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Type1 collagen as a potential component of niche for CD133-positive glioblastoma cells

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Running title: Type1 collagen as a niche for GBM

Abstract
Cancer stem cells are thought to be closely related to tumor progression and recurrence, making them attractive therapeutic targets. Stem cells of various tissues exist within niches maintaining their stemness. Glioblastoma stem cells (GSCs) are located to tumor capillaries, perivascular niche, which are considered to have important role in maintaining GSCs. There were some extracellular matrixes (ECM) on the perivascular connective tissue, including type1 collagen. We here evaluated whether type1 collagen has a potential of niche for GSCs. Imunohistochemical staining of type1 collagen and CD133, one of the GSCs markers, on glioblastoma (GBM) tissues showed CD133-positive cells were located in immediate proximity to type1 collagen around tumor vessels. We cultured human GBM cell lines, U87MG and GBM cells obtained from fresh surgical tissues, T472 and T555, with serum-containing medium (SCM) or serum-free medium with some growth factors (SFM) and in non-coated (Non-coat) or type1 collagen-coated

plates(Col). The RNA expression levels of CD133 and Nestin as stem cell markers in each condition were examined. The Col condition not only with SFM but SCM made GBM cells more enhanced the RNA expression of CD133, compared to Non-coat/SCM. Semi-quantitative measurement of CD133-positive cells by immunocytochemistry showed a statistically significant increase of CD133-positive cells in Col/SFM. In addition, T472 cell line cultured in the Col/SFM had Capabilities of sphere formation and tumorigenesis. Type1 collagen was found in the perivascular area and showed a possibility to maintain GSCs. These findings suggest that type1 collagen could be one important component of niche for CD133 positive GSCs and maintain GSCs in adherent culture.

Key words: Glioblastoma, Stem cell, Type1 collagen, Stem cell niche, CD133

Introduction

Glioblastoma (GBM) is one of the most malignant brain tumors, usually causing a fatal outcome within two years despite maximal tumor resection and concurrent radio-chemotherapy. A novel treatment modality must therefore be developed. Recently, the existence of cancer stem-like cells (CSCs) in various tumors, including GBM, was indicated [1-4] and led to new concepts and targets in treatment. Calabrese et al. reported that GSCs were located to tumor capillaries and perivascular niche[5] maintaining brain tumor stem-like cells. There were some extracellular matrixes (ECM) in the perivascular area including type1 collagen[6]. Type1 collagen exists in the parenchyma of the subependymal layer and blood vessels[7] that maintains neural stem cells (NSCs)[8]. We therefore hypothesized that type1 collagen would also maintain and propagate GSCs as well in adherent culture.

Materials and methods

Immunohistochemical assessment of the original tumor tissues

We carried out immunohistochemical experiments on fresh tumor tissues giving origin to primary GBM cell cultures at our institution. These fresh tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Two micrometer serial sections were cut on a microtome and dried overnight at 37°C, followed by dewaxing in xylene. Hematoxylin and eosin (H&E) staining and immunohistochemical staining of the following antibodies were performed on the sections: type1 collagen (I-8H5, MP Biomedicals, Solon, OH; diluted 1:250) and CD133 (W6B3C1, Miltenyi Biotech, Bergisch Gladbach, Germany; diluted 1:50). Dehydrated sections were treated using a pressure cooker with citrate buffer (pH 6.0, 3min). Endogenous peroxidase was blocked with Envison Kit (DAKO, Copenhagen, Denmark). These sections were incubated for 30 minutes at 37°C with antibodies against CD133 or type1 collagen. The primer antibodies were detected with biotin conjugated rabbit anti-mouse (IgG + IgA + IgM) antibody, incubated with avidin peroxidase (Vecstain ABC kit, Vector Labs, Burlingame, CA) for 30 minutes at room temperature and with diaminobenzidene (DAB, Dako, Glostrup, Denmark) for 3 minutes.

Glioblastoma cell lines

The established GBM cell line U87MG, which was obtained from ECACC (Wiltshire, UK), and the primary GBM cell cultures diagnosed according to World Health Organization guidelines, were prepared as approved by the Hokkaido University Hospital Ethical Committee. All tissues were transported in 4°C in Dulbecco's modified Eagle's medium/F-12 medium (DMEM/F12, 1:1; Invitrogen, Life Technologies Corp., Carlsbad, CA, USA).

Resected segments were minced and dissociated enzymatically with 0.5% hyaluronidase (Sigma, St Louis, MO, USA) and 0.05% collagenase (Wako Pure Chemical Industries Ltd, Osaka, Japan) for 30 minutes. Then, cells were filtrated through a 40-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Primary cultures were resuspended in serum-contained medium (SCM; 10% fetal bovine serum in DMEM/F12) or serum-free medium (SFM; DMEM/F12 with human recombinant epidermal growth factor (20ng/ml EGF; Miltenyi Biotec, Bergisch Gladbach, Germany), human recombinant fibroblast growth factor (20ng/ml FGF2; Miltenyi Biotec), leukemia inhibitory factor (10ng/ml LIF; Miltenyi Biotec), and N2 supplement (Invitrogen, Life Technologies Corp., Carlsbad, CA), plated on a non-coated plate (Non-coat; Nunc, Roskilde, Denmark) or a type1 collagen-coated plate (Col; Beckton Dickinson Labware, Bedford, UK). These cultures were incubated in 21% O_2 and 5% CO_2 at 37°C. T472 and T555 cell lines which repeatedly-subcultured from GBM specimens for more than half a year were mainly used and cloning procedure was not performed in sphere formation and tumorigenicity assay.

Reverse transcription-PCR analysis

Total RNA was extracted by RNeasy® Plus Mini Kit (Qiagen, Valencia, CA, USA). Strand complementary DNA (cDNA) was synthesized from 500 ng RNA using PrimeScript™ RTreagent Kit (Takara, Shiga, Japan). Primers as stem-cell marker for semi-quantitative RT-PCR were: CD133 forward 5'-TCACTGAGCACTCTATACCAAAGCG-3' and reverse 5'-TTGCACGATGCCACTTTCTCACTG-3'; human Nestin forward 5'-CTACCAGGAGCGCGTGGC-3' and reverse 5'-TCCACAGCCAGCTGGAACTT-3'. Human actin-β forward 5'-CCCAGCACAATGAAGATCAA-3' and reverse 5'-GATCCACACGGAGTACTTG-3' were using as internal control.

Semiquantitative analysis of CD133 expression with immunocytochemical staining

Cells cultured on collagen-coated culture slides (Beckton Dickinson Labware, Bedford, UK) were rinsed in PBS and fixed cold acetone at -20°C for five minutes. First, culture slides were preincubated in Peroxidase Block (Dako, Glostrup, Denmark) for 60min and then blocked with BlockAce (DS pharma biomedical, Osaka, Japan) for 20 min at 37°C.

Next, to detect CD133, slides were hybridized with anti-CD133 antibody (CD133/1, 1:10; Miltenyi Biotec, Bergisch Gladbach, Germany) overnight at 4°C. Then, slides were incubated at 37°C for 1hr with biotin-labeled anti-mouse (IgG+IgA+IgM) (Nichirei, Tokyo, Japan) and sensitivity was enhanced using Vecstatin ABC kit (Vector Laboratories, Burlingame, CA). The staining immunoproducts were visualized with 3,3,2-diaminobenzidine (DAB; Dako, Copenhagen, Denmark) and counterstained with hematoxylin to visualize the cell nuclei.

Cell count was performed blindly in three random high-power fields, and the mean ±SD of each condition was calculated.

Sphere formation assay

A limiting-dilution assay was performed as described previously[3]. T472 cell line subcultured over 10 times in Col/SFM was dissociated and seeded in 96-well non-coated microwell plates in 200µl volumes of SFM. Final cell dilutions ranged from 2000 cells/well to 1 cell/well in 200µl volumes. The percentage of wells without spheres for each cell density was calculated after 7 days. Control cells passaged over 10 times in non-coat/SCM were also seeded.

Evaluation of Tumorigenicity by Orthotopic Injection

5-8 week old female nude mice (BALB/c, nu/nu; CREA Japan Inc., Tokyo, Japan) were anesthetized under 2% isoflurane in N_2O/O_2 (70:30). Tumorigenicity was evaluated by injecting cultured T472 cells with Non-coat/SCM or Col/SFM. Cells (1×10^4 or 1×10^5) in 2μl of PBS were delivered into the right putamen with coordinates 1mm rostral to Bregma, 2mm lateral, and 2.5mm deep using a Hamilton microsyringe (10 μl) with 26g needle.

Statistical analysis

All data were expressed as mean ±standard deviation (SD). Statistical analysis was performed with Statcel2 software (OMS publishing Inc, Saitama, Japan). Continuous data were compared by unpaired t-test between two groups and with one-way analysis of variance (ANOVA) followed by Bonferroni test between more than 3 groups for the post hoc determination of significant differences. Values of $p<0.05$ or $p<0.01$ were considered statistically significant.

Results

Type1 collagen existing in perivascular area and CD133 located next to the collagen

In order to survey whether type1 collagen have some relations with CD133 positive cells and perivascular niche, immunohistochemical assessment of the GBM specimens were conducted.

Type1 collagen was detected in the perivascular area of various tumor vessels, thin-walled capillary vessels, thickened and multilayered vessels, and glomeruloid vascular structure (Fig1A-D). CD133-positive cells were

localized to type1 collagen in the perivascular areas and perinecrotic areas (Fig2).

Type1 collagen-coated plate enables adhesive culture and enhances glioblastoma stem-like cell markers

To assess for appropriate and effective culture conditions, the conditions of non-coat/SCM, non-coat/SFM, Col/SCM, and Col/SFM were evaluated. U87MG and T472 cells showed adhesiveness in Non-coat/SCM (Fig. 3B), Col/SCM (Fig. 3C), and Col/SFM (Fig. 3D), but sphere formation in Non-coat/SFM (Fig. 3A).

In cell-adhesive conditions, Col/SCM increased CD133 expression by over 4 times and Col/SFM by over 11 times compared to Non-coat/SCM (Fig. 4: $p<0.01$). This indicates that Col/SFM is the most suitable condition for enhancing CD133 in adherent cultures.

In GBM cells from fresh surgical tissues, T472 and T555 cells were also adherent in the Col/SFM condition, furthermore, in the Col/SFM, CD133 (Fig. 5A and 5C) and Nestin (Fig. 5B and 5D) expression were enhanced with increasing passages ($p<0.01$).

In regards to protein level, there were few CD133-positive cells in the Non-coat/SCM condition but many in the Col/SFM (Fig. 6A, B). The average of CD133-positive cells in three arbitrary high-power fields were 22% ±1.0 in Non-coat/SCM, but 73% ±3.2 (Fig.6C) in Col/SFM, and this result was statistically significant ($p<0.01$).

Glioblastoma cells cultured in type1 collagen-coated plates retain sphere-formation capability

In order to check potential stemness of GBM cell lines, sphere formation

capability of T472 cells cultured over 10 passages in Col/SFM or Non-coat/SCM, both adherent conditions, were replaced into Non-coat/SFM of sphere forming condition. As for cells from the Col/SFM condition, sphere formation was seen in every well with more than 32 cells, but there was no sphere formation in wells with 1 cell. Regarding wells with 2 cells to 16 cells, Sphere formation was observed in 12.5%～75% of wells (Fig. 7). On the other hand, there was no sphere formation at even 2000 cells/well which from Non-coat/SCM. This indicates that GBM cells cultured in Col/SFM partially maintain capability of sphere formation and its stemness.

Glioblastoma cells cultured in type1 collagen-coated plate are capable of forming tumors in vivo

To determine whether T472 cell line cultured in the Col/SFM and Non-coat/SCM condition have tumorigenic potential, we injected them into the right putamen of nude mice. There was no tumor detected 20 weeks after 1×10^5 cells-transplantation group of Non-coat/SCM-cells (n=3). However, six weeks following transplantation, both 1×10^4 and 1×10^5 Col/SFM-cells showed successful engraftment (Fig. 8A, B) in all the mice(n=3). At 20 weeks, tumor masses were found (Fig.8C, D). Some parts of the tumor showed infiltration of surrounding brain tissue (Fig.8D).

Discussion

Glioma stem-like cells (GSCs) are located in specialized microenvironments, niche, of the perivascular area and hypoxic area (perinecrotic area)[5, 9, 10]. In our study, one of the major GSCs[3, 4], CD133 positive cells were found in the perinecrotic area and perivascular area (Fig1 and 2). In the photomicrographs from T472 and T555 specimen, CD133 positive cells exist around various types of tumor vessels, and CD133-positive tumor cells seemed to be in

contact with type1 collagen (Fig. 1A-D). It seems difficult to distinguish GSCs from NSCs but tumorigenicity of T472 cell line cultured in the Col/SFM condition (Fig. 8) supported that CD133 positive cells found perivascular areas were GSCs. Type1 collagen intensified CD133, one of the major GSCs markers, expression not only in SFM but also in SCM (Fig. 4). This suggests that type1 collagen is one of the components of GSC's niche.

Type1 collagen might have another supportive role as a scaffold of CD133 positive GSCs to invade surrounding tissue. Type1 collagen enhances invasion of some other cancers via integrin-stimulated phosphoinositide 3-kinase (PI3K)/Akt pathway[11, 12]. Akt signal is deeply-involved in cell migration with Matrix metalloprotease 9 in GBM[13]. Girdin, an actin binding protein identified as a novel substrate of Akt and involved in cell invasion, is strongly expressed in GBM and especially correlates with CD133 positive GBM cells[14]. Type1 collagen could play an important role as a scaffold of CD133-positive GSCs and accelerate cell invasion through PI3K/Akt/Girdin pathway.

Kirkland reported [15] that type1 collagen inhibits differentiation and promotes expression of the stem cell markers CD133 and Bmi1 in colorectal carcinoma. Furthermore, Lee et al. [16] reported bone marrow stromal cells cultured in Col/SCM expressed neuronal and astrocytic markers by injection to the brain of cerebral infarct rat. These reports and our study support that type1 collagen is suitable for culture and maintenance of undifferentiated cells.

Pollard et al. [17] reported GSC lines propagated in adherent culture with laminin and are suitable for drug screening. Specifically laminin alpha 2 produced by brain tumor endothelial cell maintain GSCs and promote GSCs radioresistance via integrin β1 in the perivascular niche[18]. Type1 collagen mediates integrin β1/Akt resulting in tumor progression in ovarian cancer[12]. Not only laminin but type1 collagen also might contribute stemness via

interaction integrin β1 to GSCs.

Drug screening is difficult in a sphere condition because each cell is not exposed to the medical substance equally[17, 19]. For this reason it is also difficult to evaluate cell viability appropriately with MTT or WST assay. Adherent culture enables GSCs to be exposed to various reagents equally. Additionally, drug screening would be more appropriate in the Col/SFM condition as a better approximated niche for GSCs.

In our experience, GSCs were stably propagated in Col/SFM over 20 passages, but it was unstable to passage several cell lines in sphere condition (data not shown). Type1 collagen was widely applied for expanding various cell lines in an adherent condition [20]. Some of ECMs like type1 collagen or laminin may behave as a component of the niche and offer GSCs sites for stable propagation. This idea could be applied to other tumors, in cases where it is difficult to culture tumor cells in sphere condition, or if there are few available specimen materials to achieve CSCs.

Conclusion

Type1 collagen has a potential of niche for GBM. GBM stem-like cell lines could propagate and maintain stemness and tumorigenicity on type1 collagen. This could be applied to other tumors in order to culture CSCs.

Acknowledgments

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Figure Legends

Fig.1

Type1 collagen (A, C) and CD133 (B, D) immunohistochemical staining on paraffin serial sections from glioblastoma patient-specimens, T472 (A, B) and T555 (C, D), which applied to GBM cell lines. Lumen in vessel indicated by "V". Type1 collagen-positive areas were seen around various types of tumor vessels, glomerular-like vascular structures (white arrow head), thickened vessels (black arrow heads), and capillaries (arrows). CD133-positive cells were localized around tumor vessels and sometimes along intravascular lumen (B, D). Bar = 50μm(A, B), 30μm(C, D)

Fig.2

CD133 immunohistochemical staining on a paraffin section from T472 specimen. Lumen in vessel indicated by "V" and Necrosis indicated by "N". CD133-positive cells were localized in a perivascular area and a perinecrotic area. Bar = 100μm

Fig.3

Photographs showing various growth morphologies of T472 cell line in different cultures. (Media types: serum-containing medium (SCM) or serum-free medium (SFM); plate types: non-coated plate (Non-coat), type1 collagen-coated plate (Col))

Col plate with SFM and each plate with SCM were appropriate for maintaining cell adhesion. Non-coat plate created cell spheres in SFM. Bar = 250μm

Fig.4

The RNA expression level of CD133 of U87MG cultured in three types of adherent cultures, non-coated plate (Non-coat) with serum-containing medium (SCM), type1 collagen-coated plate (Col) with SCM, or serum-free medium

with some growth factors (SFM). The level of RNA expression of CD133 significantly increased in SFM as compared to that in SCM with both types of plates. Interestingly, CD133 expression was also significantly increased in the Col/SCM condition relative to the Non-coat/SCM. All data was normalized to Actin-β.

Fig.5

The RNA expression level of CD133 and Nestin of T472 and T555 cell lines (A and C: CD133, B and D: Nestin), a primary cultures of glioblastoma, in non-coated plate (Non-coat) with serum-containing medium (SCM) after 3 passages, or type1 collagen-coated plate (Col) with serum-free medium with some growth factors (SFM) after 3 or 5 passages. Both T472 and T555 cell lines, the level of RNA expression of CD133 significantly increased in the Col/SFM condition as compared to that in the Non-coat/SCM after 3 and 5 passages (Fig.5A and 5C), and more after 5 passages than 3 passages. Nestin expression was also significantly increased in the Col/SFM after 5 passages compared to Non-coat/SCM in both cell lines (Fig.5B and 5D). All data were normalized to Actin-β.

Fig.6

Microphotographs of immunocytochemistry and semi-quantitative measurement of CD133 positive cells of U87MG in non-coated plate/serum-containing medium (Non-coat/SCM) or type1 collagen-coated plate/serum-free medium (Col/SFM).

Fig6A and 6B: The number of CD133-positive cells cultured in Col/SFM was markedly increased relative to that in Non-coat/SCM. Bar = 50μm

Fig.6C: The ratios of CD133-positive cells in any three microscopic fields were 22% (±1.0%) in Non-coat/SCM and 73% (±3.2%) in Col/SFM, respectively, and were statistically higher in Col/SFM than in non-coat/SCM.

The nuclei of U87MG were countestained by hematoxylin.

Fig.7

A limiting-dilution assay was performed to investigate capability of sphere formation of a GBM cell line. T472 cells cultured in non-coated plate/serum-containing medium (Non-coat/SCM) or type1 collagen-coated plate/serum-free medium (Col/SFM) over 10 passages were replaced into Non-coat/SFM of the sphere formation condition. There was no sphere formation in Non-coat/SCM, but in Col/SFM all wells over 32 cells had tumor spheres.

Fig.8

Glioblastoma cells from T472, cultured in type1 collagen-coated plate/serum-free medium (Col/SFM) over 10 passages formed tumors upon orthotopic transplantation into the nude mice. All images were HE. Microphotographs of Fig.8A and B show tumor initiating at 6 weeks after transplantation. Tumor grew (C) and infiltrated the surrounding brain (D) at 20 weeks after transplantation. Bar = 200μm (A), 100μm (B, C, D)

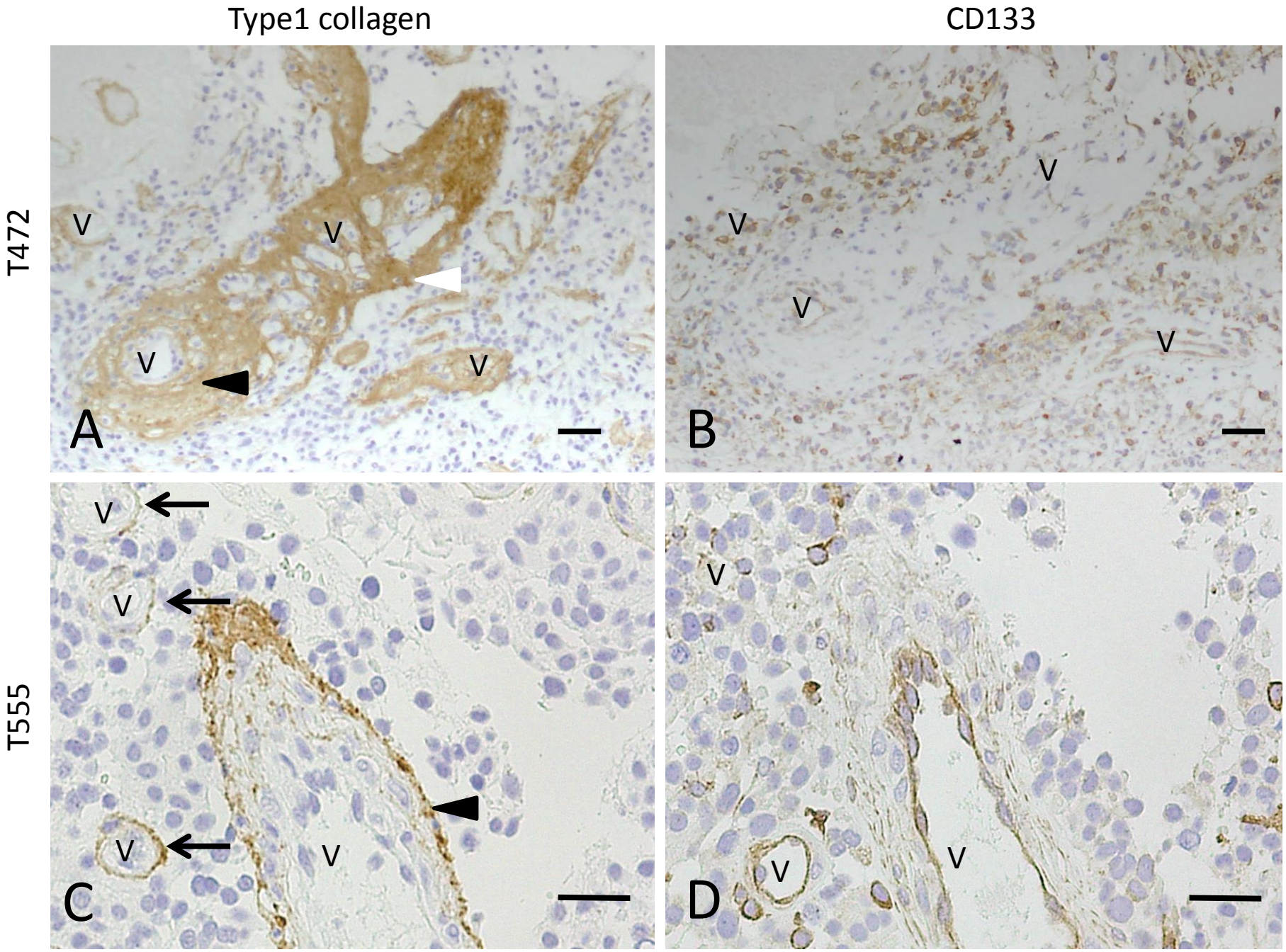


Fig.1

CD133

T472

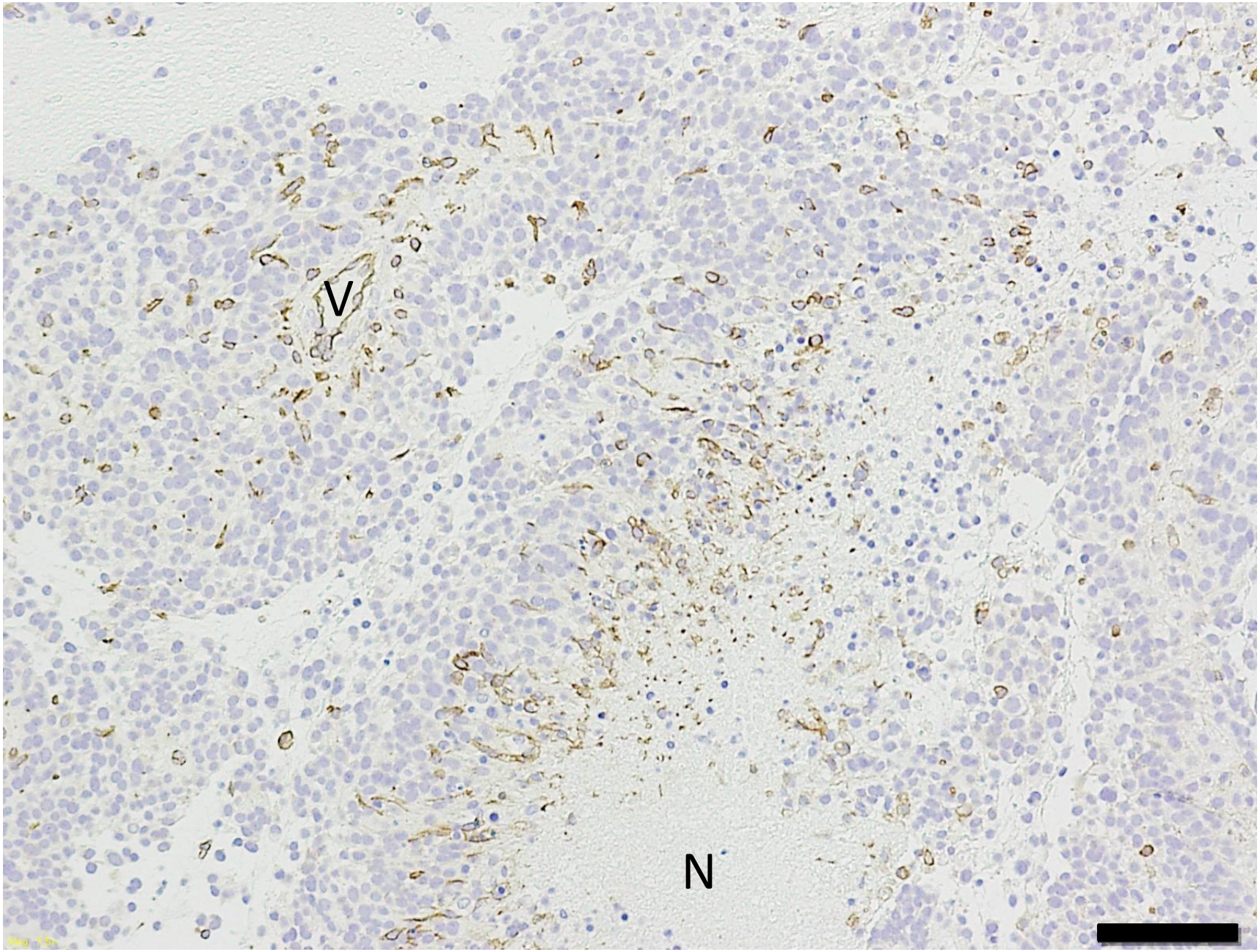
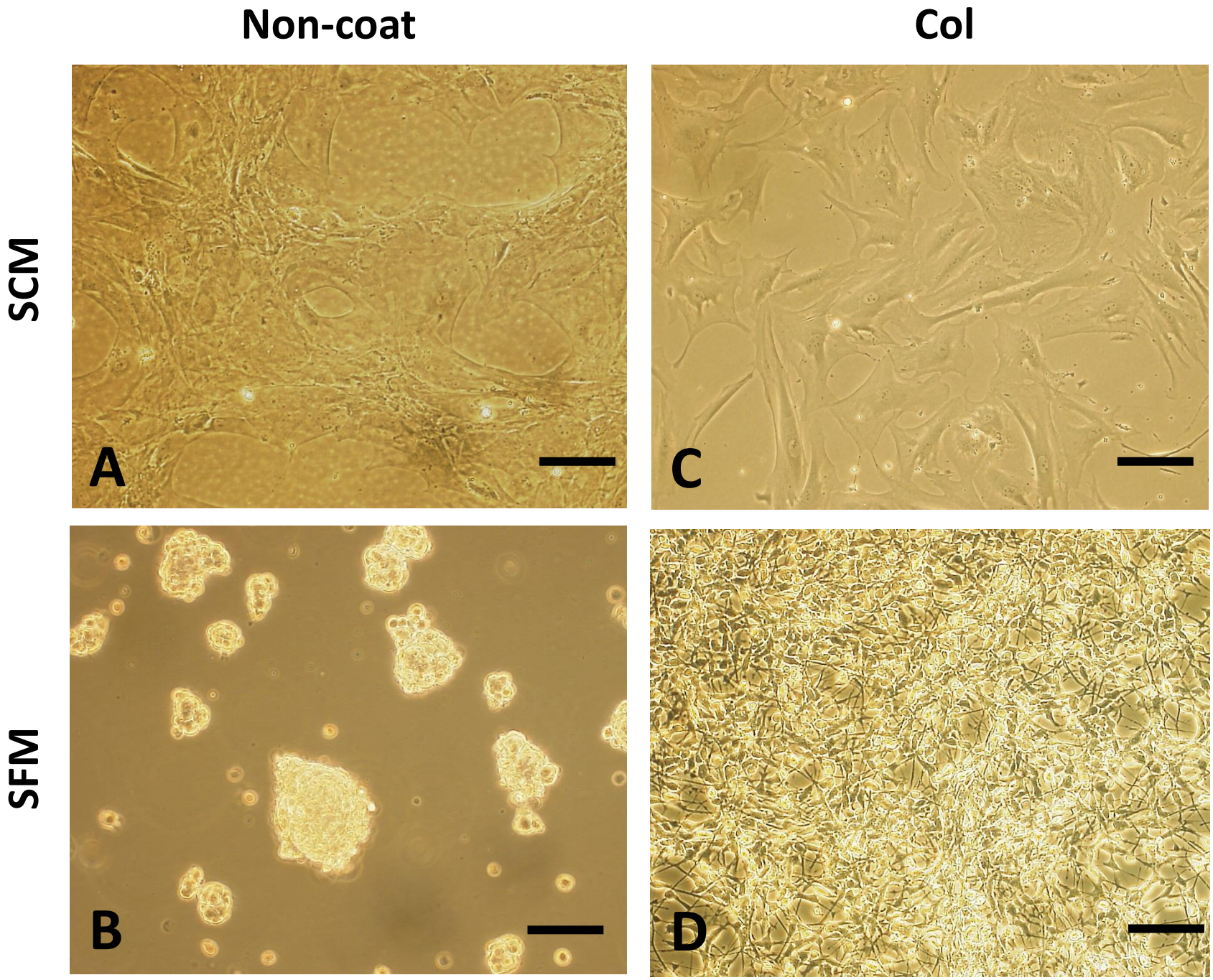
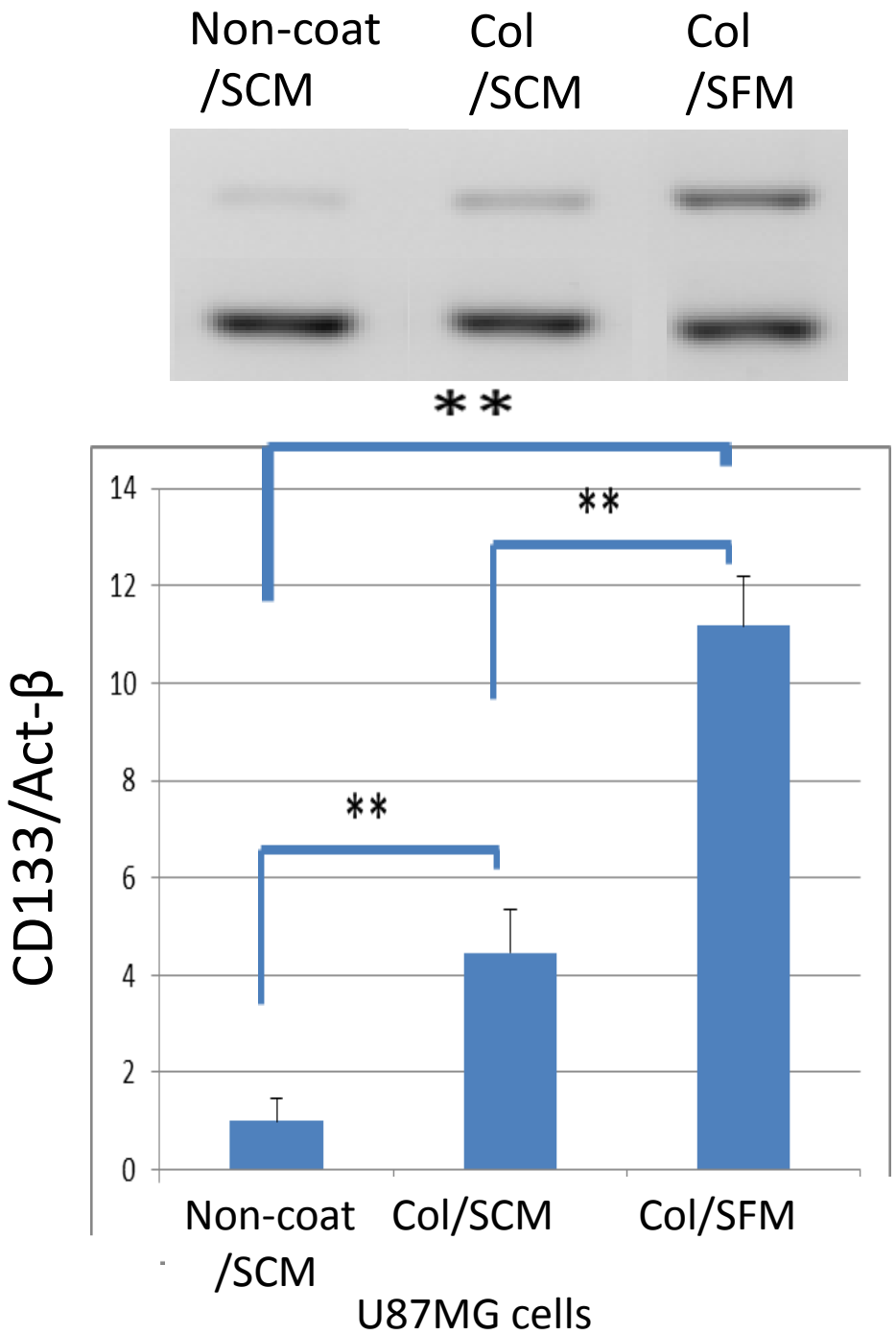


Fig.2



T472 cells

Fig.3


Non-coat /SCM
Col /SCM
Col /SFM
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**
CD133/Act-β
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10
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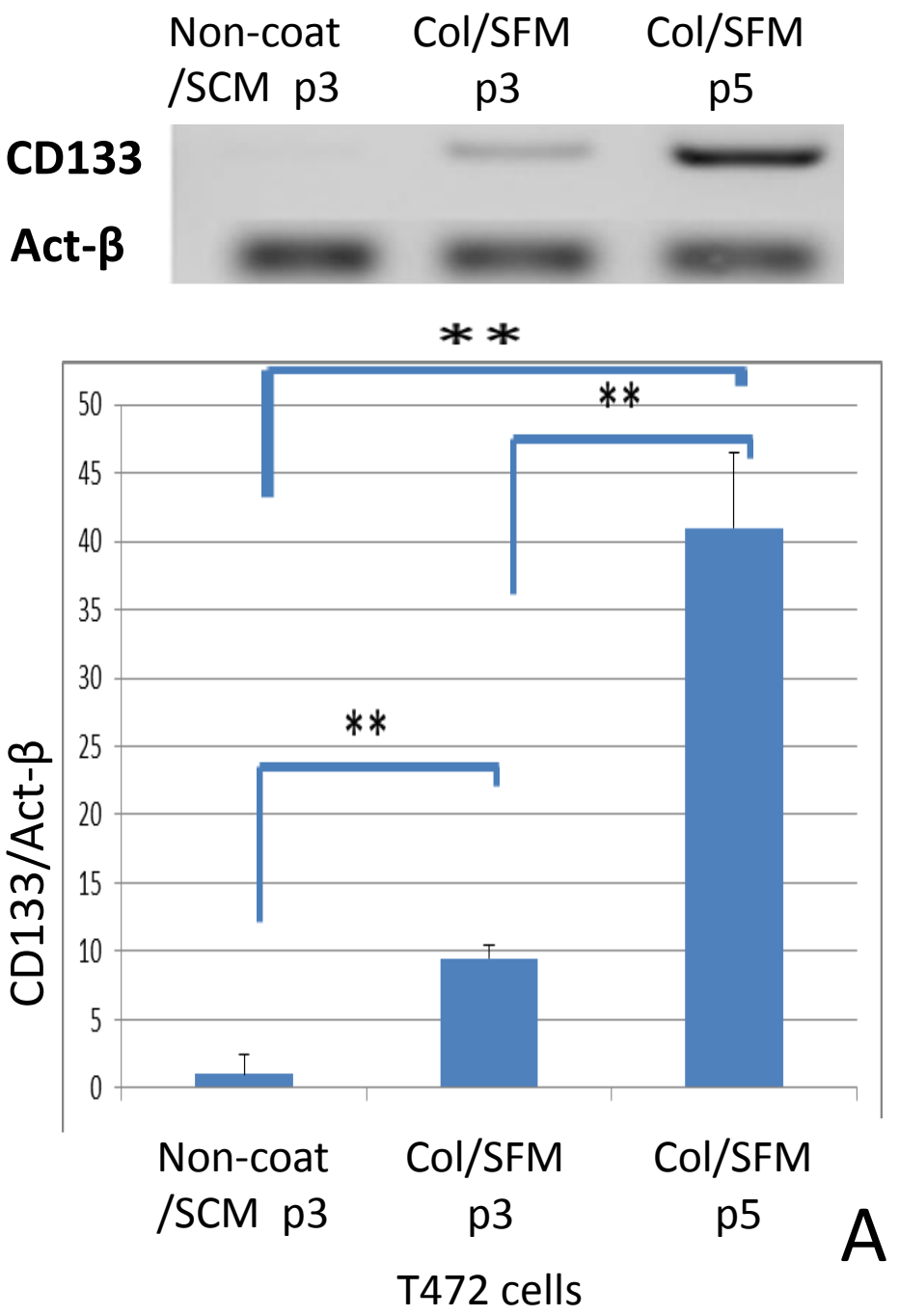
2
0
Non-coat /SCM
Col/SCM
Col/SFM
U87MG cells

Fig.4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Act-β
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CD133/Act-β
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10
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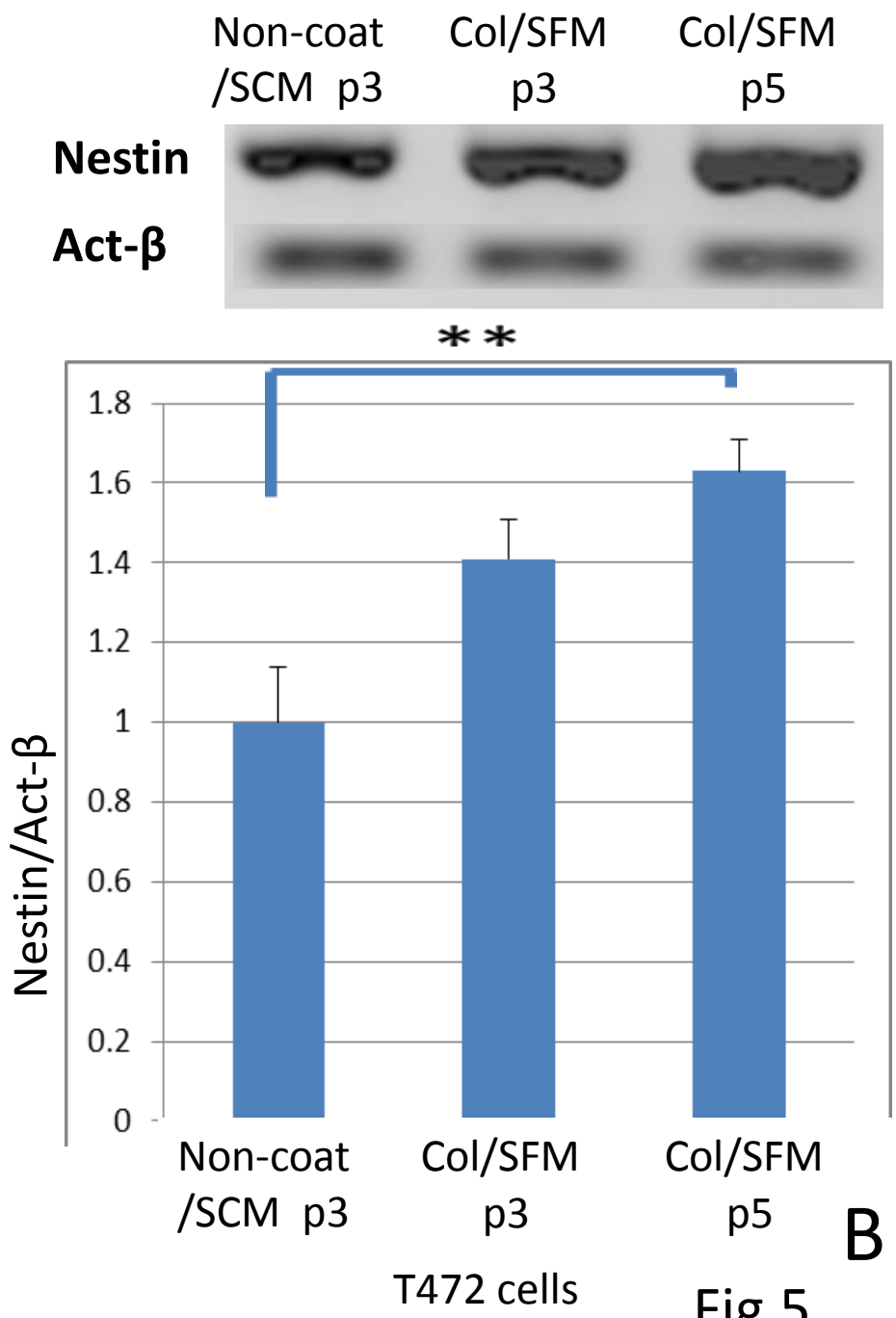
Col/SFM p3
Col/SFM p5
T472 cells
A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Col/SFM p5
Nestin
Act-β
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Nestin/Act-β
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1.6
1.4
1.2
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0.8
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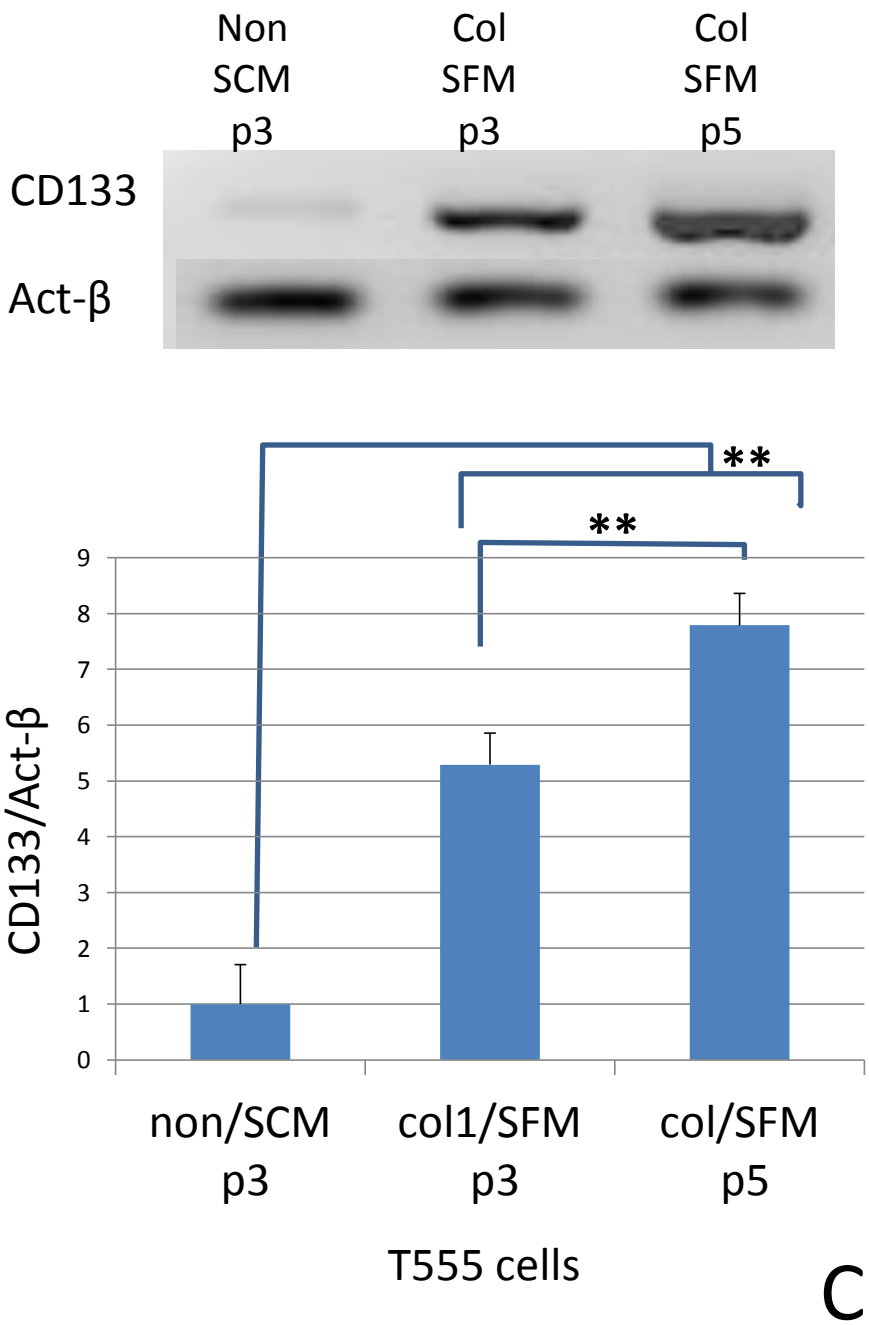
Non-coat /SCM p3
Col/SFM p3
Col/SFM p5
T472 cells
B

Fig.5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Col SFM p5
CD133
Act-β
**
**
CD133/Act-β
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2
1
0
non/SCM p3
col1/SFM p3
col/SFM p5
T555 cells
C

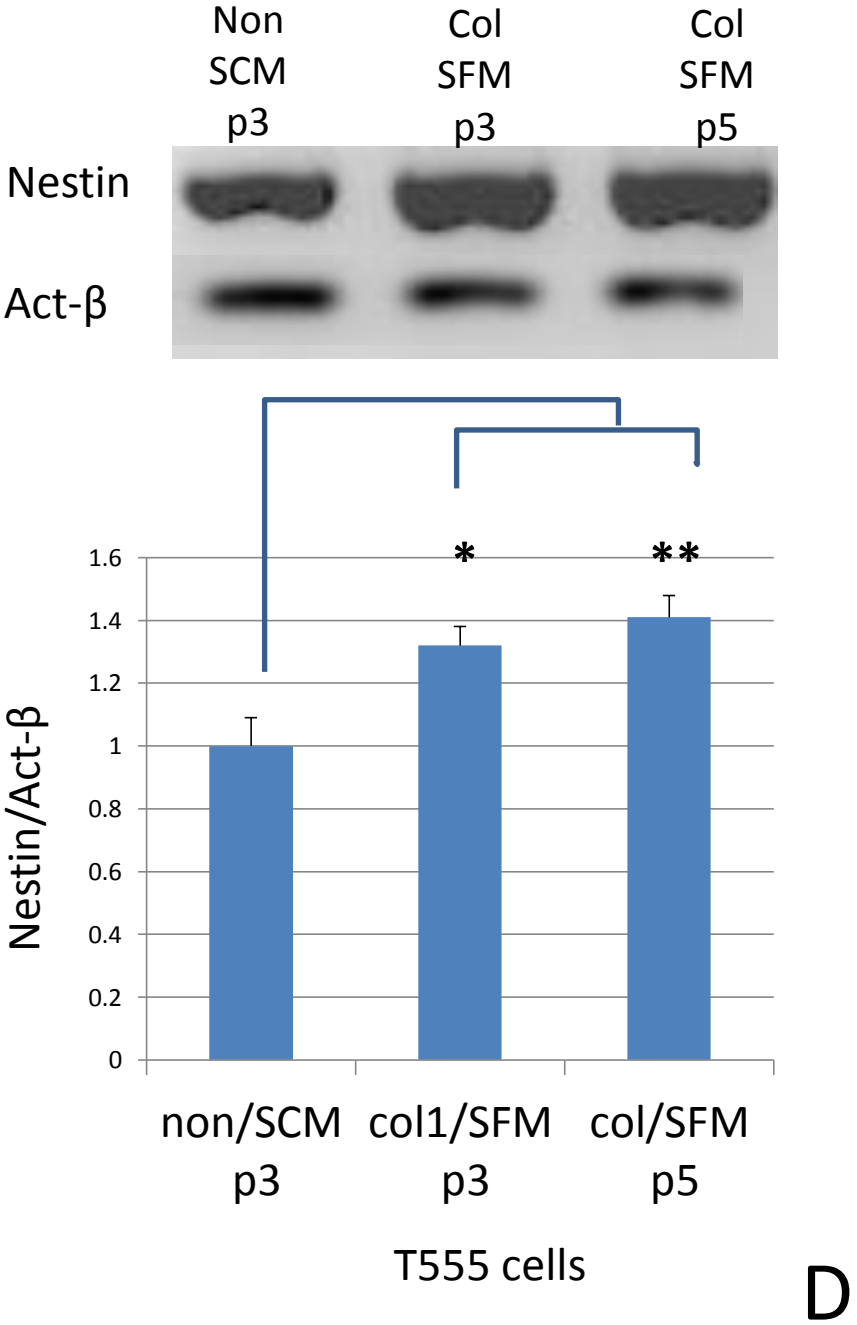
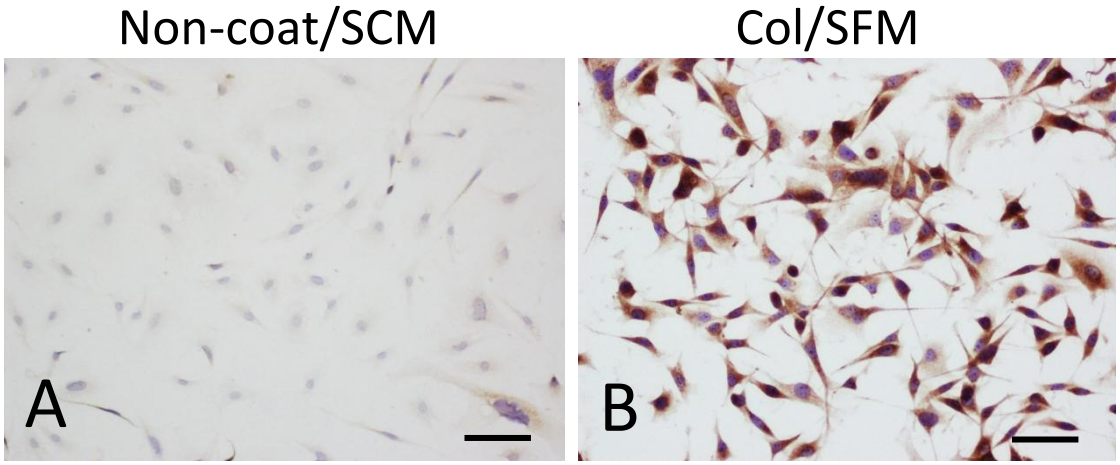
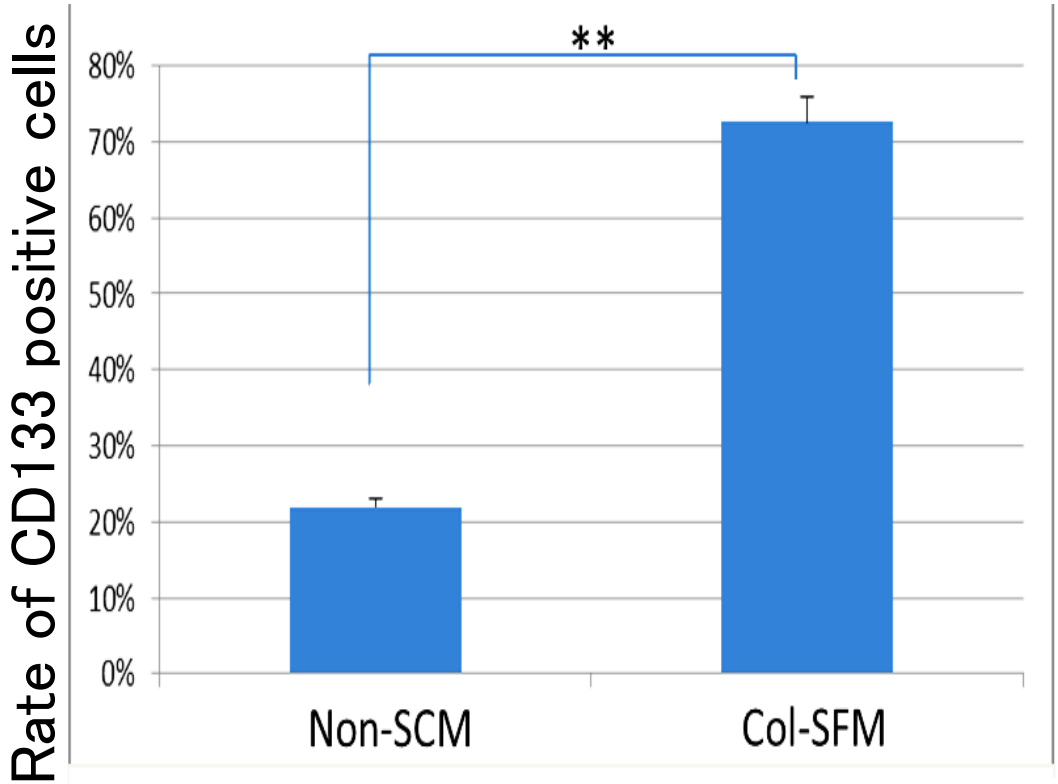

Non SCM p3
Col SFM p3
Col SFM p5
Nestin
Act-β
*
**
Nestin/Act-β
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
non/SCM p3
col1/SFM p3
col/SFM p5
T555 cells
D

Fig.5





U87MG cells

Fig.6

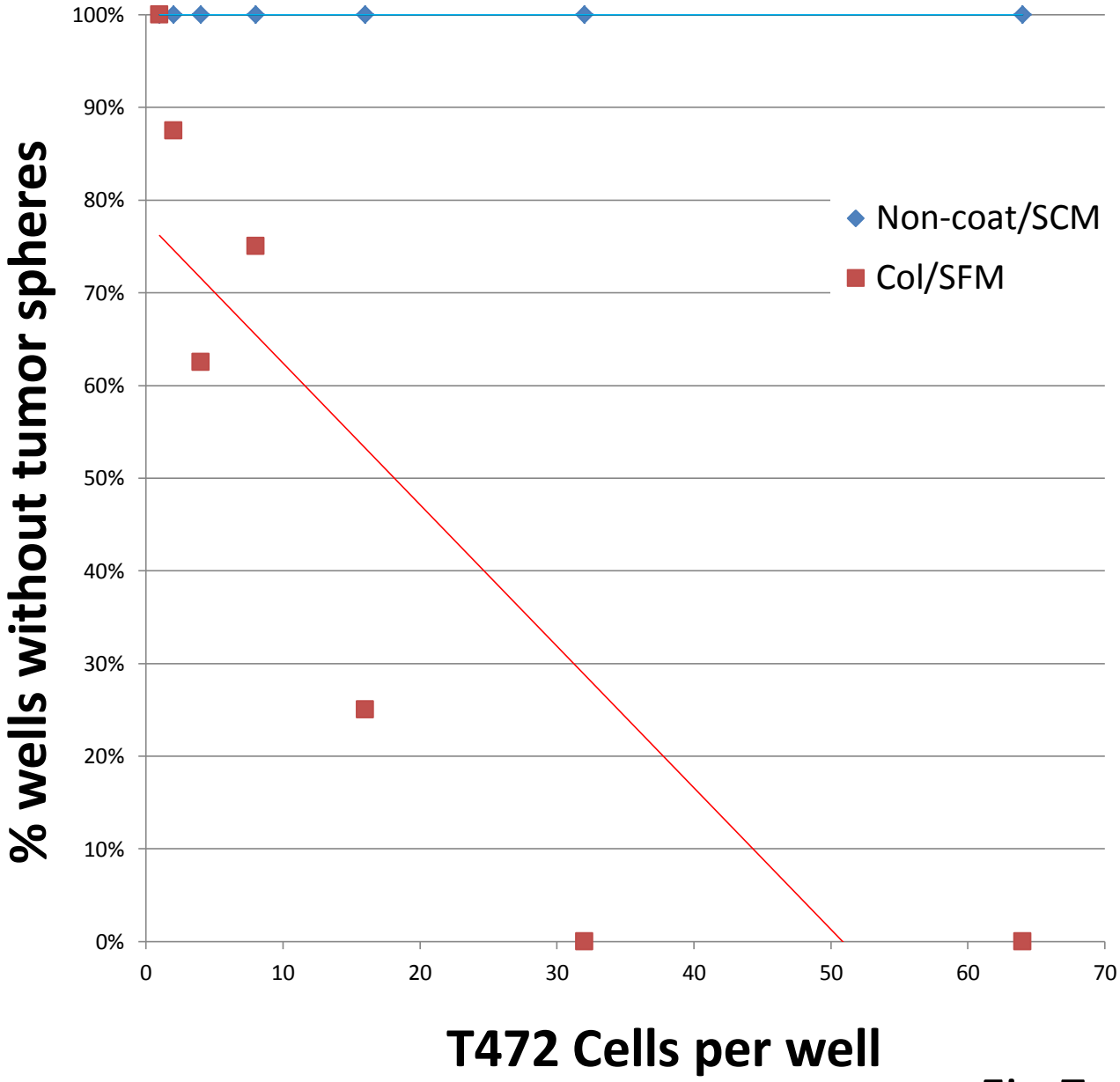

% wells without tumor spheres
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
10
20
30
40
50
60
70
Non-coat/SCM
Col/SFM
T472 Cells per well

Fig.7

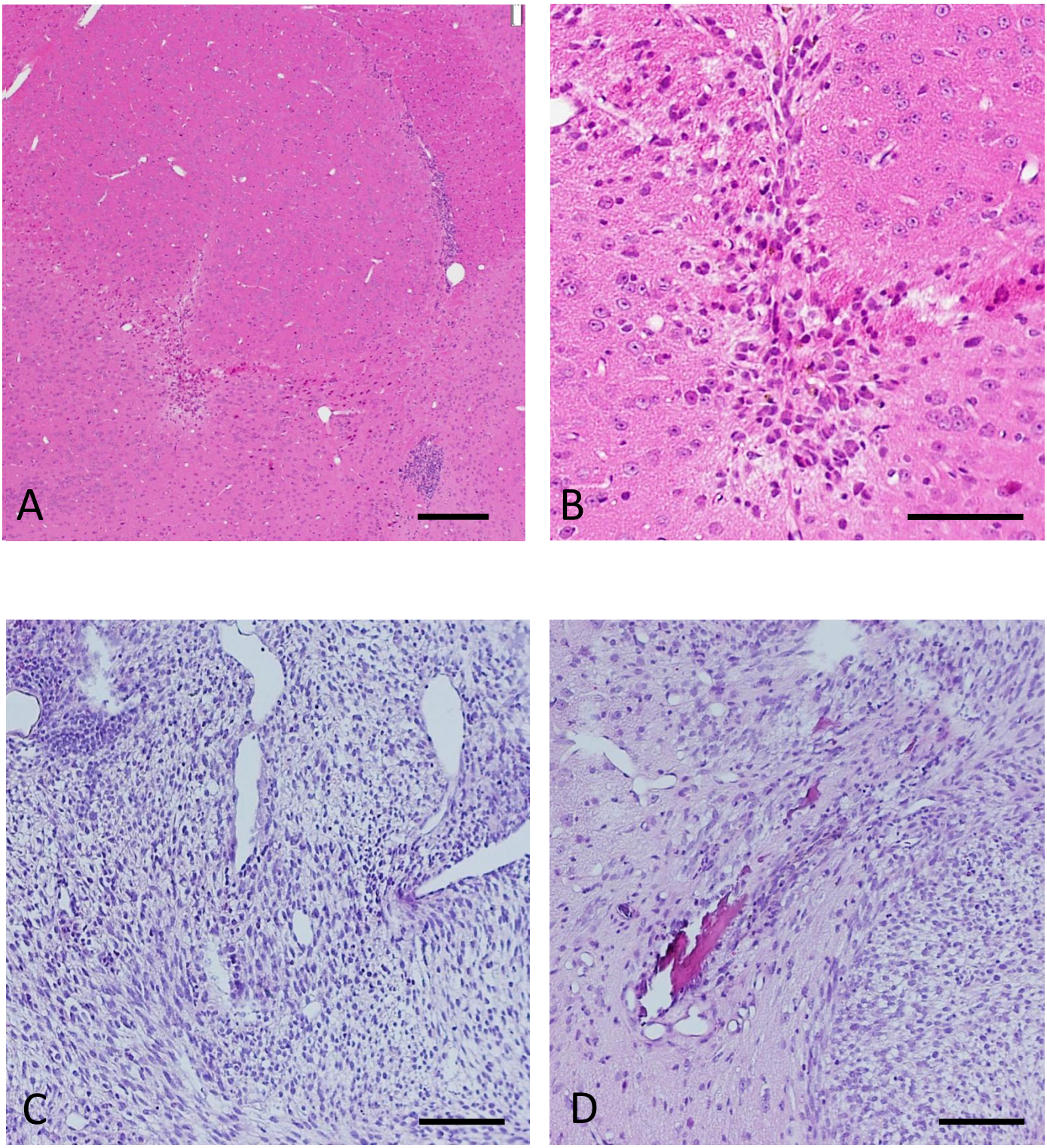


Fig.8