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$CD57^{high}$ neuroblastoma cells have characteristics of tumor-initiating cells

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I dedicate this thesis to my parents

Contents

List of Abbreviations

AKT	v-akt Murine Thymoma Viral Oncogene Homolog
ATRA	All-trans retinoic acid
bFGF	Human basic fibroblast growth factor
Bmi-1	Polycomb ring finger protein
BSA	Bovine Serum Albumin
B27	Stem cell culture supplement
° C	Grad Celsius
CD57	Cluster of differentiation 57
CD133	Cluster of differentiation 133
c-kit	Cluster of differentiation 117
CO_2	Carbon Dioxide
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium and Nutrient Mixture F12
EMEM	Eagle's Minimal Essential Medium
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Fig.	Figure
FISH	Fluorescence in situ hybridization
GFAP	Glial fibrillary acidic protein
G^+	Glial fibrillary acidic protein^{positive} (GFAP^{positive})
G^+P^+	Glial fibrillary acidic protein^{positive}Peripherin^{positive} (GFAP^{positive}Peripherin^{positive})
Ham's F12	Nutrient Mixture F12 Ham
HeLa	a cervical carcinoma cell line
HNK-1	Human natural killer 1
I.U.	International Units

IgG	Immunglobulin G
IgG1	Immunglobulin G1
IgM	Immunglobulin M
I-type	Intermediate-type
LIF	Recombinant human leukemia inhibitory factor
LMP	Low melting point
ml	milliliter
mm	millimeter
mM	millimolar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
MYCN	myc myelocytomatosis viral related oncogene, neuroblastoma derived
µg	mikrogramm
µl	mikroliter
µM	mikromolar
µm	micrometer
NB	Neuroblastoma
NCSCs	Neural crest stem cells
NDS	Normal donkey serum
Neuro-2a	a murine neuroblastoma cell line
ng	nanogramm
NGS	Normal goat serum
NIH/3T3	murine fibroblasts
NRG-1-ß1	Human neuregulin1-ß1
N-type	Neuroblastic-type
P^+	$Peripherin^{positive}$
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
P^-G^-	$Peripherin^{negative}$Glial fibrillary acidic protein^{negative} ($Peripherin^{negative}GFAP^{negative}$)

RAG$^{-/-}$/common γ-chain$^{-/-}$ mice	recombinase activating gene$^{-/-}$/common γ-chain$^{-/-}$ mice
rhEGF	recombinant human epidermal growth factor
RPMI 1640	Roswell Park Memorial Institute 1640
SK-N-BE(2)-C	a neuroblastoma cell line
S-type	substrate-adherent-type
U87-MG	a human glioblastoma cell line
U-NB1	a low passage neuroblastoma culture
USA	United States of America
w/v	Mass-voulme percentage

1. Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood. Advanced patient age and tumor stage are clinical predictors of poor outcome while amplification of myc myelocytomatosis viral related oncogene – neuroblastoma derived – (MYCN) and undifferentiated histology constitute well-established biological parameters for poor prognosis. Loss of 1p36, gain of 17q and loss of 11q have been delineated as additional molecular indicators for poor prognosis, amongst others [37]. While patient stratification has become more precise, outcome for high risk NB has improved little for decades despite intensive multimodal therapy. Thus, new biological markers and new therapeutic targets are needed. The concept of cancer stem cells – or tumor-initiating cells – holds some promise to provide these. As laid out by seminal investigations [2, 6, 24, 28, 29, 31, 46, 47] this concept stipulates that only a fraction of the cells within a tumor is endowed with the potential to initiate tumors, to self-renew, and to differentiate into all the different cells composing a heterogeneous tumor. There is increasing evidence that tumor-initiating cells are less susceptible to conventional chemo- and radiotherapy [4, 17, 18, 21, 35, 38, 41, 42] while tumor-initiating cells of some cancers may be specifically targeted by more recent kinase inhibitors [35, 54]. While the tumor-initiating cell concept does not require that the cancer's cell-of-origin is a tissue stem cell, there is evidence for this, such as in acute leukemia [31, 6, 5, 27]. Intuitively, malignancies arising from tissue stem cells may be predisposed to harbor tumor-initiating cells since tissue stem cells already possess many characteristics of tumor-initiating cells, such as self-renewal and the capacity to differentiate, and because both types of stem cells use similar signaling pathways [9, 24, 33, 36, 50, 53].

In NB the so-called side population has been described to contain tumor-initiating cells [25]. More recently, neuroblastoma cells isolated from bone marrow metastases have been reported to be enriched for tumor-initiating cells [23]. These studies show that tumor-initiating cells exist in neuroblastomas. However, the nature of these cells is still elusive.

NB is an embryonic tumor thought to arise from immature derivatives of neural crest stem cells (NCSCs) [23, 40, 52].

It thus appears reasonable to assume that markers, which define neural crest stem cells and their derivatives, and that are expressed in NB, may constitute markers for NB-initiating cells. Surface antigens are particular important markers for tumor-initiating cells since they allow isolation of viable cells. For these reasons we considered that cluster of differentiation 57 (CD57) may serve as a marker for NB-initiating cells. CD57 is a carbohydrate epitope detected by the human natural killer 1 (HNK-1) antibody [1]. It is expressed on adhesion molecules of several cell types, in particular migrating neural crest cells [7, 8]. The sequence of CD57 expression during development of the human sympathetic ganglia and the adrenal medulla has been described [11, 26]. In neuroblasts of sympathetic ganglia and the adrenal medulla expression of CD57 is detectable at 8 weeks of gestation, becomes intense between 13 and 24 weeks and decreases to undetectable levels thereafter. In contrast, CD57 is not detectable in adrenal chromaffin cells up to 15 weeks of gestation, while becoming detectable at relatively low intensity thereafter and well into childhood. Taken together, CD57 expression is associated with neural crest stem cells and sympathetic neuroblasts, potential cells-of-origin of NB.

In NB strong expression of CD57 is associated with histologically more immature NB cells [26]. Given that CD57 is expressed in stem and progenitor cells that are potential cells-of-origin of NB with – NCSCs and immature neuroblasts, respectively – and that expression of CD57 may be correlated with aggressiveness of NB cells, we investigated, whether CD57 expression in NB segregates with characteristics of a tumor-initiating cell. To this end we investigated both a low-passage NB culture we established, U-NB1, as well as the SK-N-BE(2)-C neuroblastoma cell line. This NB cell line is paradigmatic for the so-called intermediate-type (I-type) of NB. As other I-type NB the SK-N-BE(2)-C cell line contains cells with clonogenic self-renewal capacity that differentiate spontaneously and upon induction into the two lineages composing heterogeneous neuroblastomas - neuroblasts and Schwann-like cells -, and which are tumorigenic [51, 13]. In addition, these cells express cluster of differentiation 133 (CD133) as well as cluster of differentiation 117 (c-kit) and polycomb ring finger protein (Bmi-1) [51, 13], markers for tumor-initiating cells in brain and other solid tumors and for neural crest stem cells, respectively. Furthermore, as in other tumor-initiating cells and tissue stem cells, self-renewal and differentiation of SK-N-BE(2)-C cells is regulated by Bmi-1 [13, 14].

Taken together, the SK-N-BE(2)-C cell line, as other NB cell lines of the I-type, contains cells with the characteristics of neuroblastoma stem cells [44].

This is in contrast to NB cell lines of the neuroblastic- (N) and substrate-adherent- (S) types that differentiate solely along either the neuronal lineage (N-type) or the glial/Schwannian cell lineage (S-type) and show decreased (N-type) or absent (S-type) clonogenicity and tumorigenicity [44]. Accordingly, I-type cells loose their clonogenicity when differentiated into N-type or S-type cells [13]. Of note, CD57 expression is prominent in some of the SK-N-BE(2)-C cells and decreases upon differentiation to peripheral neuronal cells or glial/Schwann cells [13].

Using the low-passage NB culture U-NB1 and the SK-N-BE(2)-C NB cell line, we now show that neuroblastoma cells with high expression of CD57 have characteristics of tumor-initiating cells.

2. Materials and Methods

2.1 Cell Culture

The human neuroblastoma cell line SK-N-BE(2)-C (LGC Promochem, Teddington, United Kingdom) was cultured in a 1:1 mixture of Eagle's Minimum Essential Medium (EMEM; ATCC, Manassas, United States of America (USA)) and Nutrient Mixture F12 Ham (Ham's F12; PAA, Pasching, Austria) supplemented with 10 % heat inactivated fetal bovine serum (FBS; Biochrom, Berlin, Germany), 2 mM L-glutamine and penicillin/streptomycin (Invitrogen, Carlsbad, USA) and 0.1 mM nonessential amino acids (Invitrogen) in an atmosphere of 5 % CO_2 at 37 C°.

U-NB1 cells were maintained in Dulbecco's Modified Eagle Medium and Nutrient Mixture F12 (DMEM/F12), 2 % B27 (Invitrogen), 20 ng/ml recombinant human epidermal growth factor (rhEGF), 20 ng/ml recombinant human basic fibroblast growth factor (bFGF; Strathmann Biotech, Hamburg, Germany), 20 ng/ml recombinant human leukemia inhibitory factor (LIF; Millipore, Temecula, USA), 10 I.U./ml (5 µg/ml) Heparin (Roche Diagnostics, Mannheim, Germany) and 2 mM L-glutamine and penicillin/streptomycin in a humidified incubator at 37 C° in 5 % CO_2. RhEGF and bFGF were added twice a week. Within 1 week after tumor isolation, nonadherent spheres were observed. Cells were passaged once a week by mechanical dissociation and split 1:5.

Fluorescence in situ hybridization (FISH) analysis showed 2 copies of MYCN and no deletion of 1p36 (data not shown). Experiments with U-NB1 cells were done between passages 3 to 13. Adherently growing U-NB1 cells were maintained in Roswell Park Memorial Institute 1640 Medium (RPMI 1640; Invitrogen) with 10 % heat inactivated FBS, L-glutamine and antibiotics as above on collagen type I-coated flasks (BD Biosciences, Heidelberg, Germany).

2.2 FACS cell-sorting

$CD57^{high}$ and $CD57^{low}$ subpopulations of SK-N-BE(2)-C and U-NB1 cells were isolated using phycoerythrin (PE) -conjugated mouse monoclonal CD57 antibody (Abcam) and the fluorescence activated cell sorting system Aria™ (FACS; BD Biosciences). SK-N-BE(2)-C or U-NB1 cells were detached by trypsin, washed in Phosphate buffered saline (PBS; Biochrom) and resuspended in growth medium. 10^7 cells were incubated for 25 minutes at 4 C° in PBS containing 5 µl CD57 antibody or unspecific PE-conjugated immunoglobulin M (IgM) isotype control (BD Biosciences). Cells above the 90^{th} percentile of CD57 expression were sorted by flow cytometry into a $CD57^{high}$ fraction, cells below the 30^{th} percentile of CD57 expression into a $CD57^{low}$ fraction. The purity of sorted fractions was monitored by FACS reanalysis and viability of cells was determined by trypan blue (Sigma) exclusion method. All analyses and sorts were repeated at least 5 times.

2.3 Soft agar clonogenicity assay

1.2 % mass-volume percentage (w/v) low melting point agarose (LMP agarose; Sigma) in sterile double distilled water (ddH_2O; DeltaSelect, Pfullingen, Germany) was melted in a microwave oven and cooled to 40 C° in a water bath (Grant Instruments Ltd, Cambridgeshire, Great Britain). 2 x concentrated growth medium containing 2 x Ham's F12 (Invitrogen), 2 x EMEM (Invitrogen), 20 % FBS and 2 x additives were prewarmed in the water bath. Equal volumes of both solutions were mixed to produce 0.6 % agar. For bottom agar, 500 µl of the media-agar mix were gently pipetted into each well of a 24-well plate. SK-N-BE(2)-C cells were separated by flow cytometry into $CD57^{high}$ and $CD57^{low}$ fractions. Trypan blue exclusion was used to determine the number of viable cells and to ascertain that all cells were single cells. 0.6 % (w/v) LMP agarose was prepared as described above and mixed 1:1 with double concentrated growth medium containing 2 x 10^3 cells/ml. 1 ml of this top agar was pipetted on the solidified bottom agar and allowed to gel. Cells were fed with growth medium that was replaced every 3 days. Plates were incubated at 37 C° in a humidified incubator for 28 days until colony formation was observed and stained with 5 mg/ml 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromid (MTT; Sigma) to visualize colonies.

2.4 Serial sphere - forming assay

U-NB1 and SK-N-BE(2)-C cells growing as spheres were sorted into $CD57^{high}$ and $CD57^{low}$ fractions as described above. Sorted cells were pelleted and resuspended at a density of 1 viable cell/µl in 24-well plates ($1x10^3$ cells per well were seeded) with 14 mm cover slips to avoid adherence in serum-free DMEM/F12 containing 2 % B27, 20 ng/ml rhEGF, 20 ng/ml bFGF, 20 ng/ml LIF, 10 I.U./ml (5 µg/ml) heparin and 2 mM L-glutamine and penicillin/streptomycin. Spheres were cultured in a humidified incubator with 5 % CO_2. One week after plating spheres were photographed and counted. For the formation of second-generation spheres $CD57^{high}$ neurospheres were expanded, separated into the different CD57 fraction, counted and re-plated at clonal density. A third sort was performed in the same manner and tertiary spheres were identified after ten days.

2.5 Cytology

For detection of CD57 expression, SK-N-BE(2)-C cells were seeded at a concentration of $5x10^4$ cells/100 µl in PBS on adhesive slides (Dianova, Hamburg, Germany) and allowed to adhere for three hours. Cells were fixed with 2 % formaldehyde solution (Sigma) for 10 minutes, permeabilized with 0.1 % Triton X-100 (Sigma), blocked with 0.3 M glycine (Baker, Phillipsburg, USA) for 10 minutes and with 1 % Bovine Serum Albumin (BSA; Serva, Heidelberg, Germany) in PBS for 30 minutes. Cells were incubated at 4° C overnight with mouse monoclonal CD57 antibody diluted 1:100 in PBS and 1 % BSA. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, USA) diluted 1:5000 in PBS to visualize nuclei. HeLa cervical carcinoma cells (DSMZ, Braunschweig, Germany) were used as negative control. Cells were mounted with fluorescent mounting medium (DAKO, Hamburg, Germany) and covered with cover slips (Marienfeld, Lauda, Germany). SK-N-BE(2)-C and U-NB1 cells were stained with antibodies against peripherin and glial fibrillary acidic protein (GFAP). We used the murine neuroblastoma Neuro-2a (DSMZ) and the human glioblastoma U87-MG cell line (LGC Standards, Wesel, Germany) as positive controls and HeLa cervical carcinoma cells and NIH/3T3 fibroblasts (DSMZ) as negative controls. Cells were seeded at clonal density (1 cell/µl) or at a concentration of $5x10^4$ cells onto poly-L-lysine coated (SK-N-BE(2)-C) or on collagen type I-coated (U-NB1) glass cover slips.

Cells were allowed to adhere overnight in growth medium, supplemented with 10 % heat-inactivated FBS and the appropriate antibiotics. Cells were fixed with 95 % ethanol and 5 % glacial acetic acid at -20° C for 30 minutes. Cells were blocked with 0.3 M glycine for 45 minutes followed by blocking with 10 % normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, USA), 10 % normal goat serum (NGS; Sigma) and 0.5 % BSA in PBS for 30 minutes at room temperature. Cells were incubated with a 1:1000 dilution of rabbit anti-peripherin polyclonal primary antibody (Invitrogen) and a 1:100 dilution of monoclonal anti-glial-fibrillary acidic protein (clone G-A-5; Sigma) in PBS with 0.5 % BSA, 1 % NDS and 1 % NGS for one hour at room temperature. The following secondary antibodies were used: Alexa 488-conjugated donkey anti-rabbit Immunoglobulin G (IgG; Invitrogen) 1:500 and Alexa Fluor 594-conjugated goat anti-mouse Immunoglobulin G1 (IgG1; Invitrogen) 1:100 in PBS with 0.5 % BSA, 1 % NDS and 1 % NGS for one hour at room temperature in the dark. Nuclei were stained with DAPI and mounted on slides (Roth, Karlsruhe, Germany) as described above. Cells were washed 5 times with PBS for 5 minutes and immunofluorescence reactivity was analyzed using an Olympus AX70 PROVIS microscope (Olympus, Hamburg, Germany).

2.6 Forced differentiation assay

Human neuregulin1-β1 (NRG-1-β1; R&D Systems, Minneapolis, USA) was dissolved in sterile PBS containing 0.1 % BSA and 5 μM stock solutions were prepared. All-trans retinoic acid (ATRA; Sigma, Taufkirchen, Germany) was dissolved in chloroform to a concentration of 20 mM and stored at -20° C. Just before use, drugs were diluted into growth medium as indicated above containing 2 % FBS. SK-N-BE(2)-C and U-NB1 cells, growing as neurospheres, were stained with anti-CD57 antibody (5 μl/10^7 cells) and sorted into $CD57^{high}$ and $CD57^{low}$ fractions. Cells were plated either on poly-L-lysine-coated (SK-N-BE(2)-C) or on collagen type I-coated glass cover slips (U-NB1) at a concentration of 10^3 cells/ml. Cells were allowed to adhere overnight in growth media supplemented with 10 % FBS. The following day, growth media were replaced with media supplemented with 2 % FBS and differentiation-inducing cytokines. To differentiate, SK-N-BE(2)-C cells were treated with ATRA in a concentration of 20 μM or with NRG-1-β1 in a final concentration of 5 nM for 10 days.

For forced differentiation of U-NB1 cells ATRA in a concentration of 1 µM for 14 days was used. Cells treated with growth medium supplemented with 2 % FBS, and PBS/BSA or chloroform were used as negative control.
Differentiation medium was changed every 3 days. Differentiation was confirmed by immunofluorescence cytology.

2.7 Repopulation assay

To determine the differentiation capacity of CD57 fractions adherently growing SK-N-BE(2)-C cells and spheres of U-NB1 cells were labeled with anti-CD57 (5 µl/10^7 cells) and separated by flow cytometry into $CD57^{high}$ and $CD57^{low}$ fractions. Cells were plated in growth medium either on poly-L-lysine coated (SK-N-BE(2)-C) or on collagen type I-coated glass cover slips (U-NB1) at a concentration of 10^3 cells/ml. The presence of a single cell suspension was verified microscopically. Cells were incubated for seven days at 37 C° in a humidified atmosphere with 5 % CO_2, stained for peripherin and glial fibrillary acidic protein and analyzed by immunofluorescence microscopy.
To investigate the ability of the CD57 fractions to reconstitute *in vitro* the parental cell line in regard to CD57 cell surface expression cells were sorted into $CD57^{high}$ and $CD57^{low}$ populations, plated in 6-well plates and allowed to expand for 5 days in serum-supplemented media. Cells were analyzed daily for CD57 expression by flow cytometry.
To analyse *in vivo* reconstitution of CD57 expression $1x10^5$ $CD57^{high}$ and $CD57^{low}$ isolated cells were suspended in 10 µl PBS containing 25 % Geltrex™ (Invitrogen) and transplanted into the adrenal gland of $RAG^{-/-}$/common γ-$chain^{-/-}$ mice as described below. 7 weeks after the transplantation mice were sacrificed. Tumors were procured, minced and digested with Liberase Blendzyme 1 (Roche Diagnostics GmbH, Mannheim, Germany). Single cells were stained with PE-conjugated anti-CD57 antibody and analyzed by FACS.

2.8 Neuroblastoma mouse model

6 - 8 week old male recombinase activating $gene^{-/-}$/common γ-$chain^{-/-}$ mice ($RAG^{-/-}$ /common γ-$chain^{-/-}$ mice) bred in the Animal Research Center of the University of Ulm and housed in sterile isolators under pathogen-free conditions were engrafted orthotopically into the left adrenal gland under sterile conditions with $1x10^4$ viable U-NB1 cells.

Tumors were resected and single cell suspensions were generated by mincing and chemical dissociation using Liberase Blendzyme 1 (Roche). Cells were sorted by FACS into $CD57^{high}$ and $CD57^{low}$ fractions as described above.

Mice were anesthetized with ketamin (25 mg/ml; Pfizer, Berlin, Germany)/rompun (20 mg/ml; Bayer, Leverkusen, Germany). A left lateral incision to the abdomen was made to visualize the adrenal gland. Tumor cells suspended in 50 µl of a 1:3 mixture of BD Matrigel™ High Concentration (BD Biosciences) and DMEM/F12 or RPMI 1640 medium without supplements were injected with a 27-gauge needle into the adrenal gland. The peritoneum and the skin were sutured. Each group included 10 mice monitored twice a week for the presence of tumors by palpation, and for evidence of morbidity. Mice were sacrificed when tumors became palpable, id est after 4-7 weeks. For serial orthotopic transplantation of fractionated cells tumors were procured and the procedure of tumor cell dissociation, fractionation and orthotopic injection repeated twice.

2.9 Statistical analysis

Quantitative results are specified as mean values ± standard deviation (Excel 2004, Microsoft Deutschland GmbH, Unterschleißheim, Germany) or as box plots (GraphPad Prism 4, San Diego, California, USA). Student's two-tailed *t*-test was used to test for differences in the experimental groups (Excel 2004). A $*p < 0.05$ was considered statistically significant.

3. Results

3.1 Clonogenicity of SK-N-BE(2)-C NB cells corresponds to expression of CD57

We first confirmed expression of CD57 in the SK-N-BE(2)-C cell line, an I-type NB cell line known to contain cells with stem cell-like activity. Immunofluorescence cytology revealed that 50 ± 5 % of all cells expressed CD57 to varying degree (Figure 1A) (Fig.). Flow-cytometric analysis, being more sensitive, showed that 70 % of SK-N-BE(2)-C cells expressed CD57 (Fig. 1B, upper left panel). To determine the role of CD57 in the aggressiveness of NB cells we sorted SK-N-BE(2)-C cells by flow cytometry into a $CD57^{high}$ and a $CD57^{low}$ fraction (Fig. 1B, upper central and right panel, respectively). Anchorage-independent clonal growth, as determined in soft agar, is an *in vitro* feature closely associated with the ability of tumor cells to initiate tumor growth *in vivo*. Unfractionated SK-N-BE(2)-C cells were clonogenic in soft agar (Fig. 1B). Clonogenicity of SK-N-BE(2)-C cells segregated with expression of CD57, as clonogenic growth was confined to the $CD57^{high}$ fraction, with nearly no colonies growing in the $CD57^{low}$ fraction (Fig. 1B). These data indicate that expression of CD57 determines clonogenicity of SK-N-BE(2)-C cells.

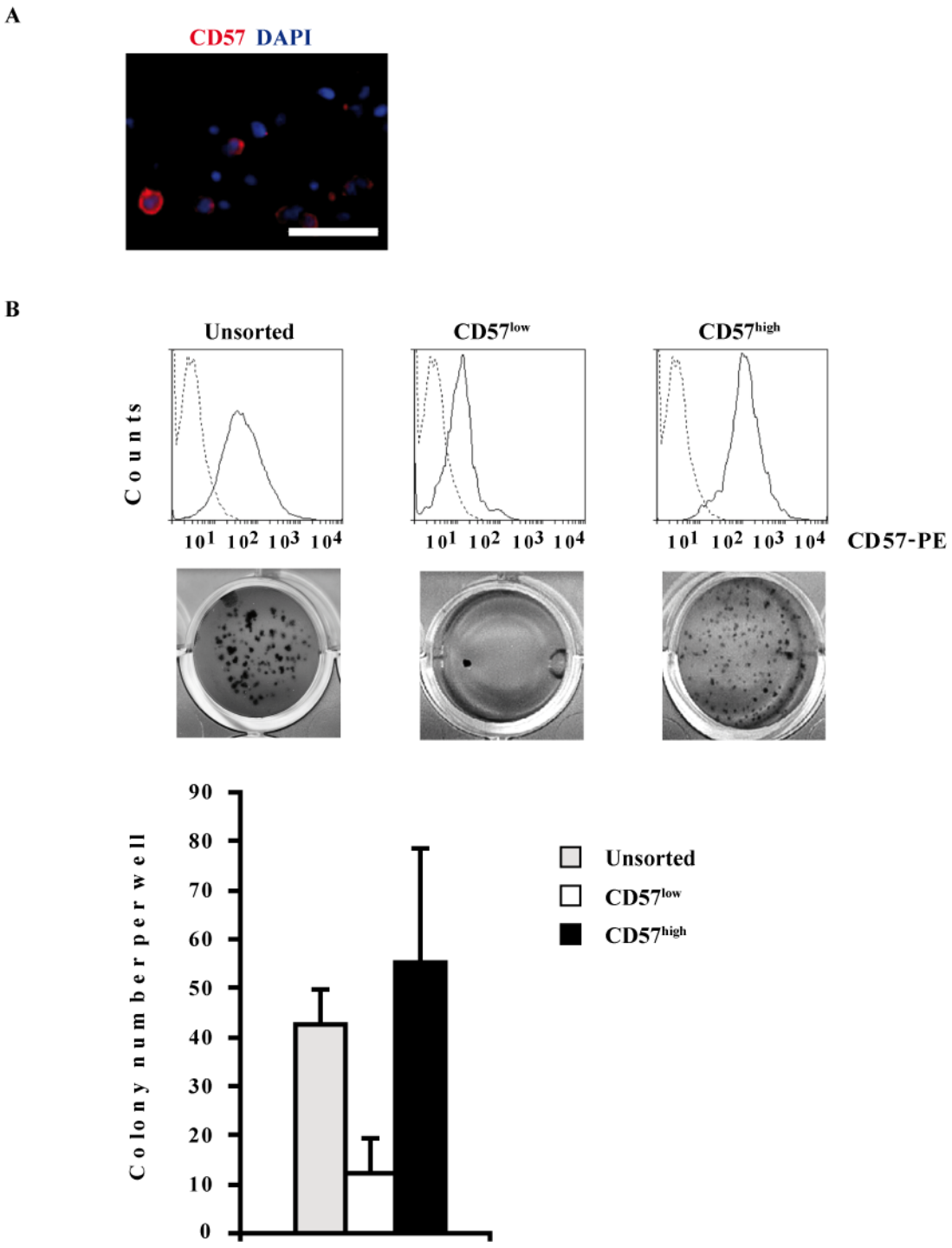

Figure 1: Expression of CD57 determines clonogenicity of SK-N-BE(2)-C neuroblastoma cells

(A) A subset of SK-N-BE(2)-C cells expresses CD57. SK-N-BE(2)-C cells grown on coverslips were stained with PE-labeled anti CD57 antibody (red). Nuclei were visualised by DAPI (blue). Scale bar corresponds to 50 µm. The experiment was repeated five times with similar results.

(B) SK-N-BE(2)-C cells with high expression of CD57 are clonogenic. SK-N-BE(2)-C cells were Separated by flow cytometry into a $CD57^{high}$ fraction (cells above the 90^{th} percentile of CD57 expression) and a $CD57^{low}$ fraction (cells below the 30^{th} percentile). CD57 expression of unsorted and sorted cells is shown in histograms. For soft agar clonogenicity assays fractionated SK-N-BE(2)-C cells were seeded at 1000 cells per well into 24-well plates. 28 days after plating, colonies were visualised by MTT-staining. The efficiency of colony formation of the fractions was calculated and is depicted in the Graph. Experiments were repeated three times with similar results.

CD57 = Cluster of differentiation 57; DAPI = 4',6-diamidino-2-phenylindole; MTT = 3-(4, 5-Dimethylthiazol-2-yl)-2; µm = micrometer, PE = Phycoerythrin; SK-N-BE(2)-C = a neuroblastoma cell line

3.2 Induction of NB spheres correlates with expression of CD57

High-passage adherently growing cell lines, such as SK-N-BE(2)-C NB cells, may be prone to artefacts stemming from prolonged in vitro culture. We therefore aimed at establishing a stable sphere culture containing I-type NB cells from a patient with NB. In Figure 2A such a culture - called U-NB1 - is shown, which under adherent growth conditions displayed a morphology typical for I-type NB cells, id est intermediate between neuronal appearing N-type and cytoplasma-rich S-type NB. As U-NB1 cells also fullfilled the other critereria for I-type NB cells (see below), these low-passage sphere cultures were used in all subsequent experiments.

We investigated, whether expression of CD57 in NB cells is associated with induction of spheres. 50 % of U-NB1 cells expressed CD57, as determined by flow cytometry (Fig. 2B, upper left panel). U-NB1 cells were fractionated by FACS according to CD57 expression. $CD57^{high}$ U-NB1 cells induced spheres at a 2.8-fold higher frequency than did $CD57^{low}$ cells (Fig. 2B). Upon dissociation of $CD57^{high}$ spheres recloned cells induced new spheres with increased frequency (Fig. 2B), suggesting that cells growing within spheres become adept at inducing new spheres. Cells from these second-generation spheres maintained the capacity to induce new, third-generation spheres. $CD57^{high}$ cells retained their increased capacity to induce spheres throughout recloning. Similar results were observed with SK-N-BE(2)-C cells (data not shown). Thus, expression of CD57 marks a population within U-NB1 and SK-N-BE(2)-C NB cells with a sustained disposition to induce spheres.

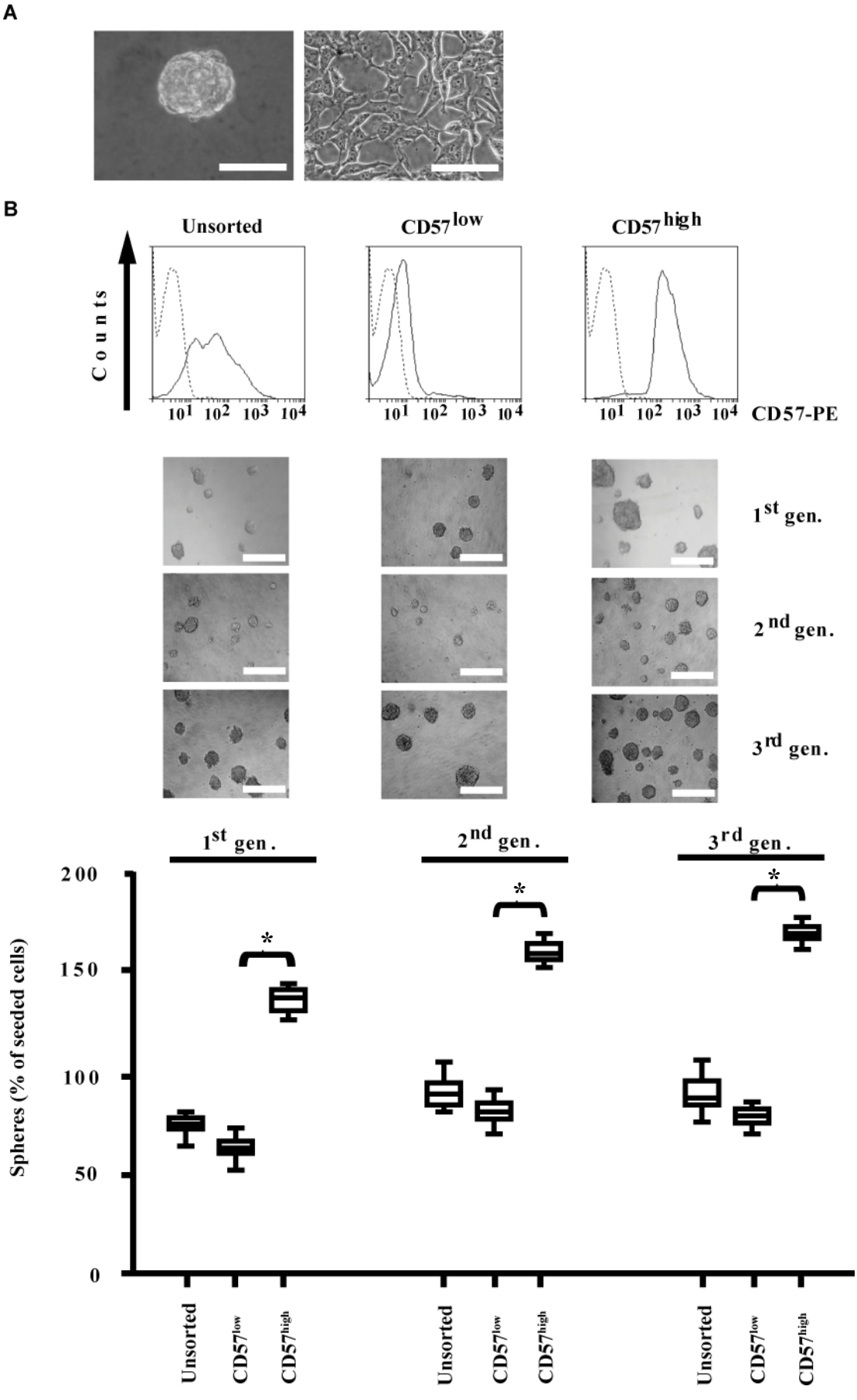
A
B
Unsorted
CD57low
CD57high
Counts
CD57-PE
1st gen.
2nd gen.
3rd gen.
Spheres (% of seeded cells)
200
150
100
50
0
*
Unsorted
CD57low
CD57high

Figure 2: Expression of CD57 correlates with induction of spheres in the U-NB1 neuroblastoma culture

A) U-NB1, a low-passage neuroblastoma culture established and maintained as spheres. U-NB1 cells were generated from a patient with NB after several blocks of chemotherapy. To initiate and propagate tumor spheres single cells were plated in non-adherent plates and grown in serum-free medium containing EGF and bFGF (left picture). For adherent growth cells were cultured on collagen-coated plastic in serum-supplemented medium (right picture). Bars correspond to 100 µm.

(B) Enhanced and stable induction of spheres by U-NB1 cells strongly expressing CD57. $CD57^{high}$ and $CD57^{low}$ U-NB1 cells were isolated by FACS sorting (upper panel). Solid lines correspond to CD57, dashed lines to isotype controls. Fractionated cells were plated at very low density in serum-free medium and the appearance of spheres was documented (middle panel). The number of "first generation" spheres was determined. After dissociation of spheres this procedure was repeated twice ("second" and "third generation" spheres, respectively). Results are depicted as box plots (lower panel), *$p < 0.05$. Experiments were performed twice, with similar results. Bars correspond to 100 µm.

bFGF = recombinant human basic fibroblast growth factor; CD57 = cluster of differentiation 57; EGF = recombinant human epidermal growth factor; FACS = fluorescence activated cell sorting; Gen. = generation; µm = micrometer, NB = Neuroblastoma; *p = p-value; U-NB1 = a low passage neuroblastoma culture

3.3 Increased incidence of peripherin$^-$ GFAP$^-$ cells in $CD57^{high}$ U-NB1 and SK-N-BE(2)-C cells

As U-NB1 cells have an I-type NB cell morphology and because I-type cells coexpress peripherin, a marker for mature peripheral neurons, and GFAP, a marker for glial and Schwann cells, we determined expression of these proteins in U-NB1 cells. Indeed, the majority of U-NB1 coexpressed peripherin and GFAP (Fig. 3, upper panel). Thus, both morphology and marker expression suggest that U-NB1 cells are of the I-type.

Next, we investigated, how expression of CD57 correlates with the expression of peripherin and GFAP. In U-NB1 cells significantly more $CD57^{high}$ cells were negative for both peripherin and GFAP compared to $CD57^{low}$ cells (19 % versus 2.5 %; Fig. 3A). Similar results were obtained in SK-N-BE(2)-C cells (data not shown). Thus, the $CD57^{high}$ fraction of U-NB1 and SK-N-BE(2)-C cells contains more undifferentiated cells than the $CD57^{low}$ fraction.

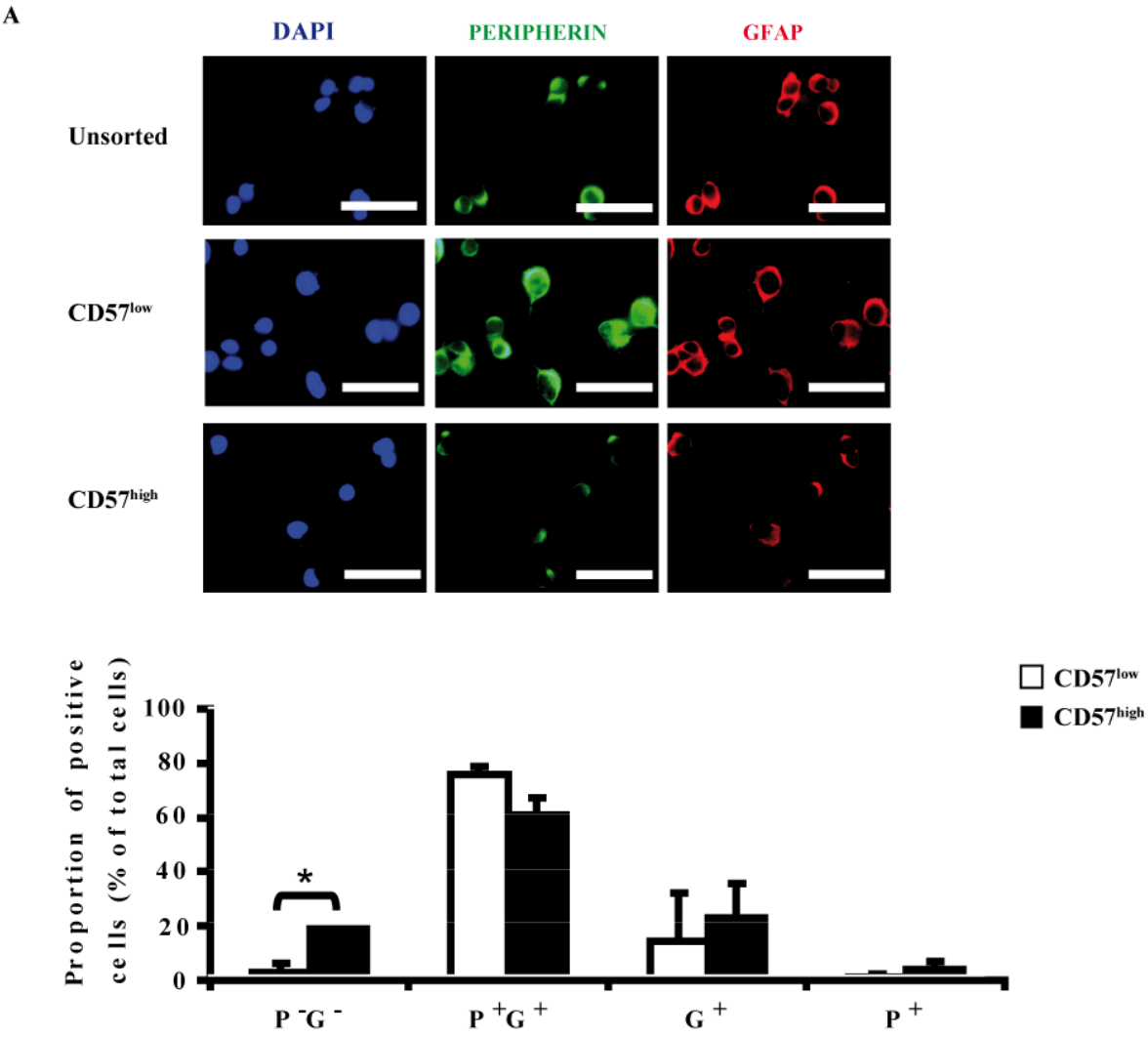

Figure 3: Increased incidence of lineage-negative cells in the CD57^{high} fraction of U-NB1 cells

(A) CD57^{high} and CD57^{low} U-NB1 cells were isolated by FACS sorting. Cells were plated on collagen-coated coverslips and stained for peripherin (green) and GFAP (red). Nuclei were visualized with DAPI (blue). Scale bars correspond to 50 µm. The results of the staining are quantitated in the graph, *p < 0.05. Three independent experiments were performed, with similar results.

CD57 = cluster of differentiation 57; DAPI = 4',6-diamidino-2-phenylindole; FACS = fluorescent activated cell sorting; GFAP = glial fibrillary acidic protein; G^+ = GFAP^{positive}; µm = micrometer, P^-G^- = Peripherin^{negative}GFAP^{negative}; P^+ = Peripherin^{positive}; P^+G^+ = Peripherin^{positive}GFAP^{positive}; *p = p-value (statistical hypothesis testing), U-NB1 = a low passage neuroblastoma culture

3.4 CD57^{high}, but not CD57^{low}, U-NB1 and SK-N-BE(2)-C cells can be differentiated to a predominant neuronal or glial phenotype

Tumor-initiating cells can differentiate to the various cell lineages that constitute a heterogeneous tumor. As differentiated NB consist of cells with phenotypes of peripheral sympathetic neurons and glial Schwann cells we investigated the differentiation capacity of CD57^{high} and CD57^{low} NB cells toward these cells types. Clones of CD57^{high} and CD57^{low} U-NB1 cells were subjected to all-trans-retinoic acid and neuregulin for neural and glial differentiation, respectively. CD57^{low} U-NB1 cells were unresponsive to these differentiation cues, as their biphenotype did not change (Fig. 4). A similar non-response was seen in SK-N-BE(2)-C cells (data not shown). In stark contrast, CD57^{high} U-NB1 cells responded to ATRA by decreasing expression of GFAP while maintaining peripherin expression and displaying neurite outgrowth. More pronounced was the response to neuregulin, as expression of peripherin was nearly abrogated while GFAP expression was maintained (Fig. 4). Similar results were seen in SK-N-BE(2)-C cells (data not shown). Thus CD57^{high}, but not CD57^{low}, U-NB1 and SK-N-BE(2)-C cells have to certain extent the differentiation capacity expected for NB-initiating cells.

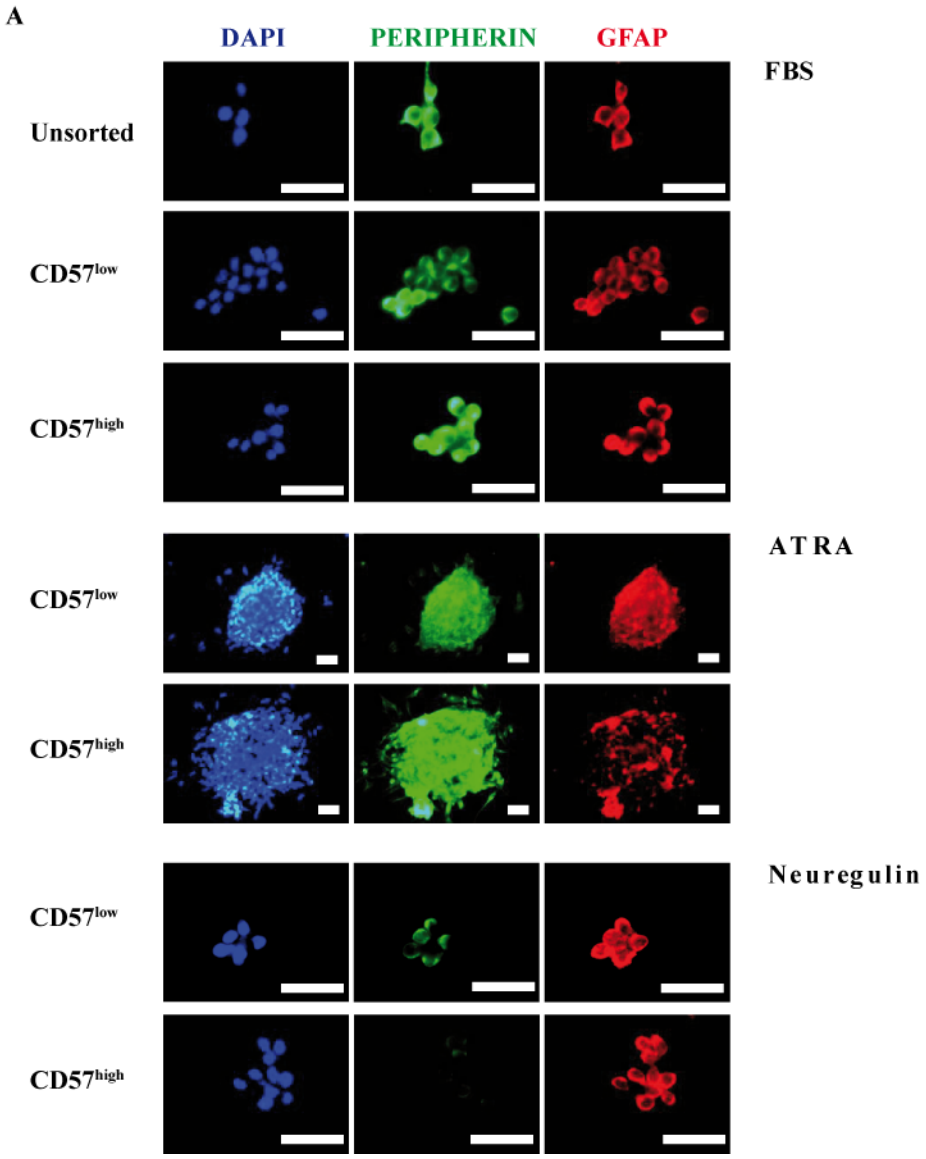

Figure 4: CD57^{high}, but not CD57^{neg}, U-NB1 cells are responsive to cues for preferential neuronal or glial differentiation

(A) CD57^{neg} and CD57^{high} U-NB1 cells were isolated on the basis of CD57 cell surface expression via FACS-separation. The cell fractions were plated in clonal density onto collagen coated coverslips and differentiation-inducing cytokines were added to RPMI 1640 medium supplemented with 2 % FBS. For the reconstitution assay 10 % FBS was added to the cell culture medium. Cells were treated over a ten day period with 1 µM ATRA or cultured in glial lineage specific medium containing NRG1-ß1 for 14 days or cultured for one week in 10 % FBS followed by double- immunofluorescence staining for monitoring of the expression of GFAP (red) and peripherin (green). Cell nuclei were stained with DAPI (blue). Scale bars correspond to 50 µm. All experiments were repeated at least three times, with similar results.

ATRA = all-trans retinoic acid; CD57 = cluster of differentiation 57; DAPI = 4',6-diamidino-2-phenylindole; FACS = fluorescent activated cell sorting; FBS = fetal bovine serum; GFAP = glial fibrillary acidic protein; µM = micromolar, NRG1-ß1 = human neuregulin1-β1; RPMI 1640 = Roswell Park Memorial Institue 1640; U-NB1 = a low passage neuroblastoma culture

3.5 $CD57^{high}$, but not $CD57^{low}$, U-NB1 cells reconstitute the heterogeneity of CD57 expression

Reconstitution of a tumor's heterogeneity is a hallmark of tumor-initiating cells. If high expression of CD57 marks NB-initiating cells, $CD57^{high}$ NB should generate both $CD57^{high}$ and $CD57^{low}$ cells, whereas $CD57^{low}$ cells should only be capable of generating $CD57^{low}$ cells. Indeed, *in vitro* $CD57^{high}$ U-NB1 cells reconstituted the heterogeneity of CD57, whereas $CD57^{neg}$ cells did not (Fig. 5A). *In vivo*, orthotopic tumors generated from $CD57^{high}$ cells contained both $CD57^{high}$ and $CD57^{low}$ cells whereas tumors from $CD57^{low}$ cells only harbored $CD57^{low}$ cells (Fig. 5B). That $CD57^{high}$ but not $CD57^{low}$ cells reconstitute the heterogeneity of CD57 expression supports the notion of $CD57^{high}$ U-NB1 representing tumor-initiating cells.

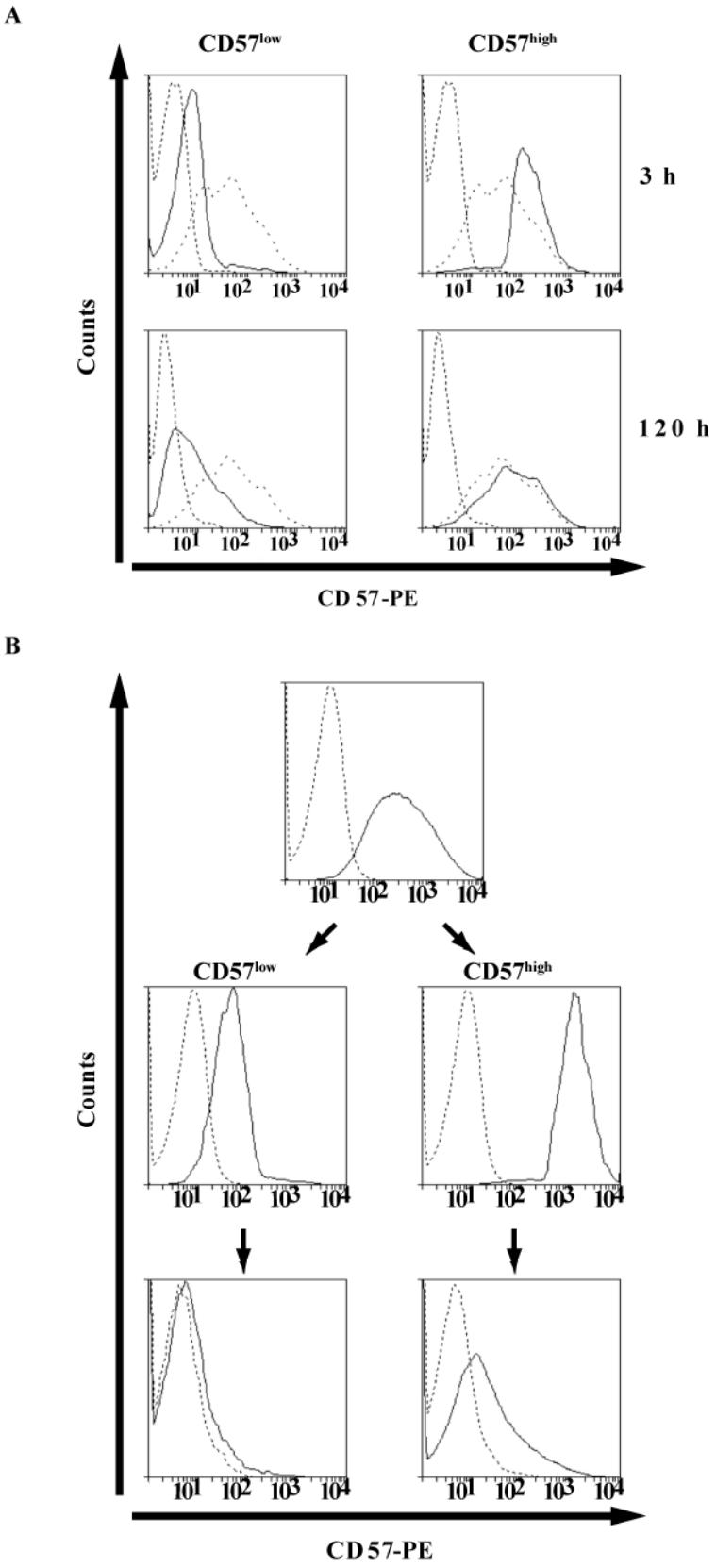

Figure 5: CD57^{high}, but not CD57^{low}, U-NB1 cells generate both CD57^{high} and CD57^{low} cells

(A) CD57^{high} cells reconstitute the heterogeneity of CD57 expression *in vitro*. FACS-fractionated CD57^{low} and CD57^{high} U-NB1 populations were collected and seeded onto collagen coated plastic in RPMI 1640 medium supplemented with 10 % FBS. The fractions were again stained and analyzed for CD57 expression on days 1 and 5 after FACS-fractionation. Solid lines show CD57 expression of the fractions, dashed lines of the unsorted parental U-NB1 culture and dotted lines represent isotype controls.

(B) CD57^{high} cells reconstitute the heterogeneity of CD57 expression in tumors. U-NB1 cells (top) were separated into CD57^{low} and CD57^{high} cells (middle panel). 1 x 10^4 cells were transplanted into the left adrenal gland of RAG$^{-/-}$/common γ-chain$^{-/-}$ mice. Mice were sacrificed 7 weeks post-transplantation, tumors were dissociated and tumor cells were analysed for expression of CD57 (lower panel). Solid lines show CD57 expression of the fractions, dotted lines represent isotype controls.

CD57 = cluster of differentiation 57; FACS = fluorescent activated cell sorting; FBS = fetal bovine serum; h = hours; PE = Phycoerythrin; RAG$^{-/-}$/common γ-chain$^{-/-}$ mice = recombinase activating gene$^{-/-}$/common γ-chain$^{-/-}$ mice; RPMI 1640 = Roswell Park Memorial Institute 1640; U-NB1 = a low passage neuroblastoma culture

3.6 CD57^{high} U-NB1 cells initiate more orthotopic tumors with shorter latency than CD57^{low} cells

For investigating tumorigenicity an orthotopic model was employed. To decrease the likelihood of culture-associated artifacts U-NB1 cells from dissociated U-NB1 tumors rather than from cultures were used. CD57^{high} and CD57^{low} cells were surgically transplanted into the adrenal glands of mice. CD57^{high} cells showed a decreased latency of tumor formation compared to CD57^{low} cells (Fig. 6A). To investigate self-renewal CD57^{high} and CD57^{low} cells were isolated from CD57^{high}-derived tumors and retransplanted orthotopically. CD57^{high} cells formed tumors with decreased latency and with increased frequency compared to CD57^{low} cells. Autopsy confirmed the findings of palpation in regard to tumor frequency (data not shown). The increased tumorigenicity upon retransplantation suggests that CD57^{high} U-NB1 cells self-renew more efficiently than CD57^{low} cells.

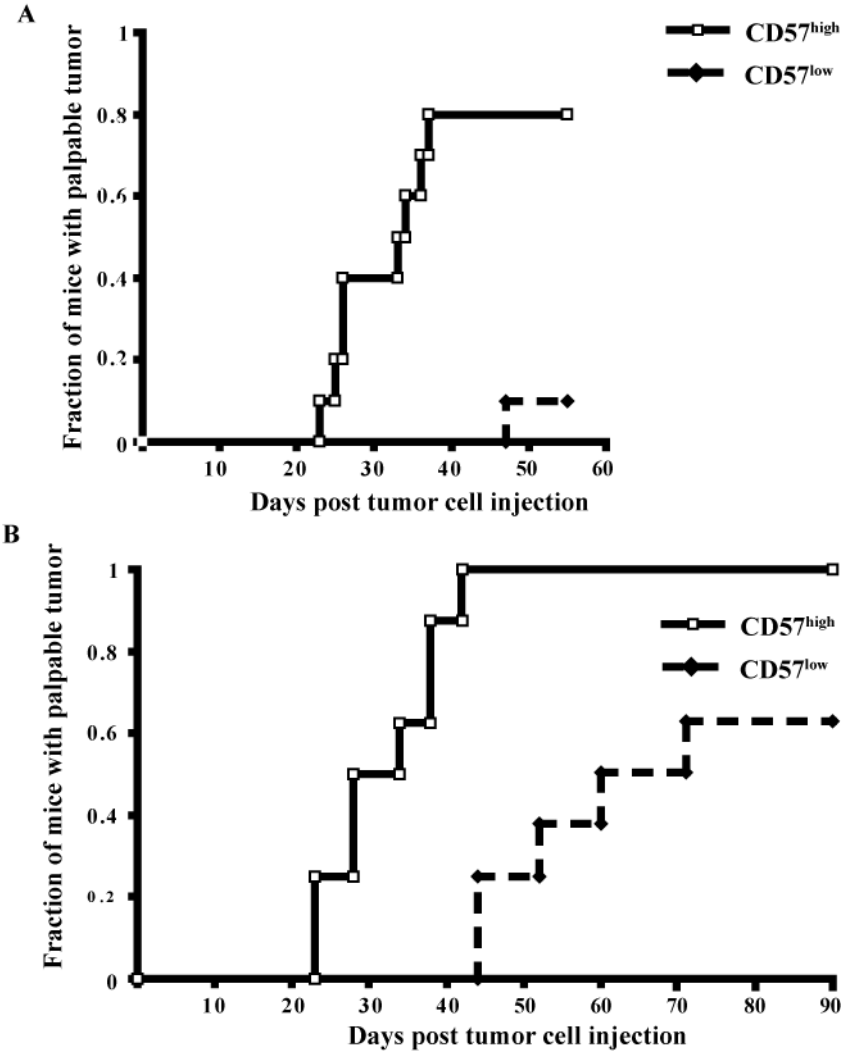

Figure 6: Increased frequency and decreased latency of tumor formation of CD57^{high} U-NB1 cells

(A) CD57^{high} U-NB1 cells generate orthotopic tumors with shorter latency than CD57^{low} cells. U-NB1 tumors were dissociated to single cells. CD57^{high} and CD57^{low} cells were isolated by FACS. 10^4 cells were surgically transplanted into the left adrenal glands of RAG$^{-/-}$/common γ-chain$^{-/-}$ mice (10 animals per group). Mice were monitored weekly by palpation to detect development of tumors. Mice were sacrificed at 56 days when mice in the CD57^{high} group became sick. Autopsies were performed in all mice to verify the presence or absence of tumors.

(B) Upon repeated injection CD57^{high} U-NB1 cells initiate more and faster appearing tumors than CD57^{low} cells. For repeat tumor cell transplantation cells from tumors initiated by CD57^{high} cells were dissociated, sorted into CD57^{high} and CD57^{low} fractions and injected intraadrenally (10 animals per group). The development of tumors was monitored by palpation and autoptically verified when mice had to be killed because of excessively large tumors or when the experiment was terminated at 90 days.

CD57 = cluster of differentiation 57; FACS = fluorescent activated cell sorting; $RAG^{-/-}$/common γ-$chain^{-/-}$ mice = recombinase activating $gene^{-/-}$/common γ-$chain^{-/-}$ mice; U-NB1 = a low passage neuroblastoma culture

4. Discussion

We have described a subpopulation of NB cells with high expression of CD57 and characteristics of tumor-initiating cells. Thus, we show that $CD57^{high}$ NB cells have increased clonogenicity, enhanced sphere-inducing capacity, a more undifferentiated phenotype, an increased response to differentiation cues and the ability to reconstitute cellular heterogeneity. Most important, $CD57^{high}$ NB cells possess enhanced tumorigenicity and self-renewal.

4.1 U-NB1, a new low-passage NB sphere culture of the I-type

We first established a NB sphere culture that by morphology, coexpression of peripherin and GFAP, high clonogenicity in soft agar and marked tumorigenicity fullfilled the criteria of I-type NB cells that are known to include as yet undefined tumor-initiating cells [10, 13, 43, 44, 51]. By virtue of limited passage and of being maintained as a sphere culture U-NB1 cells are akin to primary NB cells and were thus used to complement the established I-type NB cell line SK-N-BE(2)-C. Cell lines have been shown to retain and even amplify a stem cell hierarchy [25, 30, 34, 55], making them a suitable model to characterize NB-initiating cells. Clearly, however, $CD57^{+}$ tumor-initiating cells should also be sought for in cells acutely dissociated from primary NB.

4.2 Increased clonogenicity and sphere-inducing capacity of $CD57^{high}$ NB cells

Anchorage-independent clonogenic growth, a defining in vitro property of aggressive cancer cells, almost exclusively segregated with the $CD57^{high}$ fraction of SK-N-BE(2)-C cells. Similarly, spheroid growth in low-serum conditions was induced and maintained more efficiently by the $CD57^{high}$ fraction of U-NB1 cells. As with other cancer cells the ability of SK-N-BE(2)-C cells to grow as suspended spheroids in low-serum conditions may reflect v-akt Murine Thymoma Viral Oncogene Homolog (AKT) -mediated anoikis resistance [22], enhanced growth factor independence or other, yet to be identified mechanisms. Intercellular adhesion mediated by homophilic interaction of CD57 [16] may add to the propensity of $CD57^{high}$ to form spheres.

Independent of the mechanisms underlying spheroid growth, NB spheroids growing in low-serum are, like other cancer spheroids, enriched with tumor-initiating cells [22]. Thus, the enhanced ability of $CD57^{high}$ U-NB1 cells to grow as spheres suggested that this population contains NB-initiating cells.

4.3 Increased response of $CD57^{high}$ NB cells to differentiation cues

A major reason why we chose to investigate CD57 as a potential marker for NB-initiating cells is the strong expression of CD57 in migrating NCSCs [7, 8].

Such NCSCs give rise to the sympathetic neuroblasts and glia cells of peripheral sympathetic ganglia [48]. These NCSCs, or the sympathetic neuroblasts derived from them, are potential, although as yet unproven, cells of origin of NB [23, 40, 52]. While the transformed cell of origin of NB does not necessarily constitute its tumor-initiating cell, it is reasonable to assume that both share common features. NB can contain, in addition to neuroblasts or more mature ganglion cells, a stromal component consisting of glial or Schwann cells [45]. While there is strong evidence that this stromal component consists of non-malignant cells that have migrated into the tumor [3], there is also evidence that these stromal cells may have the same clonal derivation as the neuroblastic or ganglionic cells [39]. The latter would support the notion of the NCSC being the cell-of-origin of NB. Given these cues we reasoned that NB-initiating cells may not only be susceptible to peripheral neuronal but also to glial differentiation. U-NB1 and SK-N-BE(2)-C are I-type NB cells, which characteristically coexpress the peripheral neuronal maker peripherin and the glial marker GFAP. Thus, differentiation into either lineage becomes evident by maintenance of one and loss of the other lineage marker. We show that $CD57^{high}$ but not $CD57^{low}$ NB cells can be differentiated into either lineage. This supports the notion that $CD57^{high}$ cells are NB-initiating cells, and is in line with NCSCs being potential cells of origin of NB.

In addition to being more responsive to differentiation cues the $CD57^{high}$ fraction contained more cells neither expressing peripherin nor GFAP. These cells disappeared upon forced differentiation, consistent with being of a less differentiated phenotype than $CD57^{high}$ $peripherin^{+}$ $GFAP^{+}$ cells.

4.4 Reconstitution of heterogeneity of CD57^{high} NB cells

Reconstitution of tumor heterogeneity is a hallmark of tumor-initiating cells. U-NB1 and SK-N-BE(2)-C cells have an undifferentiated homogenous phenotype. Therefore we investigated reconstitution of the heterogeneity of CD57 expression by CD57^{high} versus CD57^{low} cells. Consistent with CD57^{high} U-NB1 and SK-N-BE(2)-C cells representing tumor-inititating cells only the CD57^{high} fractions reconstituted *in vitro* and *in vivo* the distribution of CD57 expression found in the unfractionated cells.

4.5 Enhanced self-renewal of CD57^{high} NB cells

The defining characteristic of a tumor-initiating cell is its enhanced ability to self-renew. One manifestation of self-renewal is the ability of a cancer cell to generate tumors upon serial transplantation. After orthotopic transplantation into the adrenal gland tumorigenicitiy of CD57^{high} U-NB1 cells formed tumors faster than CD57^{low} cells. Upon retransplantation of cells from CD57^{high}-derived tumors, tumorigenicity was increased in the CD57^{high} fraction. Thus, the CD57^{high} fraction of U-NB1 cells contains cells with enhanced self-renewal ability characteristics of NB-initiating cells. This conclusion is also supported by the fact that CD57^{high} but not CD57^{low} cells generate both the CD57^{high} tumor-initiating cell and the CD57^{low} transit-amplifying cell, which suggests asymmetric division of CD57^{high} cells. Tumorigenicity was not confined to CD57^{high} cells. This shows that the CD57^{high} fraction is enriched with, but does not contain all, NB-initiating cells.

4.6 Potential link between strong expression of CD57 and tumor initiation in U-NB1 and SK-N-BE(2)-C cells

Compared to their CD57^{low} counterparts CD57^{high} U-NB1 and SK-N-BE(2)-C cells show enhanced tumorigenicity, self-renewal, differentiation capacity, reconstitution of heterogeneity, sphere induction and clonogenicity, id est characteristics of NB-initiating cells. The question arises why strong expression of CD57 is associated with NB-initiating cells.

CD57 is known to mediate cell-cell interactions [16], adherence of cells to laminin of the extracellular matrix via binding to integrins [19, 20], invasion and migration [7, 8], and metastasis [49]. Many of these mechanisms would also be important for NB-initiating cells, it remains to be explained how CD57 mediates self-renewal and differentiation capacity, the two hallmarks of tumor-initiating cells. As CD57 defines migrating NCSCs [7, 8] from which NB is thought to arise [23, 40, 52] it is plausible that some NB cells have coopted CD57-associated traits from NCSCs, thus becoming endowed with some of the features defining NB-initiating cells.

4.7 Prognostic implications of CD57^{high} NB cells

We show that CD57^{high} NB cells have attributes of tumor-initiating cells. This is consistent with data indicating that NB cells strongly expressing CD57 have an immature phenotype [26]. However, our data may appear to be at odds with findings showing that NB with less than 5 % CD57-positive cells have a poorer prognosis than those with more CD57-positive cells [12]. However, these data do not contradict ours, which predict that the number of cells strongly expressing CD57 rather than the number of NB cells expressing any amount of CD57 determines the aggressiveness of NB. An alternative reconciliation may lie in the proclivity of CD57^{high} NB cells to differentiate. This may enhance therapy-induced differentiation, thus overriding the poor prognosis features associated with CD57^{high} NB-initiating cells.

In conslusion, we have provided evidence that CD57^{high} NB cells have characteristics of tumor-initiating cells. This warrants further study of clinically annotated neuroblastoma samples to determine the prognostic significance of CD57^{high} NB-initiating cells.

5. Summary

Neuroblastoma, an extracranial solid tumor of childhood, is thought to originate from neural crest-derived cells and is known to contain tumor-initiating cells. Cluster of differentiation 57 (CD57) defines migratory neural crest stem cells in normal development and is expressed in neuroblastoma. Thus it is possible that markers which define neural crest stem cells and their derivatives and that are expressed in Neuroblastoma (NB) may serve as markers for potential NB stem cells. Surface antigens are particular important markers for cancer stem cells because they allow the isolation of viable tumor cells. For these reasons we considered that CD57 may serve as a marker for NB stem cells.

We show that a low passage neuroblastoma culture (U-NB1 cells) and a neuroblastoma cell line (SK-N-BE(2)-C cells) contain a fraction of cells that strongly expresses CD57. In order to explore whether $CD57^{high}$ SK-N-BE(2)-C cells were enriched for cancer stem-like cells, we performed soft agar clonogenicity assays and showed that only $CD57^{high}$ SK-N-BE(2)-C cells were clonogenic. $CD57^{high}$ SK-N-BE(2)-C as well as U-NB1 cells showed increased tumor sphere-inducing capacity and clonogenicity.

$CD57^{high}$ cells also displayed enhanced tumorigenicity and self-renewal capacity when transplanted into adrenal glands of mice, an orthotopic location for neuroblastoma.

To assess the multilineage differentiation potential of NB cells, differentiation was induced by addition of differentiation-inducing cytokines. $CD57^{high}$ cells could be bidirectionally differentiated towards a predominant neuronal and glial phenotype. $CD57^{high}$ NB cells can spontaneously differentiate into cells exhibiting the biphenotype and reconstitute cellular tumor heterogeneity as well as the $CD57^{low}$ tumor cell fraction.

Taken together, $CD57^{high}$ NB cells have attributes of tumor-initiating cells. This may have important implications for the prognosis of neuroblastoma.

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Anne-Marie Schlitter, February 2010

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Doctoral Thesis

since 02/2007 **"CD57^{high} neuroblastoma cells have characteristics of tumor-initiating cells"**

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