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Heterogeneity in retinoic acid signaling in neuroblastomas; role of matrix metalloproteinases in retinoic acid-induced differentiation

Suchitra Joshi[#], Rakeshwar S. Guleria, Jing Pan, Donald DiPette and Ugra S. Singh*

From

Department of Internal Medicine, The Texas A&M University System Health Science Center; Central Texas Veterans Health Care System; Scott & White Clinic, 1901 South 1st Street, Temple, Texas, 76504. # New address: Department of Neurology, University of Virginia Health Science Center, Charlottesville, VA 22908-0394.

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*Corresponding author:

Division of Molecular Cardiology, Cardiovascular and Cancer Research Institute, Building 205, 1901 South 1st Street, Temple, Texas 76504; Tel.: 254-743-2465; Fax: 254-743-0165; email: Usingh@medicine.tamhsc.edu

ABSTRACT:

Causes of retinoid resistance often observed in neuroblastomas are unknown. We studied all trans-retinoic acid (RA) signaling in neuroblastoma cells differing in N-myc levels in terms of neurite formation, expression of tissue transglutaminase, neuronal marker proteins, matrix metalloproteinases (MMPs), and activation of Rac1 and Cdc42. Poor invasiveness observed in SH-SY5Y, LA-N-5, and SMS-KCNR cells was associated with RA-induced neurite formation, Cdc42 activation and N-myc down regulation; expression of constitutively active Cdc42 down regulated N-myc expression and reduced invasion in RA-resistant SK-N-BE(2) and IMR32 cells. RA treatment for 24 h transiently increased invasion and expression of MMP9 in SH-SY5Y, LA-N-5 and MMP2 in SMS-KCNR cells. MMP inhibition prevented RA-induced neurite formation indicating a role in differentiation. Variation in RA signaling thus follows a defined pattern and relates to invasive potential. A defective RA signaling might result in retinoid resistance and unpredictable clinical outcome observed in some neuroblastomas.

INTRODUCTION

Neuroblastoma the childhood solid tumor of peripheral nervous system accounts for 8-10% of all childhood cancers [1]. It originates due to improper differentiation of the neural crest cells which possess inherent tendency to migrate and invade during the course of development [2, 3] and consists of tumorigenic 'N' type (neuronal) and non-tumorigenic 'S' type (Schwannian) cells [4]. This cellular heterogeneity and occasional spontaneous regression are the unique features of this tumor that make prognosis difficult and clinical outcome unpredictable [5, 6]. Neuroblastoma aggressiveness and poor prognosis is associated with N-myc amplification [7, 8], that also confers resistance to retinoids used in neuroblastoma therapy [9, 10]. All trans-retinoic acid (RA)-induced differentiation of the 'N' type cells involves N-myc down-regulation however, some of the N-myc amplified neuroblastoma cells differentiate in response to RA, while others do not [11-14].

Signaling involved in RA induced differentiation has been extensively studied however; a comparative analysis of RA responses in cell lines differing in N-myc amplification has not been attempted. Such a comparison would reveal probable components associated with retinoid resistance. RA induces activation of tissue-transglutaminase and Rho GTPases (Rac1 and Cdc42) during neurite formation in SH-SY5Y cells [15, 16], which represent important components of RA signaling.

Recently we also reported transient increase in invasion after RA treatment in SH-SY5Y cells [11]. Search for possible mechanism prompted us to study expression of matrix metalloproteinases (MMPs). MMPs are a family of zinc dependent proteinases that degrade the extracellular matrix surrounding tumor cells [17] and are divided into

membrane type MMPs (MT-MMPs), collagenases, gelatinases, stromelysins and others [18, 19]. MMP2 and MMP9 are predominantly expressed MMPs in neuroblastomas; mediate invasion and angiogenesis [20, 21] and show increased expression in advanced stage disease [22-24]. MMP expression is also regulated by N-myc indicating a link between MMPs, N-myc and tumor spread [25].

To compare activation of RA signaling components, we studied formation of neurite outgrowth, expression of neuronal marker proteins (GAP43 and total tau), tissue transglutaminase (TGase) and MMPs as well as activation of Rho GTPases (Rac1 and Cdc42) in six neuroblastoma cell lines differing in N-myc levels. Our results show heterogeneity in RA responses that follows a definite pattern, a differential RA signaling thus might result in spontaneous regression and retinoid resistance observed in some neuroblastomas.

MATERIALS AND METHODS

Cell culture and RA treatment:

Neuroblastoma cell lines SH-SY5Y, IMR32, SK-N-BE(2) and SK-N-FI (ATCC, Manassas, VA), and SMS-KCNR and LA-N-5 (a kind gift from Dr. Reynolds, University of Southern California and Children's Hospital Los Angeles, CA) were grown in the medium containing 10% serum and 1% streptopenicillin at 37⁰C in a humidified incubator with 5% CO₂. Treatment with RA (5 μM; Sigma, St. Louis, MO) was given in the medium containing 1% serum and the cells were incubated in the medium containing 1% serum for 12 h prior to addition of RA. Cells treated with DMSO (vehicle)

were used as a control to compare RA responses. To study expression of MMPs, RA treated cells were incubated in serum free medium for additional 12 h.

Adenoviral infections:

Cells were infected with adenoviruses expressing constitutively active Cdc42 (Cdc42 V12) (a kind gift from Dr. James Bamberg, Colorado State University, Fort Collins, CO) or GFP (control) in the medium containing 3% serum at MOI of 50 (SK-N-BE(2) cells) or 25 (IMR32 cells) for 8 h. Activation and expression of Cdc42 as well as invasion potential was studied after 24 h (IMR32) or 48 h (SK-N-BE(2)).

Western blot analysis and PBD assay:

Cell lysis and Western blotting was performed as described previously [26]. The cell lysates corresponding to 20 µg proteins were separated by SDS-PAGE and Western blotted using antibodies against TGase (1:1000; Neomarkers, Newington, NH), GAP43 (1:500) and N-myc (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), total tau (1:1000; Cell Signaling Technology, Beverly, MA) or actin (1:5000; Sigma, St. Louis, MO). To study expression of MMPs, 20 µl conditioned medium was separated by SDS-PAGE and Western blotted with anti-MMP9 (1:500) or anti-MMP2 (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Activation of Rac1 or Cdc42 was studied by affinity precipitation of GTP bound forms of these proteins using GST (Glutathione s-transferase) fused PAK binding domain (GST-PBD) immobilized to agarose beads as described previously [15]. Briefly, the cell lysates were incubated with GST-PBD beads for 1 h, washed thrice with lysis buffer, separated on SDS-PAGE and Western blotted

for Rac1 (1:1000; Cell Signaling Technology, Beverly, MA) or Cdc42 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Levels of Rac1 and Cdc42 in the cell lysates were studied by Western blotting for normalization. Activation of Rac1/Cdc42 was expressed as the ratio of GTP-Rac1/Cdc42 to total Rac1/Cdc42 after densitometric scanning of corresponding bands.

Cell invasion assay:

Cell invasion was studied using matrigel-coated transwell inserts (8 μm pore size, 10,000 pores/ cm^2) as described previously [11]. Cells (2.5×10^5) suspended in serum free medium were added to the upper well of transwell inserts coated with 0.7 mg/ml matrigel matrix (BD Biosciences, Franklin Lakes, NJ). Serum (10%) containing medium was added to the lower chamber and cells were allowed to invade for 48 h. Invaded cells were stained, photographed using Nikon Digital Camera under Zeiss microscope (40X magnification) and counted (10 random fields from each transwell insert).

Gelatin zymography:

Expression of MMPs was studied in the conditioned medium by zymography as described before [12]. Briefly, 20 μl of conditioned medium was subjected to non-reducing SDS-PAGE gels co-polymerized with 0.1% gelatin (Sigma, St. Louis, MO). Gels were washed with 2.5% Triton X-100 for 3 h, rinsed with distilled water and incubated overnight in the buffer containing 50 mM Tris-Cl pH 7.5, 0.2 M NaCl and 5 mM CaCl_2 at 37 $^\circ\text{C}$. Enzyme activity was visualized by staining gels with Coomassie

Brilliant Blue R250. MMP activity was quantified by densitometric scanning of corresponding signals.

Analysis of cell morphology:

To study neurite outgrowth cells were treated with RA or MMP inhibitors (Calbiochem, La Jolla, CA) in 1% serum containing medium. The medium was replaced everyday. The cells were photographed using Nikon Digital Camera under Zeiss microscope (40X magnification). Five random fields were photographed and neurite outgrowth was determined by measuring length of longest neurite per cell (n=50) using Image tool program (University of Texas Health Science Center, San Antonio, TX).

Statistical analysis:

Statistical analysis of the data was determined by one way ANOVA, paired t-test, and Tukey-Kramer multiple comparison test. The results were considered significant at $P < 0.05$.

RESULTS

Neuroblastoma cells show heterogeneity in RA responsiveness

Some neuroblastoma cells are resistant to RA-induced differentiation

To study relation between N-myc amplification and RA responsiveness in the 'N' type neuroblastoma cells we treated N-myc non-amplified (SH-SY5Y and SK-N-FI) (Fig. 1B) [27, 28] and N-myc amplified neuroblastoma cell lines (SMS-KCNR, IMR32, SK-N-BE(2) and LA-N-5) (Fig. 1B) [10, 29, 30] with RA for 2 days. RA induced neurite length

in SH-SY5Y, LA-N-5 and SMS-KCNR cells (Fig. 1A) but not in IMR32, SK-N-BE(2) and SK-N-FI cells. RA treatment for 24 h drastically reduced N-myc expression in SMS-KCNR and LA-N-5 cells, while in SK-N-BE(2) cells N-myc levels reduced after 48 h of RA treatment. In contrast, N-myc levels remained unchanged in IMR32 cells. TGase is shown to regulate neurite formation in SH-SY5Y cells [16] and RA induced TGase expression in all the cell lines except IMR32 (Fig. 1B). Two days of RA treatment did not induce expression of neuronal marker proteins GAP43 and total tau in SH-SY5Y, SMS-KCNR, SK-N-BE(2) and IMR32 cells (Fig. 1B). In LA-N-5 cells, basal expression of neuronal marker protein tau was high and expression of GAP43 increased after RA treatment (Fig. 1B), which indicated higher differentiated state of these cells. RA also induced GAP43 and total tau expression in SK-N-FI cells (Fig. 1B). DMSO treatment had no effect on the expression of N-myc, TGase, GAP43 or total tau (data not shown).

RA induced Cdc42 activation is associated with differentiation

Rac1 and Cdc42 regulate neurite formation [15, 31, 32], however RA induced Rac1 activation only in SH-SY5Y cells (Fig. 2A, B), while basal GTP-Rac1 was higher in IMR32 cells. Activation of Cdc42 on the other hand increased with RA treatment in SH-SY5Y, SMS-KCNR, LA-N-5 and SK-N-FI cells but not in SK-N-BE(2) and IMR32 cells (Fig. 2C, D). Activation of Rac1 and Cdc42 in cells treated with DMSO for 2 days remained unchanged (data not shown). These studies indicated that cell lines that differentiate in response to RA show increased expression of TGase and activation of Cdc42 (Table-1)

Neuroblastoma cells differ in the invasive potential

Invasive potential and MMP expression was studied in neuroblastoma cell lines. IMR32 and SK-N-BE(2) cells were more invasive, while SH-SY5Y, SMS-KCNR and SK-N-FI were less invasive (Fig. 3A, B). LA-N-5 cells though N-myc amplified were least invasive which was in accordance with highly differentiated status of these cells (Fig. 3A, B). Differential invasion potential within N-myc amplified cells indicated that some factors other than N-myc amplification were involved in invasion. All the cell lines showed expression of MMP2, however, no correlation between MMP-2 expression and invasive potential was observed (data not shown).

Overexpression of constitutively active Cdc42 down regulates N-myc expression and reduces invasion

To study whether RA-induced Cdc42 activation is important for N-myc down-regulation, we infected SK-N-BE(2) and IMR32 cells with adenovirus expressing constitutively active Cdc42 (Cdc42 V12). A 5-6 fold higher activation of Cdc42 and reduced N-myc expression was observed in cells expressing Cdc42 V12 (Fig. 4A, B). Constitutive activation of Cdc42 also reduced invasion potential by 2-3 folds (Fig. 4C). Although activation of Cdc42 is involved in cell death [33, 34], over-expression of Cdc42 did not induce cell death within the 48 h time frame used in these studies (data not shown). Morphology of these cells remained unaltered (data not shown) indicating that constitutive activation of Cdc42 was not sufficient to induce neurite formation.

RA induces invasion and MMP expression in SH-SY5Y and SMS-KCNR cells

We have previously shown that 24 h RA treatment induces invasion in SH-SY5Y cells [11]. RA also induced invasion in SMS-KCNR cells but not in LA-N-5, IMR32, SK-N-BE(2) and SK-N-FI cells (Fig. 5A) (data not shown). In SH-SY5Y and LA-N-5 cells RA-induced MMP9 was first detected after 16 h and 8 h of RA treatment respectively, peaked at 24 h and decreased subsequently, while in SMS-KCNR cells increased MMP2 expression was observed after 16 h of RA treatment and remained high till 48 h (Fig. 5B-D).

MMP inhibition blocks RA-induced invasion in SH-SY5Y and SMS-KCNR cells

Invasion in SH-SY5Y and SMS-KCNR cells after RA treatment was studied by inhibiting MMP9 and MMP2 respectively. Addition of anti-MMP9 antibody (50 µg/ml) inhibited RA-induced invasion in SH-SY5Y cells (Fig. 6A, B). Similarly, addition of anti-MMP2 antibody (50 µg/ml) inhibited RA-induced invasion in SMS-KCNR cells (Fig. 7A, B). To confirm the role of MMPs in RA-induced invasion, assay was also performed in the presence of MMP9 inhibitor (2 µM) in SH-SY5Y cells (Fig. 6A, B) and MMP2 inhibitor (5 µM) in SMS-KCNR cells. In the presence of MMP9 and MMP2 inhibitors there was a reduced invasion in SH-SY5Y and SMS-KCNR cells respectively (Fig. 7A, B), confirming role of MMPs in RA-induced invasion.

RA-induced MMPs are involved in formation of neurite outgrowth

Since RA-induced MMP expression was observed in SH-SY5Y, SMS-KCNR and LA-N-5 cells which also form neurites after RA treatment, we studied involvement of MMPs in

neurite formation. In SH-SY5Y and LA-N-5 cells when treated with RA in the presence of MMP9 inhibitor, neurite length was significantly reduced (Fig. 8A, B). Inhibition of MMP2 similarly inhibited RA-induced neurite formation in SMS-KCNR cells (Fig. 8C), however cells treated with the inhibitor rounded up and were less adherent indicating that MMP2 inhibition also affected some of the functions related to cell survival or adhesion.

DISCUSSION

Neuroblastomas are unique type of tumors, some of which spontaneously regress, some respond to retinoid therapy and undergo regression, while others show retinoid resistance [1, 35] often due to N-myc amplification [9, 36, 37]. This differential behavior might be due to cellular heterogeneity in retinoid signaling. To identify components of heterogeneity, we studied responses of six 'N' type cell lines to RA. RA induced differentiation was associated with increased expression of TGase as well as activation of Cdc42 in RA-responsive cells and these responses were associated with reduced N-myc expression in the N-myc amplified SMS-KCNR and LA-N-5 cells. Reduction in the N-myc expression is known to be a prerequisite for RA-induced differentiation [38, 39].

N-myc amplification is associated with neuroblastoma aggressiveness, metastasis and resistance to chemotherapy [9, 40, 41] and N-myc amplified SK-N-BE(2) and IMR32 cell lines were more invasive in nature and resistant to RA-induced differentiation. However, this N-myc associated invasiveness was not observed in SMS-KCNR and LA-N-5 cells, which could be due to activation of Cdc42 after RA treatment.

Recently an opposing action between N-myc and Cdc42 has been reported [42] and over-expression of Cdc42 V12 in SK-N-BE(2) and IMR32 cells indeed reduced invasion along with down-regulation of N-myc expression. SK-N-BE (2) and IMR32 cells however did not form neurites after expression of Cdc42 V12 indicating that additional components are involved in neurite formation. Sensitivity to retinoids is determined by key signaling molecules such as cellular retinoid binding proteins, retinoid receptors and defective retinoid signaling can result in RA resistance [43-45]. Resistance shown by cell lines SK-N-BE(2) and IMR32 could be due to such defects in retinoid signaling. A balance between N-myc signaling and retinoid signaling might play an important role in neuroblastoma progression/regression.

Similar RA responses (TGase and MMP expression as well as Cdc42 activation) were also observed during RA induced neurite formation in N-myc non-amplified SH-SY5Y cells. Involvement of TGase and Cdc42 in neurite formation is well known [16, 31, 32] and these proteins appear to be common determinants for RA induced differentiation. Further, cell line SK-N-FI showed only part of the RA responses; Cdc42 was activated but TGase expression increased only slightly. These cells did not form neurites upon RA treatment which could be due to the absence of one of the components (TGase) necessary for neurite formation. SK-N-FI cells are known to possess multidrug resistance [28, 46] and an altered signaling mechanism involved in differentiation might be responsible for the observed drug resistance.

We further studied a correlation between expression of MMPs and invasive potential in these cell lines, however neither found such a correlation nor found any correlation between N-myc amplification and MMP expression which was in accordance

with studies using clinical neuroblastoma samples [22]. On the other hand RA treatment increased MMP expression in cell lines SH-SY5Y, SMS-KCNR and LA-N-5 and increased MMP expression was associated with increased invasion in SH-SY5Y and SMS-KCNR cells. Though RA also induced MMP9 expression in LA-N-5 cells a corresponding increase in invasion was not observed. LA-N-5 cells are more differentiated than the other two cell lines as indicated by extensive neurite network formed after 24 h of RA treatment and higher expression of neuronal marker proteins GAP43 and total tau (Figure 1A-C). Differentiated neuroblastoma cells are known to lose their invasion potential [47] and hence though MMP expression increased, LA-N-5 cells remained non-invasive. RA-induced MMP expression has also been observed in the growth plate chondrocytes, pluripotent P19 cells, pancreatic cancer cells Dan-G, chondrogenic progenitor cells ATDC5, [48-51] and neuroblastoma cells SK-N-BE [52]. Role of MMP9 and MMP2 in neurite process formation is well known in peripheral neurons, Schwann cells and oligodendrocytes [53-58]. Increased MMP expression is proposed to confer invasiveness to growing neurite [52, 59] and inhibition of neurite formation in SH-SY5Y, SMS-KCNR and LA-N-5 cells after treatment with MMP inhibitors indicates similar involvement of MMPs in RA-induced differentiation. During development, neural crest cells migrate and invade under regulation of RA giving rise to peripheral ganglia [3, 60, 61]. The transient increase in invasion potential in SH-SY5Y and SMS-KCNR cells might mimic *in vivo* differentiation process.

In conclusion, our studies show heterogeneity in RA responses within the 'N' type neuroblastoma cell lines. In addition to conventional classification based on N-myc amplification, these studies show that the cell lines could also be classified based on RA

responses. Cell lines poor in invasion showed RA-induced neurite formation, TGase expression and Cdc42 activation which represent markers of RA-sensitivity. Further, RA-induced neurite formation was accompanied by increased MMP expression and inhibition of MMPs inhibited neurite formation. The heterogeneity observed in the present study might explain the ineffectiveness of the drug (retinoid) regimen followed in some neuroblastomas.

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LEGENDS

Figure 1:

RA induces neurite formation in SH-SY5Y, SMS-KCNR and LA-N-5 cells

Neuroblastoma cells were treated with 5 μ M RA or vehicle (control) for 2 days in the medium containing 1% serum. (A) Average neurite length in control and RA-treated neuroblastoma cells (n=50). (B) Western blots showing expression of N-myc, TGase, neuronal marker proteins GAP43 and total tau in control and RA-treated cells. Total cellular proteins (20 μ g) were used for Western blotting. Blots were reprobed with anti-actin antibody for normalization. Control shows expression levels in cells treated with DMSO for 2 days (maximum time point of RA treatment).

Figure 2:

RA differentially activates Rac1 and Cdc42 in neuroblastoma cells

Rac1 and Cdc42 activation was studied by GST-PBD pull down assay. (A) Western blots showing levels of GTP-Rac1 in the pull down samples and total Rac1 in the cell lysates. (B) Histogram showing Rac1 activation. Intensity of signals corresponding to Rac1 was estimated by densitometric scanning and mean Rac1 activation (ratio of GTP-Rac1 to total Rac1) was calculated from three separate experiments. (C) Western blots showing RA-induced Cdc42 activation. Cdc42 activation was studied similar to Rac1 activation using GST-PBD pull down assay. (D) Histogram showing Cdc42 activation (ratio of GTP-Cdc42 to total Cdc42) calculated from three separate experiments. Control shows activation of Rac1 and Cdc42 in cells treated with DMSO for 2 days (maximum time point of RA treatment).

Figure 3:

Neuroblastoma cell lines differ in invasive potential

Neuroblastoma cells were incubated in 3% serum containing medium for 12 h after which their invasion capacity was studied using transwell inserts coated with matrigel matrix as described in the materials and methods. (A) Cells invaded through transwell inserts (40X magnification). (B) Histogram showing average number of invaded cells counted from 10 random fields from each transwell insert. Experiment was repeated three times with 2 replicates each.

Figure 4:

Expression of constitutively active Cdc42 reduces N-myc expression and invasion potential

SK-N-BE(2) and IMR32 cells were infected with adenovirus expressing constitutively active Cdc42 (Cdc42 V12) at MOI 50 and MOI 25 respectively for 8 h. (A and B) Western blots showing expression and activation of Cdc42. Activation of Cdc42 was studied by GST-PBD pull down assay 48 h after adenoviral infections. Cells infected with adenovirus expressing GFP were used as a control. Cdc42 activation (ratio of GTP-Cdc42 to total Cdc42) was determined by densitometric scanning. (C) Histogram showing number of invaded cells. Invasive potential of the cells expressing constitutively active Cdc42 was studied 48 h after infection with adenoviruses as described in the materials and methods. Cells expressing GFP were used as control. The values represent mean \pm SD from two separate experiments with three replicates each.

Figure 5:

RA induces invasion and MMP expression in SH-SY5Y and SMS-KCNR cells

SH-SY5Y, SMS-KCNR and LA-N-5 cells were treated with RA (24 h) in the medium containing 1% serum. (A) Histogram showing number of cells invaded after RA treatment. Invasion assay was performed as described in the materials and methods and invaded cells were counted from 10 random fields from each transwell insert. The values represent mean \pm SD from three separate experiments with two replicates each. Controls represent cells treated with DMSO for 2 days. (B) Zymogram (upper panel) and Western blot (lower panel) showing MMP activity and expression after RA treatment. Gelatin zymography and Western blotting was performed using conditioned medium (20 μ l) as described in the materials and methods. (C and D) Histograms showing mean MMP activity. Intensity of the signal in the zymogram was estimated using scanning densitometry and expressed relative to signal intensity in DMSO treated control samples. Values represent mean \pm SD from three separate experiments.

Figure 6:

Inhibition of MMP9 inhibits RA-induced invasion in SH-SY5Y cells

SH-SY5Y cells were treated with RA for 24 h and invasion capacity was studied as described in the materials and methods in the presence of 50 μ g/ml anti-MMP9 antibody or MMP9 inhibitor (2 μ M). (A) Cells invaded through the transwell inserts in the presence of anti-MMP9 antibody, or normal mouse IgG (50 μ g/ml, used as a control) or MMP9 inhibitor. (B) Histogram showing average number of invaded cells. Number of

invaded cells was counted from 10 random fields from each transwell insert. The experiment was repeated three times with 2 replicates each.

Figure 7:

Inhibition of MMP2 inhibits RA-induced invasion in SMS-KCNR cells

SMS-KCNR cells were treated with 5 μ M RA for 24 h and allowed to invade matrigel coated transwell inserts for 48 h in the presence of anti-MMP2 antibody (50 μ g/ml) or MMP2 inhibitor (5 μ M). (A) Cells invaded in the presence of anti-MMP2 antibody or mouse IgG (50 μ g/ml, used as a control) or MMP2 inhibitor. (B) Average number of cells that invaded matrigel matrix counted from 10 random fields from each transwell insert. The experiment was repeated three times with two replicates each.

Figure 8:

MMPs are required for RA-induced neurite formation in SH-SY5Y, SMS-KCNR and LA-N-5 cells

Neuroblastoma cells were treated with RA in the presence of MMP9 or MMP2 inhibitor in 1% serum containing medium and formation of neurite outgrowth was studied. (A) Histogram showing neurite length in SH-SY5Y cells treated with RA for 4 days in the presence of MMP9 inhibitor (2 μ M). Neurite length was estimated as described in materials and methods from 5 random fields. (B) Histogram showing neurite length in LA-N-5 cells treated with RA in the presence of 2 μ M MMP9 inhibitor for 2 days. Neurite length was estimated as described in materials and methods from 5 random fields. (C) Histogram showing neurite length in SMS-KCNR cells treated with RA and MMP2

inhibitor (2.5 μ M) for 1 day. Neurite length was estimated as described in materials and methods. The experiment was repeated twice with similar results.

Table 1:

Tabulated representation of RA responsiveness and invasive properties of various neuroblastoma cell lines.

Table 1

Cell lines	Level of N-Myc after RA treatment	Invasiveness	Expression of TGase after RA treatment	Activation of Rac1 after RA treatment	Activation of Cdc42 after RA treatment	Neurite outgrowth after RA treatment
SH-SY5Y		+	+++	+++	+++	+++
LA-N-5	+	+	++		++	+++
SMS-KCNR	+	+	++		++	++
IMR32	+++	+++				
SK-N-BE(2)	++	++	+++			
SK-N-F1		+	+		++	

Figure 1

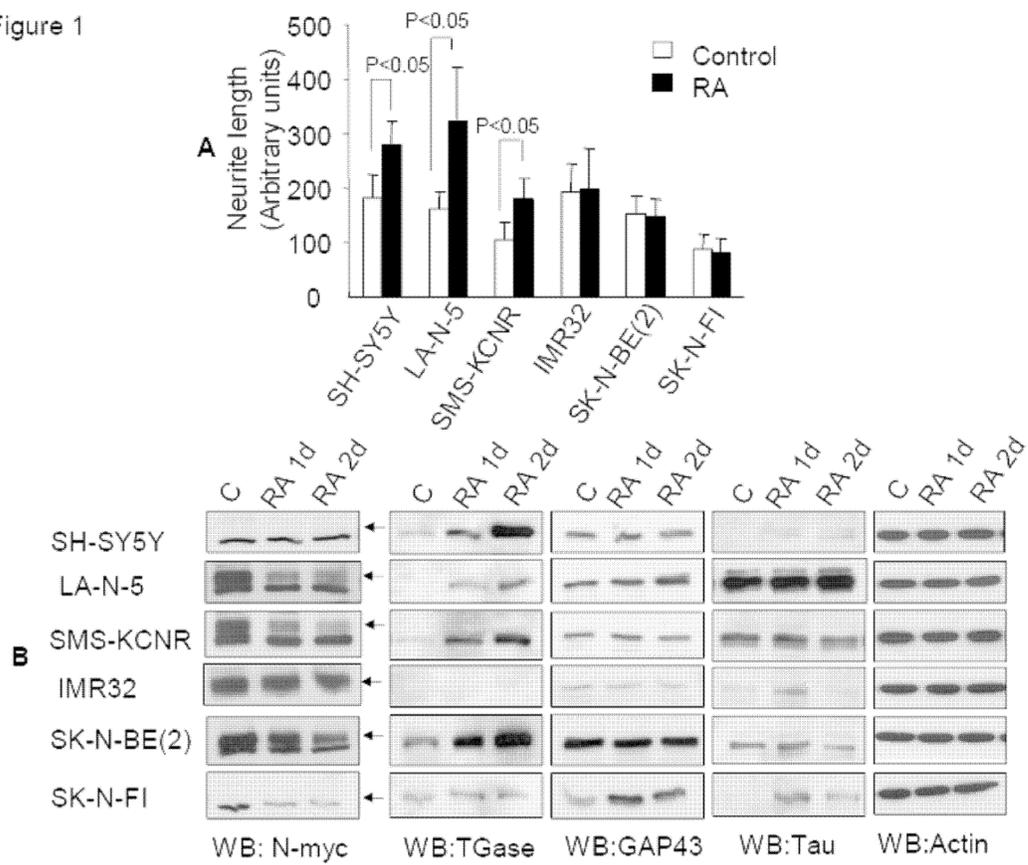


Figure 2

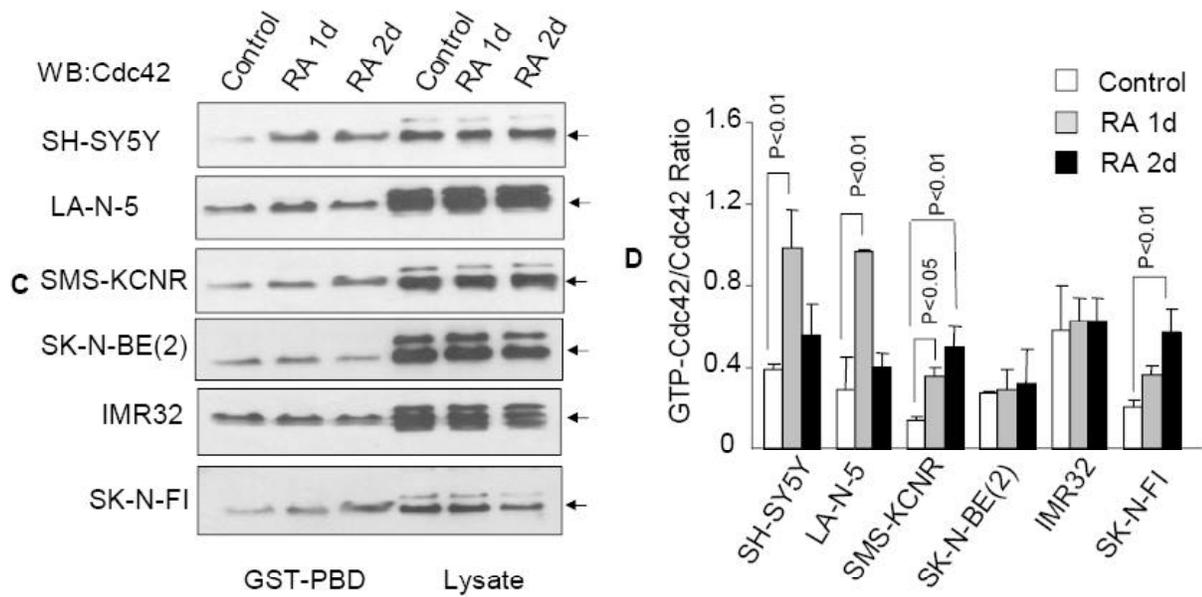
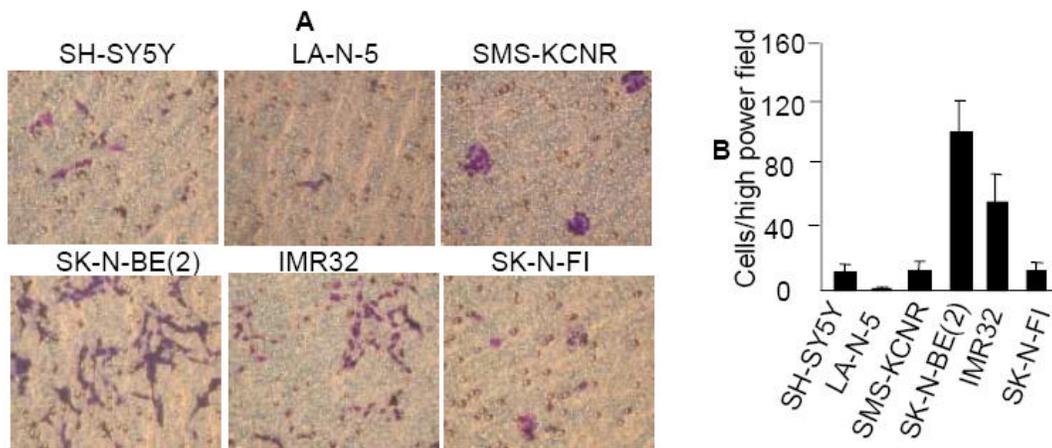
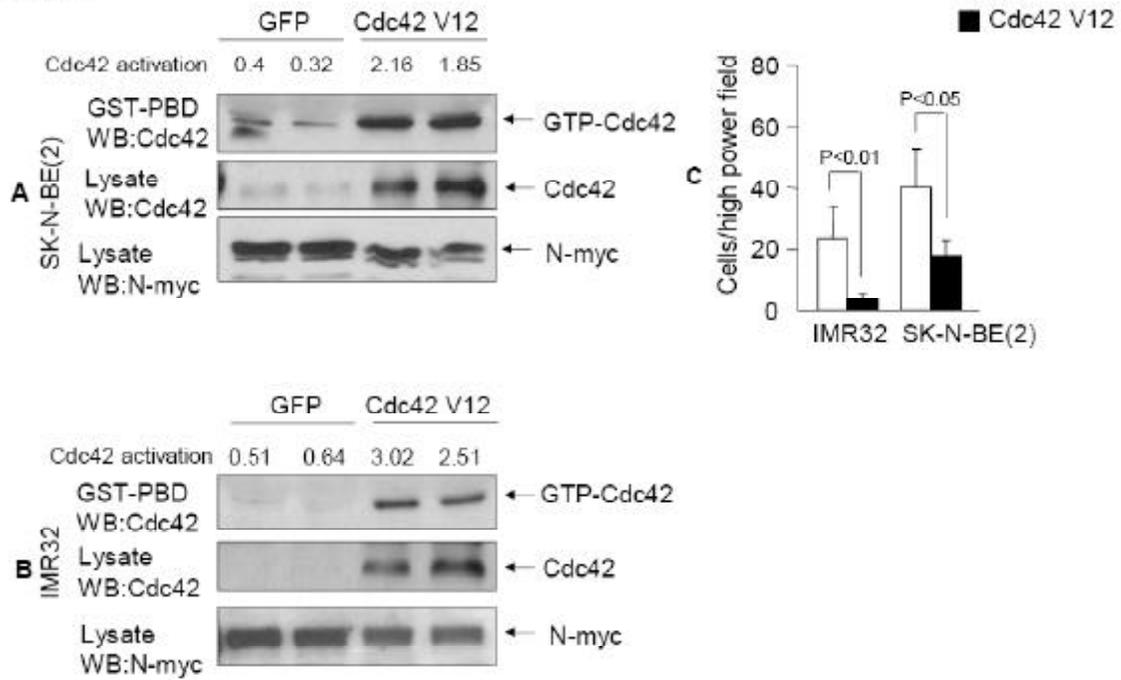


Figure 3



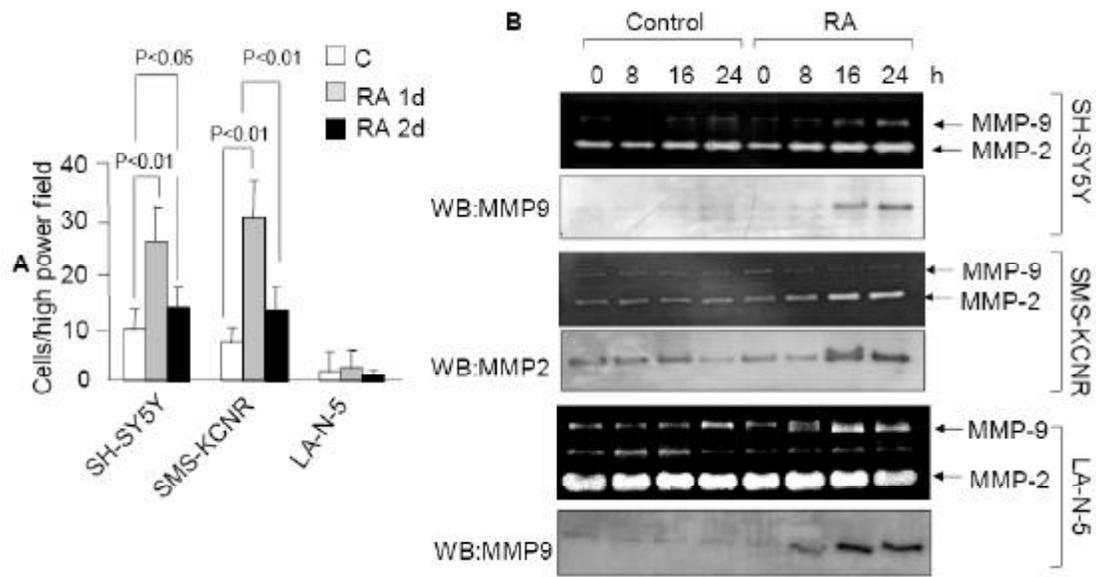
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Figure 4



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Figure 5



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Figure 6

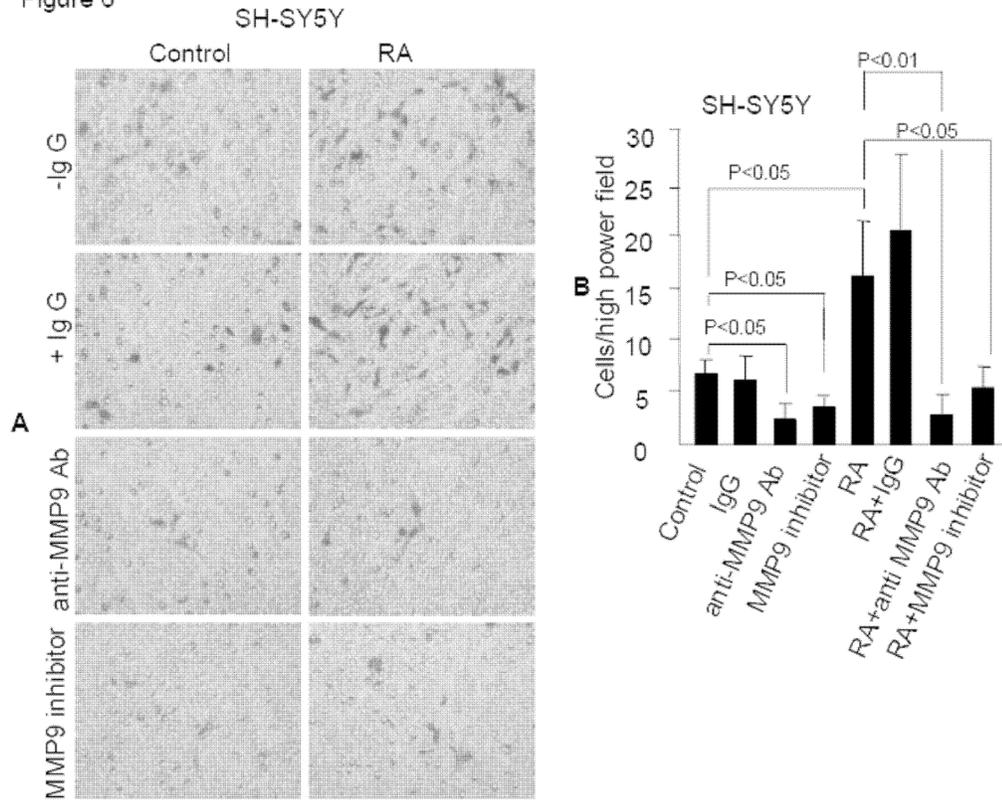


Figure 7

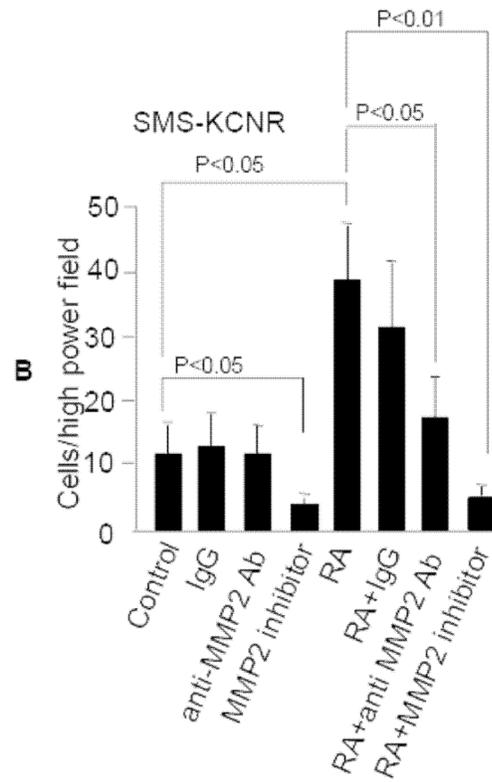
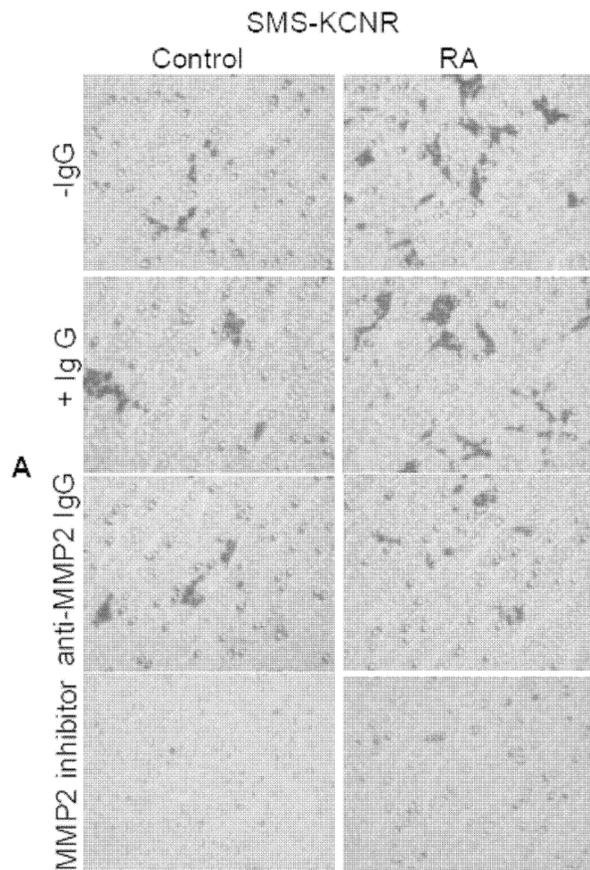


Figure 8

