに再発腫瘍や転移部位のプロファイルを取得し、I-type との比較を進める予定である。

4. 神経芽腫由来 iPC 細胞の解析：
主任研究者において培養された神経芽腫 I-type cell 由来 iPC 細胞についてアレイ CGH 解析ならびに mRNA 発現プロファイルを行い、iPC 化による特徴的な遺伝子を抽出した。 その結果、ゲノム異常の変化はほとんどなく、全体として予後不良型のプロファイルから予後良好型への変化が見られた。

考察：
I-type の細胞株はこれまでにヌードマウスへの移植実験における高い腫瘍形成能、軟寒天培地における足場非依存性増殖能の亢進などの結果から幹細胞様の性質を持つことが報告されており(Ross RA et al, 2003)、その特徴的な発現を明らかにすることは今後の実験系、ならびに臨床検体の結果の解釈において重要なヒントとなると考えられる。神経芽腫サンプルを用いた Neurospere 形成のシステムも同様に癌幹細胞を含む細胞塊の取得を通した難治性癌のモデルとして、今後複数の試料に対して網羅的に分子プロファイリングを行い、今回のデータと比較する予定である。また一方で、iPC 化によりリプログラミングされた I-type 神経芽腫細胞のゲノムならびにトランスクリプトーム解析の結果から、iPC 化細胞が予後良好タイプの遺伝子発現プロファイルを示したことから、これらの再現性を検証するために今後さらに複数種類の細胞株のデータを蓄積することが重要と考えられる。以上の手順で治療標的となる分子パスウェイ探索を助けるとともに、これらの分子生物学的プロファイルに基づく予後層別化された各サブセット毎に関与する分子パスウェイの特徴を明らかにし、治療の層別化につなげることを目指す。
さらには、次世代シーケンサーを用いたエクソンシーケンシングによる新規関連遺伝子の同定等を目指し、主任研究者により選出された難治性症例群について遺伝子変異検索、網羅的アレイ CGH 解析、遺伝子発現解析を進め一部症例に *ALK*、*PHOX2B* などの遺伝子変異等の解析を進めている。

D. 研究発表

1. 論文発表

1) Akter J, Takatori, A, Hossain S, Ozaki T, Nakazawa A, **Ohira M**, Suenaga Y, Nakagawara A. Expression of NLRR3 orphan receptor gene is negatively regulated by MYCN and Miz-1, and its down-regulation is associated with an unfavorable outcome in neuroblastoma. *Clin Cancer Res*, 17(21):6681-6692, 2011.

2) Kimura H, Nakajima T, Takeuchi K, Soda M, Mano H, Iizasa T, Matsui Y, Yoshino M, Shingyoji M, Itakura M, Itami M, Ikebe D,

Yokoi S, Kageyama H, **Ohira M**, Nakagawara A. ALK fusion gene positive lung cancer and 3 cases treated with an inhibitor for ALK kinase activity. *Lung Cancer* 75(1):66-72, 2012.

3) Isogai E, **Ohira M**, Ozaki T, Oba S, Nakamura Y, Nakagawara A. Oncogenic LMO3 collaborates with HEN2 to enhance neuroblastoma cell growth through transactivation of *Mash1*. *PLOS One* 6(5):e19297, 2011.

4) Takenobu H, Shimozato O, Nakamura T, Ochiai H, Yamaguchi Y, **Ohira M**, Nakagawara A, Kamijo T. CD133 suppresses neuroblastoma cell differentiation via signal pathway modification. *Oncogene* 30(1):97-105, 2011.

2. 学会発表

1) **Ohira M**, Isogai E, Nakamura Y, Kamijo T, Nakagawara A.: Identification of prognosis-related miRNA expression profiles in neuroblastoma. 第 70 回日本癌学会学術総会, 名古屋, 10 月 3 日-5 日, 2011.

2) Isogai E, **Ohira M**, Haraguchi S, Nakagawara A.: The LIM-only protein LMO3 could regulate growth and proliferation in neural crest cells. 第 70 回日本癌学会学術総会, 名古屋, 10 月 3 日-5 日, 2011.

3) Iwata S, **Ohira M**, Kamijo T, Yonemoto T, Ishii T, Tatezaki S, Nakagawara A.: Genomic approach to the prediction of chemotherapeutic sensitivity in osteosarcoma. 第 70 回日本癌学会学術総会, 名古屋, 10 月 3 日-5 日, 2011.

4) **大平美紀**、磯貝恵理子、中村洋子、上條岳彦、中川原章. 神経芽腫の予後に相関するマイクロ RNA 発現パターンの検索. 第 53 回日本小児血液・がん学会学術集会, 新前橋, 11 月 25 日-27 日, 2011.

E. 知的財産権の出願・登録状況

1. 特許取得
   無し

2. 実用新案登録
   無し

3. その他

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

遺伝子改変マウスを用いた神経芽腫発がんに関わる新規遺伝子の解析

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研究要旨
脱ユビキチン化酵素であるUSP7/HAUSPが、ポリコム群、Ink4a、p53、MDM2などを介した細胞増殖や細胞死を制御することが考えられている。このメカニズムを解明するために、本年度はUSP7/HAUSPと結合するタンパクであるRing1BとMBLR/Pcgf6をコンディショナルに欠損しうるマウス系統をそれぞれ作成し、コンディショナルKO-ES細胞を樹立した。

A．研究目的

ポリコム群複合体(PRC１)を精製したところ、そこにユビキチン特異的分解酵素７（USP7/HAUSP）を見出した。USP7/HAUSPは、p53とMDM2を脱ユビキチン化し、それらを機能的にバランスさせて、がん細胞の増殖を制御する。一方、PRC1とMBLR複合体とをバランスさせることで、ホメオボックス遺伝子の転写抑制を制御することを我々は最近明らかにした。本研究では、USP7/HAUSPがどのようにがん抑制遺伝子であるInk4aに作用するかを明らかにする。これにより、USP7/HAUSPを介した制御が、Ink4a、p53、MDM2をどのようにバランスさせ、正常細胞あるいはがん細胞の増殖や細胞死をバランスさせているのかを明らかにしうる。

B．研究方法

・USP7/HAUSPと複合体を構成するRing1BとMBLR（Pcgf6）について薬剤依存的に欠損するマウスから、ES細胞を樹立し、ES細胞における、USP7/HAUSPを含むタンパク複合体の増殖への作用を解析する。
・USP7/HAUSPを薬剤依存的に欠損するマウスに、MLL-A9融合タンパクを発現するレトロウイルスを感染させ、白血病誘導を試みた。

C．研究結果

・Ring1BとMBLR/Pcgf6コンディショナル欠損マウスを作製し、そこからタモキフェン依存的にそれぞれを欠損するマウス系統を作出し、そこからそれぞれをタモキシフェン誘導的に欠損するES細胞を作製した。Ring1BとMBLR/Pcgf6はES細胞の生存や増殖には必須ではないことが示された。これらの結果は、USP7/HAUSP欠損ES細胞では、PRC1の発現が顕著に減少したにも関わらず、ES細胞がほぼ正常に増殖する事と一致する。しかしながら、Ring1-KOとMBLR/Pcgf6-KOにおける遺伝子発現プロファイルは大きく異なっており、USP7/HAUSP-KOはMBLR/Pcgf6-KOに似た遺伝子発現プロファイルを示すことが明らかになった。
・現在のところ移植可能な白血病株の樹立には至っていない。

D．考察

USP7/HAUSPは、PRC1そのものではなく、PRC1関連複合体であるMBLR/Pcgf6複合体の機能発現制御に寄与することが明らかになり，その経路を介してink4aの発現制御に寄与する可能性が示された。

E．結論

USP7/HAUSPの新たな機能的標的の候補としてMBLR/Pcgf6複合体を同定することに成功した。

G．研究発表

論文発表

1. Li X, Isono KI, Yamada D, Endo TA, Endoh M, Shinga J, Mizutani-Koseki Y, Otte AP, Casanova M, Kitamura H, Kamijo T, Sharif

J, Ohara O, Toyada T, Bernstein BE, Brockdorff N, **Koseki H.** Mammalian Polycomblike Pcl2/Mtf2 is a novel regulatory component of PRC2 that can differentially modulate Polycomb activity at both the Hox gene cluster and at Cdkn2a genes. *Mol Cell Biol.* 31:351-364 (2011)

2. Sharif J, Endoh M, **Koseki H.** Epigenetic memory meets G2/M: to remember or to forget? *Dev Cell.* 20(1):5-6 (2011)
3. Kimura W, Machii M, Xue X, Sultana N, Hikosaka K, Sharkar MT, Uezato T, Matsuda M, **Koseki H**, Miura N. Irx11 mutant mice show reduced tendon differentiation and no patterning defects in musculoskeletal system development. *Genesis.* 49(1):2-9 (2011)
4. Kimura M, Takenobu H, Akita N, Nakazawa A, Ochiai H, Shimozato O, Fujimura YI, **Koseki H**, Yoshino I, Kimura H, Nakagawara A, Kamijo T. Bmi1 regulates cell fate via tumor suppressor WWOX repression in small cell lung cancer cells. *Cancer Sci.* 102(5):983-90 (2011)
5. Oshima M, Endoh M, Endo TA, Toyoda T, Nakajima-Takagi Y, Sugiyama F, **Koseki H**, Kyba M, Iwama A, Osawa M. Genome-wide analysis of target genes regulated by HoxB4 in hematopoietic stem and progenitor cells developing from embryonic stem cells. *Blood.* 117(15):e142-50 (2011)
6. Casanova M, Preissner T, Cerase A, Poot R, Yamada D, Li X, Appanah R, Bezstarosti K, Demmers J, **Koseki H**, Brockdorff N. Polycomblike 2 facilitates the recruitment of PRC2 Polycomb group complexes to the inactive X chromosome and to target loci in embryonic stem cells. *Development.* 138(8):1471-82 (2011)
7. Hojyo S, Fukada T, Shimoda S, Ohashi W, Bin BH, **Koseki H**, Hirano T. The zinc transporter SLC39A14/ZIP14 controls G-protein coupled receptor-mediated signaling required for systemic growth. *PLoS One.* 6(3):e18059 (2011)
8. Sharif J, ***Koseki H***. Recruitment of Dnmt1 roles of the SRA protein Np95 (Uhrf1) and other factors. *Prog Mol Biol Transl Sci.* 101:289-310 (2011) Review.
9. Nishida K, Yamasaki S, Hasegawa A, Iwamatsu A, **Koseki H**, Hirano T. Gab2, via PI-3K, regulates ARF1 in FcεRI-mediated granule translocation and mast cell degranulation. *J Immunol.* 187(2):932-41 (2011)
10. Mishima Y, Miyagi S, Saraya A, Negishi M, Endoh M, Endo TA, Toyoda T, Shinga J, Katsumoto T, Chiba T, Yamaguchi N, Kitabayashi I, **Koseki H,** Iwama A. The Hbo1-Brd1/Brpf2 complex is responsible for global acetylation of H3K14 and required for fetal liver erythropoiesis. *Blood.*118(9):2443-53 (2011)
11. Takada Y, Naruse C, Costa Y, Shirakawa T, Tachibana M, Sharif J, Kezuka-Shiotani F, Kakiuchi D, Masumoto H, Shinkai Y, Ohbo K, Peters AH, Turner JM, Asano M, **Koseki H.** HP1γ links histone methylation marks to meiotic synapsis in mice. *Development.* 138(19):4207-17 (2011)
12. Mochizuki-Kashio M, Mishima Y, Miyagi S, Negishi M, Saraya A, Konuma T, Shinga J, **Koseki H**, Iwama A. Dependency on the polycomb gene Ezh2 distinguishes fetal from adult hematopoietic stem cells. *Blood.* 118(25):6553-61 (2011)
13. Zhang J, Gao Q, Li P, Liu X, Jia Y, Wu W, Li J, Dong S, **Koseki H**, Wong J. S phase-dependent interaction with DNMT1 dictates the role of UHRF1 but not UHRF2 in DNA methylation maintenance. *Cell Res.* 21(12):1723-39 (2011)
14. Tan J, Jones M, **Koseki H**, Nakayama M, Muntean AG, Maillard I, Hess JL. CBX8, a polycomb group protein, is essential for MLL-AF9-induced leukemogenesis. *Cancer Cell.* 20(5):563-75 (2011)
15. Hisada K, Sánchez C, Endo TA, Endoh M, Román-Trufero M, Sharif J, **Koseki H,** Vidal M. RYBP represses endogenous retroviruses and preimplantation- and germ line-specific genes in mouse embryonic stem

cells. *Mol Cell Biol.* 32(6):1139-49 (2012)
16. T Takagi S, Saito Y, Hijikata A, Tanaka S, Watanabe T, Hasegawa T, Mochizuki S, Kunisawa J, Kiyono H, **Koseki H**, Ohara O, Saito T, Taniguchi S, Shultz LD, Ishikawa F. Membrane-bound human SCF/KL promotes in vivo human hematopoietic engraftment and myeloid differentiation. *Blood.* 119(12):2768-77 (2012)
17. Watarai H, Sekine-Kondo E, Shigeura T, Motomura Y, Yasuda T, Satoh R, Yoshida H, Kubo M, Kawamoto H, **Koseki H**, Taniguchi M.Development and function of invariant natural killer T cells producing t(h)2- and t(h)17-cytokines. *PLoS Biol.* 10(2):e1001255 (2012)
18. Oguro H, Yuan J, Tanaka S, Miyagi S, Mochizuki-Kashio M, Ichikawa H, Yamazaki S, **Koseki H,** Nakauchi H, Iwama A. Lethal myelofibrosis induced by Bmi1-deficient hematopoietic cells unveils a tumor suppressor function of the polycomb group genes. *J Exp Med.* 209(3):445-54 (2012)

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平成２３年度 分担研究報告書

難治性神経芽腫における特異的ヒストン修飾の解析とその臨床応用に関する研究

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研究要旨

ポリコーム阻害剤の抗腫瘍剤として有用性が確認された。神経芽腫の癌幹細胞におけるゲノムワイドなヒストン修飾と遺伝子発現制御を明らかにし、難治性神経芽腫の治療に有効なエピジェネティクス治療の開発につなげたい。

A．研究目的

遺伝子異常に加えて、エピジェネティックな転写制御の異常が癌の悪性化に関わることが明らかになりつつある。難治性神経芽腫におけるエピジェネティック異常はまた解析されておらず、基盤となる情報が必要である。本研究では難治性神経芽腫におけるヒストン修飾のゲノムワイドな解析を通して難治性神経芽腫におけるエピジェネティック異常の一端を明らかにするとともに、得られた知見をもとに、難治性神経芽腫の治療に有効なエピジェネティック治療の可能性を探る。

B．研究方法

神経芽腫患者検体を培養して作製したスフェア細胞におけるゲノムワイドなヒストン修飾状態をクロマチン免疫沈降とDNAマイクロアレイを組み合わせたChIP-chip法により検証する。また、エピジェネティック治療の可能性をマウスモデルを用いて検証する。

（倫理面への配慮）

特になし。

C．研究結果

神経芽腫患者検体を培養して作製したスフェア (sphere) は未分化な幹細胞を多く含むことが明らかになっている。このスフェアと切除直後の未処理の患者検体から RNA を抽出し、DNA マイクロアレイによる遺伝子発現解析を行っている。さらに、神経芽腫細胞株の未分化状態（スフェア形成状態）と通常培養による分化度の高い状態の２群に関してゲノムワイドなヒストン修飾を ChIP-Chip 法 により解析中である。また難治性神経芽腫における MYCN の標的遺伝子としてポリコーム群遺伝子の重要性が確認された (Ochiai et al, Oncogene 29:2681, 2010)。そこで、ポリコーム群蛋白 EZH2 の低分子化合物阻害剤(DZNep)の効果を、まず、肝細胞がん細胞株 Huh7 細胞で検証した。EZH2 はヒストン H3K27 のトリメチル化活性を有するが、DZNep 添加によりその活性が著明に抑制されることが確認された。また DZNep は Huh7 細胞のスフェア形成を有意に抑制するとともに免疫不全マウス皮下における Huh7 細胞の腫瘍形成を明らかに抑制した。

D．考察

ポリコーム阻害剤は抗腫瘍剤として有用であることが確認された。難治性神経芽腫へも応用できる可能性があると考えられる。

E．結論

ポリコーム阻害剤の抗腫瘍剤として有用性が確認された。神経芽腫の癌幹細胞におけるゲノムワイドなヒストン修飾を解析し、神経芽腫の癌幹細胞特異的なヒストン修飾と遺伝子発現制御を明らかにし、難治性神経芽腫の治療に有効なエピジェネティクス治療の開発につなげたい。

G．研究発表

1.論文発表

1. Chiba T, Suzuki E, Negishi M, Saraya A, Miyagi S, Konuma T, Tanaka S, Tada M, Kanai F, Imazeki F, **Iwama A**, and Yokosuka O. 3-deazaneplanocin is a promising therapeutic agent for the eradication of tumor-initiating hepatocellular carcinoma cells. *Int J Cancer* 2011 Jun 29. doi: 10.1002/ijc.26264. [Epubahead of print].
2. Yuan J, Takeuchi M, Negishi M, Oguro H, Ichikawa H, and **Iwama A.** Bmi1 is essential for leukemic reprogramming of myeloid progenitor cells. *Leukemia* 25, 1335-1343, 2011.
3. Oguro H, Yuan J, Tanaka S, Miyagi S, Mochizuki-Kashio M, Ichikawa H, Yamazaki S, Koseki H, Nakauchi H, and **Iwama A.** Lethal myelofibrosis induced by *Bmi1*-deficient hematopoietic cells unveils a tumor suppressor function of the polycomb group genes. *J Exp Med* 209, 445-454, 2012.

2.学会発表

1. **岩間厚志**（2011）関連分野セミナーエピジェネティクス update」第 40 回日本免疫学会学術集会 11 月（千葉）
2. **岩間厚志**（2011）教育講演「血液疾患と Epigenetics」第 96 回近畿血液地方会 11 月（大阪）
3. **岩間厚志**（2011）教育講演「造血器腫瘍のエピゲノム」 第 73 回日本血液学会総会 10 月（名古屋）
4. **岩間厚志**（2011）「造血幹細胞のエピジェネティクス」 第３２回日本炎症・再生医学会シンポジウム「疾患と再生における幹細胞生物学の新展開」 ６月（京都）

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
Takehiko Kamijo	Neuroblastoma: Role of MYCN/Bmi1 Pathway in Neuroblastoma.		Pediatric Cancer, Volume 1, Neuroblastoma	Springer Science+Business Media B.V.		2012	

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Takenobu H, Shimozato O, Nakamura T, Ochiai H, Yamaguchi Y, Ohira M, Nakagawara A, Kamijo T.	CD133 suppresses neuroblastoma cell differentiation via signal pathway modification.	Oncogene	30	97-105	2011
Zhang L, Haraguchi S, Koda T, Hashimoto K, Nakagawara A.	Muscle atrophy and motor neuron degeneration in human NEDL1 transgenic mice. .	J. Biomed. Biotechnol.	2011	831092	2011
Iwama E, Tsuchimoto D, Iyama T, Sakumi K, Nakagawara A, Takayama K, Nakanishi Y ,Nakabeppu Y.	Cancer-related PRUNE2 protein is associated with nucleotides and is highly expressed in mature nerve tissues.	J. Mol. Neurosci.	44	103-114	2011
Ryu M, Hamano M, Nakagawara A, Shinoda M, Shimizu H, Miura T, Yoshida I, Nemoto A, Yoshikawa A.	The benchmark analysis of gastric, colorectal and rectal cancer pathways: toward establishing standardized clinical pathway in the cancer care.	Jpn. J. Clin. Oncol.	41	2-9	2011
Ozaki T, Nakagawara A.	p53: the attractive tumor suppressor in the cancer research field.	J. Biomed. Biotechnol.	2011	603925	2011
Okoshi R, Kubo N, Nakashima K, Shimozato O, Nakagawara A, Ozaki T.	CREB represses p53-dependent transactivation of MDM2 through the complex formation with p53 and contributes to p53-mediated apoptosis in response to glucose deprivation.	Biochem. Biophys. Res. Commun.	406	79-84	2011

Kimura M, Takenobu H, Akita N, Nakazawa A, Ochiai H, Shimozato O, Fujimura YI, Koseki H, Yoshino I, Kimura H, Nakagawara A, Kamijo T.	Bmi1 regulates cell fate via tumor suppressor WWOX repression in small cell lung cancer cells.	Cancer Sci.	102	983-990	2011
Kawahara N, Sugimura H, Nakagawara A, Masui T, Miyake J, Akiyama M, Wahid IA, Hao X, Akaza H.	The 6th Asia Cancer Forum: What Should We Do to Place Cancer on the Global Health Agenda? Sharing Information Leads to Human Security.	Jpn. J. Clin. Oncol.	1	723-729	2011
Ozaki T, Nakagawara A.	Role of p53 in cell death and human cancers.	Cancers	3	994-1013	2011
Takahashi A, Tokita H ,Takahashi K, Takeoka T, Murayama K, Tomotsune D, Ohira M, Iwamatsu A, Ohara K, Yazaki K, Koda T, Nakagawara A, Tani K,	A novel potent tumour promoter aberrantly overexpressed in most human cancers,	Scientific Reports	1,15		2011
Isogai E, Ohira M, Ozaki T, Oba S, Nakamura Y, Nakagawara A.	Oncogenic LMO3 collaborates with HEN2 to enhance neuroblastoma cell growth through transactivation of Mash1.	PLoS ONE	6	e19297	2011
London WB, Castel V, Monclair T, Ambros P F, Pearson AD, CohnS L, Berthold F, Nakagawara A, Ladenstein R L, Iehara T, MatthayK K.	Clinical and Biologic Features Predictive of Survival After Relapse of Neuroblastoma: A Report From the International Neuroblastoma Risk Group Project.	J. Clin. Oncol.	29	3286-3292	2011
Nakajima T, Yasufuku K, Nakagawara A, Kimura H, Yoshino I.	Multi-gene mutation analysis of metastatic lymph nodes in non-small cell lung cancer diagnosed by EBUS-TBNA.	Chest	140	1319-1324	2011

Kawahara N, Roh JK, Akaza H, Inoue H, Shibuya K, Iwasaki M, Tsuji T, Nishiyama M, Nakagawara A, Watanabe K, Nozaki S, Inoue M, Sugimura H, Miyake J, Li F.	The 7th Asia Cancer Forum: from the perspective of human security, how can we collaborate as Asians in order to place cancer on the global health agenda? How can we fill in the gaps that exist among us?	Jpn. J. Clin. Oncol.	41	825-831	2011
Shih YY, Lee H, Nakagawara A, Juan HF, Jeng YM, Tsay YG, LinDT, Hsieh FJ, Pan CY,Hsu WM, Liao YF.	Nuclear GRP75 Binds Retinoic Acid Receptors to Promote Neuronal Differentiation of Neuroblastoma.	PLoS One.	6	e26236	2011
Taggart DR, London WB, Schmidt ML, Dubois SG, Monclair TF, Nakagawara A, De Bernardi B, Ambros PF,Pearson AD, Cohn SL, Matthay KK.	Prognostic Value of the Stage 4S MetastaticPattern and Tumor Biology in Patients With Metastatic Neuroblastoma Diagnosed Between Birth and 18 Months of Age.	J. Clin. Oncol.	29	4358-4364	2011
Ozaki T, Yamada C, Nakagawara A.	A novel role of RUNX3 in the regulation of p53-mediated apoptosis in response to DNA damage.	Seikagaku	83	751-754	2011
Akter J, Takatori A, Hossain S, Ozaki T, Nakazawa A, Ohira M, Suenaga Y, Nakagawara A.	Expression of NLRR3 orphan receptor gene is negatively regulated by MYCN and Miz-1, and its down-regulation is associated with unfavorable outcome in neuroblastoma.	Clin. Cancer Res.	17	6681-6692	2011
Shih YY, Nakagawara A, Lee H, Juan HF, Jeng YM, Lin DT, YangYL, Tsay YG, Huang MC, Pan CY, Hsu WM, Liao YF.	Calreticulin Mediates Nerve Growth Factor-Induced Neuronal Differentiation.	J. Mol. Neurosci.	Epub ahead of print		2011
Li X, Isono K, Yamada D, Endo TA, Endoh M, Shinga J, Koseki YM, Otte AP, Casanova M, Kitamura M, Kamijo T, Sharif J, Ohara O, Toyada T, Bernstein BE, Brockdorff N and Koseki H,	Mammalian Polycomblike Pcl2/Mtf2 is a novel regulatory component of PRC2 that can differentially modulate Polycomb activity at both the Hox gene cluster and at Cdkn2a genes.	Mol. Cell Biol.	31	351-364	2011

Sasaki M, Kawahara K, Nishio M, Mimori K, Kogo R, Hamada K,Itoh B, Wang J, Komatsu Y, Yang YR, Hikasa H, Horie Y, Yamashita T, Kamijo T, ZhangY, Zhu Y, Prives C, Nakano T, Mak TW, Sasaki T, Tomohiko Maehama T, Mori M, and Suzuki A.	Regulation of the MDM2-P53 Pathway and Tumor Growth by PICT1/GLTSCR2 via Nucleolar RPL11	Nat. Med.	17	944-951	2011
Sharif J, Endoh M, Koseki H.	Epigenetic memory meets G2/M: to remember or to forget?	Dev. Cell	20	5-6	2011
Kimura W, Machii M, Xue X, Sultana N, Hikosaka K, Sharkar MT ,Uezato T, Matsuda M, Koseki H, Miura N.	Irxl1 mutant mice show reduced tendon differentiation and no patterning defects in musculoskeletal system development.	Genesis	49	2-9	2011
Oshima M, Endoh M, Endo TA, Toyoda T, Nakajima-Takagi Y, Sugiyama F, Koseki H, Kyba M, Iwama A, Osawa M.	Genome-wide analysis of target genes regulated by HoxB4 in hematopoietic stem and progenitor cells developing from embryonic stem cells.	Blood	117	e142-50	2011
Casanova M, Preissner T, Cerase A, Poot R, Yamada D, Li X, Appanah R, Bezstarosti K, Demmers J, Koseki H, Brockdorff N.	Polycomblike 2 facilitates the recruitment of PRC2 Polycomb group complexes to the inactive X chromosome and to target loci in embryonic stem cells.	Development	138	1471-1482	2011
Hojyo S, Fukada T, Shimoda S, Ohashi W, Bin BH, Koseki H, Hirano T.	The zinc transporter SLC39A14/ZIP14 controls G-protein coupledreceptor-mediated signaling required for systemic growth.	PLoS One.	6	e18059	2011
Sharif J, Koseki H.	Recruitment of Dnmt1 roles of the SRA protein Np95 (Uhrf1) and other factors.	Prog. Mol. Biol. Transl. Sci.	101	289-310	2011
Nishida K, Yamasaki S, Hasegawa A, Iwamatsu A, Koseki H, Hirano T.	Gab2, via PI-3K, regulates ARF1 in FcεRI-mediated granule translocation and mast cell degranulation.	J. Immunol.	187	932-941	2011

Mishima Y, Miyagi S, Saraya A, Negishi M, Endoh M, Endo TA, Toyoda T, Shinga J, Katsumoto T, Chiba T, Yamaguchi N, Kitabayashi I, Koseki H, Iwama A.	The Hbo1-Brd1/Brpf2complex is responsiblefor global acetylation of H3K14 and required for fetal liver erythropoiesis.	Blood	118	2443-2453	2011
Takada Y, Naruse C, Costa Y, Shirakawa T, Tachibana M, Sharif J, Kezuka-Shiotani F, Kakiuchi D, Masumoto H, Shinkai Y, Ohbo K, Peters AH, Turner JM, Asano M, Koseki H.	HP1γ links histone methylation marks to meiotic synapsis in mice.	Development	138	4207-4217	2011
Mochizuki-Kashio M, Mishima Y, Miyagi S, Negishi M, Saraya A, Konuma T, Shinga J, Koseki H, Iwama A.	Dependency on the polycomb gene Ezh2 distinguishes fetal from adult hematopoietic stem cells.	Blood	118	6553-6561	2011
Zhang J, Gao Q, Li P, Liu X, Jia Y, Wu W, Li J, Dong S, Koseki H, Wong J.	S phase-dependent interaction with DNMT1 dictates the role of UHRF1 but not UHRF2 in DNA methylation maintenance.	Cell Res.	21	1723-1739	2011
Tan J, Jones M, Koseki H, Nakayama M, Muntean AG, Maillard I, Hess JL.	CBX8, a polycomb group protein, is essential for MLL-AF9-induced leukemogenesis.	Cancer Cell	20	563-575	2011
Chiba T, Suzuki E, Negishi M, Saraya A, Miyagi S, Konuma T, Tanaka S, Tada M, Kanai F, Imazeki F, Iwama A, and Yokosuka O.	3-deazaneplanocin is a promising therapeutic agent for the eradication of tumor-initiating hepatocellular carcinoma cells.	Int. J. Cancer	130	2557-2567	2011
Yuan J, Takeuchi M, Negishi M, Oguro H, Ichikawa H, and Iwama A.	Bmi1 is essential for leukemic reprogramming of myeloid progenitor cells.	Leukemia	25	1335-1343	2011
Kimura H, Nakajima T, Takeuchi K, Soda M ,Mano H, Iizasa T, Matsui Y, Yoshino M, Shingyoji M, Itakura M, Itami M, Ikebe D, Yokoi S, Kageyama H, Ohira M, Nakagawara A.	ALK fusion gene positive lung cancer and 3 cases treated with an inhibitor for ALK kinase activity.	Lung Cancer	75	66-72	2012

Hisada K, Sánchez C, Endo TA, Endoh M, Román-Trufero M, Sharif J, Koseki H, Vidal M.	RYBP represses endogenous retroviruses and preimplantation- and germ line-specific genes in mouse embryonic stem cells.	Mol. Cell Biol.	32	1139-1149	2012
T Takagi S, Saito Y, Hijikata A, Tanaka S, Watanabe T, Hasegawa T, Mochizuki S, Kunisawa J, Kiyono H, Koseki H, Ohara O, Saito T, Taniguchi S, Shultz LD, Ishikawa F.	Membrane-bound human SCF/KL promotes in vivo human hematopoietic engraftment and myeloid differentiation.	Blood	119	2768-2777	2012
Watarai H, Sekine-Kondo E, Shigeura T, Motomura Y, Yasuda T, Satoh R, Yoshida H, Kubo M, Kawamoto H, Koseki H, Taniguchi M.	Development and function of invariant natural killer T cells producing t(h)2- and t(h)17-cytokines.	PLoS Biol.	10	e1001255	2012
Oguro H, Yuan J, Tanaka S, Miyagi S, Mochizuki-Kashio M, Ichikawa H, Yamazaki S, Koseki H, Nakauchi H, Iwama A.	Lethal myelofibrosis induced by Bmi1-deficient hematopoietic cells unveils a tumor suppressor function of the polycomb group genes.	J. Exp. Med.	209	445-454	2012
Yoshihara Y, Wu D, Kubo N, Sang M, Nakagawara A, Ozaki T.	Inhibitory role of E2F-1 in the regulation of tumor suppressor p53 during DNA damage response.	Biochem. Biophys. Res. Commun.	Epub ahead of print		2012
Takehiko Kamijo	Role of stemness-related molecules in neuroblastoma.	Pediatric Research	In press		2012

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ORIGINAL ARTICLE

CD133 suppresses neuroblastoma cell differentiation via signal pathway modification

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CD133 (prominin-1) is a transmembrane glycoprotein expressed on the surface of normal and cancer stem cells (tumor-initiating cells), progenitor cells, rod photoreceptor cells and a variety of epithelial cells. Although CD133 is widely used as a marker of various somatic and putative cancer stem cells, its contribution to the fundamental properties of cancer cells, such as tumorigenesis and differentiation, remains to be elucidated. In the present report, we found that CD133 was expressed in several neuroblastoma (NB) cell lines/tumor samples. Intriguingly, CD133 repressed NB cell differentiation, for example neurite extension and the expression of differentiation marker proteins, and was decreased by several differentiation stimuli, but accelerated cell proliferation, anchorage-independent colony formation and *in vivo* tumor formation of NB cells. NB cell line and primary tumor-sphere experiments indicated that the molecular mechanism of CD133-related differentiation suppression in NB was in part dependent on neurotrophic receptor RET tyrosine kinase regulation. RET transcription was suppressed by CD133 in NB cells and glial cell line-derived neurotrophic factor treatment failed to induce RET in CD133-expressing cells; RET overexpression rescued CD133-related inhibition of neurite elongation. Of note, CD133-related NB cell differentiation and RET repression were mainly dependent on p38MAPK and PI3K/Akt pathways. Furthermore, CD133 has a function in growth and RET expression in NB cell line- and primary tumor cell-derived tumor spheres. To the best of our knowledge, this is the first report of the function of CD133 in cancer cells and our findings may be applied to improve differentiation induction therapy for NB patients.
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Introduction

CD133 (AC133; human prominin-1) belongs to a family of cell-surface glycoproteins harboring five transmembrane domains (Corbeil *et al.*, 2001) and was originally found as a hematopoietic stem cell marker (Yin *et al.*, 1997). CD133 was subsequently shown to be expressed by a number of progenitor cells, including those of the epithelium, where it is expressed on the apical surface (Corbeil *et al.*, 2000). Previously, it was found that CD133-expressing cells in brain tumors have the capacity for unlimited self-renewal, as well as the ability, in small numbers, to initiate tumor formation and progression in immuno-deficient mice (Singh *et al.*, 2004), suggesting that CD133-expressing cells satisfy the important criteria required for tumor-initiating cells (TICs) (Reya *et al.*, 2001; Jordan *et al.*, 2006). Using similar methods, CD133 has recently been designated as a marker associated with TICs in the colon (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007), pancreatic (Olempska *et al.*, 2007), liver (Yin *et al.*, 2007), skin (Monzani *et al.*, 2007) and prostate (Collins *et al.*, 2005; Miki *et al.*, 2007) cancers. Maw *et al.* (2000) reported homozygosity for a 1-bp deletion (1878delG) in exon 16 of the CD133 gene predicted to cause a frameshift at codon 614 and a prematurely truncated protein lacking about half of the second extracellular loop, the final membrane-spanning segment and the cytoplasmic-C-terminal domain; this missense mutation caused retinal degeneration in four affected members of a consanguineous Indian family. This finding was further confirmed by an article describing that loss of Prom-1 in genetically modified mouse results in the progressive degeneration of mature photoreceptors with complete loss of vision (Zacchigna *et al.*, 2009); however, to the best of our knowledge, no reports have studied the function of CD133 in tumorigenesis.

Neuroblastoma (NB) is the most common pediatric solid malignant tumor derived from the sympathetic nervous system. Unlike the many childhood malignancies for which survival has been improved by recent therapies, high-risk NB is still one of the most difficult tumors to cure, with only 30% long-term survival despite intensive multimodal therapy (Maris *et al.*,

2007). The clinical presentation and treatment response of advanced NB, which results in relapse and a refractory state after a good responsive to the initial chemotherapy, suggest that TICs likely exist in NB tumors. A previous report indicated the isolation and characterization of putative TICs using primary-sphere formation with tumors and bone marrow metastases from NB patients, although CD133 expression was not detected in a bone marrow-derived high-risk NB tumor-sphere sample (Hansford *et al.*, 2007). On the other hand, it was reported that sub-cloned NB cells (designated 'intermediate type'), which have a significantly more malignant phenotype, with four- to fivefold greater plating efficiencies in soft agar and sixfold higher tumorigenicity in athymic mice, expressed high amounts of CD133 mRNA compared with less malignant subclones (Walton *et al.*, 2004); therefore, the function of CD133 in NB tumorigenesis and aggressiveness remains unresolved.

Previous reports about CD133 expression in NB and its function as a stem cell marker in several tumors prompted us to study the function of CD133 in NB cells (Walton *et al.*, 2004; Hansford *et al.*, 2007). Our results clearly indicated that CD133 also seems to regulate cell proliferation and tumorigenesis in NB cells. Importantly, CD133 represses NB cell differentiation and is decreased by several differentiation stimulators. We studied the molecular mechanism of CD133-related differentiation inhibition in NB cells and found that it was in part dependent on RET tyrosine kinase receptor regulation via signal pathway modification. Furthermore, CD133 is expressed in NB cell spheres and has a function in sphere growth and RET regulation.

In specific malignancies, for example NB and acute promyelocytic leukemia, differentiation induction therapy using retinoic acid is clearly effective. *In vitro* experiments indicated that all-*trans*-retinoic acid (ATRA) treatment induced morphological and biochemical differentiation in these cancer cells, suggesting that the induced differentiation seems to repress the tumorigenic activity of cancer cells (Brodeur *et al.*, 2000; Weinberg, 2006). Together, CD133 may regulate NB tumorigenesis and proliferation by preventing differentiation.

Results

CD133 has a function in NB cell proliferation

First, we checked the expression of CD133 in NB cell lines and found its expression in 7 out of 20 (53%) cell lines (Figure 3d and Supplementary Figure 1S). A high level of cell-surface expression of *CD133* was detected in TGW and SK-N-DZ cells, and modest expression was found in IMR32 (Figure 1a; Supplementary Figure 1Sa). Next, we knocked down *CD133* in highly expressing NB cells and analyzed the knockdown-induced phenotype. Figure 1b shows that infection of shRNA-reduced CD133 mRNA and protein and *CD133* knockdown in TGW cells effectively resulted in significant growth retardation. Inhibition of cell proliferation by CD133 small-interference RNA was also observed in SK-N-DZ cells (Supplementary Figure S1). Furthermore, stable knockdown of *CD133* in TGW cells suppressed cell proliferation under anchorage-independent conditions (Figure 1c). To test tumorigenicity *in vivo*, *CD133*-silenced TGW cells were injected subcutaneously into nude mice. Mock shRNA lentivirus-infected cells formed large tumors within 9 days post-injection; CD133 shRNA lentivirus-infected cells formed very small tumors (Figure 1d). Next, we examined the effect of CD133 on NB cell proliferation (Supplementary Figure 2S). CD133 was successfully expressed in SH-SY5Y cells by lentivirus. The proliferation rate of CD133-expressing SH-SY5Y cells was 2–2.5-fold greater than mock cells. Moreover, a soft agar colony formation assay showed that CD133-expressing cells formed more and bigger colonies than mock-control cells.

CD133 knockdown induces NB differentiation

In NB cells, differentiation into a neuronal phenotype is induced when cells are treated with several stimulations. Glial cell line-derived neurotrophic factor (GDNF) induced neurite outgrowth in TGW cells (Figure 2a, center). In CD133 knocked-down TGW cells, neurite formation was observed even under normal culture conditions (Figure 2a, KD). We scored cells with neurite length longer than the cell body diameter as neurite positive (Figure 2b). CD133 knocked-down cells showed intensified neurite extensions when compared with mock cells. Mock-infected and CD133 knocked-down cells were collected at the end of the experiment, and mRNA was extracted and subjected to RT–PCR (Figure 2c). With *GAP43/neurofilament (NF) 68* as neuronal differentiation markers, these expressions were constitutively upregulated in CD133 knocked-down cells. Along with differentiation induced by treatment with ATRA or phorbol-12-myristate-13-acetate (TPA) in parental TGW cells, CD133 expression was suppressed at both protein and mRNA levels (Supplementary Figure 3S). These results indicated that CD133 may suppress the differentiation of NB cells.

CD133 regulates RET expression in NB cells

To identify the mechanism of CD133-related cellular differentiation, we studied the expression of several neurotrophic receptors and RET receptors because they are the important signal transduction pathway molecules, which have important functions in sympathetic nerve and NB cell differentiation (Kaplan *et al.*, 1993; Klein, 1994; D'Alessio *et al.*, 1995; Enomoto *et al.*, 2001). We introduced *CD133* cDNA into several NB cell lines (Figure 3a), and checked the effect of CD133 overexpression on *RET* expression using a primer pair recognizing all *RET* isoforms, *RET51*, *RET9* and *RET43*, formed by alternative splicing of C-terminal exon cassettes (Myers *et al.*, 1995; Enomoto *et al.*, 2000). Intriguingly, in *RET* and all *RET* isoforms, transcriptions were suppressed in CD133-overexpressing NB cells (*RET* reduction was 1.3–3.8-fold by qPCR); however,

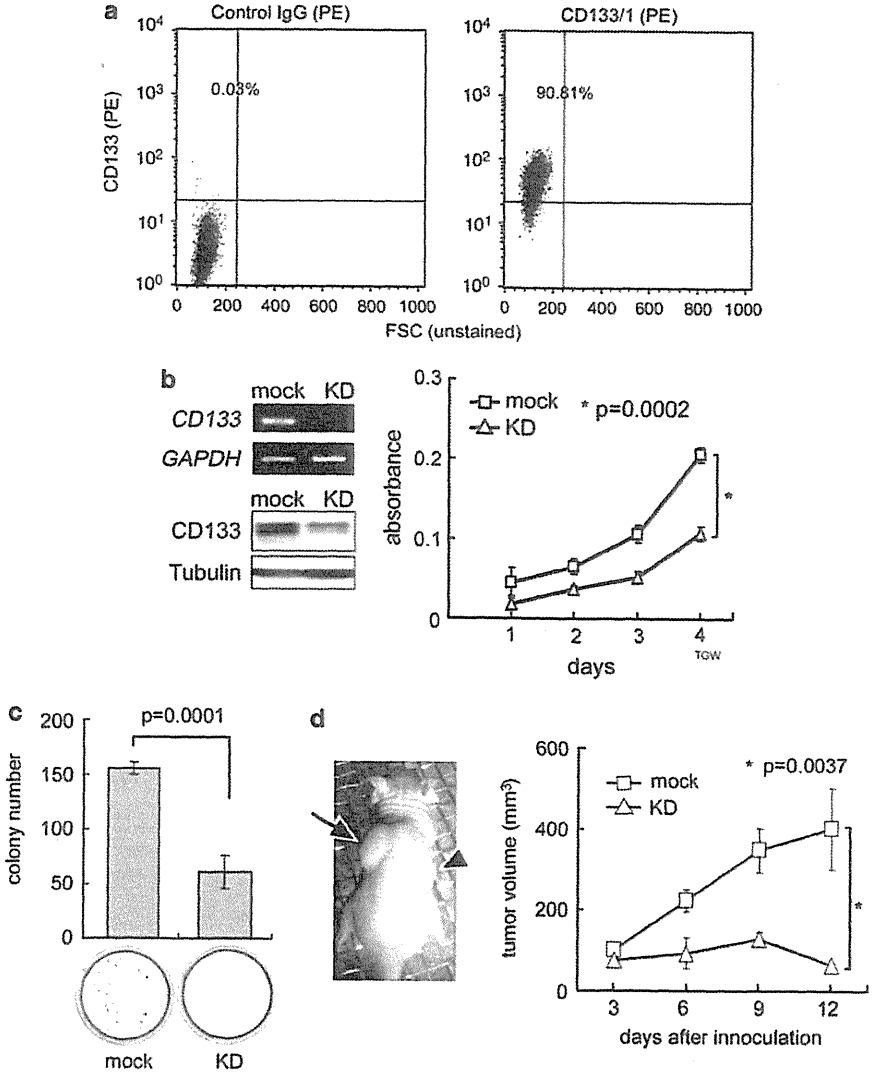

Figure 1 *CD133* knockdown inhibits the growth of human neuroblastoma (NB) cells. (**a**) Flow cytometric analysis of CD133 expression profiles in TGW cells. CD133 fluorescence is depicted on the *y* axis, and the percentage of CD133-positive cells is shown in the left upper corner of each plot. (**b**) Stable knockdown of *CD133* by lentivirus-mediated shRNA was performed as described in Materials and methods. *CD133* expression was detected by semi-quantitative RT–PCR and western blotting analysis in TGW cells. Growth curves were obtained by WST-8 assay. Anchorage-independent colony formation (**c**) and *in vivo* tumorigenic assay (**d**). TGW cells were stably transduced with shRNA against mock or CD133 (KD). (**c**) Colonies were stained with MTT dye and directly counted under a phase contrast microscope. (**d**) Tumor development in BALB/c AJcl *nu/nu* mice on injection of TGW cells stably infected with shRNA against mock (arrow) and CD133 (KD, arrowhead) cells. Tumor volume was measured every 3 days. Data are presented as the mean ± s.d. of tumors in four mice.

the effects of CD133 on *TrkA/B/C, p75NGFR* and *GDNF* expressions did not show a specific tendency. CD133 knockdown clearly increased *RET* mRNA (*RET* induction was 2.5–3.0-fold by qPCR). CD133-mediated RET downregulation was also observed at the protein level (Figure 3b). Furthermore, CD133 expression in primary NB spheres resulted in transcriptional suppression of *RET* (Figure 3c). These results suggest that CD133 suppresses *RET* gene transcription in NB cells.

To study the expression pattern of *CD133* and *RET* mRNA in human NBs, we performed semi-quantitative RT–PCR. *CD133* was expressed in 7 of 20 NB cell lines tested (Figure 3d), and only 1 NB cell line was *RET* positive in the 7 cell lines. We further studied *CD133* and *RET* expression in unfavorable patient-derived tumors (stages 3 and 4, TrkA(−), *MYCN* amplified). Again, *RET* expression was profoundly repressed in CD133-expressing NB tumors (Figure 3e). Finally, we studied the transcriptional activity of *RET* promoter in CD133-expressing cells. *RET* promoter reporter-derived luciferase activity was significantly suppressed in CD133-expressing cells (Figure 3e).

CD133 regulates NB cell differentiation in a RET-dependent manner

We investigated the biological effects of CD133 overexpression on *RET* downregulation in SH-SY5Y cells.

Significant neurite outgrowth was observed when mock-infected cells were stimulated with GDNF (Figure 4a). At the same time, no obvious difference was observed between mock- and GDNF-treated CD133-expressing cells. These results implied that CD133 overexpression inhibited NB cell differentiation.

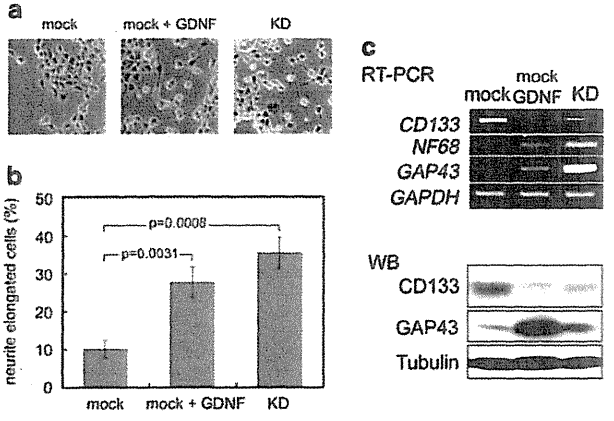

Figure 2 CD133 silencing induces differentiation in TGW cells. TGW cells were infected with lentivirus vectors encoding shRNA against *CD133* (right) or a mock (left) as a negative control. Ten days after infection, cells were treated with buffer (mock and KD) or GDNF (10 ng/ml, middle). Cells were scored for the presence of neurites longer than one cell diameter 72 h after treatment (photo: (**a**), bar graphs: (**b**)). Data are presented as the mean ± s.d. from at least three independent experiments. Statistical analysis was performed by Student's *t*-test. (**c**) NB differentiation-related molecule *neurofilament 68 (NF68)* and *GAP43* expressions in RT–PCR and WB. NF68 protein was not detected by WB in TGW cells.

We examined the effect of the co-expression of CD133 and RET (RET9) on SH-SY5Y cells. RET-expressing lentivirus was co-infected into stably CD133-expressing SH-SY5Y cells. Ten days after infection, ectopic RET and CD133 expressions were observed both at protein

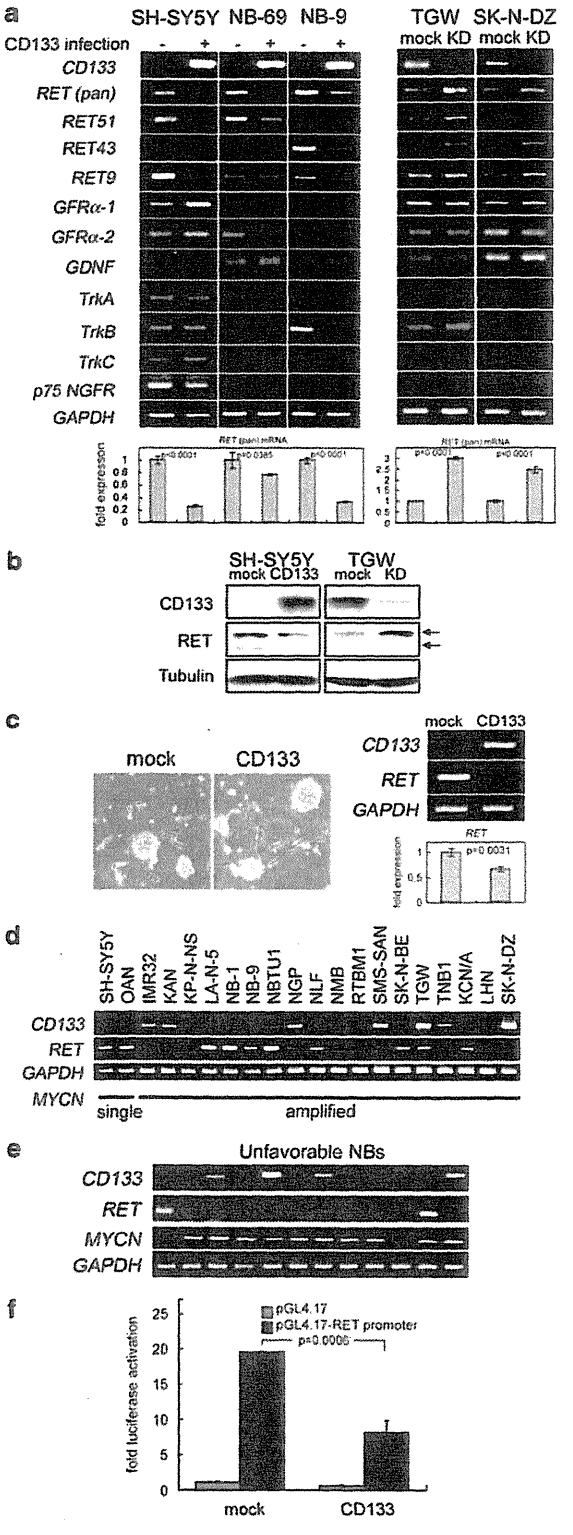

Figure 3 CD133 inhibits RET expression in NB cells. (**a**) SH-SY5Y, NB-69 and NB-9 cells were infected with mock or CD133-expressing lentivirus, and TGW and SK-N-DZ cells were stably infected with shRNA against mock or CD133 (KD) lentivirus. Semi-quantitative RT–PCR analyses were performed with CD133-modified NBs using specific primers against each *RET* isoform, *Trk* families, *GFRα-1/2* and *GDNF*. *GAPDH* was used as a loading control. Expression level of *RET* (pan) was analyzed by qPCR. In qPCR, relative *RET* values were normalized by *GAPDH*. Data are representative results of at least three independent experiments. (**b**) CD133-expressing SH-SY5Y or CD133 knocked-down TGW cell lysates were subjected to western blotting for CD133 and pan-RET expression. Pan-RET antibody detected two bands corresponding to RET isoforms (arrows). (**c**) Primary sphere from a stage 4 NB patient was infected with mock or CD133-expressing lentivirus. Five days after infection, RNA was extracted for semi-quantitative RT–PCR of *CD133*/*RET* and qPCR of *RET*. GAPDH was used as an internal control. Data are representative of three tumor samples. (**d**) Expression of *CD133* and *RET* mRNA in NB cell lines. In all, 18 NB cell lines with amplified *MYCN* and 2 cell lines with a single copy of *MYCN* were used for semi-quantitative RT–PCR analysis. (**e**) Semi-quantitative RT–PCR analysis in unfavorable primary NBs. The results of 12 NBs are shown. Unfavorable NBs: International NB Staging System (INSS) stage 3 or 4, *TrkA* (−), with *MYCN* amplified. (**f**) Effects of CD133 on *RET* promoter (0.8 kb) activity in SH-SY5Y cells. pGL4.17-*RET* promoter-driven luciferase activities were normalized to pRL-SV40 early enhancer/promoter-driven *Renilla* luciferase activities as the transfection control and expressed as relative values.

and mRNA levels (Figure 4b and data not shown). As seen in Figure 4a, GDNF significantly induced neurite outgrowth of CD133/RET co-expressing SH-SY5Y cells. CD133 single-infected cells did not respond to GDNF, suggesting that the response was dependent on RET receptor expression. However, the expression of neuronal cell differentiation markers induced by GDNF was not recovered by *RET* in CD133-expressing cells (Figure 4b). These findings indicated that CD133 inhibits GDNF-promoted neuronal differentiation via not only by *RET* but also by the other signal pathways.

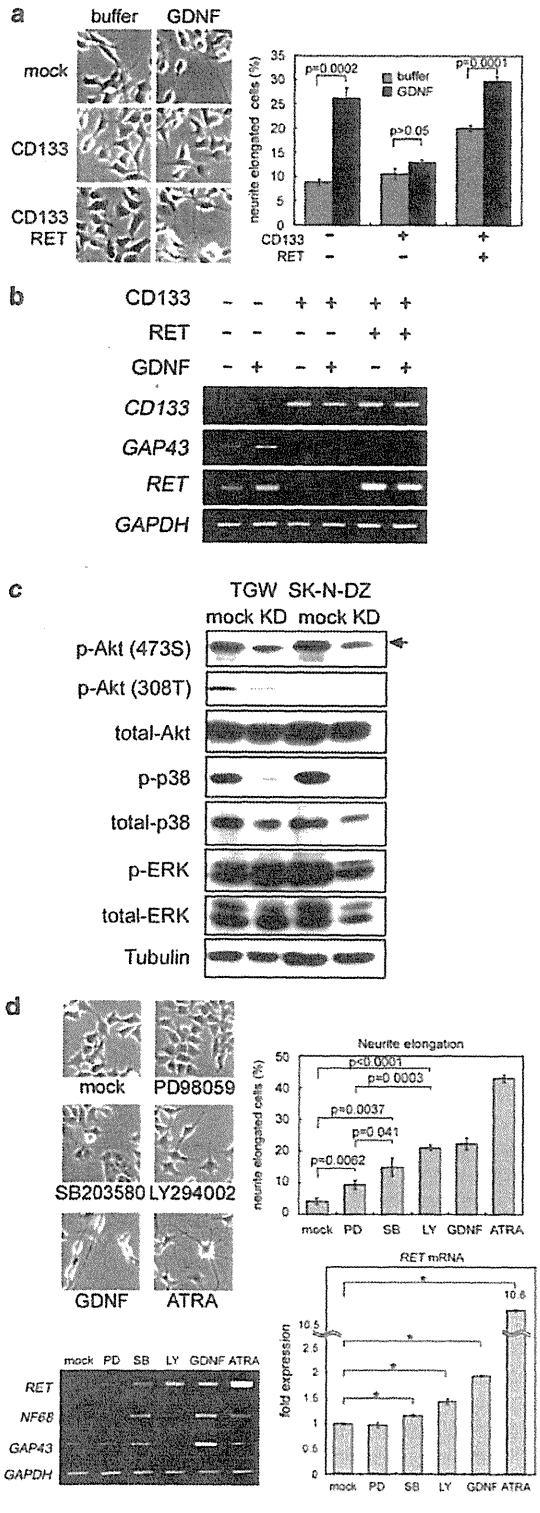

Figure 4 NB cell differentiation was regulated by CD133-dependent RET suppression via signal pathway modification. (**a**) Mock, CD133 and/or RET9 co-expressing SH-SY5Y cells were treated with GDNF (50 ng/ml) for 72 h. Cells were scored for the presence of neurites longer than one cell diameter after GDNF treatment. (**b**) CD133 and/or RET9 co-infected SH-SY5Y cells were cultured with or without GDNF treatment. Semi-quantitative RT–PCR analyses of *CD133*, *GAP43*, *RET* and *GAPDH* were performed. (**c**) The levels of phospho-Akt (p-Akt(473S) and p-Akt(308 T)), total-Akt, phospho-p38MAPK (p-p38), total-p38MAPK, phospho-ERK (p-ERK), total-ERK and tubulin were analyzed by western blot analysis. (**d**) TGW cells were cultured with DMSO (mock, 0.1%), PD98059 (PD, 5 μM), SB203580 (SB, 5 μM), LY294002 (LY, 5 μM), GDNF (50 ng/ml) or ATRA (5 μM) for 96 h. Cells were scored for the presence of neurite longer than one cell diameter after treatments. Semi-quantitative RT–PCR analysis of *RET*/*NF68*/*GAP43*/*GAPDH*, and qPCR of *RET* were performed.

CD133 regulates RET expression and NB cell differentiation by modification of signaling pathways

To identify the mechanism of *RET* downregulation in CD133-expressing cells, we studied the signaling molecule status in CD133 knocked-down cells (Figure 4c) and found a strong suppression of Akt (473S, 308T) and p38MAPK phosphorylation, but not ERK1/2 in both TGW and SK-N-DZ cells. To confirm the Akt and p38MAPK phosphorylation status caused by CD133 downregulation, we treated TGW cells with kinase inhibitors. MEK1 inhibitor (PD98059, PD), p38MAPK inhibitor (SB203580, SB) and PI3K inhibitor (LY294002, LY) induced neurite elongation in NB cells, and SB and LY were more effective for neurite elongation than PD. *RET* induction by kinase inhibitors was correlated with neurite elongation; however, differentiation markers *NF68* and *GAP43* were significantly induced by SB treatment. These results suggest that downregulation of p38MAPK and PI3K/Akt pathways has a function in CD133-related neurite elongation and differentiation marker expression is affected mainly by the p38MAPK pathway.

CD133 has a function in tumor-sphere growth and cell survival

It was previously reported that NB TICs were accumulated in NB spheres in serum-free media (SFM) (Hansford *et al.*, 2007). These observations prompted us to study the function of CD133 in tumor-sphere formation of NB cells. In IMR32 cells, only a small fraction of cells expressed CD133 (Supplementary Figure 1Sa). IMR32 cells were cultured in SFM with epidermal growth factor and fibroblast growth factor for a week, and sphere formation, upregulation of *CD133* (11.8-fold induction) and suppression of *RET* (2.8-fold reduction) were observed (Figure 5a). In primary NB cells from bone marrow metastasis,