The Role of Wnt-5a and MMP-2 in Human Glioma

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Summary

Wnts are secreted ligands which consist of 19 members in humans, regulate cell proliferation, differentiation, motility, and fate in many stages including embryonic stage and tumorigenesis. Wnts bind to cell surface receptors named Frizzleds and LRPs, and transduce their signals through β-catenin-dependent and -independent intracellular pathways.

Gliomas are one of the most common intracranial tumors. Gliomas exhibit a progression associated with widespread infiltration into surrounding neuronal tissues. However, the molecular mechanisms that stimulate the invasion of glioma cells are not fully understood. We established two cell lines from human glioma cases and analyzed the expression of all Wnt and Frizzled members in these cell lines and other well-known glioma cell lines, by real-time PCR study. The mRNA of Wnt -5a, -7b, Frizzled -2, -6, and -7 were over-expressed in glioma cells. The elevation of Wnt-5a expression was most remarkable. Although Wnt-5a is reported to have oncogenic and antioncogenic activity in several cancers, the role of Wnt-5a signaling in human glioma cells remains unclear.

Immunohistochemical study also revealed high expression of Wnt-5a in 26 (79%) of 33 human glioma cases. The positivity of Wnt-5a expression was

correlated with clinical grade.

Knockdown of Wnt-5a expression suppressed migration, invasion, and expression of matrix metalloproteinase-2 of glioma cells. Reciprocally, treatment with purified Wnt-5a ligand resulted in stimulation of cell migration and invasion. MMP-2 inhibitor suppressed the Wnt-5a-dependent invasion of U251 cells. These results suggested that Wnt-5a is not only a prognostic factor but also a therapeutic target molecule in gliomas to prevent tumor cell infiltration.

Introduction

Gliomas of astrocytic, oligodendroglial, and ependymal origin account for more than 70% of all brain tumors. The most frequent (65%) and most malignant histological type is glioblastoma. Despite advances in surgical and clinical neuro-oncology, their prognosis remains poor. In a meta-analysis of 12 randomized clinical trails, the overall survival rate of patients with glioblastomas (grade IV) and anaplastic astrocytomas (grade III) was 40% at one year and only slightly higher (46%) after combined radio- and chemo-therapy.[1] Glioblastomas may develop *de novo* (primary glioblastoma) or by progression from low-grade or anaplastic astrocytoma (secondary glioblastoma). Various genetic alterations were reported in glioblastoma.[2] Furthermore, glioblastomas are characterized by marked invasiveness and vascularity. Glioma tumor cells often invade beyond the main tumor mass at diagnosis, and this makes surgical treatment difficult. Clarification of the molecular mechanism of invasion could be useful to understand the pathogenesis of glioma.

Wnt proteins are a large family of cysteine-rich secreted molecules, consisting of at least 19 members in mammals to date. Wnt regulates cell growth, cell motility, and cell fate.[3,4] There are at least three distinct intracellular pathways activated by Wnt proteins. The canonical pathway is identified as a β-catenin-dependent pathway that is highly conserved among species.[5] When Wnt acts on its cell-surface receptors, which consist of Frizzled (Fz) and

LRP5/6, β-catenin is stabilized by release from the Axin-mediated degradation complex. The accumulated β-catenin is translocated to the nucleus, where it binds to the translocation factor T-cell factor/lymphoid enhancer factor and thereby stimulates the expression of various target genes.[6] Some Wnts, including Wnt-1, -3a, and -7a activate the β-catenin-dependent pathway. Furthermore, accumulation of β-catenin in the cytosol has frequently been observed in many malignant tumors.[7,8]

Another class of Wnts, including Wnt-2, -4, -5a, -5b, -6, and -11, activate β-catenin-independent pathways including planar cell polarity and Ca^{2+} pathway. These pathways partially overlap and primarily regulate cell motility and polarity.[9] In these pathways, Wnt binds to Fz and activates downstream effectors including Ca^{2+}/calmodulin-dependent protein kinase II, protein kinase C, c-Jun N-terminal kinase (JNK), nuclear factor of activated T cells, and small GTPases.[5]

Wnt-5a is known to activate β-catenin-independent pathways [9-11] in various cell lines and shows contrary functions. Previous studies indicated that Wnt-5a stimulates cell motility, invasiveness, and aggressiveness in some cancer cells.[9,11-15] These reports suggested that Wnt-5a has oncogenic properties. On the other hand, some reports indicated that Wnt-5a acts as a tumor suppressor owing to its ability to inhibit proliferation, motility, and invasiveness in thyroid tumor and colorectal cancer cell lines.[16,17]

Taken together, these findings indicated that the action of Wnt-5a should be analyzed in each cell type. Marked expression of Wnt-5a was already reported but its roles in glioma cells are still unclear.

In this study, we found that Wnt-5a is highly expressed in advanced stages of glioma and that its expression is involved in invasive activities and the transcription of matrix metalloproteinase-2 (MMP-2), which is highly expressed in glioblastoma and responsible for the degradation of surrounding tissues.

Materials and Methods

Materials and chemicals. The dsRNA for Wnt-5a and scramble were purchased from Santa Cruz Biotechnology, Inc. The universal probes® for real-time PCR were provided by Roche Diagnostics GmbH. Neo conditioned medium (CM) and Wnt-3a or Wnt-5a CM was prepared as described.[10,18] Mouse Wnt-5a protein was purified to homogeneity from CM produced by mouse L overexpressing mouse Wnt-5a as described.[10] Anti-Wnt-5a antibody was purchased from LIFESPAN BIOSCIENCE (Seattle, WA, USA). Anti-β-catenin and -GSK-3β antibodies were purchased from BD Biosciences. Anti-Dvl antibody was prepared in rabbits by immunization with recombinant protein of Dvl-1-(1-140).[19] Normal human brain total RNA was purchased from Clontech Laboratories, Inc. MMP-2 inhibitor (MMP-2 inhibitor III) was purchased from Calbiochem® (Darmstadt, Germany).

Tissue samples. Patients were treated at Kagoshima University Hospital. In total, 33 primary tumors were collected from patients diagnosed with glioma. Patients provided written informed consent. The research protocol and consent form were approved by the Institutional Review Board of Kagoshima University in advance.

Immunohistochemistry and immunocytometry. The tissue samples were sectioned and stained with hematoxylin and eosin, anti-Wnt-5a or -β-catenin antibody as described previously.[20] The stainings for Wnt-5a were visually classified into four grades according to

the percentage of the Wnt-5a-positive tumor cells (positive tumor cells/ total tumor cells) by two authors (Drs Hirano H. and Arita K., Department of Neurosurgery, Kagoshima University) as follows. Score 0, Wnt-5a positive tumor cell were less than 1%; Score 1, Wnt-5a positive tumor cells were 1-9%; Score 2, Wnt-5a positive tumor cells were 10-49%; Score 3, Wnt-5a positive tumor cells were more than 50%. The procedures were executed by these two authors independently and there were no discrepancy between their decisions.

Glioma-derived cell lines. U87MG and U251 glioblastoma-derived cell lines were purchased from ECACC and RIKEN BIORESOURCE CENTER (Tsukuba Science city, Ibaraki, Japan), respectively. U105 cells were provided by Dr. Kuratsu Jun-ichi (Department of Neurosurgery, Kumamoto University, Japan). To establish G24M and G5M cell lines, primary culture tumor cells from glioblastoma cases were prepared as described.[21]

Real-time RT-PCR. Total RNA was extracted from cells as described [22,23] and reverse-transcribed using a First-strand cDNA Kit (Roche Diagnostics). Expressions of Wnt and Fz member mRNA were estimated by real-time PCR using LightCycler® TaqMan® Master, LightCycler 1.5®, and LightCycler software version 3.5 (Roche Diagnostics) according to the manufacturer's instructions. Expression of each mRNA was normalized using β-actin as a loading control. Each mRNA expression was compared with that of normal human brain. The series of primers used in this study are listed in Table 1.

RNA interference. Eighty pmol of dsRNA for Wnt-5a or scramble was transiently transfected into U87MG, U105, U251 and G24M cells (60-mm-diameter dish) using Lipofectamine2000® (Invitrogen). After 24-72 h transfection, cellular responses were analyzed.

Preparation of soluble fractions. For the isolation of the soluble fractions of U251 cells, cells were suspended in an isotonic buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 2 μg/ml leupeptin, 1 mM PMSF, and 50 mM sodium β-glycerophosphate) and homogenized by sonication. The homogenates were centrifuged at 100,000 X *g* for 30 min at 4°C.

Western blot analysis. Samples were subjected to SDS-PAGE, probed with antibodies, and visualized with ECL system® (GE Healthcare).

Cell migration and invasion activity. To measure cell migration activity, a modified Boyden-chamber (tissue culture treated, 6.5 mm in diameter, 10-μm-thickness, 8-μm-pores; Transwell (Corning, Inc.) was used as described.[24,25] The lower surface of the filters was coated with 10 μg/ml fibronectin. Cells (2.5 x 10^4 cells) were suspended in RPMI1640 containing 0.1% BSA and applied to the upper chamber. The same medium was applied to the lower chamber. After several hours, the cells on the upperside of the filters were wiped off and the cells that migrated to the lower side of the upper chamber were fixed with 4% paraformaldehyde in PBS, stained with propidium iodide (1 μg/ml), and counted.

The invasive potential of the cells was analyzed using a Matrigel-coated modified Boyden -chamber as described above.

To perform wound healing assay, the cells were plated onto 35-mm-diameter dishes and the monolayer cells were manually scratched with a plastic tip; then, after being washed with PBS, the wounded monolayers of the cells were allowed to heal for 6-24 h in RPMI1640 containing 10% FBS. The width of the wounds is expressed as a percentage of the initial width at zero time.[26] When necessary, cells were transfected with dsRNA for Wnt-5a or scramble in advance or simultaneously treated with mouse Wnt-5a (500 ng/ml) or control buffer (1 mM Tris/HCl (pH7.5), 10 mM NaCl, and 0.05% (w/v) CHAPS) in migration, invasion, and wound healing assays. The effect of MMP-2 inhibitor to the invasion activity was analyzed using a Matrigel-coated modified Boyden-chamber as described above in the presence or absence of MMP-2 inhibitor III (50 nM). These experiments were performed at least four times.

In-gel-gelatinase assay. Isolation of membrane vesicles from cell-CM and in-gel-gelatin zymography was performed as described.[27] In brief, after 24 h of incubation with subconfluent cells in RPMI1640 containing 1% FBS, CM was collected and centrifuged at 100,000 X *g* for 1 h at 4°C. Precipitated fractions (1.3 or 2.6 μg of protein/lane) were developed on SDS-polyacrylamide gel containing gelatin (1 mg/ml) from swine (Bio-Rad

Laboratories, Inc.). After electrophoresis, gels were rinsed with distilled water twice, immersed in 2.5% (w/v) Triton-X100 for 2 h, distilled in water three times, incubated in 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 10 mM $CaCl_2$ at 37°C for 48 h, and stained with 1% Coomassie Brilliant Blue and destained.

Statistical analyses. Statistical analyses were carried out with nonparametric Wilcoxon rank sum test using R software (The R Foundation for Statistical Computing) or linear-by-linear association test (exact *P* value, two-sided) using StatXact-4 (Cytel Software Corporation).

Other assays. Protein concentrations were determined using BCA protein assay (Thermo SCIENTIFIC) with BSA as a standard.

Results

Expression of Wnt and Fz family genes in glioma-derived cells. Real-time RT-PCR studies revealed that Wnt-5a was predominantly and commonly overexpressed among all 19 Wnt families in glioma-derived cell lines. Wnt-7b were moderately expressed. Wnt-1, -2, -5b, -9a and -11 were weakly expressed (Fig. 1a). The expressions of the remaining Wnts were not increased (data not shown).

Fz- 2, - 6, and -7 were dominantly expressed among 10 Fz members in glioma-derived cell lines (Fig. 1b). Expressions of Fz-1, -3, -4, -5, and -8 were not increased. Expressions of Fz-9 and -10 were not detected in glioma cells (data not shown). Furthermore, expression of Ror2, a novel Wnt-5a receptor, was very low (data not shown). These findings suggested that signaling pathways could be activated in glioma cells through overexpression of Wnts or Fzs.

Immunohistochemical analysis of Wnt-5a and β-catenin in human glioblastoma tissue. In this study Wnt-5a was focused on because its expression was greatest and it was the most commonly elevated Wnt (Fig. 1a). Normal brain and glioma tissues were stained with hematoxylin and eosin, anti-Wnt-5a or anti-β-catenin antibody (Fig. 2a-i).

Expression of Wnt-5a was elevated in higher grade glioma (Fig. 2d-f). There was a significant positive relationship between clinical grading and Wnt-5a staining positivity (Table 2, $P<0.05$) in accordance with previous report.[28] The stainings of β-catenin were weak or

localized mainly to the plasma membrane. Although the stainings of β-catenin were slightly enhanced in accordance with clinical grades, apparent cytosolic or nuclear accumulation of β-catenin-staining were not observed in these cases (Fig. 2g-i).

Furthermore, we found an exceptional case of giant cell glioblastoma (grade IV) without infiltration, Wnt-5a positivity nor nuclear β-catenin accumulation (Fig. 2j-m). Generally, giant cell glioblastoma cases showed atypical growth with clear margin between tumors and normal tissues, and could have potency to have different characterisitics and genetical backgrounds from normal type glioblastomas.

It was reported that clinical grades of glioma correlated with infiltrative activity. [29] Taken together, we hypothesized the possibility that Wnt-5a is involved in regulating not only of cell growth, as previously reported, [28] but also of infiltration in human glioma cells through β-catenin-independent pathways.

Response of U251 glioma cells to Wnt-5a protein. Wnt-5a CM stimulated the phosphorylation of Dvl of U251 cells in the presence of 2% (v/v) FBS as well as Wnt-3a CM (Fig. 3a). Wnt-3a could stabilize the soluble fraction of β-catenin but Wnt-5a could not stabilize β-catenin (Fig. 3b). These data suggested that the canonical Wnt/β-catenin pathway was present in U251 cells and that Wnt-5a did not stimulate this pathway. Transfection of dsRNA for Wnt-5a into U251 cells resulted in 75% knockdown of the expression of Wnt-5a (Fig.

3c). Similar efficiency of Wnt-5a knockdown were performed in U87MG, U105 and G24M cells (data not shown). We also examined the cell proliferation of U87MG, U105, U251, and G24M cells in a condition with or without knockdown of Wnt-5a. No significant differences in cell growth were observed by Wnt-5a knockdown up to 96 h (Fig. 3d).

Wnt-5a-dependent cellular migration of glioma cells. To analyze the role of Wnt-5a in glioma cells, we performed wound healing and transwell assays. When Wnt-5a was knocked down in U251 cells, the cells were allowed to migrate in scratch-wound cultures for 12 h. The migration of Wnt-5a knockdown U251 cells was retarded in comparison with that in control experiments (Fig. 4). Furthermore, purified Wnt-5a protein stimulated the migration of U251 cells in scratch-wound cultures (Fig. 5). When Wnt-5a was knocked down in U87MG, U105, U251, and G24M cells, migration of cells was reduced in comparison with the control cells (Fig. 6a,b). In contrast, when purified Wnt-5a protein was added to the lower chamber, migration of U87MG, U105, U251, G5M, and G24M cells was stimulated (Fig. 7a,b). Taken together, these results suggested that Wnt-5a is associated with the cell motility of glioma cells.

Wnt-5a-dependent MMP-2 induction and invasion activity of human glioblastoma cells. Several gene families including Wnts and MMPs are reported to be associated with aggressiveness of cancer. Previous reports indicated the existence of MMP-2

and -9 activity in glioblastoma.[30] It was reported that MMP-2 is extremely upregulated in malignant gliomas and correlated with the malignant progression of human gliomas in vivo.

To estimate the role of Wnt-5a in the induction of MMPs, we transfected dsRNA for Wnt-5a into U251 cells. The expression and activity of MMP-2 were down-regulated in response to knockdown of Wnt-5a. Gelatinase activity due to MMP-9 (90K) was not detected (Fig. 8a,b). Reciprocally, MMP-2 activity was increased by treatment of purified Wnt-5a (Fig. 8c). Expressions of MMP-1 and -9 were low and MMP-13 was not detected (data not shown) in U251 cells with/without treatment of purified Wnt-5a in comparison with that for MMP-2.

These findings suggested that Wnt-5a stimulated expression of MMP-2 but not MMP-1, -9, or -13 in terms of their transcription level.

Then, we performed matrigel invasion assays. Invasion of Wnt-5a knockdown U87MG, U105, U251, and G24M cells were reduced in comparison with the control cells, while treatment with purified Wnt-5a protein stimulated the invasion of U87MG, U105, U251, G5M, and G24M cells (Fig. 9a, b, Fig.10a, b).

Treatment with MMP-2 inhibitor suppressed the Wnt-5a-dependent invasive activity of U251 cells (Fig. 11).

These results suggested that Wnt-5a stimulated invasive activity of glioblastoma cells through MMP-2.

Discussion

Glioma is one of the commonly diagnosed brain tumors. Surgical therapy of glioma is difficult owing to its infiltration into surrounding neuronal tissue. Although mutations in glioma have been well studied, most of them are correlated with gene instability, cell cycle regulation, and abnormal cell growth.[2]

MMPs are zinc-containing endopeptidases that mediate cell surface-associated dissociation and degradation of the extra-cellular matrix (ECM); this could cause differences in morphogenic tissue interactions during the growth, invasion, metastasis of malignant tumors, and tumor-induced angiogenesis.[31]

MMP-2 and -9 were reported to be expressed in glioma and thought to be important for the invasive characteristics of glioma.[32] Activities of MMPs were suppressed by the tissue inhibitor of metalloproteinase (TIMP) family, soluble inhibitors of MMP, and TIMPs are released from MMPs in response to biological events such as inflammation, ECM turnover, tissue remodeling, and cell behavior.[33] Other potential biochemical functions of the TIMPs have also been elucidated, including a role for TIMP-2 in binding to membrane type-1 MMP (MMP-14) on cell surfaces, which is critical for activation of proMMP-2.[34] The molecular mechanisms that induce activation or production of MMPs remained to be clarified.

In this study, we observed predominant expression of Wnt-5a in glioma cells. Wnt-5a

showed high expression in 30% of 237 gastric cancer cases and 28% of 98 prostate cancer cases.[14,25] Reportedly, Wnt-5a stimulated proliferation of human glioblastoma cells and immunohistochemical studies have shown a correlation between glioma stages and Wnt-5a positivity.[28] We also found a positive correlation between tumor grading and Wnt-5a staining in 33 human glioma cases (Table 2). β-catenin is known to accumulate in cytosol or nuclei of cancer cells.[28] However, histological analyses also revealed that β-catenin was not overexpressed or accumulated in glioma cells in this study.

Since several reports mentioned the relationship Wnt/β-catenin pathways and medulloblastoma[35] or glioblastoma,[36,37] neuro-oncologists seemed interested in this issue. We have shown that U251, U87MG, and U105 cells (data not shown) did not accumulate cytosolic or nuclear β-catenin in the endogenous level and in response to Wnt-5a, and focused our analyses on β-catenin-independent role of Wnt-5a in the glioma cells.

We found no publication in Pubmed database using keyword of "Wnt-5a and giant cell glioma" and only one publication in Pubmed database using keyword of "Wnt and giant cell glioma".[38] Their report was about giant cell astrocytoma concomitant with hereditary disease, and should not be applied to other sporadic giant cell glioblastoma cases. Although the expression or physiological function of Wnt-5a and β-catenin expression remain to be explored, our giant glioblastoma case (grade IV) with low staining of Wnt-5a and invasion prompted us

to study a role of Wnt-5a in the cell motility and inlfiltrative activity of glioma cells.

Wnt-5a is reported to have tumor suppressive activity [16,17] and oncogenic activity, as well as to regulate several cellular responses including convergent extension, cell polarity, inhibition of β-catenin signaling cell motility, and axonal repulsion.[9,11-15,25] Wnt-1 and -3a activate MMP expressions and migration through the canonical β-catenin pathway in T cells.[39] Up-regulation of MMP-2 in human glioma has been reported and suggested to deteriorate tumor invasiveness.[32] However, it was not clear whether other Wnts could induce MMP production.

In this study, we have shown that knockdown of Wnt-5a reduced cell motility and invasion activity of glioblastoma cells. The knockdown of Wnt-5a resulted in mild decrease of MMP-2 mRNA level and strong suppression of the MMP-2 activity. MMP-2 activity is thought to be dependent not only on the amount of mRNA, but also on the speed of translation of mRNA, protein degradation, and secretion of MMP-2s, and with its activation by being released from TIMPs. TIMPs are inhibitor familiy of MMPs. We could not rule out the possibility that knockdown of Wnt-5a influences these steps in a complicated manner not by suppressing only MMP-2 mRNA expression. Judging from the result that MMP-2 inhibitor suppressed the Wnt-5a-dependent invasion activity of U251 cells, Wnt-5a could regulate infiltration activity of glioblastoma cells through MMP-2 activity.

Wnt-5a is known to bind Fz-2, -5, -8, and Ror2 but not Fz-6 and -7.[40,41] Yamamoto *et al*. reported that binding of Wnt-5a to Fz-2 or Ror2 induces MMP-1 expressions by recruitment of JunD to the AP-1 binding site of the promoter region of the *MMP-1* gene through the activation of PKC and JNK in prostate cancers and that Wnt-5a promoted cell motility through activation of Rac.[25] Minami *et al*. have shown that Wnt-5a promotes MMP-13 transcription through Ror2 in sarcoma cells.[42]

In this study, elevated expressions of Fz-2, -6, and -7 were observed but no remarkable expressions of Ror2, MMP-1, -9, and -13 (data not shown) were detected in human glioma cell lines.

Fz-2 was reported to transduce Wnt-5a signals, while Fz-6 and -7 did not.[40,41] These reports make us speculate that Wnt-5a might promote MMP-2 by activation of PKC and JNK through Fz-2, and that Wnt-5a might increase cell motility through activation of Rac in U251 glioblastoma cells. Besides Fz-2 and Ror-2, RYK is known to mediate Wnt-5a signals for axon guidance.[43] However, the role of RYK is still unknown in glioma. Given that infiltration is the major therapeutic problem of the gliomas, Wnt-5a-mediated pathway could not only be a marker for prognosis but also a therapeutic target in glioma.

In this study, we analyzed the β-catenin-independent role of Wnt-5a in invasion of glioma. Further studies will be necessary to understand the physiological and pathological roles of

Wnt-5a in glioma cells.

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FIGURE LEGENDS

Fig. 1. The mRNA expression of core components of Wnt pathways. (a) The mRNA levels of various Wnts in U87MG, U105, U251, G5M, and G24M cells were quantified by real-time RT-PCR. (b) The mRNA levels of various Fz components in the cells were quantified by real-time RT-PCR. Relative mRNA levels were expressed as proportions of the mRNA levels of various Wnts or Fzs in normal human brain tissues.

Fig. 2. Imaging analyses of Wnt-5a and β-catenin in glioma. Representative images of glioma cases and human brain tissue. (a,d,g) Normal human brain tissue; (b,e,h) astrocytoma (gradeII); (c,f,i) glioblastoma (grade IV); (j-m) giant cell glioblastoma (grade IV). (a-c,k) Normal human brain tissue or glioma regions stained by hematoxylin and eosin. (d-f,l) Microscopic images stained by anti-Wnt-5a antibody; (g-i, m) microscopic images stained by anti-β-catenin antibody. (j) Computer tomography image. Bar, 50 μm (a-i, l, m); 200 μm, (k). Arrow indictaed the margin between giant cell glioblastoma tissue and normal neuronal tissue. Insets, high-magnification images of the fields indicated by the arrowheads. Note that some cells showing Wnt-5a antibody-reactive staining in Fig 2d were small blood vessels.

Fig. 3. Activity of Wnt-5a. (a) Phosphorylation of Dvl stimulated by Wnt-5a CM. After U251 cells were treated with various concentrations of control (lanes 1,2), Wnt-3a (lanes 3,4), or Wnt-5a (lanes 5,6) CM for 1 h, the lysates were subjected to western blotting and probed with the indicated antibodies. p-Dvl, phosphorylated Dvl. (b) Accumulation of soluble β-catenin induced by Wnt-3a but not by Wnt-5a. After U251 cells were treated with Control, Wnt-3a, or Wnt-5a CM for 6 h, the lysates were subjected to the procedure described above. The blotting of GSK-3β was used as a loading control. (c) Efficiency of Wnt-5a knockdown. U251 cells were transfected with Wnt-5a or scramble dsRNA. After 48 h, the amounts of Wnt-5a mRNA were analyzed by real-time RT-PCR. The results shown are means ± s.e. from five independent experiments. $*P < 0.05$. (d) Effect of Wnt-5a knockdown on the growth of glioblastoma cells. Squares, U87MG cells; triangles, U105 cells; circles, U251 cells; daiamond, G24M cells. Open characters, scramble dsRNA transfected; closed characters, Wnt-5a knockdown.

Fig. 4. Effect of Wnt-5a knockdown on glioma motility. (a) Inhibition of cell migration by Wnt-5a dsRNA. U251 cells were transfected with scramble or Wnt-5a dsRNA for 72 h and then the cells were wounded. The culture was continued for 12 h. Bars, 400 μm. (b) Migration distances were measured and are expressed as percentages of the

migration transfected with scramble dsRNA at 12 h. The results shown are means ± s.e. from at least five independent experiments. $^{**}P < 0.01$.

Fig.5. Wnt-5a-dependent cell motility in glioma cells. (a) Stimulation of cell motility by Wnt-5a (500ng/ml) treatment. U251 cells treated with growth medium containing control buffer or Wnt-5a were wounded. The culture was continued for 12 h. Bars, 400 μm. (b) Migration distances were measured and are expressed as percentages of the migration in the presence of control buffer at 12 h. The results shown are means ± s.e. from seven independent experiments. $^{**}P < 0.01$.

Fig. 6. Wnt-5a is involved in migration of U87MG, U105, U251, and G24M glioblastoma cells. (a) Inhibition of cell migration by Wnt-5a knockdown. The cells were allowed to migrate for 2.5 h. These images were representative. Bars, 100 μm. (b) Relative migration activities of U251 glioblastoma cells in Fig. 6a are expressed as proportions of migration of control cells. The results shown are means ± s.e. from five independent experiments. $^{**}P < 0.01$.

Fig. 7. Wnt-5a is involved in migration of U87MG, U105, U251, G24M, and G5M glioblastoma cells. (a) Stimulation of cell migration by Wnt-5a (500ng/ml) treatment. The cells were allowed to migrate for 2.5 h. These images were representative. Bars, 100 μm. (b) Relative migration activities of U251 glioblastoma cells in Fig. 7a are expressed as proportions of migration of control cells. The results shown are means ± s.e. from five independent experiments. $^{**}P < 0.01$.

Fig. 8. Wnt-5a-dependent MMP-2 activity in U251 glioblastoma cells (a) The decrease of mRNA levels of MMP-2 in Wnt-5a-knockdown U251 cells were quantified by real-time RT-PCR. The results shown are means ± s.e. from five independent experiments. $^{*}P < 0.05$. (b) In-gel-gelatinase assay. Membranous vesicles of CM (1.3 or 2.6 μg of protein) from control or Wnt-5a-knockdown U251 cells were subjected to in-gel-gelatinase assay. Arrow indicates the position of MMP-2 activity. (c) Effect of Wnt-5a (500ng/ml) treatment to mRNA level of MMP-1, -2, and -9. White bars indicated each mRNA levels in normal human brain as controls. Shaded bars, untreated U251 cells; black bars, Wnt-5a treated U251 cells.

Fig. 9. Wnt-5a is involved in invasion of glioma cells. (a) Invasion activity of U87MG, U105, U251, and G24M cells by Wnt-5a. The cells were allowed to invade in Wnt-5a knockdown condition. The cells were allowed to invade for 24-36 h. These images were representatives. Bars, 100 μm. (b) Relative invasion activities of Wnt-5a-knockdown U251 glioblastoma cells are expressed as proportions of invasion of control cells. These results shown are means ± s.e. from four independent experiments. *$P < 0.05$.

Fig.10. Wnt-5a is involved in invasion of glioma cells. (a) The cells were allowed to invade in Wnt-5a (500ng/ml) treated condition. The cells were allowed to invade for 12-36 h. These images were representatives. Bars, 100 μm. (b) Relative invasion activities of Wnt-5a-treated U251 glioblastoma cells are expressed as proportions of invasion of control cells. These results shown are means ± s.e. from five independent experiments. **$P < 0.01$.

Fig.11. MMP-2-dependent stimulation of invasion activity by Wnt-5a treatment in U251 cells. The cells were allowed to invade for 24 h. Invasion activity was measured under the similar conditions as Fig. 10b with or without MMP-2 inhibitor (50nM). The results shown are means ± s.e. from four independent experiments. *$P < 0.05$.

Table 1. Primer sets used in this study for real-time RT-PCR.

Wnt1-#81-1F	5’-cgctggaactgtcccact-3’
Wnt1-#81-2R	5’-aacgccgtttctcgacag-3’
Wnt2-#4-1F	5’-ccagagccctgatgaatctt-3’
Wnt2-#4-2R	5’-ttgtttcaagaaccgcttaca-3’
Wnt2b-#34-1F	5’-gccgtgtcatgctcagaa-3’
Wnt2b-#34-2R	5’-gtggactacccctgctgatg-3’
Wnt3-#81-1F	5’-ctcgctggctacccaattt-3’
Wnt3-#81-2R	5’-gcccagagatgtgtactgctg-3’
Wnt3a-#5-1F	5’-catgaaccgccacaacaac-3’
Wnt3a-#5-2R	5’-tggcacttgcacttgaggt-3’
Wnt4-#4-1F	5’-gcagagccctcatgaacct-3’
Wnt4-#4-2R	5’-cacccgcatgtgtgtcag-3’
Wnt5a-#48-1F	5’-attgtactgcaggtgtaccttaaaac-3
Wnt5a-#48-2R	5’-ccccctataaatgcaactgttc-3’
Wnt5b-#56-1F	5’-ctgctgctgctgttcacg-3’
Wnt5b-#56-2R	5’-caccgggttcaaagctaatg-3’
Wnt6-#5-1F	5’-cagttccagttccgcttcc-3’
Wnt6-#5-2R	5’-gaacacgaaggccgtctc-3’
Wnt7a-#75-1F	5’-cttcgggaaggagctcaaa-3’
Wnt7a-#75-2R	5’-gcaatgatggcgtaggtga-3’
Wnt7b-#4-1F	5’-cgcctcatgaacctgcata-3’
Wnt7b-#4-2R	5’-gctgcatccggtcctcta-3’
Wnt8a-#75-1F	5’-gggtcaaacaatggaaaaaca-3’
Wnt8a-#75-2R	5’-tccccaaattccacattgtc-3’
Wnt8b-#75-1F	5’-tgtgatgactcccgcaac-3’
Wnt8b-#75-2R	5’-tccgaagcccacattgtc-3’
Wnt9a-#8-1F	5’-tccagttccgctttgagc-3’
Wnt9a-#8-2R	5’-agccgaggagatggcatag-3’
Wnt9b-#55-1F	5’-ccccaaccttgttgaggac-3’
Wnt9b-#55-2R	5’-tcccttatttccctccatgtc-3’
Wnt10a-#16-1F	5’-atccacgcgagaatgagg-3’
Wnt10a-#16-2R	5’-ccgcatgttctccatcact-3’
Wnt10b-#27-1F	5’-aatgcgaatccacaacaaca-3’
Wnt10b-#27-2R	5’-tccagcatgtcttgaactgg-3’
Wnt11-#81-1F	5’-tgtgctatggcatcaagtgg-3’
Wnt11-#81-2R	5’-cagtgttgcgtctggttcag-3’
Wnt16-#8-1F	5’-caatgaacctacataacaatgaagc-3’
Wnt16-#8-2R	5’-cagcggcagtctactgacat-3’

Fz1-#69-1F	5’-cggcaagaccctcaactc-3’
Fz1-#69-2R	5’-ccttgtttgctgttggtgag-3’
Fz2-#1-1F	5’-ggtgtcggtggcctacat-3’
Fz2-#1-2R	5’-gagaagcgctcgttgcac-3’
Fz3-#75-1F	5’-acagcaaagtgagcagctacc-3’
Fz3-#75-2R	5’-ctgtaactgcagggcgtgta-3’
Fz4-#19-1F	5’-ttcacaccgctcatccagta-3’
Fz4-#19-2R	5’-tgcacattggcacataaaca-3’
Fz5-#2-1F	5’-tggagctgcgctaatcct-3’
Fz5-#2-2R	5’-ttggtgtgtgatccatgagg-3’
Fz6-#23-1F	5’-tgggttggaagcaaaaagac-3’
Fz6-#23-2R	5’-tcttcgactttcactgattgga-3’
Fz7-#54-1F	5’-gccagcttgtgcctaatagaa-3’
Fz7-#54-2R	5’-agccgggagaaactcacag-3’
Fz8-#18-1F	5’-cgctggtcatctacctcttca-3’
Fz8-#18-2R	5’-gatgcggaacagggacac-3’
Fz9-#44-1F	5’-cctgcatcccctagagacag-3’
Fz9-#44-2R	5’-tcacttgcctgaccttgaca-3’
Fz10-#25-1F	5’-ttggttttccagcgaagg-3’
Fz10-#25-2R	5’-ccacaaattagttacacaagaggcta-3’
MMP1-#7-1F	5’-gctaacctttgatgctataactacga-3’
MMP1-#7-2R	5’-tttgtgcgcatgtagaatctg-3’
MMP2-#70-1F	5’-ataacctggatgccgtcgt-3’
MMP2-#70-2R	5’-aggcacccttgaagaagtagc-3’
MMP9-#6-1F	5’-gaaccaatctcaccgacagg-3’
MMP9-#6-2R	5’-gccacccgagtgtaaccata-3’
MMP13-#73-1F	5’-ccagtctccgaggagaaaca-3’
MMP13 #73-2R	5’-aaaaacagctccgcatcaac-3’
Ror2-#17-1F	5’-cccctcattaaccagcacaa-3’
Ror2-#17-2R	5’-ttcccaaaccggtcctct-3’
β-actin-#64-1F	5’-ccaaccgcgagaagatga-3’
β-actin-#64-2R	5’-ccagaggcgtacagggatag-3’

Numbers included in the primer name refer to the name of Universal Probe (Roche Diagnostics) used for real-time RT-PCR.

Table 2. Relationship between WHO stage and Wnt-5a protein expression score in human glioma cases.

	Grade II	Grade III	Grade IV	subtotal
score 0	5 (15%)	4 (12%)	2 (9%)	11 (33%)
score 1	1 (3%)	2 (6%)	0 (0%)	3 (9%)
score 2	2 (6%)	5 (15%)	2 (6%)	9 (27%)
score 3	1 (3%)	1 (3%)	8 (24%)	10 (30%)
total	9	12	12	33

Statistical significance was analyzed by linear-by-linear association test (exact *P* value, two-sided, $P < 0.05$).

Fig. 1.

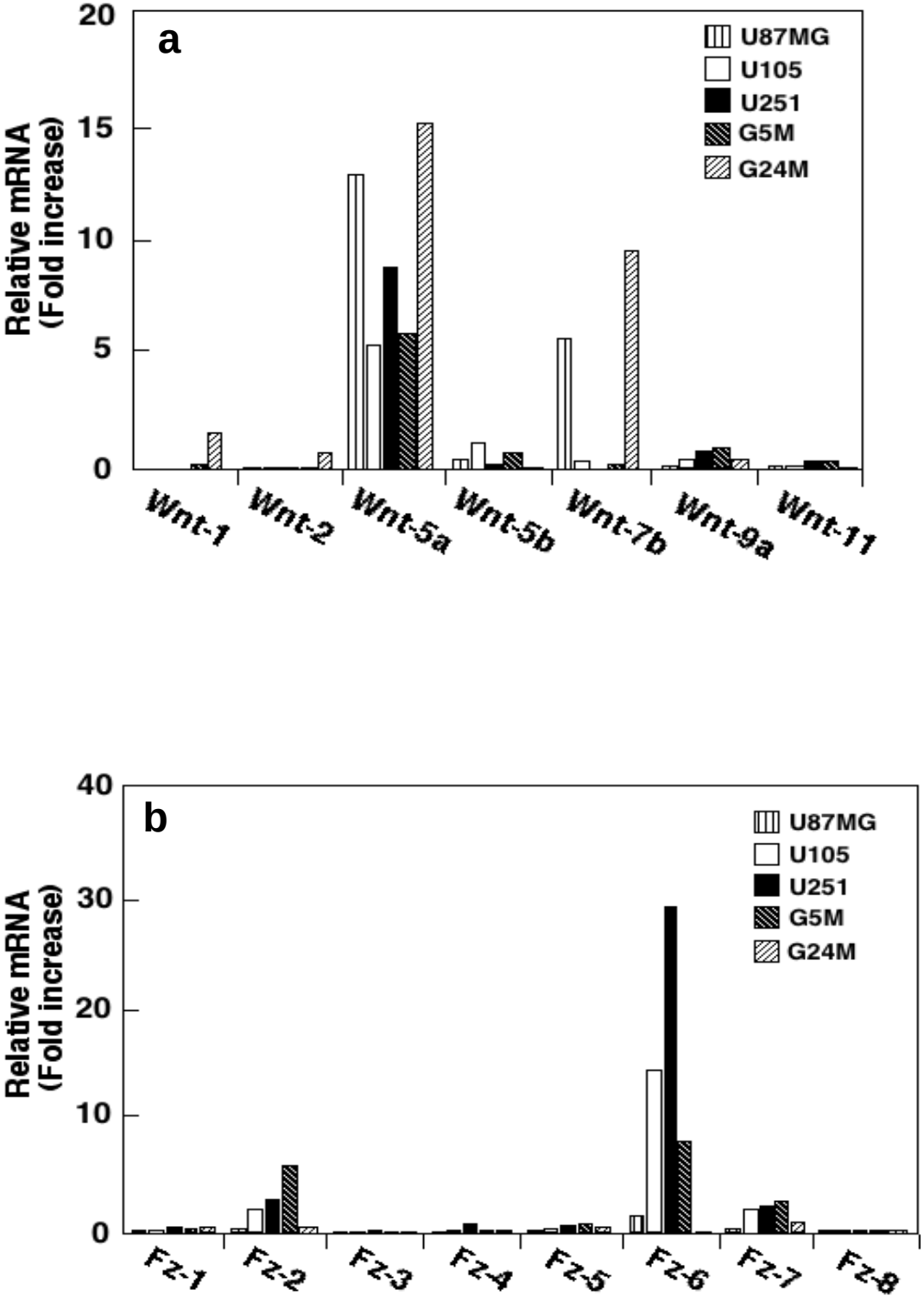
a
U87MG
U105
U251
G5M
G24M
Relative mRNA (Fold increase)
20
15
10
5
0
Wnt-1
Wnt-2
Wnt-5a
Wnt-5b
Wnt-7b
Wnt-9a
Wnt-11
b
U87MG
U105
U251
G5M
G24M
Relative mRNA (Fold increase)
40
30
20
10
0
Fz-1
Fz-2
Fz-3
Fz-4
Fz-5
Fz-6
Fz-7
Fz-8

Fig. 2.

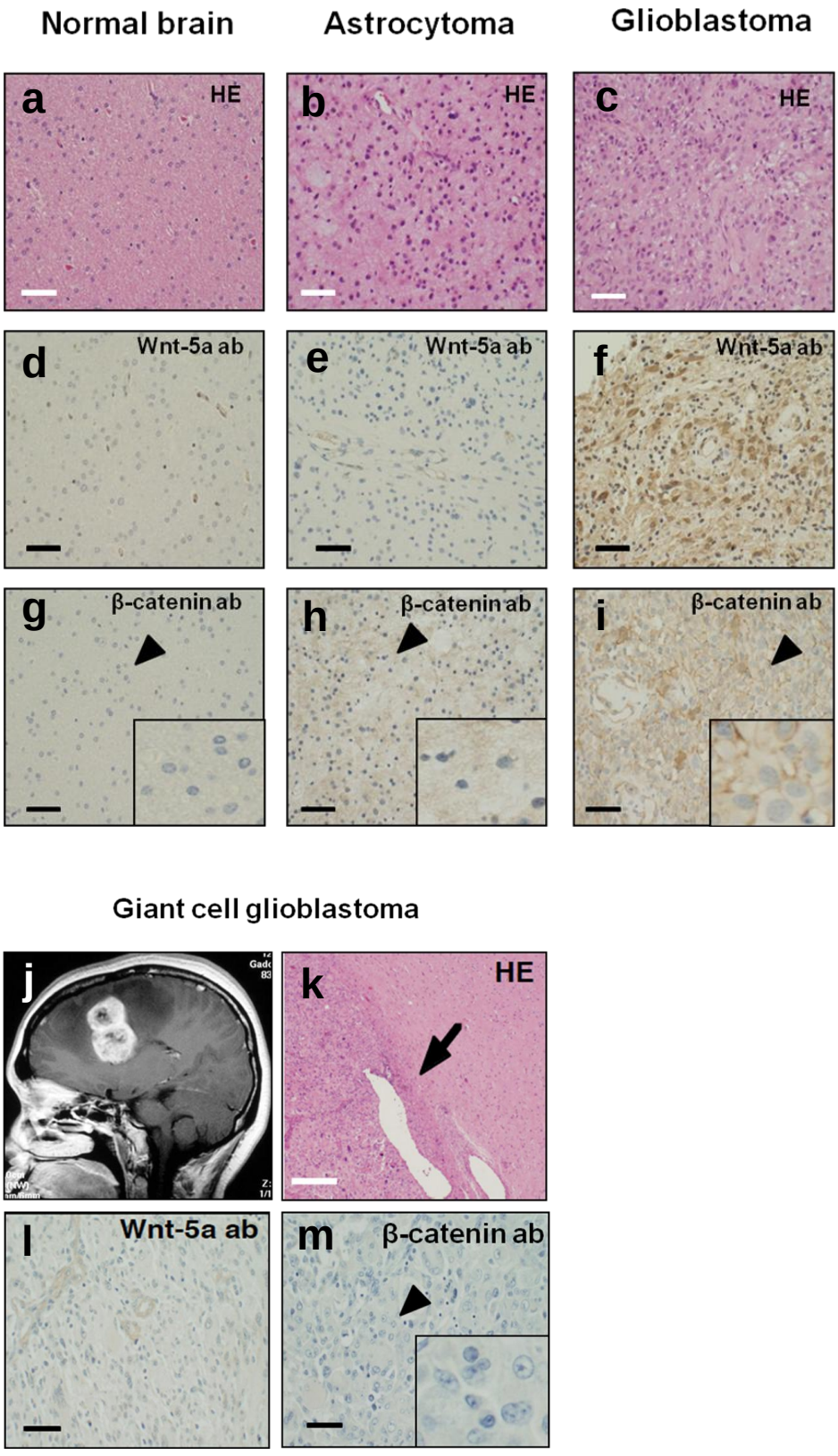
Normal brain
Astrocytoma
Glioblastoma
a
HE
b
HE
c
HE
d
Wnt-5a ab
e
Wnt-5a ab
f
Wnt-5a ab
g
β-catenin ab
h
β-catenin ab
i
β-catenin ab
Giant cell glioblastoma
j
k
HE
l
Wnt-5a ab
m
β-catenin ab

Fig. 3.

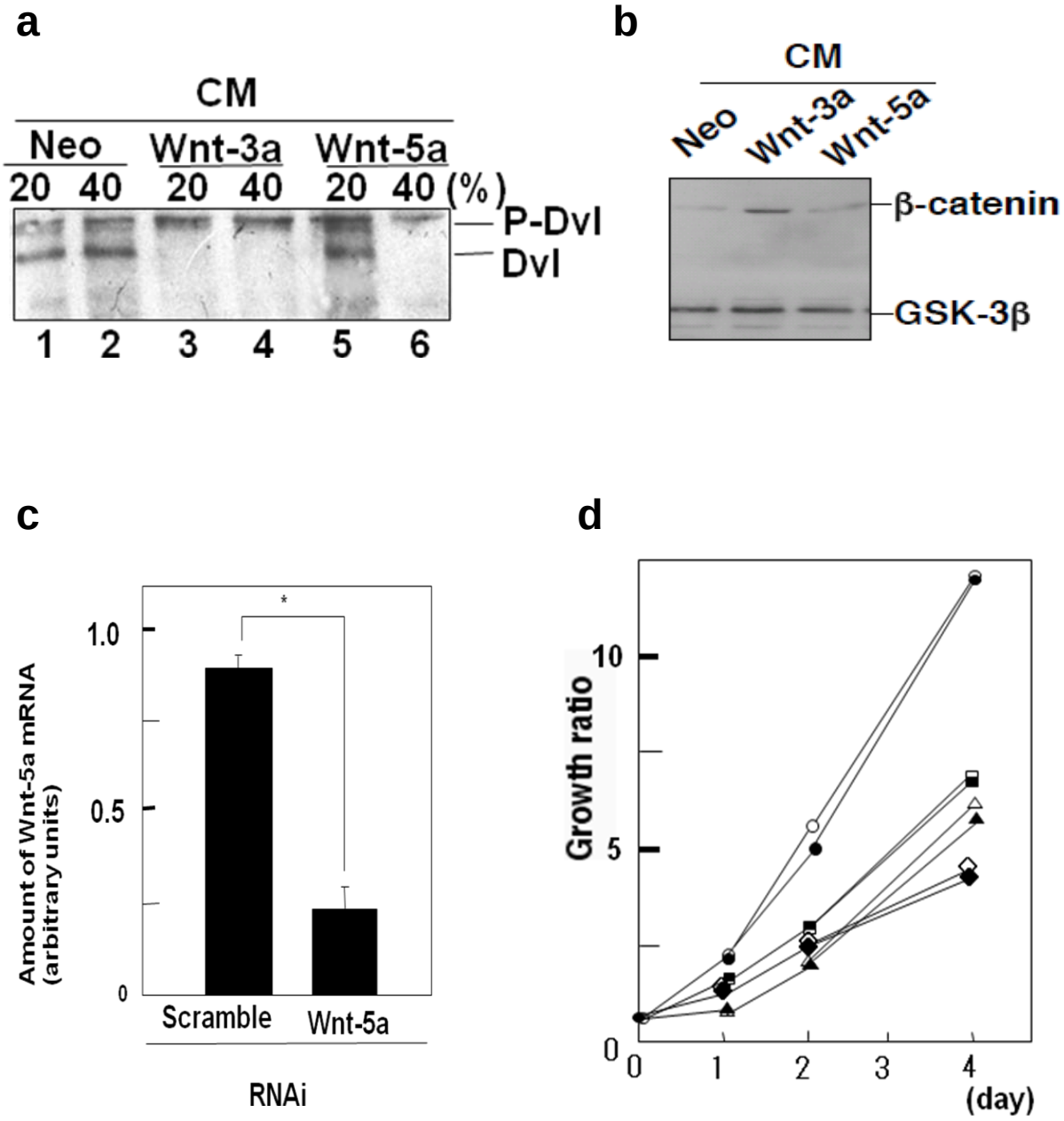
a
CM
Neo
Wnt-3a
Wnt-5a
20 40 20 40 20 40 (%)
P-Dvl
Dvl
1 2 3 4 5 6
b
CM
Neo
Wnt-3a
Wnt-5a
β-catenin
GSK-3β
c
Amount of Wnt-5a mRNA (arbitrary units)
1.0
0.5
0
*
Scramble
Wnt-5a
RNAi
d
Growth ratio
10
5
0
0 1 2 3 4
(day)

Fig. 4.

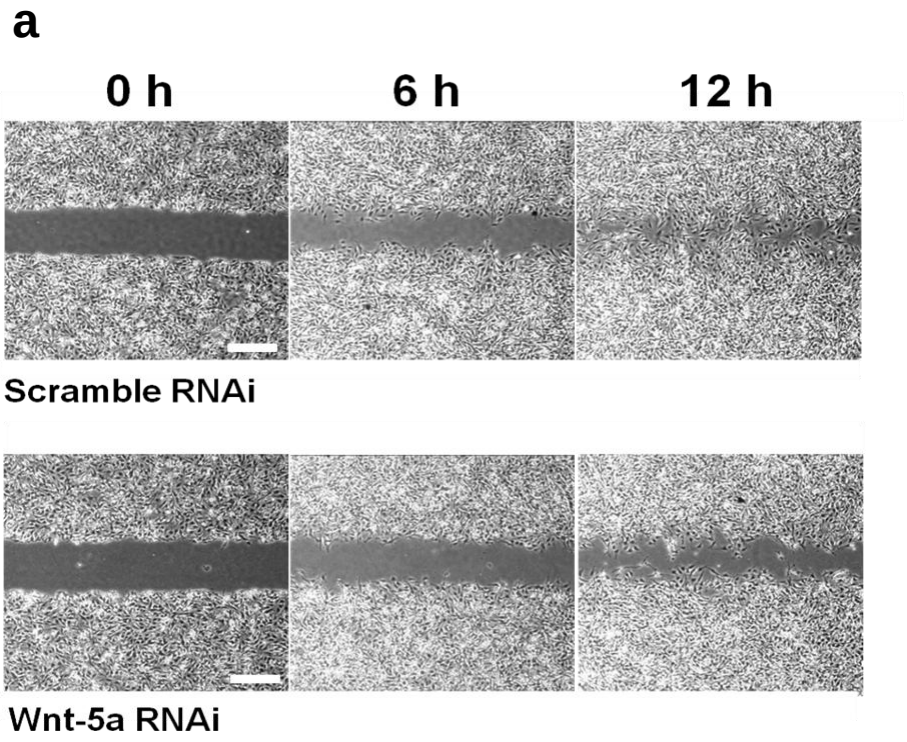
a
0 h
6 h
12 h
Scramble RNAi
Wnt-5a RNAi
b
**
Relative distance(%)
100
50
0
Scramble
Wnt-5a
RNAi

Fig. 5.

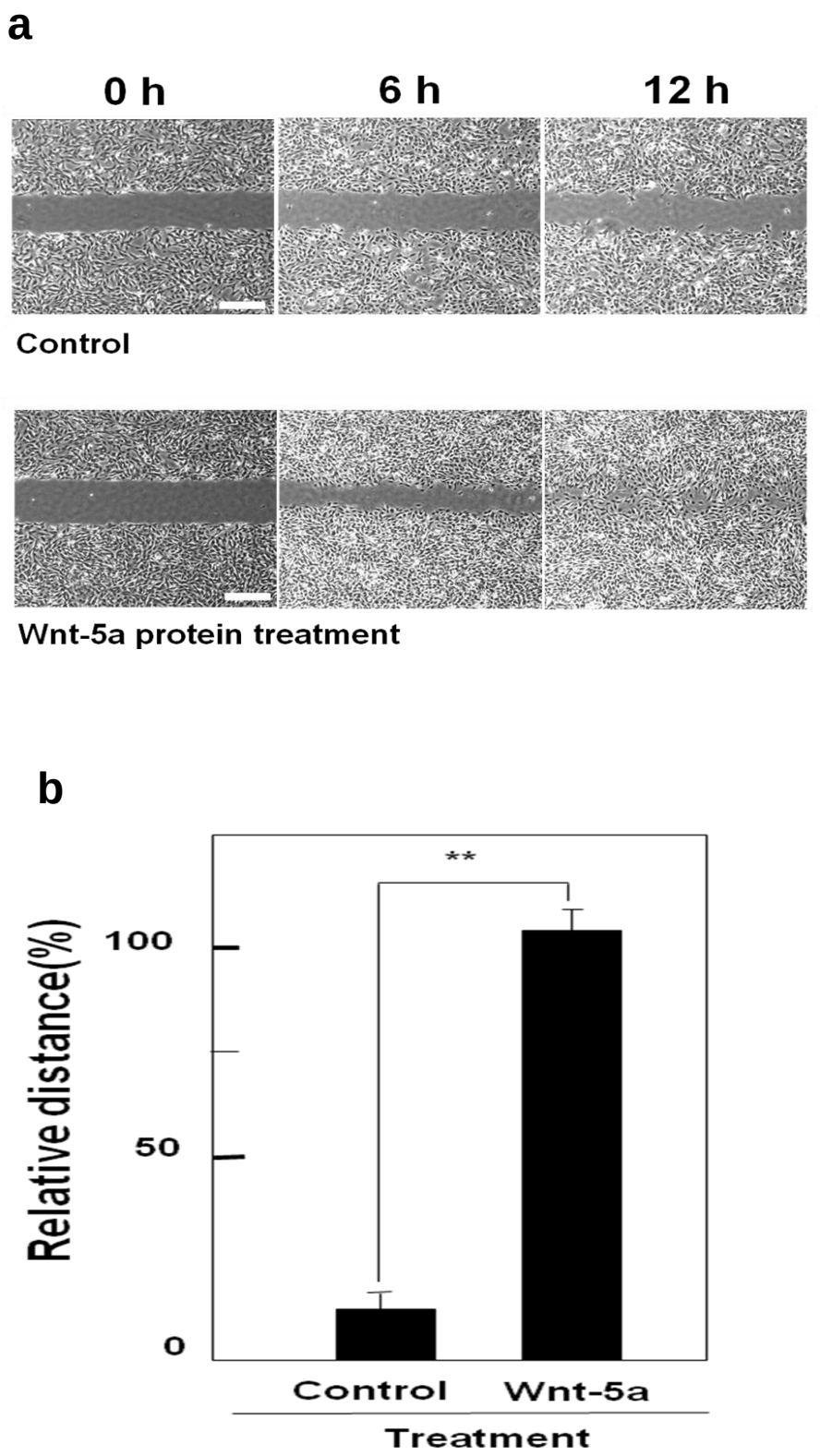
a
0 h
6 h
12 h
Control
Wnt-5a protein treatment
b
**
Relative distance(%)
100
50
0
Control
Wnt-5a
Treatment

Fig. 6.

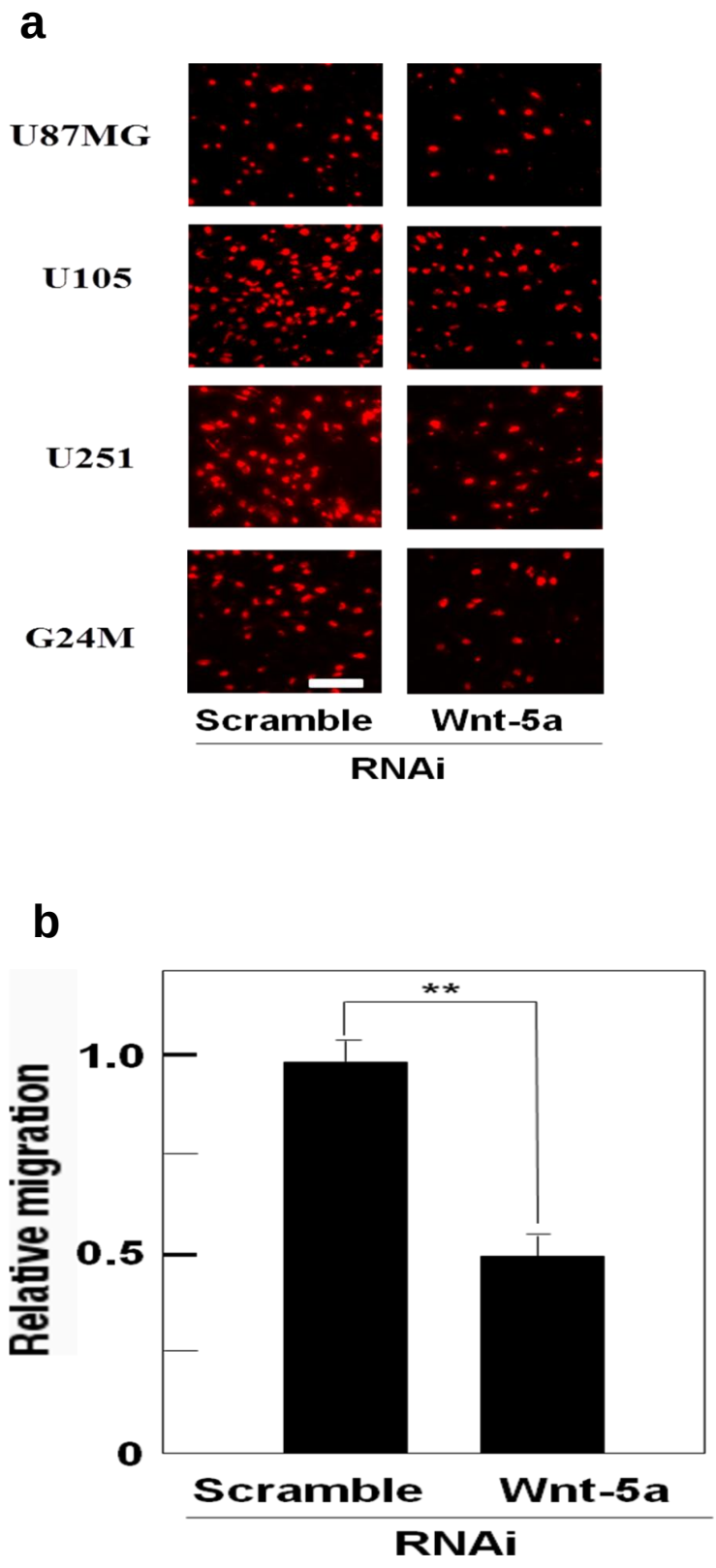
a
U87MG
U105
U251
G24M
Scramble
Wnt-5a
RNAi
b
Relative migration
1.0
0.5
0
**
Scramble
Wnt-5a
RNAi

Fig. 7.

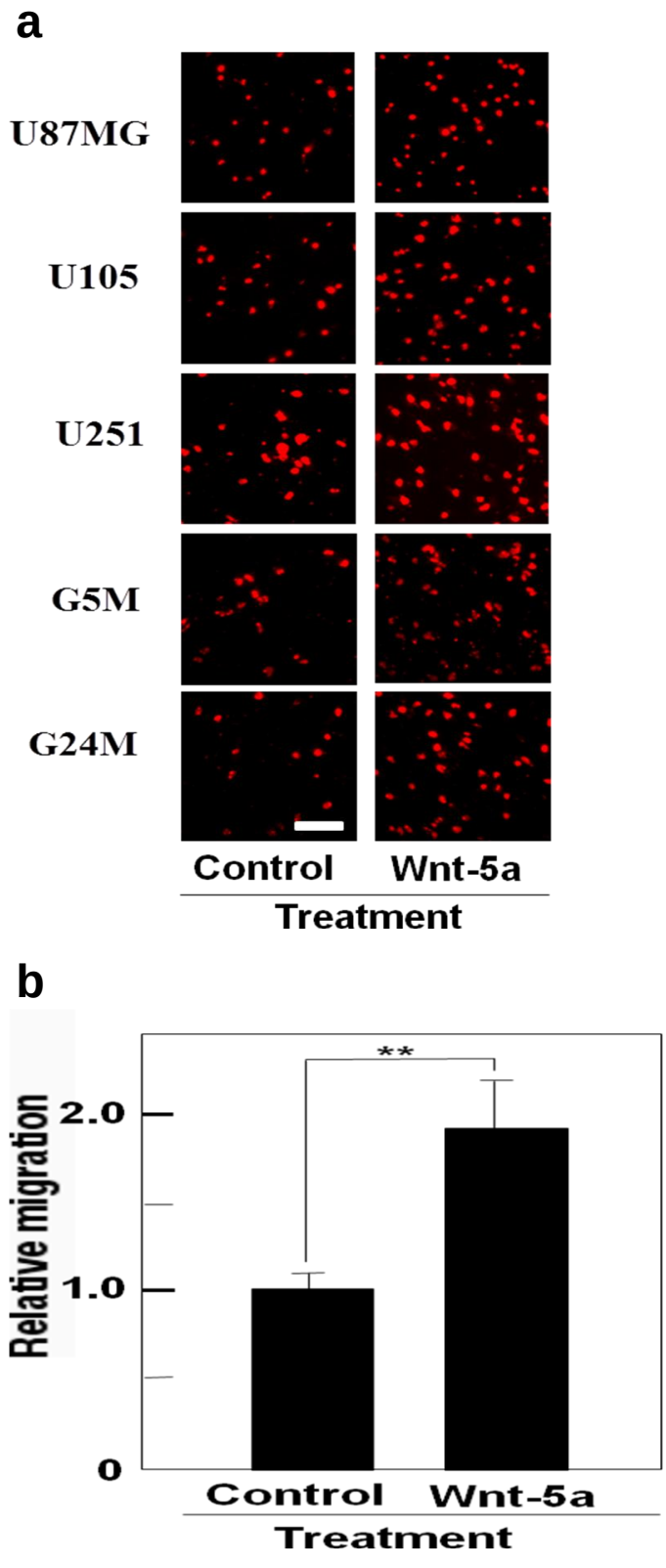
a
U87MG
U105
U251
G5M
G24M
Control
Wnt-5a
Treatment
b
**
Relative migration
2.0
1.0
0
Control
Wnt-5a
Treatment

Fig. 8.

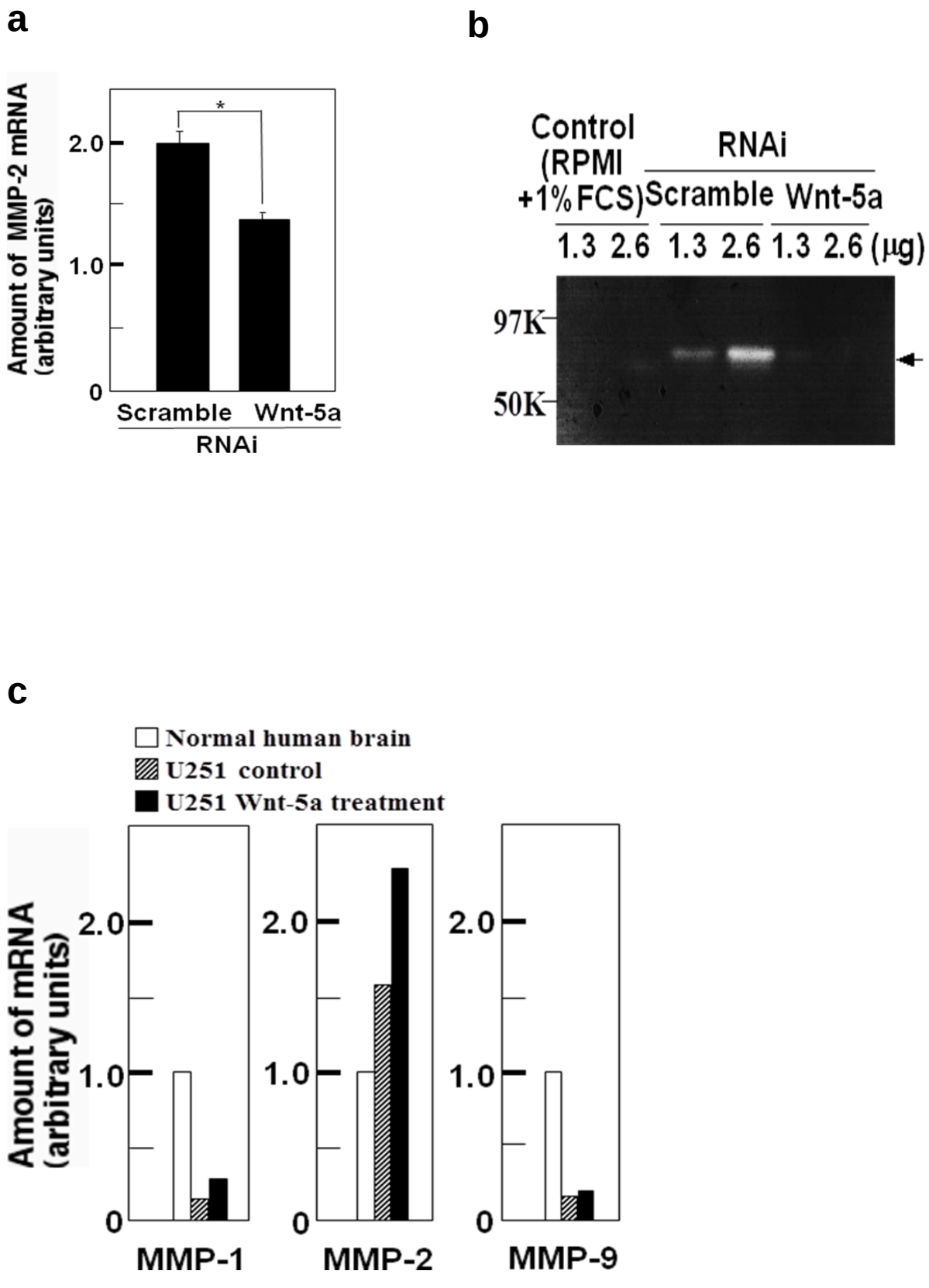
a
Amount of MMP-2 mRNA (arbitrary units)
2.0
1.0
0
*
Scramble
Wnt-5a
RNAi
b
Control (RPMI +1% FCS)
RNAi
Scramble
Wnt-5a
1.3 2.6 1.3 2.6 1.3 2.6 (μg)
97K
50K
c
Normal human brain
U251 control
U251 Wnt-5a treatment
Amount of mRNA (arbitrary units)
2.0
1.0
0
MMP-1
MMP-2
MMP-9

Fig. 9.

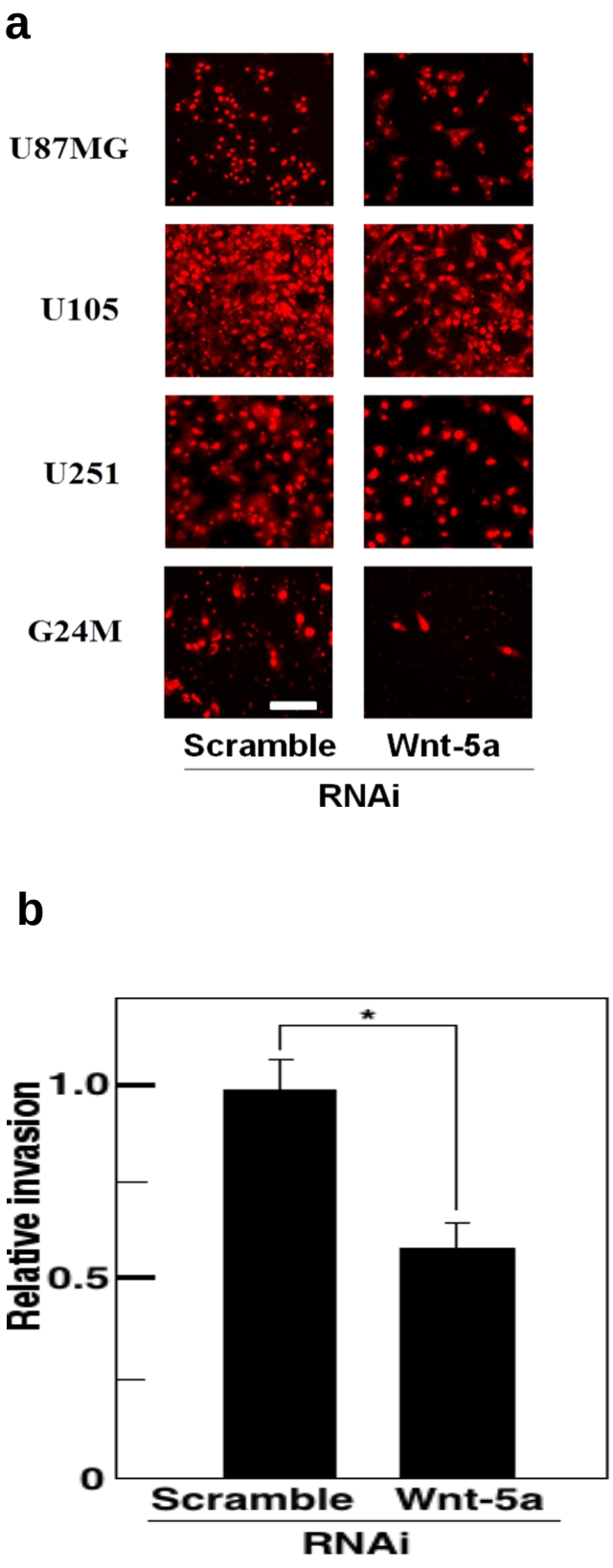
a
U87MG
U105
U251
G24M
Scramble
Wnt-5a
RNAi
b
Relative invasion
1.0
0.5
0
*
Scramble
Wnt-5a
RNAi

Fig. 10.

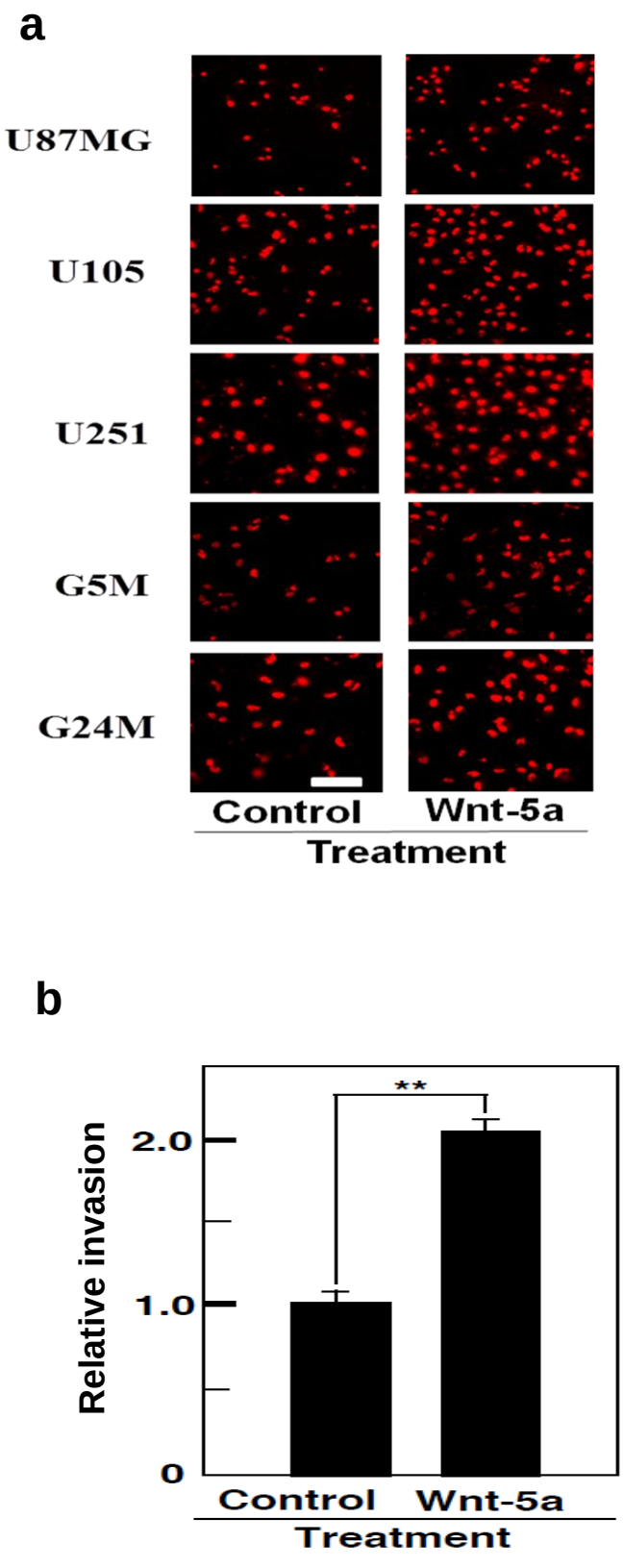
a
U87MG
U105
U251
G5M
G24M
Control
Wnt-5a
Treatment
b
Relative invasion
2.0
1.0
0
**
Control
Wnt-5a
Treatment

Fig. 11.