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Retinoic-Acid-Induced Downregulation of the 67 KDa Laminin Receptor Correlates with Reduced Biological Aggressiveness of Human Neuroblastoma Cells

Juan M. Escamilla[1], Christine Bäuerl[1], Carlos M. R. López[1], Satu P. Pekkala[1], Samuel Navarro[2] and Domingo Barettino[1]
[1]*Instituto de Biomedicina de Valencia, Consejo Superior de Investigaciones Científicas,Valencia,*
[2]*Dept. of Pathology, School of Medicine, University of Valencia, Valencia Spain*

1. Introduction

Neuroblastoma is a common tumor of the childhood arising from embryonal sympathetic neural cell precursors. Despite of the improved therapeutic strategies, the survival rate of high-risk neuroblastoma patients is poor. Although complete clinical remissions can be achieved, relapse is relatively frequent, indicating a role for the persistence of the minimal residual disease (for review, Maris, 2010). Treatments with derivatives of retinoic acid (RA), the biologically active form of vitamin A, produce significant improvements on the therapy of high-risk neuroblastoma patients, when used together with intensive multimodal therapies (Reynolds et al., 2003, for review). Despite some controversy on dosage and treatment schedules (Matthay & Reynolds, 2000; Reynolds et al., 2003), treatment with 13-*cis*-RA (isotretinoin) has been reported to produce a modest but significant increase in the event-free survival of high-risk neuroblastoma patients (Matthay et al., 2009; Matthay et al., 1999). RA and its derivatives, the retinoids, are differentiating agents that induce growth arrest, differentiation and/or apoptosis of neuroblastoma cells *in vitro* (Lovat et al., 1997; Pahlman et al., 1984; Sidell, 1982; Thiele et al., 1985). However the molecular bases of RA therapeutic actions in neuroblastoma have not been established yet.

With the aim of identifying possible molecular targets for RA treatment, we have analyzed RA-induced gene expression changes in SH-SY5Y cultured human neuroblastoma cells (Lopez-Carballo et al., 2002), by means of Ordered Differential Display RT-PCR (Matz et al., 1997). Among more than 60 genes identified, we could found *LAMR1* (also called *RPSA*), encoding the precursor for the 67-KDa Laminin Receptor (67LR) (Wewer et al., 1986; Yow et al., 1988), whose mRNA levels were remarkably reduced after RA treatment. The 67LR is a multifunctional protein which is involved in cell adhesion, required for maintaining of cell viability (Scheiman et al., 2010; Susantad & Smith, 2008), acts as receptor for infectious agents like viruses and prions (Vana et al., 2009) and mediates the actions of the green tea polyphenol epigallocatechin-3-gallate (Umeda et al., 2008). Expression of 67LR has been

correlated with the biological aggressiveness, and the invasive and metastatic capacities of tumors from diverse origin. Many examples in which the expression of 67LR is increased in a tumor in respect to the corresponding normal tissue have been reported, and elevated expression of 67LR correlates with tumor progression and has been considered as adverse prognostic factor in several human cancers (Castronovo, 1993; Menard et al., 1997; Menard et al., 1998; Viacava et al., 1997, for review). *LAMR1* encodes a 37 KDa precursor protein (Wewer et al., 1986; Yow et al., 1988), that is acylated and glycosylated to generate the mature 67LR (Buto et al., 1998; Katagiri et al., 2005; Landowski et al., 1995) A role of 67LR in stabilizing the binding of integrins to laminin has been postulated (Magnifico et al., 1996), and it has been proposed that binding of 67LR contributes to laminin remodeling, thereby facilitating laminin degradation by proteolytic enzymes and increasing the release of motility fragments (Ardini et al., 2002; Berno et al., 2005; Vande Broek et al., 2001). Therefore, 67LR could play a role in the degradation of basal membrane whose major component is laminin, a critical step of the metastasis process and a prerequisite for tumor vascularization, involving the invasion of tumor mass by endothelial cells.

We report here that RA treatment of human neuroblastoma cells induces a reduction on the mRNA and protein levels of 67LR. In parallel, RA treatment impaired neuroblastoma cell migration towards laminin in haptotaxis assays *in vitro* and reduced invasiveness through *Matrigel* in *in vitro* invasion assays, processes in which 67LR played a major role. In addition, we show by immunohistochemical methods that more than two thirds of the human high-risk neuroblastic tumors assayed expressed 67LR to certain extent. The results showed here support an important role for 67LR in migration towards and invasion through the basement membrane, critical steps on the metastasis process. RA-induced downregulation of 67LR correlates with a reduction in the biological aggressiveness *in vitro* of neuroblastoma cells, and supports the idea of 67LR as an important molecular target for the therapeutic actions of RA in neuroblastoma.

2. Experimental procedures

2.1 Cell culture and treatments

SH-SY5Y cells (ATCC no. CRL-2266) were cultured in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. LA-N-1 cells (ECACC no. 06041201) were grown in a 1:1 mixture of EMEM and Ham's F-12 medium containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids. Cell cultures were kept in a humidified incubator at 37ºC with 5% CO_2. The medium was replaced every 2 days and the cells were split before they reached confluence. Cycloheximide (CHX), all-*trans*-retinoic acid (RA), and LY294002 (LY) were purchased from Sigma. The different compounds were dissolved in Dimethyl-sulfoxide and added to the culture medium at the indicated concentrations. Peptide P11 (Ac-CDPGYIGSR-NH_2, (Graf et al., 1987b) and its Scrambled control (Ac-YCIPGDRGS-NH_2) were synthesized and purified by HPLC to >90%. Peptides were dissolved in serum-free culture medium at 60 μg/ml (60 μM).

2.2 RNA analysis

Northern Blot analysis of total RNA from SH-SY5Y cells with [^{32}P]-labeled probes were performed as previously described (Lopez-Carballo et al., 2002). *LAMR1* probe consisted of a 1 Kb fragment obtained from the EST clone IMAGE 1271323. Probes for Cyclophilin A (*PPIA)* and beta-actin (*ACTB*) consisted in cDNA fragments generated by RT-PCR. For

quantification of the *LAMR1* expression levels, duplicate Northern Blots were exposed to Fuji IP capture plates, measured using a Fuji FLA-5000 laser scanner and analyzed using Fuji ImageQuant software. *LAMR1* absolute values were normalized to the values obtained for *ACTB*.

2.3 Western blot

Whole cell extracts were obtained by lysis of the cells in RIPA buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% Glycerol) containing 0.5% Nonidet P-40, 0.1% SDS, protease inhibitors (1 mM PMSF, 40 μg/ml aprotinin and 40 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium ortovanadate, 1 mM NaF). After 10 min incubation on ice, the lysate was cleared by centrifugation (16,100xg, 10 min, 4ºC), and protein concentration determined. Extracts were diluted with 2x sample buffer containing freshly added 50 mM Dithiothreitol. Western blot analysis of proteins from whole cell extracts was performed as described (Lopez-Carballo et al., 2002). Antibodies against the 67-KDa Laminin Receptor (Wewer et al., 1987), were obtained from Abcam. Monoclonal antibodies against the 37-KDa Laminin Receptor precursor (MPLR, Buto et al., 1997) were a generous gift of Dr. S. Menard (Istituto Nazionale Tumori, Milan, Italy). Antibodies against beta-actin were purchased from Sigma. Horseradish-peroxidase-conjugated secondary antibodies were obtained from GE Healthcare, and Jackson ImmunoResearch. Chemiluminiscent signals were developed with ECL (GE Healthcare).

2.4 Immunofluorescence

SH-SY5Y neuroblastoma cells were treated with RA or vehicle for 96 h, scraped from the plate and washed in PBS. A drop of the cell suspension was applied onto poly-lysine-coated slides and let to settle for 10 min at room temperature. The slides were fixed with 4% paraformaldehyde in PBS for 20 min, and washed three times with PBS. After blocking with 3% Bovine Serum Albumin in Phosphate Buffered Saline (PBS) for 40 min, slides were sequentially incubated with Anti-67-KDa Laminin Receptor (Abcam, diluted 1:50 in the same buffer) and Fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit IgG (Jackson ImmunoResearch, diluted 1:100 in the same buffer). After washing with PBS, the slides were counter-stained with Hoechst 33258 (Scharlab, 50 mg/ml) for 5 min, rinsed with PBS and mounted with anti-fade mounting medium (Dako-Cytomation). Slides were sequentially photographed in a Leica fluorescence microscope equipped with FITC- and UV-specific excitation filters.

2.5 Migration and invasion assays

The effects of RA treatments, and Laminin β1-derived peptide P11 on the migration and invasion capacities of SH-SY5Y neuroblastoma cells were tested according to published protocols (Albini et al., 1987; McCarthy et al., 1983), with slight modifications. A modified, light-opaque Boyden chamber (Falcon HTS FluoroBlok, 8 μm pore size; Becton-Dickinson) was used in both migration and invasion assays. Cells were treated with 1 μM RA or vehicle in culture medium during the indicated times, and labeled in the plate with Calcein AM (5 μM; Molecular Probes-Invitrogen), during 30 min at 37ºC. Labeled cells were detached from the plate by scraping and counted. Migration/haptotaxis assays (see Fig. 3A) were used to measure cell movement toward an immobilized laminin-rich extracellular matrix protein gradient. For that purpose, the lower chamber of the plate was coated with BD *Matrigel*

Matrix (10 μg/cm²; Becton Dickinson) as attractant and filled with serum-free medium. 50,000 labeled cells in serum-free medium were added onto the upper chamber. As control, equivalent experiments in which coating with *Matrigel* was omitted were set. After 6h of incubation at 37ºC, the fluorescent light emitted by the cells that migrated through the opaque filter was measured with a Victor² multilabel counter (PerkinElmer Life and Analytical Sciences). The assays were performed at least in triplicate. For invasion assays (see Fig. 3C) the porous membrane of the Boyden chamber was covered with BD *Matrigel* matrix (5 μg/cm² in serum-free medium; Becton Dickinson) and allowed to gel for 30 min. 50,000 calcein-labeled cells were carefully layered on top of the *Matrigel* layer and culture medium containing 10% FBS as chemoattractant to promote cell invasion was placed into the lower well. The fluorescent light emitted by cells in the lower chamber after 6 h incubation at 37ºC was measured in triplicate assays as above. Peptide blocking of 67LR was performed by incubating 50,000 labeled cells in 500 μl of serum-free medium with P11 peptide or Scrambled control peptide (60 μg/ml; 60μM) for 30 min at 37ºC and added to the upper chamber of migration/haptotaxis or invasion assays prepared as described above. For statistical analysis, one-way ANOVA together with Tukey post-hoc test were employed.

2.6 Immunocytochemistry

Immunohistochemical study was performed following streptavidin-biotin method with antigen retrieval using citrate buffer 0.1M, pH 6.0 and heating with autoclave at 1.5 atm during 3 minutes. Primary antibody (Anti-67-KDa Laminin Receptor, Abcam) was diluted 1:100. Positive controls include internal epithelial tissues as well as one case of invasive breast carcinoma. Negative controls consisted in substitution of the primary antibody by mouse ascites and/or PBS. Immunohistochemistry was performed on a tissue microarray composed of cylinders of 1.5 mm in diameter of 49 cases of Neuroblastoma that was assembled using a manual tissue arrayer (MTA , Beecher Inc, USA) (Noguera et al., 2009).

3. Results

3.1 RA treatment of SH-SY5Y neuroblastoma cells results in downregulation of the expression of the metastasis-associated 67-KDa Laminin receptor

To identify differentially expressed genes during RA treatment of human neuroblastoma cells we have used the Ordered Differential Display RT-PCR technique (Matz et al., 1997). One of the displayed fragments, showing strong downregulation after 24 h of RA treatment, was amplified and sequenced. The sequence obtained (data not shown) corresponded to the *LAMR1* gene (also called *RPSA*), encoding the 67 KDa Laminin Receptor (67LR; Wewer et al., 1986; Yow et al., 1988). Because 67LR has been described as a metastasis-associated gene, whose expression levels are related to tumoral progression (Menard et al., 1997; Menard et al., 1998; Sobel, 1993) we decided to study the regulation of *LAMR1* transcription by RA with some detail. As shown on the Northern Blot in Fig. 1a, *LAMR1* expression levels were significantly reduced after a 24 h RA treatment. This delayed downregulation profile resembled that reported for the coordinate repression of the *ID1*, *ID2* and *ID3* HLH genes (Lopez-Carballo et al., 2002).

As was the case for the ID genes, the mechanism by which RA downregulates *LAMR1* expression appears to be complex. The reduction on the levels of *LAMR1* mRNA observed after 24 h of RA treatment did not occur when cells were pre-treated with the protein synthesis inhibitor Cycloheximide (CHX), indicating an indirect regulation that requires

newly synthesized proteins (Fig. 1b). RA activates the PI3K/Akt signaling pathway through a non-genomic mechanism (Lopez-Carballo et al., 2002; Masiá et al., 2007). Such activation is also required for the downregulation of the expression of *LAMR1*, since it did not occur in cells treated with RA in the presence of the PI3K inhibitor LY290004 (Fig. 1b).

3.2 RA treatment reduces the presence of the 67-KDa Laminin receptor at the cell surface

67LR is synthesized as a 37-KD precursor (37LRP) subunit, which is processed to the mature form of the protein on the cell surface (Buto et al., 1998; Landowski et al., 1995). Using antibodies recognizing the mature form of this protein in Western Blots (Wewer et al., 1987), we have detected that RA treatment results in a decrease on the levels of 67LR in cellular extracts of SH-SY5Y cells(Fig. 2A). By means of a monoclonal antibody specific for the 37LRP (Buto et al., 1997), we observed a parallel reduction on the expression of the precursor protein (Fig. 2B). A similar RA-induced decrease in 67LR protein expression could be observed in another neuroblastoma cell line, LA-N-1 (Fig.2C). Immunofluorescent

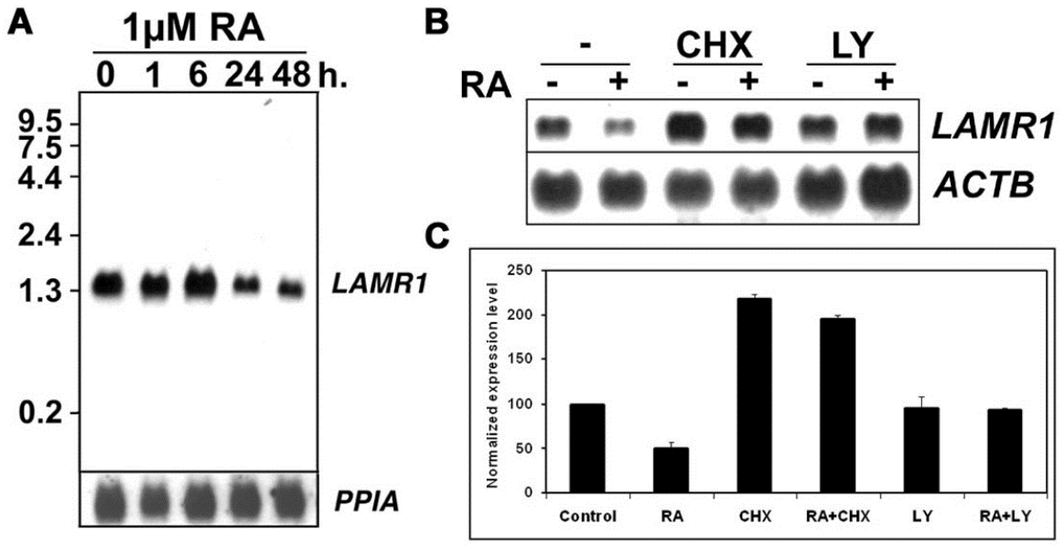

Fig. 1. (A-C) RA-induced downregulation of the expression of *LAMR1.*

A. Northern Blot. Each lane contains 15 μg of total RNA from SH-SY5Y neuroblastoma cells treated with 1 μM RA for the times indicated in the figure. The blot was sequentially hybridized with [^{32}P]-labeled DNA probes specific for the mRNAs of 67LR (*LAMR1*, 1.3 kb), and cyclophilin A (peptidyl- prolyl-isomerase A, *PPIA*, 1 Kb) as internal loading control.
B. Northern Blot. Each lane contains 15 μg of total RNA from SH-SY5Y neuroblastoma cells pre-treated during 30 min with vehicle (-), Cycloheximide (CHX) or 10 μM LY294002 (LY), and then treated with 1 μM RA (+) or vehicle (-) during 24 h in the presence of the inhibitor. The blot was sequentially hybridized with [^{32}P]-labeled DNA probes specific for the mRNAs of the 67LR (*LAMR1*, 1.4 Kb), and Actin-β (*ACTB*, 2 Kb) as internal control.
C. Quantitative analysis of the experiment shown in (B). The radioactivity present in each band on the blot was determined with a Fuji FLA5000 phosphorimager. The values obtained for *LAMR1* probe were normalized with the corresponding *ACTB* values. The graph shows the average (bars) and standard deviation (error bars) of a duplicate experiments.

staining with the same antibody used on fig 2A, showed that 67LR is less abundant on the membranes of RA treated SH-SY5Ycells, as compared with untreated cells (Fig. 2D).

3.3 RA treatment of neuroblastoma cells reduces migration towards and invasion through Matrigel

Laminin is a basal membrane specific glycoprotein. Apart from its structural function, this protein presents a biological activity linked to adhesion, migration, growth and differentiation of certain cell types. Laminin is the major component of the commercial basal membrane preparation *Matrigel* (Kleinman & Martin, 2005, for review), that was used in the following experiments as source of laminin-rich matrix. RA has been reported to reduce the migratory, invasive and metastatic capacity of neuroblastoma cells in *in vitro* assays

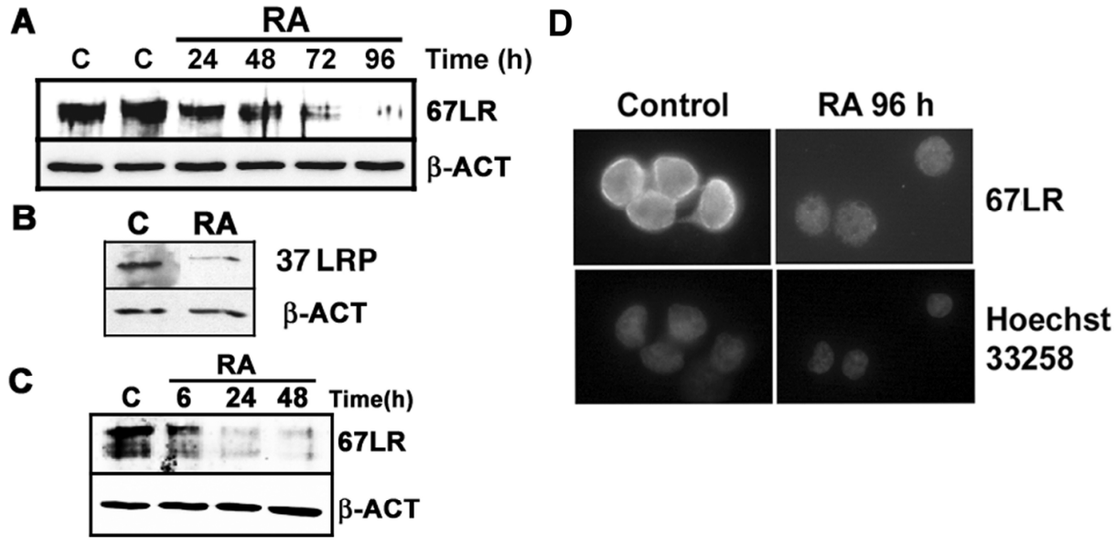

Fig. 2. (A-D) RA-induced reduction on the levels of the 67LR protein.

A. Western Blot. Each lane contains 20 μg of protein extract from SH-SY5Y neuroblastoma cells treated with 1 μM RA for the times indicated in the figure. The blot was sequentially incubated with specific antibodies raised against 67LR protein, and with β-actin antibodies (β-ACT), as loading control. B. Western Blot. Each lane contains 20 μg of protein extract from SH-SY5Y neuroblastoma cells treated with 1 μM RA or vehicle for 72 h. The blot was sequentially incubated with a monoclonal antibody against the 37 KDa Laminin Receptor Precursor protein (37LRP), and with β-actin antibodies (β-ACT), as loading control. C. Western Blot. Each lane contains 20 μg of protein extract from LA-N-1 neuroblastoma cells treated with 1 μM RA for the times indicated in the figure. The blot was sequentially incubated with specific antibodies raised against 67LR protein, and with β-actin antibodies (β-ACT), as loading control. D. Immunofluorescence Microscopy. SH-SY5Y cells were treated during 96 h with 1 μM RA or vehicle, scraped from the plate, and applied onto Poly-lysine-coated slides. The slides were fixed with 4% paraformaldehyde in PBS, and sequentially incubated with rabbit Anti-67LR and FITC-conjugated anti-rabbit IgG. After washing with PBS, the slides were counter-stained with Hoechst 33258 and sequentially photographed in a Leica fluorescence microscope equipped with FITC- and UV-specific excitation filters.

(Joshi et al., 2006; Joshi et al., 2007; Meseguer et al., 2011; Voigt & Zintl, 2003). To look for RA-induced biological effects in neuroblastoma cells that could be related to the observed downregulation of the 67LR, we used assays for migration/haptotaxis towards *Matrigel* and for invasion through *Matrigel*. Migration and invasion assays described here were based on published methods (Albini et al., 1987; McCarthy et al., 1983), and conducted measuring the proportion of calcein-labeled migrating cells in modified Boyden chamber assays, using FluoroBlok light-opaque transwell inserts. Calcein-labeled SH-SY5Y cells (50,000 cells/well) were seeded onto the upper chamber of a FluoroBlok Insert in serum-free DMEM. For migration/haptotaxis assays, the lower chamber was coated with *Matrigel* (10 µg/cm^2) and contained serum-free DMEM. (Fig. 3A). Similar experiments without *Matrigel* coating were set as negative controls for these experiments. Haptotaxis assays with neuroblastoma cells have shown increased migration when *Matrigel* is used as attractant. Surprisingly, short treatments with RA (24 h) resulted in a transient increase on cell migration that however occurred independently of the presence of chemoattractant. Treatment of SH-SY5Y cells

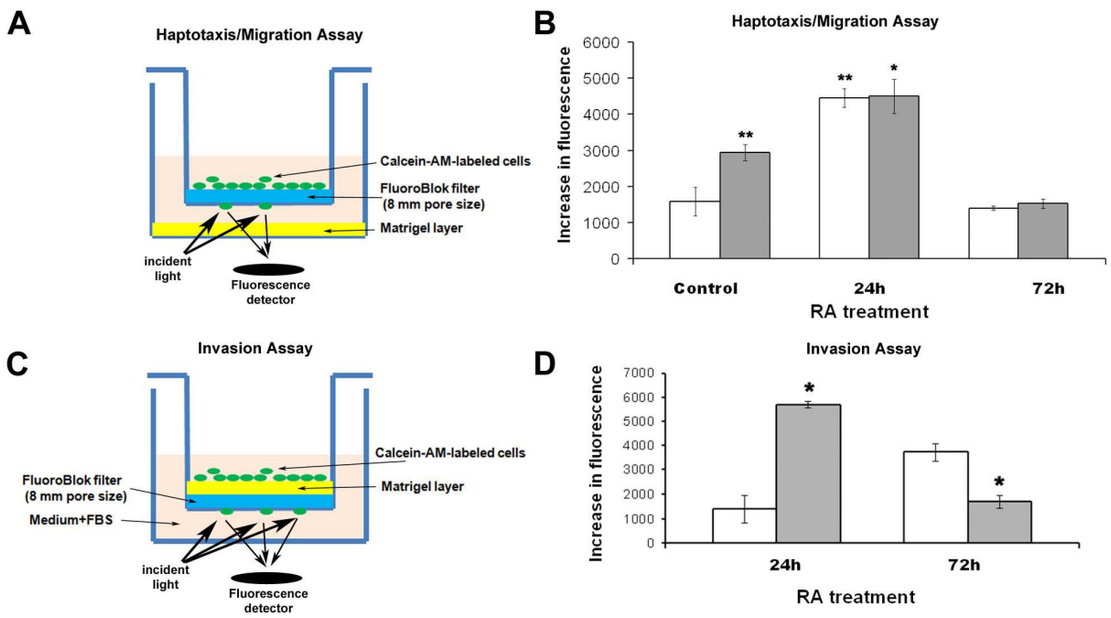

Fig. 3. (A-D) RA treatment reduces migration towards and invasion through *Matrigel*.

A. Schematic representation of the Haptotaxis/migration towards *Matrigel* assay. B. Effect of RA on cell migration towards *Matrigel*. Cells treated with 1 µM RA as indicated or untreated controls were assayed in the absence (empty bars) or in the presence (grey bars) of *Matrigel* as chemoattractant, and the increase in fluorescence was measured. The graph shows the average +/- SD of triplicate experiments. The asterisks indicate a statistically significative difference between the corresponding sample and the untreated control without chemoattractant (ANOVA test; * $p<0.05$; ** $p<0.01$). C. Schematic representation of the invasion assay. D. Effect of RA on cell invasion through *Matrigel*. Cells treated with RA as indicated (grey bars) and their corresponding untreated controls (empty bars) were assayed, and the increase in fluorescence was measured. The graph shows the average +/- SD of triplicate experiments. The asterisks indicate a statistically significative difference between the sample and its untreated control. (ANOVA test; * $p<0.05$).

with RA for 72 h reduced their migration rate and ablated the haptotactic attractive effect of *Matrigel* (Fig. 3B). For *in vitro* invasion assays the porous membrane separating both

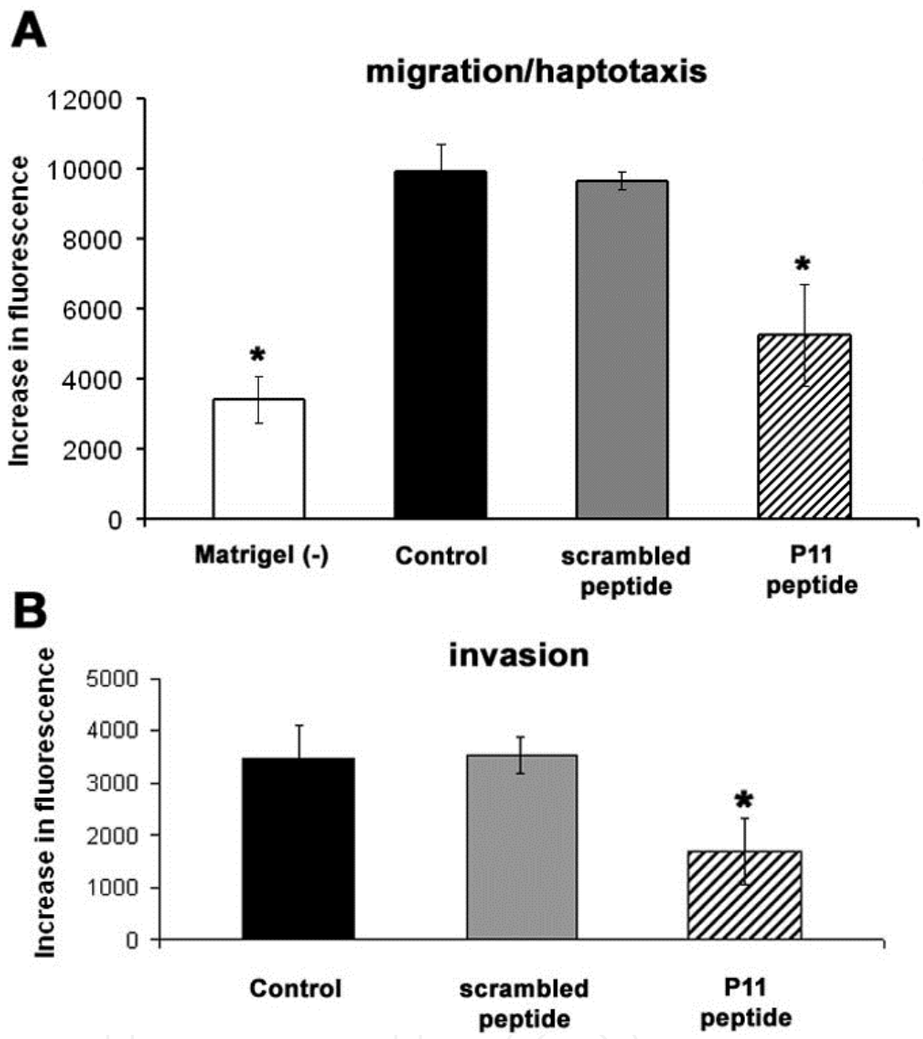

Fig. 4. (A-B) The 67-KDa laminin receptor is involved in migration towards and invasion through *Matrigel*.

A. Effect of Laminin peptide P11 on cell migration towards Matrigel. Untreated cells (black bar), as well as cells treated with scrambled peptide (grey bar) or P11 peptide (hatched bar) were assayed for migration using *Matrigel* as chemoattractant, together with a negative control experiment where the attractant was omitted (empty bar). The graph shows the average +/- SD of triplicate experiments. The asterisks indicate a statistically significative difference between the sample and its control. (ANOVA test; * $p<0.05$).

B. Effect of laminin peptide P11 on cell invasion through Matrigel. Untreated cells (black bar), as well as cells treated with scrambled peptide (grey bar) or P11 peptide (hatched bar) were used for *in vitro Matrigel* invasion assays. The graphs show the average +/- SD of triplicate experiments. The asterisks indicate a statistically significative difference between the sample and its control. (ANOVA test; * $p<0.05$).

chambers of a FluoroBlok transwell was covered with *Matrigel* (5µg/cm²), simulating the basal membrane and 5% FBS was added to the lower chamber as chemoattractant (Fig. 3C).
Similarly to what occurred with the migration assay, a transient increase on the invasive capacity of neuroblastoma cells was detected on *Matrigel* invasion assays after 24 h of RA treatment. Nevertheless, treatment with RA for 72 h resulted in strong reduction on the invasiveness of SH-SY5Y neuroblastoma cells (Fig. 3D). In the human neuroblastoma cell line LA-N-1 the effects of RA on migration/haptotaxis and in *in vitro* invasion were equivalent to those reported for SH-SY5Y cells (data not shown).

3.4 The 67-KDa Laminin receptor is involved in migration towards and invasion through Matrigel

The 67LR binds to the sequence CDPGYIGSR from the Laminin β1 chain (Graf et al., 1987b), and a peptide with that sequence (P11 peptide) impaired the activity of 67LR *in vitro* in laminin-mediated cell attachment and haptotaxis experiments (Graf et al., 1987a) and reduced the formation of experimental metastases *in vivo* (Iwamoto et al., 1987), probably by blocking the Laminin binding sites on 67LR. To assess the involvement of 67LR on the haptotaxis and invasiveness capacities of neuroblastoma cells, we have used P11 peptide to specifically block 67LR. Neuroblastoma cells were incubated with P11 peptide (60 µg/ml), or a scrambled peptide of the same chemical composition but different amino acid sequence. Afterwards the cells were used either on migration/haptotaxis experiments or in *in vitro* invasion assays as above. The results obtained showed that incubation of SH-SY5Y cells with P11 peptide significatively reduced migration towards *Matrigel*, whereas addition of the scrambled control peptide had no relevant effect. (Fig. 4A).
Similarly, addition of peptide P11 also reduced the invasion of neuroblastoma cells through *Matrigel*, whereas incubation with the control peptide had no remarkable effect on invasion (Fig. 4B). These results indicate the involvement of 67LR on laminin-induced migration/haptotaxis and in invasion through *Matrigel*-coated filters. Taken together, our results strongly support that RA-induced downregulation of 67LR contributes to the observed reduction of the biological aggressiveness of neuroblastoma cells after RA treatment.

3.5 Expression of the 67-KDa Laminin receptor in human neuroblastoma tumors

The expression of 67LR in human neuroblastoma primary tumors was tested by immunocytochemistry with the same 67LR antibody used for Western Blots and Expression of 67LR in neuroblastoma tumor samples was analyzed by immunohistochemistry with anti-67LR antibodies. The graph shows the percentage of the analyzed neuroblastoma samples (n=60) showing weak or no signal (empty sector), moderate intensity signal (hatched sector) or strong signal (black sector).
immunofluorescence. The antibody provided good results with paraffin sections, showing specific staining on epithelial tissues and invasive carcinomas used as positive controls (data not shown). Paraffin sections of a tissue microarray including 49 high-risk primary neuroblastoma tumor samples, including40 cases with *MYCN* amplification (100 to 500 copies per haploid genome) and 9 cases with *MYCN* gain (2 to 4 copies per haploid genome), and complete sections from 11 additional poorly differentiated neuroblastoma tumors were analyzed. From these 60 different human primary neuroblastoma samples 17 showed no or weak signal (28.33%), 22 showed positive staining with moderate intensity signal (36.67%), and 21 (35%) showed strong signal (Fig. 5).

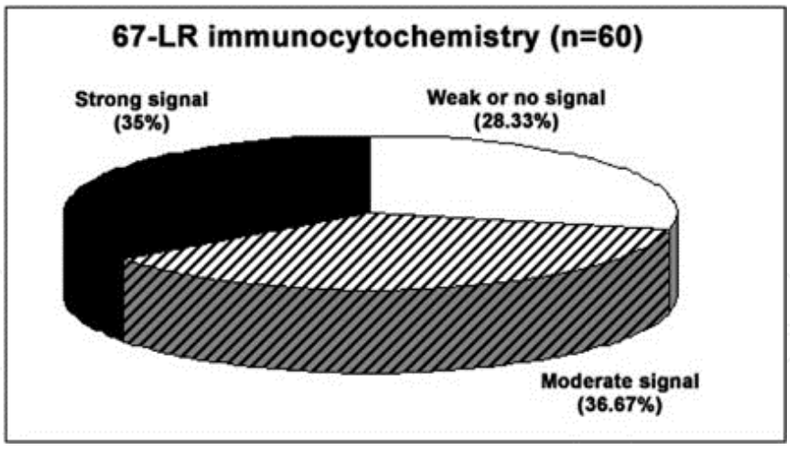

Fig. 5. Expression of 67LR in neuroblastoma tumor samples.

Photomicrographs of representative neuroblastoma tumor sections stained for 67LR are shown in Figure 6, including sections showing no signal (Fig. 6A), weak signal (Fig 6B), moderate intensity signal (Fig. 6 C and D), and strong signal (Fig. 6 E and F). Nuclear staining was detected in these strongly labeled tumor sections, which is an unexpected location for 67LR. Nevertheless, it has to be noted that shedding of 67LR by tumor cells has been reported (Berno et al., 2005; Karpatova et al., 1996; Moss et al., 2006), and it appears

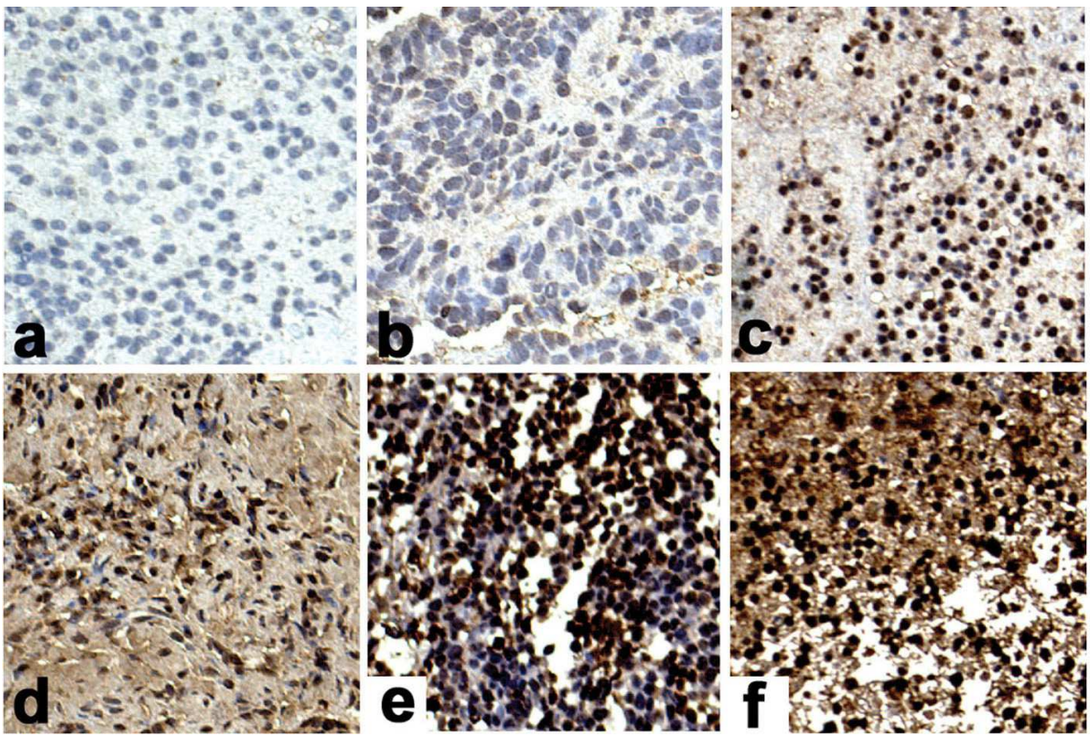

Fig. 6. (a-f) Immunohistochemical detection of 67LR in neuroblastoma tumor samples.

Photomicrographs of representative neuroblastoma tumor paraffin sections stained for 67LR antibody and counterstained with hematoxylin, including sections showing no signal (A), weak signal (B), moderate intensity signal (C and D), and strong signal (E and F).\

conceivable that artefactual location of shed 67LR molecules could occur during the fixation and inclusion procedures. In summary, over 71% of the assayed neuroblastoma samples showed expression of 67LR by immunocytochemistry, with a full range of expression levels.

4. Discussion

The 67LR has been involved in the biological aggressiveness of tumor cells, by helping to laminin remodeling and favoring its degradation by proteolytic enzymes (Ardini et al., 2002; Berno et al., 2005; Vande Broek et al., 2001). Remodeling of the tumor microenvironment and degradation of basement membrane components are two crucial steps in the development of metastases (Wang et al., 2005b). Here we show that RA treatment of neuroblastoma cells results in a reduction of the expression of 67LR. Concomitant to the downregulation of 67LR, RA treatments result in a reduction in cell migration towards *Matrigel* and an impairment in neuroblastoma cells invasiveness in *in vitro* assays. Finally, the involvement of 67LR in migration and invasion of neuroblastoma cells is supported by experiments in which impairment of the 67LR activity by laminin-derived P11 peptide results in reduced migration and invasion capacities for neuroblastoma cells. Taken together all these results support the idea that RA-induced downregulation of 67LR would account for the observed reductions of the migration capacity and invasiveness *in vitro*, and therefore would contribute of a marked reduction of the biological aggressiveness of neuroblastoma cells. RA treatments have been shown to be effective for the therapy of human neuroblastoma, especially in avoiding relapse when applied in a context of minimal residual disease after intensive therapies (Matthay & Reynolds, 2000; Reynolds et al., 2003) The reduction of the ability of migrating towards the basement membrane and degrading it would fit into the range of actions that would lead to effective therapeutic effects in that context.

Several lines of evidence suggest that the 67LR could be a useful therapeutic target for cancer treatment. The anti-cancer cytokines TNFα and IFNγ downregulate the expression of 67LR in transformed keratinocytes (Clausse et al., 1998). Blocking of 67LR with specific antibodies or laminin peptides, or downregulating its expression by anti-sense RNA leads to decreased migration and invasiveness *in vitro* to reduced metastatic and angiogenic potentials in different experimental metastasis models (Iwamoto et al., 1987; Narumi et al., 1999; Satoh et al., 1999; Tanaka et al., 2000)

Remarkably, short term RA treatments induce a transient increase in neuroblastoma cell migration and invasion *in vitro*. The increase in migration appears to be nonspecific, since also occurs in the absence of laminin chemoattractant. Similar RA-induced rapid transient increases in migration and invasion in neuroblastoma cells have been reported (Joshi et al., 2006; Meseguer et al., 2011), and correlated to the expression of tissue transglutaminase (Joshi et al., 2006). Blocking of RA signaling *in vivo* also impaired neuroblast migration from the subventricular zone of the brain to the olfactory bulb (Wang et al., 2005a), suggesting that short term RA-induced increases in migration probably reflects a physiological feature of RA action on the regulation of neurogenesis. Coupling of differentiation with migration has been described for mouse cortical neurons and the involvement of proneural bHLHs transcription factors in migration has been proposed (Ge et al., 2006). Remarkably, RA treatment of neuroblastoma cells results in changes in the expression patterns of bHLH factors including induction of proneural bHLH factors like *NEUROD6* and *NEUROD1* (Jogi et al., 2002; Lopez-Carballo et al., 2002).

Another important aspect is the mechanism through which RA induces the downregulation of *LAMR1* gene, which appears to be complex. It has reported that *LAMR1* is downregulated by p53, through an AP-2 binding element located in its first intron (Modugno et al., 2002). It

is noteworthy that RA treatment of N1E-115 neuroblastoma cells results in the induction of p73, a protein structurally and functionally related to p53 (De Laurenzi et al., 2000).In addition, accumulation of transactivation-deficient DeltaN-p73α in undifferentiated neuroblastic tumors has been reported (Douc-Rasy et al., 2002). Recently it has been reported that 67LR is a hypoxia-inducible factor target gene in gastric cancer (Liu et al., 2010). In neuroblastoma the hypoxia-inducible factors play an important role in maintaining an undifferentiated stem cell-like phenotype, that correlates with poor outcome (Holmquist-Mengelbier et al., 2006; Noguera et al., 2009; Pietras et al., 2009; Pietras et al., 2010).
Finally, we show that more than 71% of the high-grade primary neuroblastoma samples expressed 67LR as revealed by immunocytochemical analysis. The striking differences found in the expression levels of 67LR among the samples open the possibility that these differences could reflect diversity in biological aggressiveness of the tumor cells that may contribute to the differences in the clinical outcome and the heterogeneity of the therapeutic response.

5. Conclusion

RA-induced downregulation of 67LR correlates with a reduction in the biological aggressiveness *in vitro* of neuroblastoma cells, and supports the idea of 67LR as an important molecular target for the therapeutic actions of RA in neuroblastoma.

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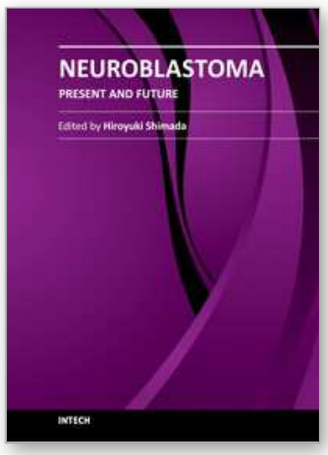

Neuroblastoma - Present and Future

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Neuroblastoma, once called "enigmatic", due to "unpredictable" clinical behaviors, is composed of biologically diverse tumors. Molecular/genomic properties unique to the individual tumors closely link to the clinical outcomes of patients. Establishing risk stratification models after analyzing biologic characteristics of each case has made a great success in patient management. However, the trend of improving survival rates in neuroblastoma over the last 30 years has started to level off, and currently available treatment modalities have almost reached to their maximized intensity. Furthermore, aggressive treatment causes significant long-term morbidities to the survivors. We really need to make the next step to the level of personalized medicine with more precise understanding of neuroblastoma biology. This book includes useful data and insights from the world's experts in this field. I believe this book can make an excellent contribution to all the investigators working hard and fighting for the children stricken by this disease.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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