Endosialin: A novel malignant cell therapeutic target for neuroblastoma

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Abstract. Endosialin emerged recently as a potential therapeutic target for sarcoma. Since some sarcoma subtypes, such as Ewing's sarcoma, show characteristics of neuroendocrine differentiation, we wondered whether cancers with neuroendocrine properties and/or neuroectodermal origin, such as neuroblastoma, small cell lung cancer and melanoma, may express endosialin. Endosialin protein expression was surveyed in neuroblastoma, small cell lung cancer and melanoma in human clinical specimens by immunohistochemistry (IHC) and in human cell lines by flow cytometry. Side population cells were examined to determine whether cancer stem cells can express endosialin. Endosialin-expressing neuroblastoma cell lines were implanted in immunodeficient mice and allowed to grow. The xenograft tumors were resected and tested for endosialin expression by IHC. In human clinical specimens, vascular endosialin staining was observed in neuroblastoma, small cell lung cancer and melanoma. Malignant cell staining was strongest in neuroblastoma, weak in melanoma and rare in small cell lung cancer. In human cell lines, endosialin was detected in neuroblastoma cell lines, including cancer stem cell-like side population (SP) cells, but was absent in melanoma and was both rare and weak in small cell lung cancer. Human neuroblastoma xenograft tumors were found to be positive for endosialin. Our work suggests that endosialin may be a suitable therapeutic target for neuroblastoma.

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Introduction

Endosialin/CD248/TEM1 was first identified as a protein expressed in tumor vasculature, with predominant expression in pericytes (1-9). Endosialin recently emerged as a potential therapeutic target for sarcoma. Our group conducted a survey of endosialin expression in 86 paraffin-embedded clinical specimens of sarcoma. Immunoreactive tissue components in sarcomas were malignant cells, stromal cells and vasculature. Seventy (81%) were positive for endosialin, with 44 (51%) reaching at least 50% coverage of immunoreactive tissue components. Staining intensity was scored on the scale 0, 1+, 2+, 3+. All 9 sarcoma subtypes included specimens with at least 50% immunoreactive tissue components positive with a minimum of 2+ staining intensity indicating the high prevalence of endosialin in sarcomas (10-12).

Ewing's sarcoma is one of the sarcoma subtypes in which endosialin can be detected in malignant cells (11,12). Since the Ewing's sarcoma family of tumors includes tumors that display microscopic and immunohistochemical evidence of neural differentiation, peripheral primitive neuroectodermal tumors (PNET), we wondered whether other tumor types with neuroendocrine features or neuroectodermal origin may also express endosialin (13,14). Neuroblastoma, a malignancy of neural crest origin for which there is anecdotal evidence of endosialin expression was examined (1,15). Melanoma, a malignancy of neuroectodermal origin, and small cell lung cancer, a disease where markers of neuronal differentiation can be found in nearly 75% of clinical specimens were also examined (16-18).

Neuroblastoma, melanoma and small cell lung cancer represent a high unmet medical need. Neuroblastoma is the most common extra-cranial cancer in childhood (19). It is a neural crest-derived peripheral nervous system tumor with varied pathobiology. While low-risk disease can be cured with surgery, high-risk disease requires intensive radiotherapy, chemotherapy and/or immunotherapy. Stage 4S neuroblastoma is defined by dissemination to liver and skin. It cannot be

treated and requires supportive care (15). The MYCN gene is amplified in 40% of high-risk neuroblastomas and expression of N-myc protein is associated with poor prognosis (20,21).

Melanoma is a neural crest-derived peripheral nervous system tumor. One of the earliest evidence of the neuroectodermal origin of melanoma is an experiment where neural crest cells isolated from quail embryos were observed to give rise to melanocytes in culture (16). Metastatic melanoma has a 10-year survival rate of only 10%. The standard of care for metastatic melanoma is dacarbazine which has a single-agent objective response rate of only 15%. The overall survival benefit of dacarbazine is unclear and its administration is essentially palliative (22).

Small cell lung cancer is distinct from other lung cancers due to its neuroendocrine features including expression of dopa decarboxylase, calcitonin, neuron-specific enolase, chromogranin A, CD56 (neural cell adhesion molecule), gastrin-releasing peptide (GRP), and insulin-like growth factor 1 (IGF1). Small cell lung cancer is initially very responsive to chemotherapy; however, most patients relapse and die within 2 years (17). A recent review of nearly three decades of small cell lung cancer phase III clinical trials for extensive disease involving over 10,000 patients found no significant improvement in patient survival times over that period of time (23).

We examined neuroblastoma, melanoma and small cell lung cancer clinical specimens and human cell lines for endosialin expression and found marked differences in endosialin expression among the three cancer types with substantial malignant cell endosialin expression in neuroblastoma. The data demonstrate that neuroblastoma is distinct from melanoma and small cell lung cancer in displaying malignant cell expression of endosialin in addition to vascular expression of endosialin. Study of two human neuroblastoma cell lines showed that side population cells from both lines maintained expression of endosialin. When grown as a subcutaneous or sub-renal capsule implant xenograft in immunodeficient mice, SK-N-AS neuroblastoma robustly expressed endosialin.

Materials and methods

Clinical specimens. Human clinical specimens were obtained from Genzyme Genetics, Analytical Services (Los Angeles, CA). All specimens were collected between 2000 and 2005.

Cell lines. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were propagated in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA).

Immunohistochemistry. The anti-endosialin antibody was generated in collaboration with Kyowa Hakko Kirin Co., Ltd, Takasaki, Japan as described previously (11). Formalin-fixed, paraffin-embedded (FFPE) tissue sprcimens were deparaffinized with 3 changes of xylene, rehydrated in baths of 95% and 100% graded ethanol and rinsed well in running distilled water. Slides were then placed in a pressurized decloaking chamber and pretreated to 125˚C for 30 sec in sodium citrate epitope retrieval solution (Invitrogen) before cooling down for 15 min to 95˚C (slides are in the chamber for a total of 30 min). The decloaking chamber was opened and the slides were cooled to room temperature (rt) for 15 min before being removed and then washed in 3 sequential baths of Tris-buffered saline/0.1% Tween-20 wash buffer (TBST) for 3 min. All subsequent washes were performed in this manner. Slides were incubated with ready-to-use peroxidase blocking reagent (Dako, Carpinteria, CA) for 5 min at rt followed by TBST washes. Slides were then incubated with primary anti-endosialin antibody (10 μg/ml) diluted in antibody diluent (Dako) for 60 min followed by TBST washes. The slides were then incubated with a Vectastain Elite ABC detection reagent (Vector Laboratories, Burlingame, CA) diluted in TBST at 1:50 for 30 min at rt followed by TBST washes. Peroxidase reaction was visualized by incubating with a 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (Dako) for 5 min at rt and then washed in distilled water for 1 min. The slides were then re-rinsed with distilled water 3-times for 30-60 sec each, counterstained with hematoxylin (Dako) for 2 min, washed in TBST, dehydrated through graded alcohols, cleared in xylene and cover-slipped. Endosialin staining was scored independently by two pathologists. Immunoreactive tissue components were vasculature, stromal cells and/or tumor cells. Staining intensity was scored on the scale 0, 1+, 2+ and 3+. Since tumors were heterogeneous, the percentage of immunoreactive tissue components staining at each intensity level was recorded. The overall percentage refers to the three immunoreactive tissue components (vasculature, stromal cells and tumor cells) as a whole. The Long H-score is a single summary value encompassing staining intensity and percent staining coverage. The Long H-score was calculated for each specimen by multiplying each staining intensity (0, 1, 2 or 3+) by the corresponding percent tissue coverage and adding the values. Long H-scores range from 0 to 300.

Flow cytometry. Analysis of endosialin expression in live cells by flow cytometry was conducted as previously described using a fully human monoclonal antibody raised against human endosialin and a fully human isotype control antibody raised against dinitrophenol (DNP) (11). Sample acquisition was conducted on a FACS Calibur instrument (Becton-Dickinson Labware, Franklin Lakes, NJ) and analyzed with Flow Jo (Tree Star Inc., Ashland, OR). For side population analysis, cells were detached in warm Versene (PBS-EDTA, Invitrogen). Warm RPMI-2% FBS-10 mM HEPES was added once the cells were detached. The cells were centrifuged at 1,000 rpm for 5 min at rt and suspended in warm HBSS-2% FBS-10 mM HEPES at a concentration of 5 million cells per 200 μl. Five million cells per 200 μl were used per tube for staining. Hoechst 33342 (10 μg/ml) was added to each tube in the presence or absence of verapamil (100 or 500 μM). The cells were incubated in the presence of Hoechst 33342 and in the presence or absence of verapamil for 2 h at 37˚C with agitation in the dark, after which the tubes were transferred to ice. Anti-endosialin antibody (20 μg/ml) was added and incubated for 45 min on ice in cold HBSS-2% FBS-10 mM HEPES. The cells were washed once in cold HBSS-2% FBS-10 mM HEPES and suspended in 200 μl cold HBSS-2% FBS-10 mM HEPES. Phycoerythrin (PE)-labeled anti-human IgG F(ab')$_2$, (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) secondary

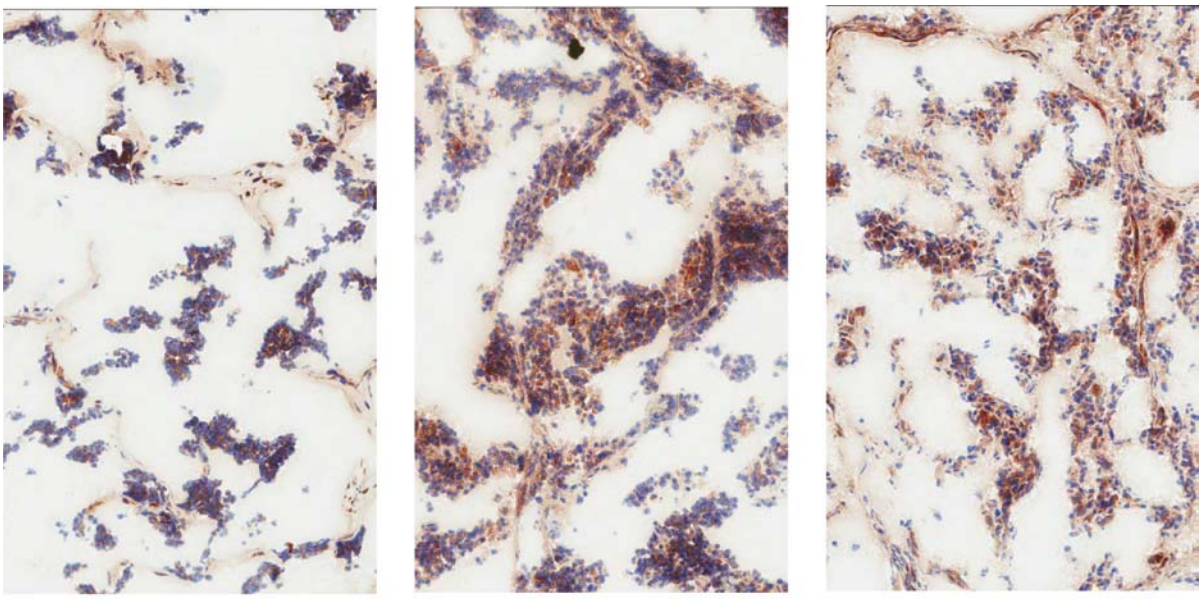
Table I. Scoring of immunohistochemical endosialin staining in human clinical specimens of neuroblastoma. Specimens 1-4 were FFPE, all others were frozen.

Specimen no.	Histologic review	Isotype control	Tumor cell intensity and percent coverage					Vascular cell intensity and percent coverage				
			3+	2+	1+	0	Long H-score	3+	2+	1+	0	Long H-score
1	Neuroblastoma	NA	0	0	0	100	0	0	0	0	100	0
2	Neuroblastoma	0	0	0	0	100	0	90	10	0	0	290
3	Neuroblastoma	NA	0	0	0	100	0	0	20	20	60	60
4	Neuroblastoma	0	0	0	0	100	0	10	20	0	70	70
5	Neuroblastoma	0	0	60	40	0	160	Too few to score				
6	Neuroblastoma	0	0	0	60	40	60	0	0	60	40	60
7	Neuroblastoma	±	0	0	20	80	20	0	0	10	90	10
8	Neuroblastoma	±	30	20	10	40	140	0	80	0	20	160
9	Neuroblastoma	±	0	40	30	30	110	0	0	30	70	30
10	Neuroblastoma	0	0	0	20	80	20	0	0	10	90	10
11	Neuroblastoma	±	0	40	30	30	110	0	60	20	20	140
12	Neuroblastoma	0	0	0	20	80	20	0	20	60	20	100
13	Neuroblastoma and adrenal	NA	0	0	0	100	0	0	0	0	100	0
14	Neuroblastoma	0	0	0	0	100	0	100	0	0	0	300
15	Neuroblastoma	0	0	20	80	0	120	70	10	10	10	240
16	Neuroblastoma	0	0	0	0	100	0	0	30	70	0	130
17	Neuroblastoma olfactory	0	0	0	10	90	10	0	80	20	0	180
18	Neuroblastoma and adrenal	0	0	0	20	80	20	10	20	60	10	100
19	Neuroblastoma in liver	0	0	0	0	100	0	0	0	5	95	5
20	Neuroblastoma*	0	0	0	10	90	10	0	40	50	10	130
21	Neuroblastoma*	0	0	0	20	80	20	30	50	10	10	200
22	Neuroblastoma	0	0	60	40	0	160	Too few to score				
23	Neuroblastoma	0	0	0	60	40	60	0	0	60	40	60
24	Neuroblastoma	±	0	0	20	80	20	0	0	10	90	10
25	Neuroblastoma	±	30	20	10	40	140	0	80	0	20	160
26	Neuroblastoma	±	0	40	30	30	110	0	0	30	70	30
27	Neuroblastoma	0	0	0	20	80	20	0	0	10	90	10
28	Neuroblastoma	±	0	40	30	30	110	0	60	20	20	140
29	Neuroblastoma	0	0	0	20	80	20	0	20	60	20	100
30	Neuroblastoma	NA	0	0	30	70	30	60	20	0	20	220

antibody was added at a dilution of 1/40 and incubated on ice for 45 min. The cells were washed once in cold HBSS-2% FBS-10 mM HEPES and suspended in 500 μl cold HBSS-2% FBS-10 mM HEPES. Propidium iodide (PI) (0.5 μg/ml) was added immediately prior to the acquisition. Cells were analyzed by flow cytometry with a 4-laser equipped SORP LSRII (Becton-Dickinson Biosciences, San Jose, CA, USA). PI was excited by 488-nm laser and the resulting fluorescence detected using a filter 610/20 with a LP dichroid mirror 610LP. Hoechst 33342 was excited with a 355 nm UV laser, and fluorescence measured dually: Hoechst Red with 605/40 filter, and Hoechst Blue with 450/40. PE was excited by 488-nm laser and the resulting fluorescence detected using a 575/26 filter with a 550LP dichroid. Data analysis was conducted in Flow Jo (Tree Star Inc.).

Chromosome analysis. Cell cultures were harvested and prepared for cytogenetic analysis using standard methods for *in situ* G-banding analysis. Cultures were exposed overnight to bromodeoxyuridine and colcemid followed by hypotonic treatment in 0.8% citrate and fixation using three changes of 3:1 (methanol/acetic acid) fixative. Coverslips were dried, removed from culture vessels, mounted on glass slides, and banded using dilute Trypsin-EDTA and Wright's stain. For each cell line, 20 metaphase cells were examined. Results are described in ISCN 2005 nomenclature.

A



B

Specimen ID	Histologic Review	Isotype control	TUMOR CELL intensity and percent coverage				
			3+	2+	1+	0	Long H-score
SK-N-DZ	Tumor	0	0	25	70	5	120
SK-N-AS	Tumor	0	76	13	8	4	260
IMR-32	Tumor	0	0	10	70	20	90
SK-N-Be(2)C	Tumor	0	0	45	50	5	140
Be(2)M17	Tumor	0	0	15	75	10	105

C

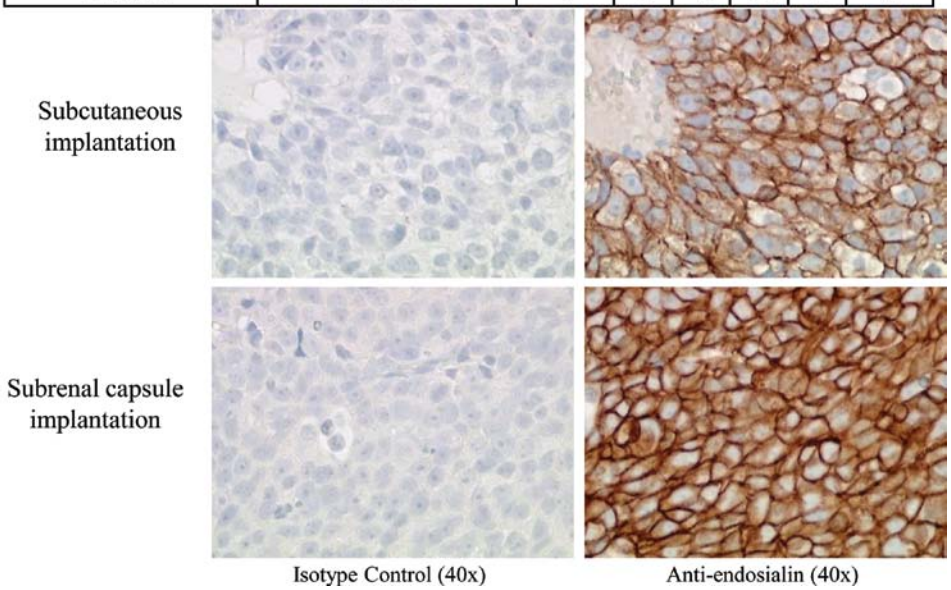


Figure 1. (A), Expression of endosialin by immunohistochemistry in three human clinical specimens of neuroblastoma is shown (Table I). (B), Scoring of immunohistochemical endosialin staining in human neuroblastoma xenograft tumors implanted subcutaneously (average scores from multiple sections). (C), Expression of endosialin staining in human SK-N-AS xenograft tumor implanted subcutaneously and in the subrenal capsule.

Fluorescence in situ hybridization. Fluorescence *in situ* hybridization was conducted on cells grown and harvested *in situ* on glass coverslips using standard cytogenetic methods. Coverslips were mounted cell-side-up on slides, pretreated, and co-denatured with a probe for N-MYC on chromosome 2 at p24 (Abbott Molecular, Des Plaines, IL). Slides were hybridized overnight at 37˚C and washed in 0.4X SSC, 0.3% NP-40 at 70˚C for 2 min followed by a room temperature wash in 2X

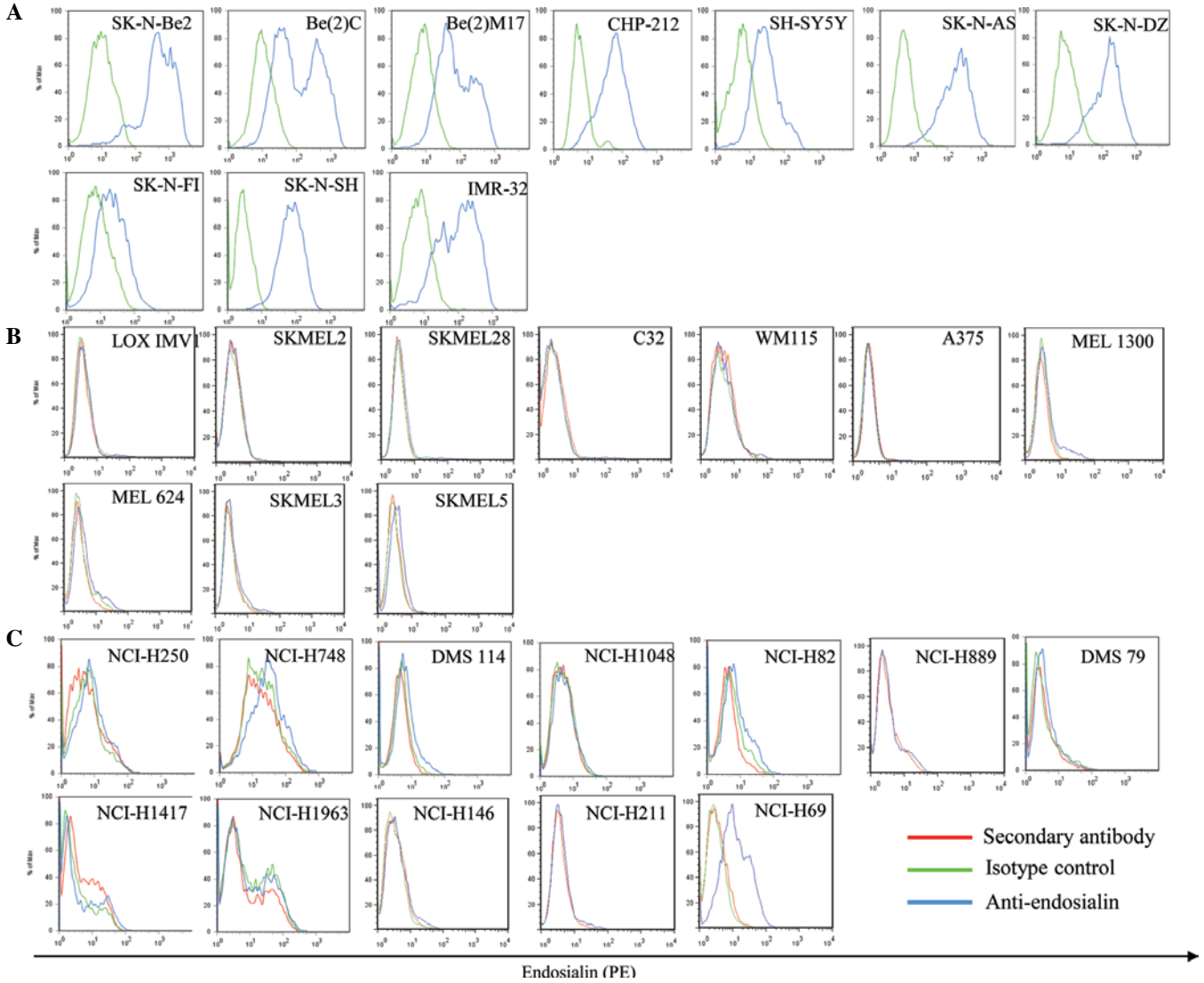


Figure 2. Expression of endosialin *in vitro* by flow cytometry in: (A), human neuroblastoma; (B), melanoma and (C), small cell lung cancer cell lines is shown.

SSC, 0.1% NP-40 for 1 min. Slides were air dried and counter-stained with diamino-2-phenylindole. Using a fluorescence microscope, 200 interphase nuclei were analyzed independently by two observers and scored for the percentage of nuclei with specific hybridization patterns.

In vivo tumor xenografts. All procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee in accordance with the Federal Animal Welfare Act (9 CFR, 1992) and conducted in an AAALAC accredited facility. Cells propagated in culture were implanted subcutaneously ($1x10^6$) in the flanks of nude mice (Harlan Laboratories, Inc., Indianapolis, IN) and passaged 4-times from mouse to mouse using trocar implants. Animals were sacrificed and tumors excised when tumor size reached 400 mm^3.

Results

Endosialin expression was surveyed by immunohistochemistry in 30 human clinical specimens of neuroblastoma. Endosialin was detected in the vasculature and in the malignant cells. Two of the 30 specimens were poorly vascular, as a result vascular endosialin could only be scored in 28/30 specimens. Vascular endosialin was detected in 26 of 28 specimens in which vasculature could be scored. Ten of 28 specimens, or approximately one third, had at least 50% coverage of the vasculature with at least 2+ staining intensity. Twenty-two of 30 specimens had endosialin-positive malignant cells, with eight, or nearly one third, having at least 40% coverage of all malignant cells with at least 2+ staining intensity (Table I and Fig. 1A). Ten human neuroblastoma cell lines were assayed for endosialin expression in culture by flow cytometry and 10 of 10 were found to be positive for endosialin, although SK-N-FI was only weakly positive (Fig. 2A).

Given the prevalence of MYCN gene amplification among neuroblastoma patients with poor prognosis, we wondered whether the 10 cell lines tested for endosialin by flow cytometry were positive for MYCN amplification (20,21). Nine of 10 lines were tested by fluorescence *in situ* hybridization (FISH). Six of 9 neuroblastoma lines were positive for MYCN amplification and 3 of 10 cell lines were negative for MYCN amplification, SK-N-AS, SK-N-FI and SH-SY5Y (Table II).

Table II. Cytogenetic analysis and MYCN amplification by fluorescence *in situ* hybridization (FISH) in human neuroblastoma cell lines are shown.

Designation	Cytogenetic analysis	MYCN FISH
SK-N-AS	44-47,XX,+1,psu dic(1;14)(p22;?q22),der(?)t(?;5)(?;q11.2), der(6)add(6)(p23)add(6)(q2?5),add(8)(q24.1),add(9)p22),add(11)(q13), add(16)(q2?4),-17,add(22)(q12),+0-4mar[cp20] Abnormalities specific to a particular neoplasm were not observed. 20 metaphases analyzed.	Negative for N-MYC. Four copies of MYCN/CEP2 were observed in 9.6% of the cells.
IMR-32	48,XY,+1,hsr(1)(p32)x2,+6,add(16)(q24)[3]/49,idem,+12[6] Homogeneously staining regions (HSR) were found in two copies of a chromosome 1 in the short arm. HSR is generally considered evidence of gene amplification, a not infrequent phenomenon in neuroblastomas. 9 metaphases analyzed.	Positive for amplification of the MYCN gene in 100% of the cells.
SK-N-FI	44-49,XY,-Y,del(1)(q42),del(2)(q33),-4,add(5)(q31),-8, del(8)(p11.2),+9,+12,del(12)(p11.2),+13,add(15)(q26),-17,-18, add(19)(p13.3)x2,add(20)(q13.3),+21,-22,+1-5mar[cp17] 17 metaphases analyzed.	Negative for N-MYC
SK-N-DZ	82-89<4n>,XXX,-X-,X,-1,der(1)t(1;1)(p36.1;q21),del(1)(q32),-2, hsr(2)(p11.2)x2,del(3)(p13),-4,-4,del(8)(q13q22),del(9)(q13q32), +11,add(11)(q23),del(11)(q13)x2,-13,-14,add(19)(q13),+2-7mar[cp20] Two chromosomes 2 have homogenously staining regions (HSR) occupying the central portion of the chromosome. HSr is usually considered evidence of gene amplification, a phenomenon that is not infrequently observed in neuroblastomas. 20 metaphases analyzed.	Positive for amplification of the MYCN gene in 99.5% of the cells.
SH-SY5Y	47,XX,dup(1)(q44q31),+7,dup(9)(q21q33),der(22)t(17;22) (q11.2;q11.2)[19]/47,idem,del(4)(q12)[1] Most of the long arm of chromosome 22 has been replaced by most of the long arm of chromosome 17. Rearrangements, such as this, that result in gain of chromatin from the chromosome 17 long arm are frequently seen in neuroblastomas. 20 metaphases analyzed.	Negative for N-MYC
Be(2)-M17	40,X,-X/Y,dup(1)(q13p36.3),der(1;15)(q10;q10),der(3)t(3;17)(p21;q21), der(7)t(1;7)(q13;q21),add(9)(p22),-10,der(11;22)(q10;q10),-16,-17,-18,-19, +mar1,+mar2(hsr)[20] A marker with a large homogeneously staining region (HSR) was found. HSRd are cytogenetic evidence of gene amplification. 20 metaphases analyzed.	Positive for amplification of the MYCN gene in 99.5% of the cells.
Be(2)-C	73-75<4n>XX,-X,-X,add(1)(p31)x2,-2,-2, add(2)(p23)x2,add(3)(p23)x2, hsr(4)(q21)x2,-5,hsr(6)(p21)x2,dup(7)(q22q31)x2,del(9)(p13)x2,-10, -11,-11,der(13;15)(q10;10)x2,del(16)(p11)x3,-17,-17,-18,-18, add(19)(p13)x2,der(20)t(11;20)(q13;p13)x2,+22,+22,+2mar[cp20] Extremely undtable karyotype. One chromosome 4 and 1 chromosome 6 have homogeneously staining regions (HSRs) in their long and short arms, respectively. HSR is generally considered evidence of gene amplification and is frequently found in neuroblastomas. 20 metaphases analyzed.	Positive for amplification of the MYCN gene in 100% of the cells.

Table II. Continued.

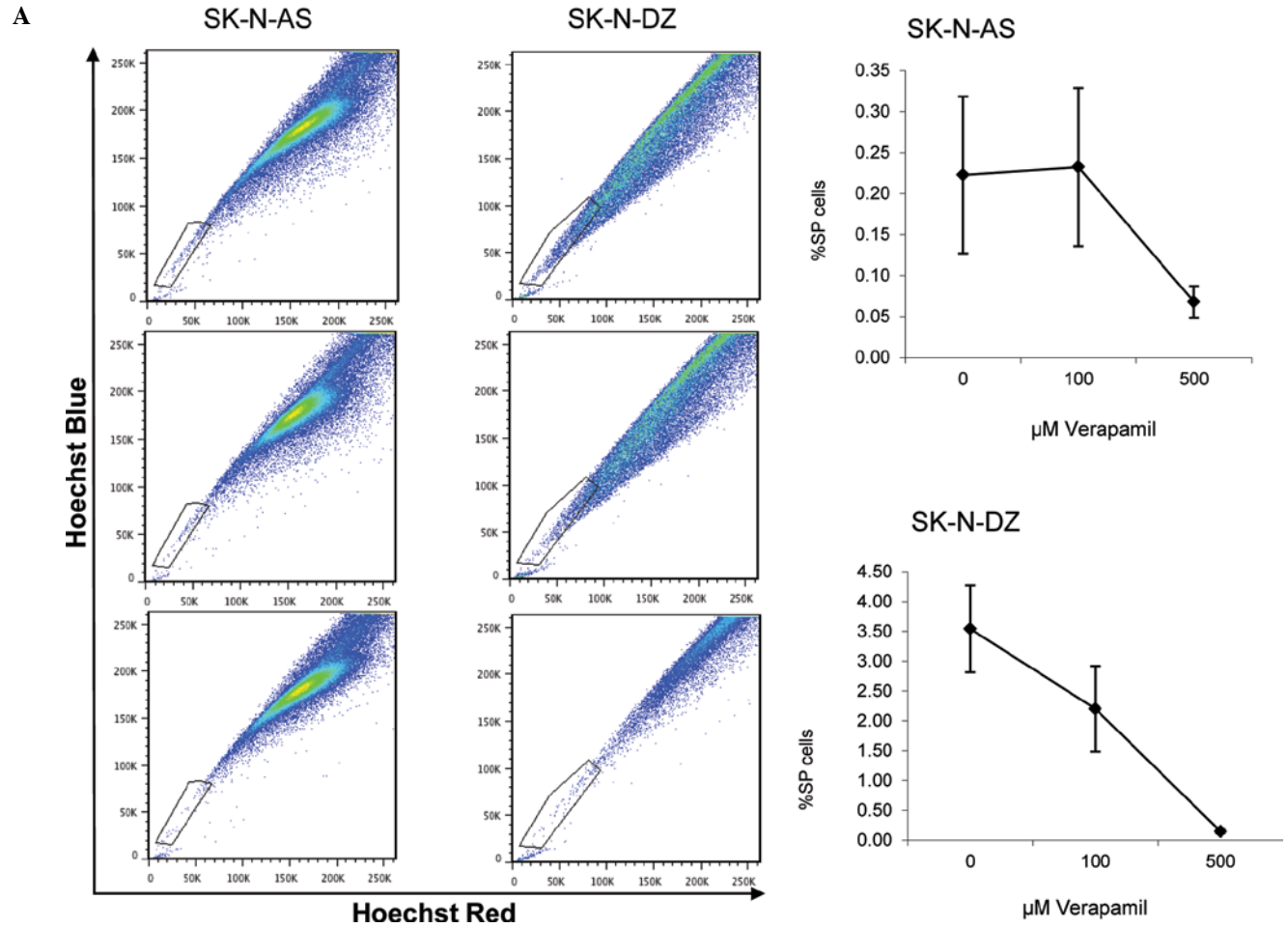
Designation	Cytogenetic analysis	MYCN FISH
SK-N-Be(2)	Four related clones were observed: 1) 46,XY,+1,add(1)(p13),der(3)t(3;17)(p21;q21),hsr(4)(q23),hsr(6)(p21.2), -18,der(20)t(11;20)(q13;p13)[6]/ 2) 44,XY,iso(1)(q10),der(3)t(3;17)(p21;q21),add(8)(p21), add(9)(p13),der(9)add(9)(p24)del(9)(q32),-10,-17,-18,+hsr mar[cp5]/ 3) 43-46,XY,+1,add(1)(p13),der(3)t(3;17)(p21;q21),hsr(6)(p21.1),+8, -13,-18[cp6]/ 4) 43,X,-Y,add(1)(p13),der(3)t(3;17)(p21;q21),hsr(4)(q23),hsr(6)(p21.1), -13,-17,-18,-19,der(20)t(11;20)(q13;p13),-21,+2-3mar[cp3] (male) Homogeneously staining regions (HSR) appear on chromosomes 4, 6 and/or a marker. HSRs are markers of gene amplification. 20 metaphases analyzed.	Positive for amplification of the MYCN gene in 99% of the cells.
CHP-212	46,XY,add(1)(p13),der(4)t(1;4)(q11;p16),t(6;7)(q25;q11.2),add(11)(p15), add(16)(q24),+6-12dmins[12]/46,idem,add(10)(p11.2)[5]/47,idem,+9[3] Double minute chromosomes (dmin) were observed - those are characteristic of neuroblastoma and usually represent N-MYC amplification. 20 metaphases analyzed.	Positive for amplification of the MYCN gene in 100% of the cells.

A side population analysis was used to determine whether tumor stem cells could be detected in the neuroblastoma cell lines and whether the side population cells would express endosialin. The Hoechst 33342 side population (SP) flow cytometry assay which identifies cells with stem cell-like properties was applied. Two human neuroblastoma cell lines were selected for side population analysis: the MYCN-amplified SK-N-DZ cell line and non-MYCN-amplified SK-N-AS cell line. A population of low-Hoechst 33342 fluorescent, verapamil-sensitive cells could be identified in both cell lines. Side population cells accounted for 3.5% of the total live (PI-negative) SK-N-DZ cell population and 0.2% of the total live (PI-negative) SK-N-AS cell population (Fig. 3A). These findings are in line with previously published data for these cell lines (31). Hoechst 33342, which is a DNA-binding dye, was toxic to the cells, an observation made by others (32,33). Overall 65% of SK-N-AS cells exposed to Hoechst 33342 were viable (PI-negative) and 40% of SK-N-DZ cells exposed to Hoechst 33342 were viable (PI-negative) at the time of flow cytometry (data not shown). In both cell lines, nearly 100% of the total cell population was endosialin-positive and nearly 100% of side population cells were endosialin-positive. In both cell lines, levels of endosialin are similar in the total cell population and in the side population cells (Fig. 3B). Thus, endosialin is a possible therapeutic target for neuroblastoma stem cell-like cells.

Five neuroblastoma cell lines were implanted subcutaneously in the flank of immunodeficient mice: SK-N-DZ, SK-N-AS, IMR-32, SK-N-Be(2)C and Be(2)-M17. All five cell lines formed endosialin-positive tumors. The highest level of endosialin staining was observed in SK-N-AS tumors where an average of 76% of tumor cells stained positive at the 3+ intensity level reaching an average Long-H score of 260/300 (Fig. 1B). The next highest level of endosialin staining was observed in SK-N-Be(2)C tumors where an average of 45% of tumor cells stained positive at the 2+ intensity level reaching an average Long-H score of 140/300 (Fig. 1B). When SK-N-AS cells were implanted subcutaneously or in the subrenal capsule of immunodeficient mice, the resulting tumors were endosialin-positive (Fig. 1C). Therefore endosialin expression detected in cell culture was maintained *in vivo* and in different tissue environments; thus providing further evidence that endosialin may be a suitable therapeutic target for advanced neuroblastoma.

Endosialin expression was also studied in melanoma which, like neuroblastoma, is a neural crest-derived malignancy (16). In clinical specimens, endosialin was detected in the vasculature in 19 of 19 specimens tested, with 10 of 19 specimens reaching 3+ staining intensity. Expression in malignant cells was rare, with only 3/19 specimens reaching 2+ staining intensity in melanoma cells. In these 3 specimens, the malignant cell coverage at the 2+ staining intensity level was weak, reaching only 10-20% of total melanoma cells (Table III). The flow cytometry data obtained from human melanoma cell lines concur with the overall weak expression or lack of malignant cell expression observed in clinical specimens. None of the 10 human melanoma cell lines tested by flow cytometry expressed detectable endosialin (Fig. 2B).

Because of the high prevalence of neuronal differentiation in small cell lung cancer, endosialin expression was examined in this tumor (17,18). Thirteen of 16 human small cell lung cancer clinical specimens tested showed vascular expression of endosialin with 6 of 16 samples reaching 10-20% vascular coverage at the 3+ intensity level (Table III). Endosialin expression in small cell lung malignant cells was very rare. Three of 16 specimens had expression in malignant cells with



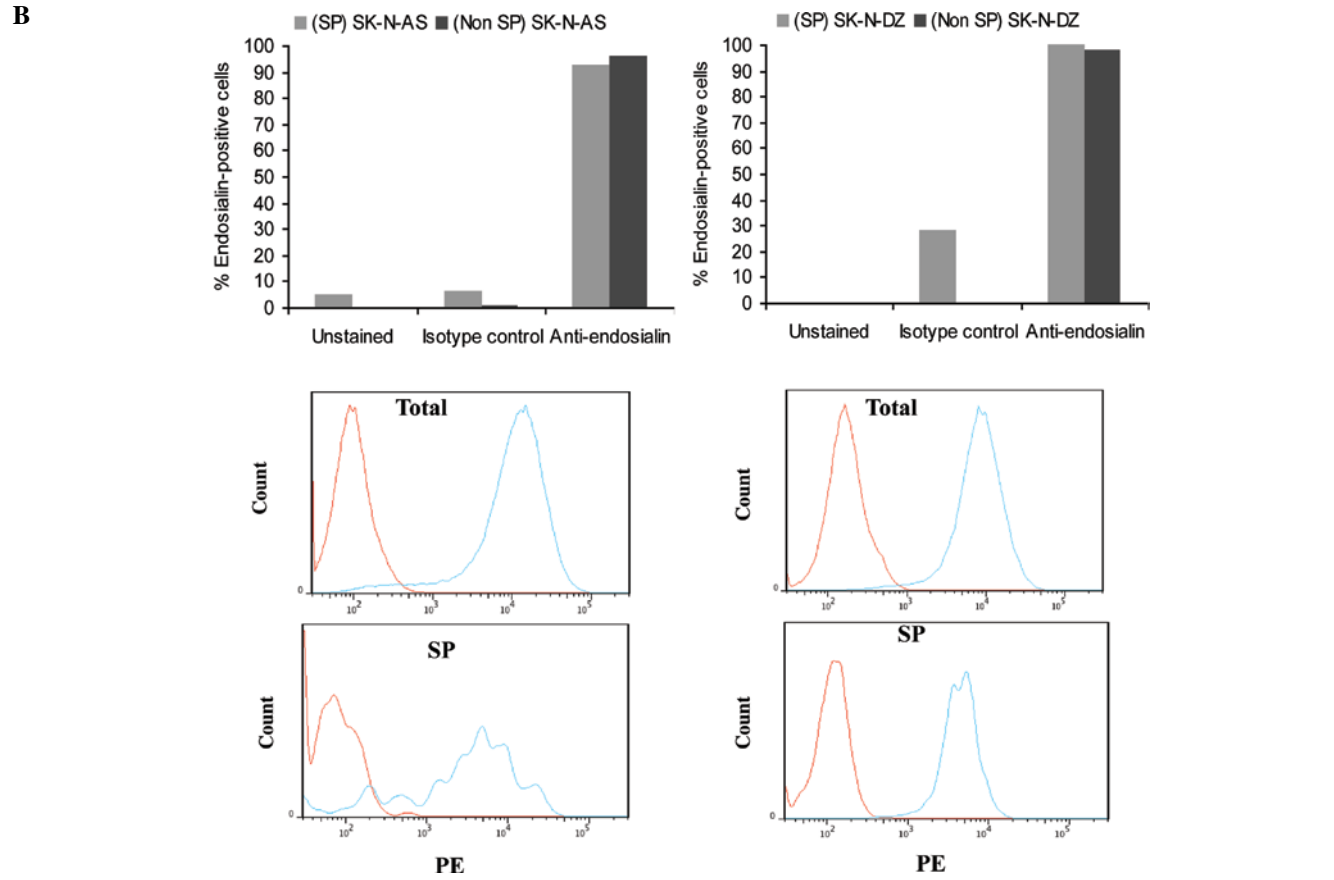


Figure 3. (A), Flow cytometric detection of side population cells in two human neuroblastoma cell lines. 0 μM verapamil (top), 100 μM verapamil (middle), 500 μM verapamil (bottom). (B), Measurement of endosialin expression in side population (SP) and the total cell population for each of two neuroblastoma cell lines by flow cytometry.

Table III. Scoring of immunohistochemical endosialin staining in FFPE human clinical specimens of melanoma and small cell lung cancer is shown.

Specimen no.	Histologic review	Isotype control	Tumor cell intensity and percent coverage					Vascular cell intensity and percent coverage				
			3+	2+	1+	0	Long H-score	3+	2+	1+	0	Long H-score
1	Melanoma	0	0	0	40	60	40	40	20	20	20	180
2	Melanoma	0	0	0	0	100	0	30	20	20	30	150
3	Melanoma	0	0	0	0	100	0	10	20	30	40	100
4	Melanoma	0	0	0	20	80	20	10	30	10	50	100
5	Melanoma	0	0	0	40	60	40	20	40	20	20	160
6	Melanoma	0	0	0	20	80	20	30	30	30	10	180
7	Melanoma	0	0	10	30	60	50	40	20	30	10	190
8	Melanoma	0	0	0	0	100	0	0	10	10	80	30
9	Melanoma	0	0	10	10	80	30	30	40	20	10	190
10	Melanoma	0	0	0	30	70	30	20	40	20	20	160
11	Melanoma, skin	0	0	0	10	90	10	0	30	40	30	100
12	Melanoma, skin	0	0	20	50	30	90	0	60	30	10	150
13	Melanoma, skin	0	0	0	0	100	0	0	10	20	70	60
14	Melanoma, skin	0	0	0	0	100	0	0	50	20	30	120
15	Melanoma, skin	0	0	0	0	100	0	0	10	20	70	40
16	Melanoma in fat tissue	0	0	0	0	100	0	0	20	10	70	50
17	Melanoma, skin	0	0	0	0	100	0	0	0	20	80	20
18	Melanoma, skin	0	0	0	10	90	10	0	0	20	80	20
19	Melanoma in brain	0	0	0	0	100	0	20	30	40	10	160
1	Small cell CA in lung	0	0	0	0	100	0	10	20	30	40	100
2	Small cell CA in lung	0	0	0	0	100	0	0	0	60	40	60
3	Small cell CA in liver	0	0	0	0	100	0	0	0	0	100	0
4	Small cell CA in liver	0	0	0	0	100	0	0	20	30	50	70
5	Small cell CA in lung	0	0	0	20	80	20	20	30	30	20	150
6	Small cell CA in fat	0	0	0	0	100	0	20	40	30	10	170
7	Small cell CA	0	0	0	0	100	0	0	40	40	20	120
8	Small cell CA	0	0	0	0	100	0	10	20	20	50	90
9	Small cell CA in lung	0	0	0	0	100	0	0	0	10	90	10
10	Small cell CA	±	0	0	20	80	20	20	40	30	10	170
11	Small cell CA in bone	0	0	0	0	100	0	0	0	0	100	0
12	Small cell CA	0	0	0	0	100	0	0	20	20	60	60
13	Small cell CA in liver	0	0	0	0	100	0	0	0	0	100	0
14	Small cell CA in lung*	0	0	0	0	100	0	0	0	10	90	10
15	Small cell CA in lung	0	0	0	0	100	0	0	0	50	50	50
16	Small cell CA	0	0	20	20	60	60	10	30	40	20	130

only 20-40% of tumor cells being positive and never exceeding 2+ staining intensity. The flow cytometry data obtained from human small cell lung cancer cell lines concur with the overall rare expression or lack of malignant cell expression observed in the clinical specimens. Twelve human small cell lung cancer cell lines were assayed for endosialin by flow cytometry, and only one was weakly endosialin positive (Fig. 2C).

Discussion

Our results show that in clinical specimens, endosialin is expressed in the vasculature of neuroblastoma, melanoma and small cell lung cancer suggesting that targeting endosialin with a therapeutic agent may have therapeutic benefit via inhibition of angiogenesis or vascular disruption. The potential for achieving therapeutic benefit seems greater in neuroblastoma

than in melanoma or small cell lung cancer since in clinical specimens of neuroblastoma there was expression of endosialin in malignant cells in addition to the tumor vasculature. The reportedly low level of expression of endosialin in normal tissues suggests the potential for achieving an acceptable therapeutic index (10). When a model anti-endosialin-toxin conjugate was tested in the endosialin-positive SK-N-AS cell line and in the endosialin-negative HT-1080 fibrosarcoma cell line, the results showed that antigen-specific growth inhibition could be achieved *in vitro* via internalization of anti-endosialin (11).

All 10 human neuroblastoma cell lines studied, both MYCN-amplified and non-MYCN-amplified, have been independently confirmed to be neuroblastoma cell lines using multiple mRNA and protein markers and clustering analysis (24). The presence of endosialin in MYCN-amplified lines is in accordance with the reported advanced clinical stage of some of the neuroblastoma lines. For example the SK-N-Be(2) cell line was derived from a bone marrow biopsy taken from a child with disseminated neuroblastoma after repeated courses of chemotherapy and radiotherapy (25-27). Similarly, the SK-N-DZ cell line, also MYCN-amplified, was derived from advanced disease, having been isolated from a bone marrow metastasis from a child with poorly-differentiated embryonal neuroblastoma (27). The presence of endosialin in such highly malignant, MYCN-amplified, resistant tumors suggests that endosialin is a possible therapeutic target for advanced disease (Table II). It should be noted that some cell lines were derived from advanced disease and yet lack MYCN amplification. For example, the SK-N-AS cell line was derived from a bone marrow metastasis and is not MYCN-amplified (27).

Side population cells were first identified by flow cytometry as a small distinct bone marrow cell subpopulation with high dye efflux capacity, enriched in hematopoietic stem cell markers and *in vivo* bone marrow reconstitution activity. The dye efflux phenomenon is verapamil-sensitive. Side population cells identified by Hoechst 33342 two color fluorescence disappear from flow cytometry detection upon exposure to verapamil (28). Side population cells have been detected in tumors and have been demonstrated to have superior tumorigenic potential compared to the bulk population in immunodeficient mice and therefore side population cells may be enriched in tumor stem cells (29,30). Both neuroblastoma cell lines tested had detectable side population cells and in both cases, the side population cells maintained expression of endosialin.

The results suggest that endosialin may be a suitable therapeutic target for advanced disseminated neuroblastoma, a disease with poor prognosis (15). Endosialin was expressed in MYCN-amplified neuroblastoma cell lines and expression was maintained in side population cells suggesting that endosialin may be expressed in neuroblastoma tumor stem cells. These observations warrant further study of endosialin in neuroblastoma, advanced neuroblastoma and neuroblastoma stem cell models, in order to fully assess the potential of endosialin as a therapeutic target.

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