Semaphorin signaling via MICAL3 induces symmetric cell division to expand breast cancer stem-like cells

Kana Tominaga,a,b,c,1,2 Hiroshi Minato,d,3 Takahiko Murayama,b Asako Sasahara,a,b Tatsunori Nishimura,b Etsuko Kiyokawab, Hajime Kanauchib, Seiichiro Shimizub, Ayaka Satob, Kotoe Nishiokab, Ei-ichi Tsujif, Masao Yano,b Toshihisa Ogawa,b Hideshi Ishib, Masaki Morib, Koichi Akashib, Koji Okamotob, Masahiko Tanabe,b Kei-ichirotad, Arinobu Tojoe, and Noriko Gotohb,c,t

aDivision of Molecular Therapy, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; bDivision of Cancer Differentiation, National Cancer Center Research Institute, Tokyo 104-0045, Japan; cDivision of Cancer Cell Biology, Cancer Research Institute, Kanazawa University, Kanazawa City, Ishikawa 920-1192, Japan; dDepartment of Pathology and Laboratory Medicine, Kanazawa Medical University, Uchinada-Machi, Ishikawa 920-0265, Japan; eDepartment of Breast and Endocrine Surgery, Graduate School of Medicine, University of Tokyo, Tokyo 112-8655, Japan; fDepartment of Breast and Endocrine Surgery, Showa General Hospital, Kodaira City, Tokyo 187-8510, Japan; gDepartment of Pathological Diagnosis, Showa General Hospital, Kodaira City, Tokyo 187-8510, Japan; hDepartment of Surgery, Minami-Machida Hospital, Machida City, Tokyo 194-0044, Japan; iDepartment of Medical Data Science, Osaka University Graduate School of Medicine, Suita City, Osaka 565-0871, Japan; jDepartment of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Suita City, Osaka 565-0871, Japan; and kDepartment of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Edited by Gregg L. Semenza, Johns Hopkins University School of Medicine, Baltimore, MD, and approved November 27, 2018 (received for review May 15, 2018)

Cancer stem-like cells (CSCs) are expanded in the CSC niche by increased frequency of symmetric cell divisions at the expense of asymmetric cell divisions. The symmetric division of CSCs is important for the malignant properties of cancer; however, underlying molecular mechanisms remain largely elusive. Here, we show a cytokine, semaphorin 3 (Sema3), produced from the CSC niche, induces symmetric divisions of CSCs to expand the CSC population. Our findings indicate that stimulation with Sema3 induced sphere formation in breast cancer cells through neuropilin 1 (NPI) receptor that was specifically expressed in breast CSCs (BCSCs). Knockdown of MICAL3, a cytoplasmic Sema3 signal transducer, greatly decreased tumor sphere formation and tumor-initiating activity. Mechanistically, Sema3 induced interaction among MICAL3, collapsin response mediator protein 2 (CRMP2), and Numb. It appears that activity of MICAL3 monooxygenase (MO) stimulated by Sema3 is required for tumor sphere formation, interaction between CRMP2 and Numb, and accumulation of Numb protein. We found that knockdown of CRMP2 or Numb significantly decreased tumor sphere formation. Moreover, MICAL3 knockdown significantly decreased Sema3-induced symmetric divisions in NPI/Numb-positive BCSCs and increased asymmetric division that produces NPI/Numb negative cells without stem-like properties. In addition, breast cancer patients with NPI-positive cancer tissues show poor prognosis. Therefore, the niche factor Sema3-stimulated NPI/MICAL3/CRMP2/Numb axis appears to expand CSCs at least partly through increased frequency of MICAL3-mediated symmetric division of CSCs.

Significance

Tumors are composed of both cancer stem-like cells (CSCs) and differentiated cancer cells. Each CSC can undergo either a symmetric cell division to produce two CSCs or an asymmetric cell division to produce one CSC and one differentiated cancer cell. It is believed that the rate of symmetric division increases as more CSCs become malignant; however, underlying molecular mechanisms remain elusive. Here we show that stimulation with a cytokine, semaphorin (Sema), activates monooxygenase of MICAL3, a cytoplasmic signal transducer, through the neuropilin (NP) receptor that is specifically expressed on the breast CSC plasma membrane. The activation of MICAL3 induces symmetric division of CSCs. Each molecule in this signaling pathway represents a promising therapeutic target for eliminating CSCs.

Author contributions: K.T. and N.G. designed research; K.T., H.M., T.M., A. Sasahara, T.N., E.K., H.K., S.S., A. Sato, K.N., E.-i.T., M.Y., T.O., H.I., M.M., K.A., K.O., M.T., K.-i.T., A.T., and N.G. performed research; K.T., H.M., K.O., and N.G. analyzed data; and K.T. and N.G. wrote the paper.

Conflict of interest statement: N.G., K.T., and A.T. applied for patent pending (2015-132122) in Japan.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1To whom correspondence may be addressed. Email: ngtokoh@staff.kanazawa-u.ac.jp or k.n.tominga22@gmail.com.
2Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.
3Present address: Department of Diagnostic Pathology, Ishikawa Prefectural Central Hospital, Kanazawa City, Ishikawa 920-8530, Japan.
4Present address: Department of Breast Surgery, National Center for Global Health and Medicine, Tokyo 162-8655, Japan.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1806851116/-/DCSupplemental.

Published online December 26, 2018.

www.pnas.org/cgi/doi/10.1073/pnas.1806851116

PNAS | January 8, 2019 | vol. 116 | no. 2 | 625–630

Breast cancer is the most common type of cancer among women throughout the world (1). The increasing rate of mortality due to breast cancer raises serious problems. Recent evidence indicates that tumor tissues are composed of heterogeneous cell populations including a relatively small number of cancer stem-like cells (CSCs) and other differentiated cancer cells (2). CSCs tend to survive irrespective of conventional chemotherapy, radiotherapy, and following treatment with molecular targeted drugs, because these treatment strategies target rapidly proliferating differentiated cancer cells but not CSCs. Targeting CSCs is thus important to improve the prognosis of cancer patients; however, molecular targeting drugs against CSCs are still unmet needs.

Stem cells have the ability to self-renew and differentiate. A stem cell divides into two daughter cells using one of two types of cell division: symmetric and asymmetric (3, 4). With symmetric division, a stem cell produces two identical daughter cells and doubles the number of self-renewing stem cells. In contrast, asymmetric cell division gives rise to two different daughter cells: one differentiated cell and one self-renewing stem cell. Recent evidence suggests that CSCs have similar characteristics regarding cell division (4, 5). Researchers believe that the more CSCs become malignant, the more they have a tendency to divide symmetrically, producing two daughter CSCs and leading to expansion of the CSC population. The molecular mechanisms of how each type of CSC division is determined remain obscure. If the mechanisms are clarified, a novel strategy for cancer therapy
may be established to reduce the CSC population by inhibiting symmetric division of CSCs.

In tumor tissues, CSCs are surrounded by a variety of cell types, including differentiated cancer cells and endothelial cells that comprise blood vessels (6). All these cells create a microenvironment that is called the CSC niche. CSCs are thought to survive by utilizing the CSC niche. We and others previously showed that breast cancer stem-like cells (BCSCs) maintain stemness for their survival in the inflammatory microenvironment by utilizing growth factors or cytokines that are produced by cancer cells in the CSC niche (6–9). By systematically analyzing the gene expression profile via the treatment of NF-κB, the inflammatory master transcription factor complex, stimulated by the growth factor hereregulin (HRG), we identified several CSC niche factors that are involved in maintenance of stemness of CSCs, including insulin-like growth factor 2 (IGF2) and growth differentiation factor 15 (10, 11).

A gene encoding the cytokine Sema3B was among the top genes in the list, and expression levels of MICAL3 were up-regulated (10).

The Sema family of membrane-bound or secreted proteins comprises 20 members in vertebrates (12). The type 3 Semas, including Sema3A and Sema3B, are secreted proteins that were originally discovered as ligands that relay repulsive signals for axon guidance during development of neuronal tissues in brain (13). Sema3A and Sema3B were subsequently shown to be involved in tumorigenesis in a context-dependent manner (14). NP and Plexin form a receptor complex for Semas (15). NP serves as the primary receptor for ligand binding, whereas the Plexin coreceptor transduces the Sema signal via the intracellular domain and activates MICAL.

MICAL is a cytoplasmic multidomain signaling protein that consists of a flavin adenine dinucleotide (FAD)-containing monooxygenase (MO) domain at the N terminus and domains for interacting with multiple proteins (16). The MICAL family of proteins comprises three major members in vertebrates: MICAL1, MICAL2, and MICAL3. MICALs have several functions that include axon repulsion via formation of a complex with CRMP (17). CRMP2 binds to tubulin heterodimers and induces microtubule polymerization (12). When Sema binds to NP, the MO domain in MICAL is activated and generates H₂O₂, a reactive oxygen species (ROS) (18). Then, CRMP2 homodimers are formed with intermolecular S–S bonds at Cys504 through oxidation, leading to the repulsion of axons (19). CRMP2 binds to several other proteins, including Numb in neurons (20). During axonal growth, CRMP2-bound Numb is involved in endocytosis at the growth cone. Numb also plays an important role in regulation of symmetric–asymmetric division of neural cells (21, 22). In Drosophila neural cells, Numb is expressed in differentiated cells and regulates asymmetric cell division (23), whereas in mammalian neural cells, Numb protein is expressed in dividing stem or progenitor cells (24, 25).

In this study, we provide evidence that Sema acts as a CSC niche factor and stimulates NP/MICAL3/CRMP2/Numb axis, leading to symmetric division and expansion of BCSCs.

**Results**

**The Sema3-NP Receptor Axis Plays Important Roles in BCSCs in Vitro.** We first examined whether the Sema3 signaling plays roles for stem-like properties of breast cancer cells (BCCs). The tumor sphere-forming ability measures properties of CSCs in vitro (26). Sphere culture medium (SCM), that contains a mixture of several growth factors or hormones, is usually used to form tumor spheres. We found that recombinant Sema3A induced tumor sphere formation of BCSCs, as a single cytokine, without other growth factors or hormones (Fig. 1 A and B). To analyze the activation of Sema3 signaling through NP1, a receptor for Sema3A, we studied the expression of NP1. Compared with MCF10A (a normal human mammary epithelial cell line), NP1 expression was elevated in all of the breast cancer cell lines that we examined (Fig. 1 C). NP1 expression was higher in breast cancer tissues than in normal breast tissues in the Oncomine database (https://www.oncomine.org/resource/login.html) (SI Appendix, Fig. S1). Plexin A, another receptor for Sema3, is expressed at similar levels in MCF10A and human breast cancer cell lines (27). The CD24−/low/CD44+ high cell population is known to be enriched by BCSCs (28). Tumor sphere formation, induced by SCM, was observed in the CD24−/low/CD44+ high patient-derived BCSC-enriched population, but not in the CD24−/low/CD44− non-BCSC population, as reported previously (8) (SI Appendix, Fig. S2). NP1 was expressed at higher levels in the CD24−/low/CD44+ high patient-derived BCSC-enriched population than in the CD24−/low/CD44− non-BCSC population (Fig. 1 D and E and SI Appendix, Fig. S3). Tumor sphere formation of patient-derived BCs significantly decreased in the presence of an NP1 neutralizing antibody in SCM (Fig. 1 F and G). We then sorted patient-derived BCs using an anti-NP1 NAb or control IgG in SCM. (Scale bar: 100 μm.) (G) Quantification of the tumor sphere-forming ability of patient-derived BCSCs in SCM. Tumor sphere-forming ability was significantly reduced by treatment with NP1 NAb or control IgG, n = 4. (H) FACS analysis of freshly obtained patient-derived BCSCs (patient 5). The cells were sorted according to the expression of CD44 and CD24 and then sorted according to the expression of NP1. (E) Percentages of NP1high cells in the CD44high/CD24−/low BCSC-enriched population or control non-BCSC populations. BCSCs derived from three patients (patients 4, 5, and 8) were used. (F) Representative phase contrast images of tumor sphere formation in patient-derived BCSCs (patient 9) in the presence or absence of an anti-NP1 neutralizing antibody (NP1 NAb) or control IgG in SCM. (Scale bar: 100 μm.) (G) Quantification of the tumor sphere-forming ability of patient-derived BCSCs in SCM. Tumor sphere-forming ability was significantly reduced by treatment with NP1 NAb or control IgG, n = 4. (H) FACS analysis of freshly obtained patient-derived BCSCs (patient 7). The cells were sorted according to the expression of NP1. (I) Representative phase contrast images of tumor sphere formation by patient-derived BCSCs. (Scale bar: 100 μm.) (J) Highly NP1high cells but not NP1low cells of patient-derived BCSCs (patient 5) formed tumor spheres by 200 ng/mL Sema3A stimulation; n = 4. (K) Expression of Nanog, ALDH1, and cytokeratin 18 (CK18) mRNA with quantitative real-time (qRT)-PCR between NP1high cells and NP1low cells in patient-derived BCSCs; n = 4. Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t tests.
antibody (Fig. 1F). We found that the NP1high cell population, but not the NP1low cell population, gave rise to tumor spheres either in Sema3A-containing medium (Fig. 1 I and J) or in SCM (SI Appendix, Fig. S4). In contrast, when patient-derived BCCs were sorted using an anti-Plexin A4 antibody to obtain cell populations with either high or low levels of Plexin A4 expression (SI Appendix, Fig. S5), both populations formed tumor spheres with similar efficiency. It is possible that other Plexin family proteins are expressed in these cells. The stemness markers Nanog and ALDH1 were expressed at higher levels, whereas the differentiation marker Cyclokinin18 (CK18) was expressed at lower levels in the NP1high cell population than in the NP1low cell population (Fig. 1K). These results suggest that Sema3A induces tumor sphere formation through NP1 receptor that is enriched in the BCSC population and that the Sema3-NP1 axis is required for efficient tumor sphere formation in SCM. Thus, NP1 would be a novel functional marker for BCSCs. In addition, we performed immunohistochemistry to examine in which cell types Sema3A and NP1 are expressed by using breast cancer tissues. Sema3A was stained in tumor cells, but not in endothelial cells or stroma cells, whereas NP1 was stained in tumor cells and endothelial cells, but not in stroma cells (SI Appendix, Fig. S6).

**MICAL3 Plays a Critical Role in BCSCs.** MICAL plays a central role in Sema-NP1-stimulated axon repulsion signaling in the nervous system (16). MICAL3 expression was higher in breast cancer tissues than in normal breast tissues in the Oncomine database (Fig. 2A). The breast cancer patients with higher levels of MICAL3 expression showed worse outcomes than those expressing lower levels of MICAL3 in the gene expression profiles (Fig. 2B). The tumor sphere-forming ability was significantly decreased by MICAL3 knockdown in BCCs (SI Appendix, Fig. S7A) cultured either in SCM (Fig. 2 C–E) or in Sema3A-containing medium (SI Appendix, Fig. S8). We also performed in vitro limiting dilution assay (LDA) for tumor sphere formation in patient-derived BCCs using two different shRNAs for MICAL3. MICAL3 knockdown significantly decreased tumor sphere-forming ability (SI Appendix, Figs. S7B and S9). However, it did not significantly affect proliferation of BCCs cultured in adherent condition in 10% serum-containing medium (SI Appendix, Fig. S10). Tumor-initiating activity measures properties of CSCs in vivo (26). We next analyzed a patient-derived xenograft (PDX) model in which patient-derived BCCs were inoculated into the mammary fat pads of immunodeficient mice. The tumor-initiating activity was markedly decreased in MICAL3 knockdown cells (Fig. 2 F and G and SI Appendix, Fig. S7B). These findings suggest that MICAL3 plays a critical role in BCSCs in vitro and in vivo.

**MO Domain of MICAL3 Plays Critical Roles in the Tumor Sphere-Forming Ability.** MICAL3 includes a FADD-containing MO domain, a calponin homology (CH) domain, a LIM domain, and a coiled-coil (CC) domain (Fig. 3A) (29). To determine which of these domains is required for tumor sphere formation in BCCs, we used cDNA constructs, MICAL3-wild type (WT), MICAL3-3G3W and MICAL3-N1 (Fig. 3A). MICAL3-3G3W is a MICAL3 mutant in which the three glycines in the FADD-binding motif GXGXXG were mutated to tryptophan, resulting in a defect in MO activity. Expression of MICAL3-3G3W abrogates the function of Drosophila MICAL in axon guidance and actin disassembly (30). MICAL3-N1 lacks the C-terminal region, which contributes to vesicle fusion to the plasma membrane (31). We transfected each construct into HEK293T cells and measured levels of H2O2 production (18, 32, 33). As expected, cells transfected with MICAL3-3G3W produced significantly lower levels of H2O2 than those with MICAL3-WT or MICAL3-N1 (Fig. 3B), indicating that the MO domain but not the C-terminal region of MICAL3 is required for production of H2O2. We next analyzed tumor sphere formation in BCCs that were transiently transfected with those constructs. When transfected cells were cultured in a floating condition, both MICAL3-WT– and MICAL3-N1–transfected cells were able to form more tumor spheres than empty vector (EV)-transfected cells either in SCM (Fig. 3 C–F and SI Appendix, Fig. S11 A and B) or in Sema3A-containing medium (Fig. 3D). In contrast, the tumor sphere-forming ability was much lower in MICAL3-3G3W–transfected cells than in MICAL3-WT– or MICAL3-N1–transfected cells. These results suggest that the activity of MICAL3 MO is required for tumor sphere formation.

**Sema3A-Stimulated MICAL3 MO Induces Interaction Between MICAL3 and CRMP2 and CRMP2 Dimerization in BCCs.** MICAL3-mediated H2O2 production contributes to CRMP2 interaction for axon repulsion (19). We next investigated the interaction between MICAL3 and CRMP2 using a Duolink in situ proximity ligation assay (PLA) (33). In situ PLA is a technique used to detect interactions between proteins inside a cell due to generation of
MO Domain of MICAL3 Is Required for Sema3A-Induced Interaction Between CRMP2 and Numb Stabilization. CRMP2 directly interacts with the amino-terminal fragment containing the phosphotyrosine binding domain of Numb during axon growth in the nervous system (20, 36). To examine whether CRMP2 interacts with Numb in BCCs, we used in situ PLA. We transected MCF7 cells with MICAL3 siRNA or transduced patient-derived BCCs with MICAL3 shRNA and cultured the sorted NP1-positive cells in the presence or absence of Sema3A-containing medium for 24 h. The number of PLA dots was greatly increased in Sema3A-containing medium but not in the medium without Sema3A (Fig. 5 A and B and SI Appendix, Fig. S17 A and B). MICAL3 knockdown significantly decreased the number of PLA dots in NP1-positive cells. To examine whether the MO domain of MICAL3 is required for the interaction between CRMP2 and Numb, BCCs were transiently transfected with CDNA constructs of MICAL3-WT or MICAL3-3G3W in Sema3A-containing medium for 24 h. The PLA dots were observed much more in EGFP-positive MICAL3-WT–transfected cells than in EGFP-positive MICAL3-3G3W–transfected cells or empty vector (EV)-transfected cells (Fig. 5 C and D and SI Appendix, Fig. S18 A and B). These results suggest that MICAL3 treatment decreases the MO domain-dependent interaction between CRMP2 and Numb in BCCs. To examine whether Sema3A signaling regulates Numb protein in BCCs, we analyzed the amount of Numb using immunoblotting. We found that stimulation with Sema3A increased the amount of Numb protein in BCCs (Fig. 5E). However, MICAL3 knockdown or EGCG treatment inhibited Sema3A-stimulated accumulation of Numb protein in BCCs (Fig. 5 E and F). These data suggest that Sema3A signaling induces accumulation of Numb protein via MICAL3. In addition, CRMP2 or Numb knockdown cells (SI Appendix, Fig. S19) showed greatly decreased tumor sphere formation cultured in Sema3A-containing medium (Fig. 5 G and H). All these results suggest that Sema3A treatment induces MICAL3 MO domain-dependent interaction between CRMP2 and Numb, leading to accumulation of Numb for sphere formation of BCCs.

**Sema3A Induces Symmetric Division of NP1/Numb-Positive CSCs Mediated by MICAL3.** To analyze symmetric–asymmetric cell division, we performed a cell pair assay using patient-derived BCCs (SI Appendix, Fig. S20). NP1-positive cells were sorted and cultured at a low density for 48 h in the medium with or without Sema3A. Single NP1-positive cells divided into pairs of daughter cells that were stuck together after 24 h. When cells were fluorescent in images. We found that the number of PLA dots was greatly increased in BCCs in Sema3A-containing medium (SI Appendix, Fig. S12 A and B). We next examined whether the Sema3A-induced interaction between MICAL3 and CRMP2 occurs in NP1-positive or NP1-negative patient-derived BCCs using anti-NP1 antibody staining. We found that Sema3A treatment greatly increased PLA dots in NP1-positive cells (NP1 pos), but not so in NP1-negative cells (NP1 neg) (Fig. 4). These results suggest that Sema3A treatment induces interaction between MICAL3 and CRMP2 in human BCCs, especially in the NP1-positive cells. We next examined whether dimerization of CRMP2 is induced by Sema3A-stimulated activity of MICAL3 MO in BCCs. When we treated MCF7 cells with 1 μM H2O2 as a control and separated the total lysates with nonreducing SDS/PAGE, intensities of the bands around 135 kDa were found, similarly observed in neuronal cells (SI Appendix, Fig. S13) (19). When BCCs were stimulated with Sema3A, the intensities of similar-sized bands were also increased (SI Appendix, Fig. S14 A and B). Furthermore, MICAL3 knockdown using shRNA significantly decreased the intensities of the bands of the homo-dimers of CRMP2 (SI Appendix, Fig. S14B). It is likely that dimerization of CRMP2 is induced by Sema3A-stimulated activity of MICAL3 MO in BCCs. To examine whether the increase of dimerization of CRMP2 is induced by MICAL3-mediated H2O2 production, we used (--)epigallocatechin gallate (EGCG) that is one of the green tea components and a selective inhibitor for MICAL MO (29, 34). In neuronal cells, EGCG treatment inhibits Sema3A-induced axon repulsion (35). We found that Sema3A-induced dimerization of CRMP2 was decreased by EGCG treatment, similarly observed in MICAL3 knockdown cells (SI Appendix, Fig. S15A). Moreover, EGCG treatment decreased tumor sphere-forming ability induced by Sema3A in a dose-dependent manner (SI Appendix, Fig. S15 B and C). Cell proliferation was not affected by EGCG treatment (SI Appendix, Fig. S16). These results further support the notion that MICAL3-mediated H2O2 production is required for Sema3A-induced CRMP2 dimerization and tumor sphere formation in BCCs. **MO Domain of MICAL3 Plays Critical Roles in Tumor Sphere Formation.** (A) Schematic of WT MICAL3 and mutant (3G3W and N1) constructs. (B) Measurement of H2O2 production using lysates of HEK293T cells transfected with the indicated constructs described in A in the presence of 200 μM NADPH. As a positive control, cells were treated with 1 μM H2O2, n = 3. (C and E) Representative phase contrast images of tumor sphere formation by MCF7 or patient-derived BCCs transiently transfected with the indicated constructs cultured in SCM. (Scale bar: 100 μm.) (D and F) Tumor sphere formation by MCF7 or patient-derived BCCs transfected with the indicated constructs cultured in SCM, n = 3. Data are shown as mean ± SD. ***P < 0.001 by Student’s t tests.

Fig. 3. MO domain of MICAL3 plays critical roles in tumor sphere formation. (A) Schematic of WT MICAL3 and mutant (3G3W and N1) constructs. (B) Measurement of H2O2 production using lysates of HEK293T cells transfected with the indicated constructs described in A in the presence of 200 μM NADPH. As a positive control, cells were treated with 1 μM H2O2, n = 3. (C and E) Representative phase contrast images of tumor sphere formation by MCF7 or patient-derived BCCs transiently transfected with the indicated constructs cultured in SCM. (Scale bar: 100 μm.) (D and F) Tumor sphere formation by MCF7 or patient-derived BCCs transfected with the indicated constructs cultured in SCM, n = 3. Data are shown as mean ± SD. ***P < 0.001 by Student’s t tests.

**Sema3A Induces Symmetric Division of NP1/Numb-Positive CSCs Mediated by MICAL3.** To analyze symmetric–asymmetric cell division, we performed a cell pair assay using patient-derived BCCs (SI Appendix, Fig. S20). NP1-positive cells were sorted and cultured at a low density for 48 h in the medium with or without Sema3A. Single NP1-positive cells divided into pairs of daughter cells that were stuck together after 24 h. When cells were...
and Numb proteins were coexpressed in only one of the daughter cells, and neither protein was expressed at a detectable level in the other daughter cell. Numb proteins tended to be localized in the region where daughter cells were attached to each other. Interestingly, we found few if any daughter cells that were single positive for either NP1 or Numb. This finding suggests that Numb proteins are stabilized in NP1-positive CSCs but not in NP1-negative differentiated cells. When cells were cultured in Sema3A-containing medium, the frequency of symmetric cell division was greatly increased compared with control medium without Sema3A [untreated (UT)] (Fig. 6B). These results suggest that Sema3A treatment gives rise to more daughter CSCs by symmetric cell division than the control medium. MICAL3 knockdown significantly decreased symmetric cell division in Sema3A-containing medium (Fig. 6C and D) up to the levels of untreated cells. These findings suggest that Sema3A induces symmetric division of NP1/Numb-positive BCSCs and that MICAL3 plays critical roles for this process. From these results, we proposed that the Sema3/NP1/MICAL3/CRMP2/Numb signaling induces symmetric division of BCSCs. By inhibition of MICAL3 MO activity or knockdown of each component in the signaling pathway, the symmetric cell division may be inhibited, leading to reduction of BCSCs.

Evaluation of Sema3/NP1/MICAL3 Expression in Breast Cancer Tissues. NP1 binds to four semaphorins, Sema3A, Sema3B, Sema3C, and Sema3D (15). To examine the significance of each semaphorin and patient outcome, we analyzed two gene expression profiles immunostained with anti-NP1 and anti-Numb antibodies after the first cell division, we found that the NP1-positive cells were cointained with the Numb protein on the cell membrane or in the cytoplasm (Fig. 6A). In cells that divided symmetrically, the NP1 and Numb proteins were strongly coexpressed and localized in both daughter cells. In cells that divided asymmetrically, the NP1
used for analysis of MICAL3 in Fig. 2B. We found that Sema3A or Sema3B, but not Sema3C or Sema3D, showed significant coexpression with MICAL3 (graphs surrounded by red frames in SI Appendix, Fig. S21). The expression levels of each Sema3, Sema3B, or Sema3D alone did not show a significant association with patient outcome, while the expression levels of both MICAL3 and Sema3A showed significantly worse outcomes in one gene expression profile. Expression levels of Sema3C showed an inverse correlation with those of MICAL3 in one gene expression profile and high expression of Sema3C was associated with favorable prognosis in the other gene expression profile (graphs surrounded by blue frames in SI Appendix, Fig. S21). To examine the association between the expression levels of NP1 in tumor cells from breast cancer tissues and the outcome of the patients, we analyzed a tissue microarray of breast cancer tissues using antibodies against human NP1 (SI Appendix, Fig. S22A). Patients with high NP1 expression in tumor cells from their breast cancer tissues showed a significantly reduced overall survival rate compared with those with low/negative NP1 expression (P = 0.0159) (Fig. 6E). NP1 expression levels were higher in tumor cells from breast cancer tissues with a triple negative subtype than those in tumor cells from breast cancer tissues with the luminal A subtype (P = 0.0179, ANOVA with Dunnett’s test) (SI Appendix, Fig. S22B). Immunohistochemistry studies using antibodies against high NP1 and Numb indicated the colocalization of NP1 and Numb in human breast cancer tissue samples (SI Appendix, Fig. S22C). Patients with higher levels of NP1 in their breast cancer tissues showed significantly reduced relapse-free survival rate and distant metastasis-free survival rate compared with those with low NP1 expression in their gene expression profiles (SI Appendix, Fig. S23) (37, 38). These results indicate that patients with breast cancer having high NP1 expression levels in cancer tissues show poor prognosis. This is consistent with the notion that NP1 signaling increases the symmetric division of CSCs, resulting in their increased number and worse prognosis.

Discussion

In the current study, we discovered that the Sema3/NP1/MICAL3/CRMP2/Numb axis may be a specific mechanism for the maintenance of the symmetric division of self-renewing CSCs. To the best of our knowledge, this report shows that CSCs and neuronal cells utilize common signaling components for the maintenance of symmetric cell division and for repulsion during axonal growth, respectively. It is possible that the Sema3/NP1/MICAL3/CRMP2/Numb axis is active in a portion of the population in any subtype of breast cancer. Breast cancer patients with NP1

Materials and Methods

All human breast carcinoma specimens were obtained from the University of Tokyo Hospital, Minami Machida Hospital, Showa General Hospital, and Kanazawa Medical University. This study was approved by the institutional review boards of the Institute of Medical Science, University of Tokyo, the University of Tokyo Hospital, Minami Machida Hospital, Showa General Hospital, Kanazawa Medical University, and Kanazawa Medical University. Written informed consent was obtained from all participants before inclusion in the study. Mice were handled according to the guidelines of the Institute of Medical Science, University of Tokyo. The experiments were approved by the committees for animal research at the Institute of Medical Science, University of Tokyo.

Tominaga et al.