

HMGI(Y) and HMGI-C Genes Are Expressed in Neuroblastoma Cell Lines and Tumors and Affect Retinoic Acid Responsiveness¹

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ABSTRACT

HMGI-C and HMGI(Y) are architectural DNA-binding proteins that participate in the conformational regulation of active chromatin. Their pattern of expression in embryonal and adult tissues, the analysis of the "pygmy" phenotype induced by the inactivation of the *HMGI-C* gene, and their frequent qualitative or quantitative alteration in experimental and human tumors indicate their pivotal role in the control of cell growth, differentiation, and tumorigenesis in several tissues representative of the epithelial, mesenchymal, and hematopoietic lineages. In contrast, very little information is available on their expression and function in neural cells. Here, we investigated the expression of the *HMGI(Y)* and *HMGI-C* genes in neuroblastoma (NB), a tumor arising from an alteration of the normal differentiation of neural crest-derived cells and in embryonal and adult adrenal tissue. Although *HMGI(Y)* is constitutively expressed in the embryonal and adult adrenal gland and in all of the NB cell lines and *ex vivo* tumors examined, its regulation appears to be associated to growth inhibition and differentiation because we observed that *HMGI(Y)* expression is reduced by retinoic acid (RA) in several NB cell lines that are induced to differentiate into postmitotic neurons, whereas it is up-regulated by RA in cells that fail to differentiate. Furthermore, the decrease of *HMGI(Y)* expression observed in RA-induced growth arrest and differentiation is abrogated in cells that have been made insensitive to this drug by *NMYC* overexpression. In contrast, *HMGI-C* expression is down-regulated during the development of the adrenal gland, completely absent in the adult individual, and only detectable in a subset of *ex vivo* NB tumors and in RA-resistant NB cell lines. We provide evidence of a causal link between *HMGI-C* expression and resistance to the growth arrest induced by RA in NB cell lines because exogenous *HMGI-C* expression in *HMGI-C*-negative and RA-sensitive cells is sufficient to convert them into RA-resistant cells. Therefore, we suggest that *HMGI-C* and *HMGI(Y)* may participate in growth- and differentiation-related tumor progression events of neuroectodermal derivatives.

INTRODUCTION

The HMG³ proteins are a heterogeneous group of nonhistone DNA-binding factors that play important architectural roles in the organization of active chromatin (1). The HMGI subfamily is composed of at least three members: HMGI, HMGY, and HMGI-C. The first two are transcribed from the same gene on chromosome 6p21, differ by the presence of an alternatively spliced exon (2, 3), and are often indicated as HMGI(Y). The *HMGI-C* gene is located on a

separate locus on chromosome 12q13–15 (4, 5). An important structural feature shared by the proteins of this family is the presence of three AT-hooks, through which they bind to A+T-rich sequences in the minor groove of the DNA helix, where they are thought to work as ancillary transcription factors (6). Indeed, HMGI(Y) participates in the transcriptional regulation of genes such as IFN- β , tumor necrosis factor- β , rRNA, interleukin 4, and IGF-binding protein-1 (7–12). HMGI-C expression is essentially absent in adult tissues (13–17), whereas it is highly expressed in most tissues of the developing mouse embryo (13). HMGI-C is thought to play important functions in the control of cell growth and differentiation. In fact, an inactivating mutation of the *HMGI-C* gene is responsible for the "pygmy" phenotype in the mouse, which is characterized by reduced size and weight of most body organs, reduced body fat, and a cell-autonomous defect in cell growth (13). Both *HMGI-C* and *HMGI(Y)* are delayed early response genes (18) with increased expression in cells transformed by oncogenes (19). The inhibition of HMGI-C protein synthesis prevents the retrovirally induced neoplastic transformation of thyroid cells (20), indicating that the anomalous HMGI-C expression may contribute to transformation. The *HMGI-C* gene is frequently expressed in chimeric proteins arising from chromosomal rearrangements in a number of benign mesenchymal tumors, including leiomyomata, lipoma, pulmonary chondroid hamartomas, fibroadenoma, and endometrial polyps (4, 21–27). However, it is also amplified or rearranged in malignant neoplasias such as human sarcomas (28), and exogenously expressed chimeric or truncated HMGI-C transforms NIH3T3 fibroblasts (29).

High expression during development and low or absent expression in adult differentiated tissues is also described for HMGI(Y). However, its augmented expression is associated with high-grade prostate and mammary cancer (30, 31) and correlates with the malignant phenotype in human thyroid and colorectal neoplasias (32–34). Rearrangements at the *HMGI(Y)* locus were observed in uterine leiomyomata (35) and pulmonary and breast hamartomas (36–38). *HMGI(Y)* expression and its functional activity were also reported in hematopoietic cells (7, 9, 11, 17), and the misexpression of alternative *HMGI-C* transcripts was recently described in leukemia samples (39). All this evidence points to a possible role for HMGI(Y) and HMGI-C proteins in the development and differentiation of several tissues and suggests that their deregulated expression may participate to the tumorigenic process in epithelia, mesenchyme, and hematological lineages. In contrast, little information is as yet available on their expression in neural cells or their relationship to events related to neuronal differentiation. By differential display analysis, we have recently found that the *HMGI-C* gene is expressed and regulated by EGF during the neurotypic differentiation of the neural crest derived TC-1S cell line,⁴ previously established in our laboratory (40, 41). This observation raised the possibility that, in addition to their func-

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³ The abbreviations used are: HMG, high mobility group; IGF, insulin-like growth factor; NB, neuroblastoma; RA, retinoic acid; EGF, epidermal growth factor; RT, reverse transcription; [³H]dThd, [³H]thymidine; DRB, doxorubicin; GFP, green fluorescent protein.

⁴ G. Giannini. EGF regulates a complex pattern of gene expression during the neurotypic conversion of TC-1S cells, manuscript in preparation.

tion in mesenchyme, epithelia, and the hematopoietic system (42), HMGI proteins might also play a role in controlling cell growth and differentiation in neural cells. Here, we focused on the expression of the *HMGI* genes in normal adrenal gland and in NB, one of the most common malignancies in children. NB is a tumor of the peripheral nervous system thought to arise from an anomalous arrest in the differentiation of the multipotent embryonal cells of the neural crest into normal adrenal medulla and postganglionic sympathetic neurons (43). Supporting this hypothesis, NB has an high rate of spontaneous regression by differentiation (44), most often observed in tumors with a systemic presentation (stage IVS). In addition, several agents can induce NB cell differentiation *in vitro* (45). RA is a quite potent inducer of NB cell differentiation, and it is currently being used in clinical trials for differentiative therapy of NB *in vivo*. However, a number of NB cell lines appear to be scarcely sensitive to the growth-inhibitory and differentiative properties of RA, and preliminary studies on the efficacy of RA in clinical trials have not yielded the expected results (46).

Here, we report of a diverging behavior of *HMGI(Y)* and *HMGI-C* gene products in NB. *HMGI(Y)* expression is readily detectable in all NB cell lines and tumors and also in normal embryonal and adult adrenal glands. However, its constitutive expression is decreased by RA in cells that undergo RA-induced growth inhibition and neuronal differentiation, but it is increased by RA in resistant NB cells. *HMGI-C* is, instead, expressed only in a subset of *ex vivo* NB tumors, RA-resistant cell lines, and embryonic adrenal gland, but it is undetectable in adult adrenal gland, suggesting that its anomalous expression might be associated with NB tumorigenesis and/or tumor progression. Indeed, the exogenous expression *HMGI-C* is sufficient to convert RA-sensitive NB cells into RA-resistant cells, thus indicating that its misexpression reduces NB cell response to growth-inhibitory stimuli.

MATERIALS AND METHODS

Cell Culture Conditions. The human NB cell lines SK-N-AS, SH-EP, KCNR, and SY5Y were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μ g/ml streptomycin at 37°C with 5% CO₂, whereas SK-N-SH required Eagle's MEM and 0.1 mg/ml sodium pyruvate. Parental SK-N-BE and the two clones 2N and 9N were cultured in RPMI supplemented with 15% fetal bovine serum and 200 μ g/ml geneticin, as described previously (47). For stimulation experiments, cells were seeded at low density. *all-trans*-RA (Sigma Chemicals Co., St. Louis, MO; 1 μ M) and/or cycloheximide (Sigma; 10 μ g/ml) was added 24 h after seeding.

Cell Cycle Analysis. Cell cycle analysis was performed as reported previously (48). Briefly, appropriately treated cells were washed in cold PBS, treated with RNase (0.5 μ g/ml), and stained with 40 μ g/ml propidium iodide. Cells were then incubated in the dark at 4°C for at least 4 h and analyzed by an Epics cytofluorometer (Coulter) using the automated computer program Mcycle (Coulter) and computer-assisted manual analysis.

RNA Preparation and Northern Blot Analysis. Total RNA was isolated according to the guanidine isothiocyanate/cesium chloride method. For Northern blot analysis, RNAs (10–20 μ g) were electrophoresed on a denaturing formaldehyde-agarose gel and transferred to Gene Screen Plus hybridization membranes (NEN Life Science Product, Boston, MA) by overnight blotting. Filters were hybridized overnight using 3 \times 10⁶ cpm/ml ³²P-labeled probes, washed to a final concentration of 0.1 \times SSC-0.1% SDS, and autoradiographed at –70°C with intensifying screens.

RT-PCR and Analysis of *HMGI-C* and *HMGI(Y)* Expression in NB Tumors. The cDNA probes for *HMGI-C* and *HMGI(Y)* were obtained by performing RT-PCR on RNA extracted from Hep 3B cells. The RT reaction was performed on total RNA (1 μ g) using the Moloney murine leukemia virus reverse transcriptase kit, according to the manufacturer's instructions (Life Technologies, Inc., Paisley, United Kingdom) in a final volume of 20 μ l for 45

min at 42°C. An aliquot (2 μ l) of the RT reaction was subjected to 35 cycles (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) of PCR amplification with *HMGI-C*- and *HMGI(Y)*-specific primers as follows: *HMGI-C*-1, 5'-AG-GAAGCAGCAGCAAGAACC-3'; *HMGI-C*-2, 5'-AGATCCAAGTCT-GCTGAGG-3'; *HMGI(Y)*-1, 5'-CCTGGACAAGGCTAACATCC-3'; and *HMGI(Y)*-2, 5'-GTGACTGCATCTCCATCACC-3'. Amplified cDNA fragments were cloned into the TA Cloning Vector (Invitrogen, San Diego, CA), and their identity was confirmed by sequencing analysis. For the analysis of *HMGI-C* and *HMGI(Y)* expression in NB tumors, total RNA (1 μ g), obtained from the tissue bank of the Italian Association of Hematology and Pediatric Oncology, was reverse transcribed as indicated above. An aliquot (5 μ l) of the RT reaction was subjected to PCR amplification as indicated above with the following *HMGI-C*-specific primers: *HMGI-C*-PR, 5'-GTTCCAGAAGAAGC-CTGCTC-3'; and *HMGI-C*-13, 5'-GTTACACACCGCTTCTTCC-3'. PCR products were electrophoresed on 1.5% agarose gel, blotted on hybridization membranes, and hybridized to the end-labeled internal primer *HMGI-C*-2. An aliquot (2 μ l) of the same RT reaction was subjected to a semiquantitative PCR (1 min at 94°C, 1 min at 58°C, and 2 min at 72°C for 20, 25, or 30 cycles) for *HMGI(Y)* amplification with the specific primers *HMGI(Y)*-1 and *HMGI(Y)*-2. PCR products were electrophoresed on 1.5% agarose gel and blotted on hybridization membranes. A second PCR-amplified fragment, obtained by means of primer *HMGI(Y)*-PR (5'-CCTCCTTCACTGTTCCTCT-3') and primer *HMGI(Y)*-3 (5'-TACGGGGACTAGGAAGTTG-3'), was cloned, labeled with ³²P, and subsequently used as internal probe for the hybridization of NB tumor samples. A fraction (1/40) of the same RT reaction was subjected to a semiquantitative PCR (1 min at 94°C, 1 min at 58°C, and 2 min at 72°C for 20, 25, or 30 cycles) to quantitatively amplify the β -actin cDNA as a control using the following specific primers: β -act-3, 5'-CTA-CAATGAGCTGCGTGTGG-3'; and β -act-4, 5'-CGGTGAGGATCTTCAT-GAGG-3'. Amplicons were electrophoresed, blotted on nylon membranes, and hybridized to a β -actin-specific probe.

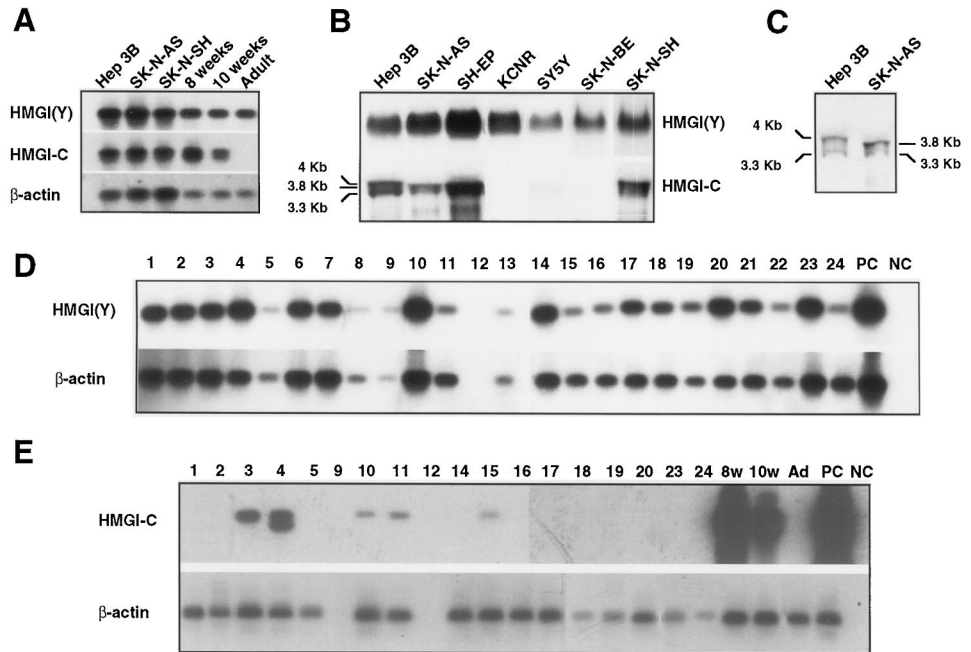
Construction of *HMGI* and *HMGI-C* Gene Expression Vectors and Cell Transfections. The entire coding sequences of the human *HMGI* and *HMGI-C* genes were cloned in frame 5' to the GFP into the pEGFP-N1 vector (Clontech, Palo Alto, CA). The pEGFP, pHMGI-GFP, and pHMGI-C-GFP plasmids were transfected into the SY5Y cells using the Lipofectamine Plus Kit (Life Technologies, Inc.) according to the manufacturer's instructions. Two days after transfection, cells were transferred in RPMI containing G418 (350 μ g/ml), and the transfected pools were selected over a period of 1 month.

[³H]dThd Incorporation. For [³H]dThd incorporation experiments the parental SY5Y cells or the transfected pools were seeded at 10⁴ cells per well in a 96-well plate. After 24 h, RA or solvent control was added to quadruplicate wells, and the plates were incubated for 4 days under standard conditions. Sixteen to 20 h prior to harvest, cells were labeled with 1 μ Ci of [³H]dThd (ICN, Costa Mesa, CA) per well. Cells were harvested using an Inotech harvester (Inotech, Lansing, MI) and counted in a Top Count NST scintillation counter (Packard Instruments Company, Downers Grove, IL).

RESULTS

***HMGI(Y)* and *HMGI-C* mRNA Expression in Normal Adrenal Gland and in NB Cell Lines and Tumor Specimens.** *HMGI-C* and *HMGI(Y)* genes are expressed at very low levels in adult differentiated tissues of mouse and human origin (14, 15). In contrast, their expression is readily detectable in embryonal and neoplastic tissues as well as in many proliferating cells *in vitro* (17, 19, 49, 50). Because *HMGI-C* is expressed and regulated by EGF during the neurotypic differentiation it induces in the neural crest-derived TC-1S cell line (40),⁴ we reasoned that the *HMGI* family members could be also expressed in other neural crest derivatives and possibly be involved in controlling their cell growth and differentiation. To test this hypothesis, we first investigated *HMGI(Y)* and *HMGI-C* expression in embryonal and adult adrenal glands as well as in NB cell lines and tumors. Using a RT-PCR approach, we found that *HMGI(Y)* mRNA is equally expressed in the adrenal glands of 8- and 10-week old human fetuses as well as in the adrenal gland of an adult individual (Fig. 1A). In contrast, *HMGI-C* expression was high in the adrenals of the

Fig. 1. Expression of *HMGI(Y)* and *HMGI-C* genes in the adrenal gland, NB cell lines, and NB tumor specimens. A, the expression of *HMGI(Y)* and *HMGI-C* was studied by RT-PCR in normal adrenal gland obtained from 8- and 10-week-old fetuses and an adult individual and compared to that of hepatoma cell line Hep 3B and NB cell lines SK-N-AS and SK-N-SH. The amplification reaction for *HMGI(Y)* and β -actin were stopped within the exponential phase (20 cycles), and the samples were blotted and hybridized to specific *HMGI(Y)* and β -actin probes; *HMGI-C* expression was analyzed by hybridization to a specific probe after 35 PCR cycles. B, total RNA (20 μ g) was extracted from the indicated NB cell lines, and the expression of *HMGI(Y)* and *HMGI-C* genes was analyzed by Northern blot. The Hep 3B hepatoma cell line was included as a positive control for *HMGI(Y)* and *HMGI-C* expression. C, the different sizes of the *HMGI-C* transcripts expressed in Hep 3B and SK-N-AS are better appreciated after a longer electrophoretic run. D, RNA from NB tumor samples was analyzed for *HMGI(Y)* (D) and *HMGI-C* (E) expression by RT-PCR as described above. 8w, 8 weeks; 10w, 10 weeks; Ad, adult; PC, Hep 3B RNA used as positive control; NC, template-free RT-PCR.



8-week fetuses, lower in the 10-week fetuses, and completely undetectable in the adult adrenal gland (Fig. 1A). We next investigated the expression of the *HMGI* genes in NB cell lines by using RT-PCR-amplified fragments to probe Northern blots containing total RNA extracted from several NB cell lines and from the Hep 3B cells as positive control (49). All of the cell lines expressed readily detectable levels of the *HMGI(Y)* transcript (Fig. 1B). On the contrary, *HMGI-C* was highly expressed only in a subset of NB cell lines (SK-N-AS, SH-EP, and SK-N-SH) in which two distinct transcripts of \sim 3.3 and \sim 3.8 kb were identified. Although the smallest *HMGI-C* transcript appeared to be common to all of the cell lines used, the higher molecular weight band observed in NB cells was clearly smaller than that expressed in Hep 3B cells (Fig. 1B). This was more clear after a longer electrophoretic run of the RNA samples extracted from the Hep 3B and SK-N-AS cells (Fig. 1C). Although the presence of two *HMGI-C* transcripts was previously noted in the Hep 3B (4) and the PLC/PRF/5 (51) human hepatoma cell lines, their precise origin and functional significance remain to be elucidated. Scant expression of the same *HMGI-C* transcripts was detectable in the SY5Y cells (Fig. 1B), whereas no *HMGI-C* mRNA expression was revealed in KCNR and SK-N-BE cells, even after long exposure of the blot. The analysis of the corresponding proteins, performed by Western blot using polyclonal antibodies developed against HMGI and HMGI-C (32) to probe properly prepared cell extracts, revealed the expression of *HMGI-C* in the SK-N-AS and SK-N-SH cell lines but not in the KCNR and SY5Y cell lines, whereas both HMGI and HMGI-Y proteins were expressed in all examined cells (data not shown).

The expression of *HMGI(Y)* and *HMGI-C* in cultured NB cell lines could reflect a feature of the tumors from which they originated, or it could be derived from the adaptation of the tumor cells to the culture conditions. To distinguish between these possibilities, we analyzed the expression of *HMGI(Y)* and *HMGI-C* genes by RT-PCR in a heterogeneous group of tumor samples. The amplification of a fragment of the β -actin cDNA from the same samples was used as a control. With this analysis, we detected *HMGI(Y)* expression in 100% of the amplifiable samples (Fig. 1D). Once the PCR results for *HMGI(Y)* were normalized for the β -actin amplification (data not shown), no significant quantitative differences were observed among

the different samples. On the contrary, *HMGI-C* expression was restricted to 5 of 18 tumors, accounting for 25% of the analyzed samples (Fig. 1E). Altogether, these results indicate that *HMGI(Y)* mRNA is constitutively expressed in the adrenal gland as well as in NB tumors and cell lines. In contrast, *HMGI-C* mRNA expression is down-regulated during adrenal gland development, totally absent in the adult adrenal gland, and restricted to a limited number of NB tumor specimens and NB cell lines.

Differential Regulation of *HMGI(Y)* Expression in RA-sensitive and -resistant NB Cell Lines. RA is known to arrest growth and induce differentiation in some but not all NB cell lines (52). Because the expression of *HMGI* genes is regulated in different cells by growth-promoting factors and in a number of tissues in association with their normal differentiation, we evaluated the expression of *HMGI(Y)* and *HMGI-C* in five different NB cell lines in response to RA. SK-N-AS (Fig. 2, A and B), SH-EP (Fig. 2, C and D), and SK-N-SH (Fig. 2, E and F) are substrate adherent (S-type) NB cell lines that did not show any major morphological change when exposed to RA up to 6 days and also failed to accumulate in the G₁ phase of the cell cycle (Fig. 2, A'-F'); these lines, therefore, represent RA-resistant cell lines. In these cells, a 48-h treatment with RA strongly increased *HMGI(Y)* expression, as detected by Northern blot analysis (Fig. 3A). In agreement with previously reported data (48, 53), RA treatment of the neuroblast-like (N-type) and RA-sensitive SY5Y and KCNR cell lines caused a marked morphological differentiation characterized by the extension of neurites exceeding at least twice the length of the cell soma clearly detectable after 4 days (Fig. 2, G-L). The flow cytometric analysis revealed that RA treatment of KCNR (Fig. 2, G' and H') and SY5Y cells (Fig. 2, I' and L') led to an increase in the number of cells in the G₀/G₁ phase of the cell cycle after 4 days of treatment, indicative of its strong antiproliferative effect. An additional consequence of RA treatment in these cell lines was the reduction of the steady-state level of the *HMGI(Y)* mRNA expression detectable after 48 h of treatment (Fig. 3B). On the contrary, we did not detect any effect of RA on *HMGI-C* gene expression in any of the cell lines analyzed (data not shown). In summary, these data suggest that RA-resistant NB cells express both *HMGI* genes and up-regulate *HMGI(Y)* gene expression in response to RA, whereas

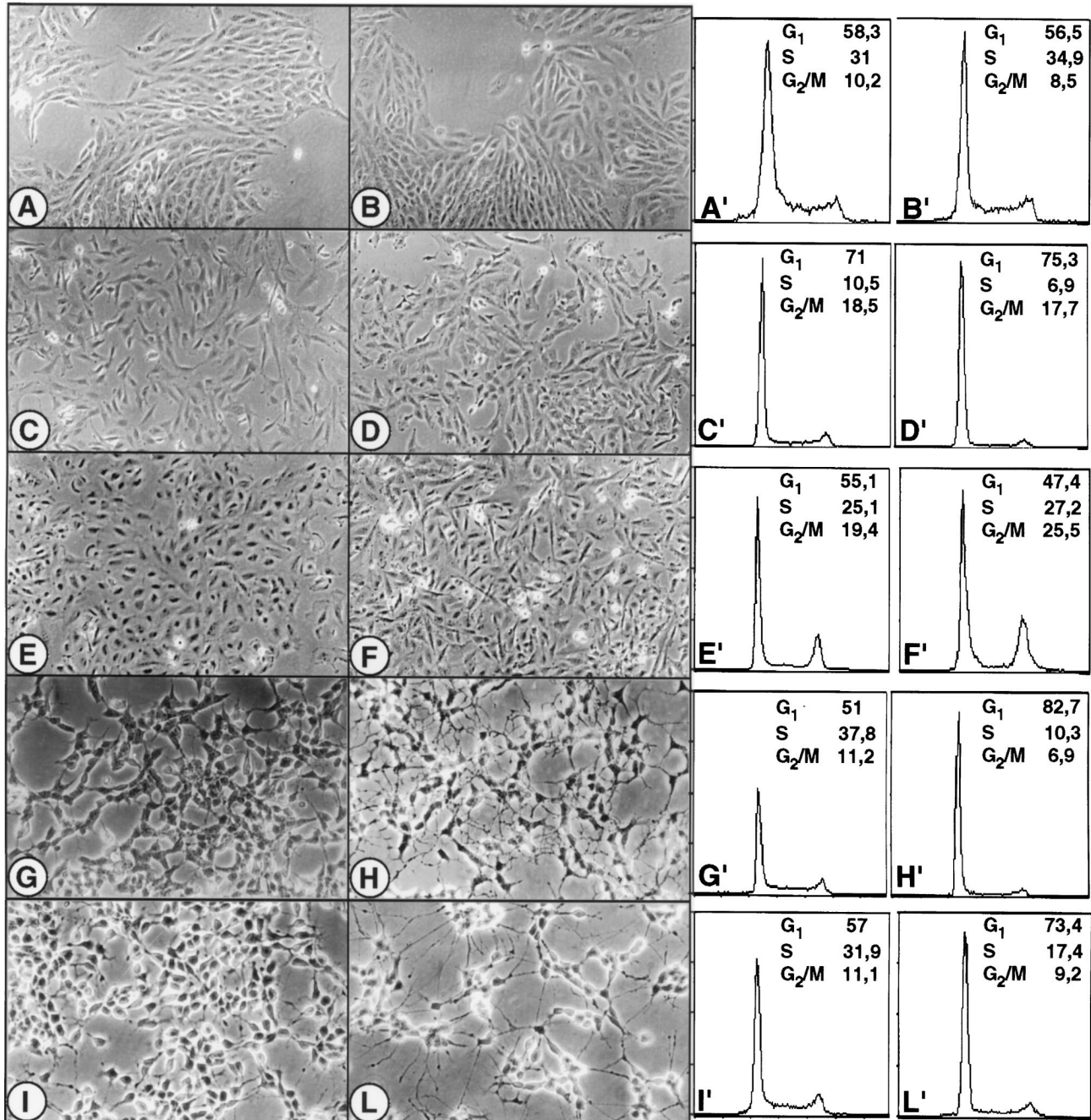


Fig. 2. RA affects differentiation and proliferation of a subset of NB cell lines. SK-N-AS (A and B), SK-N-SH (C and D), SH-EP (E and F), KCNR (G and H), and SY5Y (I and L) cell lines were treated either with RA (B, D, F, H, and L) or with solvent (A, C, E, G, and I) for 6 (SK-N-AS, SK-N-SH, and SH-EP) and 4 days (KCNR and SY5Y) and photographed to record morphological differentiation. Then cells were detached from the dish by mild trypsin digestion, washed, and processed for propidium iodide staining and cytofluorometric analysis of the DNA content (A'–I' and L'). Curves, percentages of cells in the different phases of the cell cycle.

RA-sensitive cell lines do not express *HMGI-C* gene and down-regulate *HMGI(Y)* gene expression in response to RA.

RA Stimulates *HMGI(Y)* Expression Independent of Ongoing Protein Synthesis. It has been proposed that the autocrine secretion of IGF-I and IGF-II induced by RA in several NB cell lines may provide an explanation for the resistance to RA antiproliferative effects (54–56). Because a number of growth factors, including IGFs, can promote *HMGI(Y)* expression (18), we sought to determine whether the accumulation of the *HMGI(Y)* mRNA in selected NB cell lines is directly controlled by RA or mediated by the secretion of newly synthesized growth factors. To this end, we studied the time course of induction of *HMGI(Y)* by RA in SK-N-AS cells in the

presence or absence of cycloheximide, a commonly used inhibitor of protein synthesis. RA induced a 1.5-fold increase of the *HMGI(Y)* mRNA expression by 6 h (Fig. 4 A, Lane 5, and B) and a maximum increase of 3-fold by 24 h (Fig. 4A, Lane 12). The addition of cycloheximide for up to 12 h also induced an increase in the steady-state level of *HMGI(Y)* mRNA (Fig. 4, A and B), but its effect after 24 h could not be evaluated because of its toxicity. In the same time interval, cycloheximide did not prevent RA induction of *HMGI(Y)* mRNA, and the effect of the two drugs appeared to be at least partially additive (Fig. 4, A and B). Similar experiments were also performed on SK-N-SH cells. Also in this case, cycloheximide did not prevent *HMGI(Y)* induction by RA. Addition of the RNA polymerase inhibitor

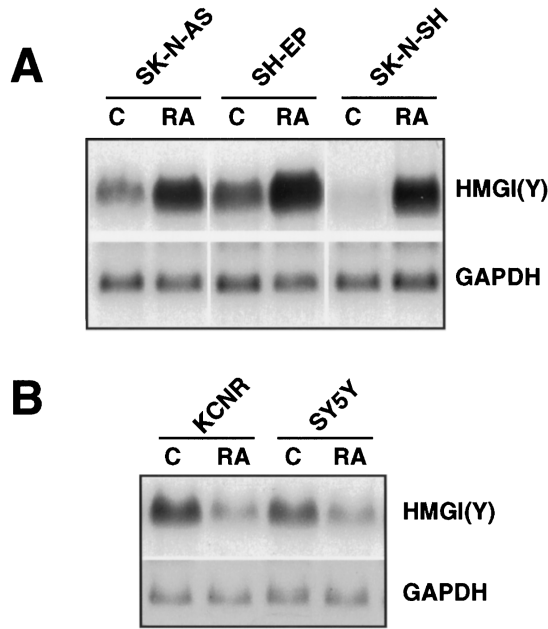


Fig. 3. Differential regulation of *HMGI(Y)* expression by RA in resistant or sensitive NB cell lines. RNA (15 μ g) extracted from the indicated NB cell lines was analyzed by Northern blot for the expression of *HMGI(Y)* gene after 48 h of RA treatment. A, SK-N-AS, SH-EP, and SK-N-SH, RA-resistant cell lines; B, KCNR and SY5Y, RA-sensitive cell lines. Glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*) hybridization to the same blots is shown as a loading control.

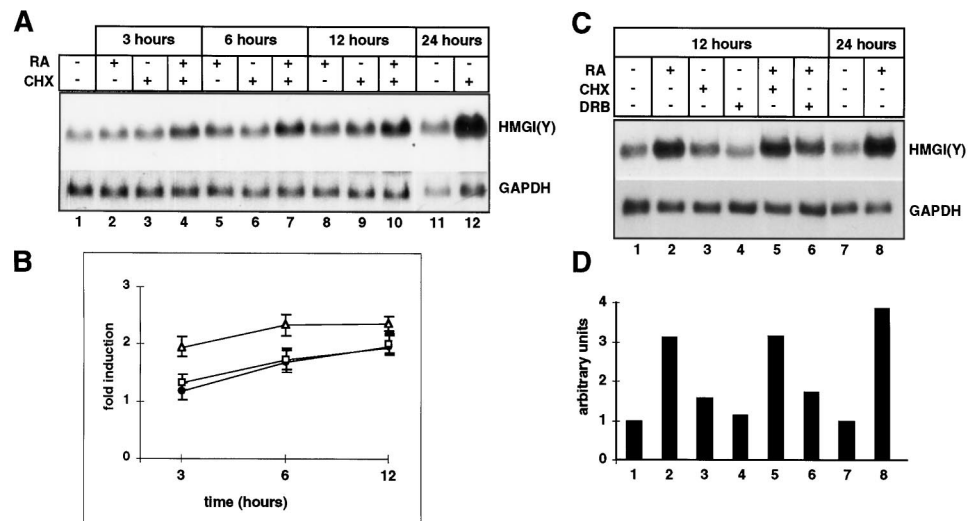
DRB to RA treatment partially inhibited its effect (Fig. 4, C, Lane 6, and D, column 6), suggesting that the increase in the steady-state levels of *HMGI(Y)* mRNA is at least in part due to a transcriptional activation. These results indicate that *de novo* protein synthesis is not necessary for induction of *HMGI(Y)* expression by RA and make the possibility that *HMGI(Y)* mRNA induction by RA is a secondary event dependent on the secretion of IGFs in RA-resistant cells unlikely.

***HMGI(Y)* Down-Regulation by RA in NB Cells Is Associated with Growth Inhibition and Differentiation.** In NB cell lines bearing *NMYC* genomic amplification, the morphological differentiation (47, 57) and growth inhibition (47) induced by RA are dependent on the down-regulation of *NMYC* and are prevented by exogenous constitutive *NMYC* expression (47, 57). A time course analysis of *NMYC* and *HMGI(Y)* mRNAs after RA treatment of the *NMYC*-

amplified *KCNR* cells showed that *NMYC* mRNA was strongly reduced after 24 h, whereas *HMGI(Y)* maximum decrease occurred at 48 h (Fig. 5, A and B). This indicated that *NMYC* down-regulation by RA precedes *HMGI(Y)* modulation as well as growth inhibition and differentiation in *NMYC*-amplified cell lines. In several model systems, the steady-state levels of *HMGI(Y)* mRNA parallel the growth and differentiation status of the cell (18, 50). In fact, *HMGI(Y)* expression is low in quiescent cells and increases during the G_1 -S transition induced by serum or distinct growth factors (18, 50). Therefore, we used exogenous *NMYC* expression as a tool to uncouple RA treatment from growth inhibition and differentiation and asked whether *HMGI(Y)* can still be repressed by RA under these conditions. To this end, we analyzed *HMGI(Y)* expression in the previously characterized *NMYC*-transfected SK-N-BE clones 2N and 9N (47), as compared to the parental SK-N-BE cells. Consistent with a previous report (47), a prolonged exposure (8–15 days) to RA induced morphological differentiation of SK-N-BE parental cells (data not shown). RA also induced differentiation in clone 2N (Fig. 6A, a and b), which fails to express the exogenous *NMYC* transcript because of a rearrangement in the promoter of the unique integrated copy of the gene (47). The morphological differentiation of both parental SK-N-BE and clone 2N was associated with a decrease in the steady state level of *HMGI(Y)* and endogenous *NMYC* mRNAs (Fig. 6, B and C). In contrast, clone 9N, which constitutively expressed the exogenous *NMYC* (Ref. 47; Fig. 6, B and C), did not morphologically differentiate in response to RA (Fig. 6A, c and d). In this clone, *HMGI(Y)* mRNA expression was not repressed by RA; on the contrary, it was slightly stimulated (Fig. 6C), which is analogous to the results obtained in spontaneously RA-resistant cell lines (Fig. 3A). These results confirm that the reduction of *HMGI(Y)* expression is linked to the growth inhibition and morphological differentiation induced by RA and also indicate that *NMYC* down-regulation is required for both *HMGI(Y)* repression and for differentiation and growth inhibition in *NMYC*-amplified cells.

Effects of the Exogenous Expression of *HMGI(Y)* and *HMGI-C* Proteins in NB Cells. The strong correlation between the expression of *HMGI-C* gene, the positive regulation of *HMGI(Y)* by RA, and the resistance of certain NB cell lines to the antiproliferative effects of this agent suggested to us that a causal link could exist between the expression and regulation of *HMGI-C* and *HMGI(Y)* and resistance to RA. To test this hypothesis, we constructed expression vectors containing the entire coding sequences of the human *HMGI* and *HMGI-C*

Fig. 4. *HMGI(Y)* expression is directly induced by RA in resistant cell lines. A, SK-N-AS cells were treated with control solvent, RA (1 μ M), or cycloheximide (*CHX*, 10 μ g/ml) for the indicated time. RNA was subsequently extracted and processed for the expression of *HMGI(Y)* gene by Northern blot. Glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*) hybridization of the same blot is shown as a loading control. B, the relative expression of *HMGI(Y)* at the different time points in three different experiments was normalized for glyceraldehyde 3-phosphate-dehydrogenase expression after densitometric analysis of the films, and the values are reported as fold induction compared to control levels. ●, RA; □, cycloheximide; △, RA plus cycloheximide. C, SK-N-SH cells were treated with control solvent, RA (1 μ M), cycloheximide (*CHX*, 10 μ g/ml), or DRB (1 μ g/ml) for the indicated time. RNA was subsequently extracted and processed as indicated above. D, the relative expression of *HMGI(Y)* was normalized for glyceraldehyde 3-phosphate-dehydrogenase expression after densitometric analysis of the films, and the values are reported as arbitrary units.



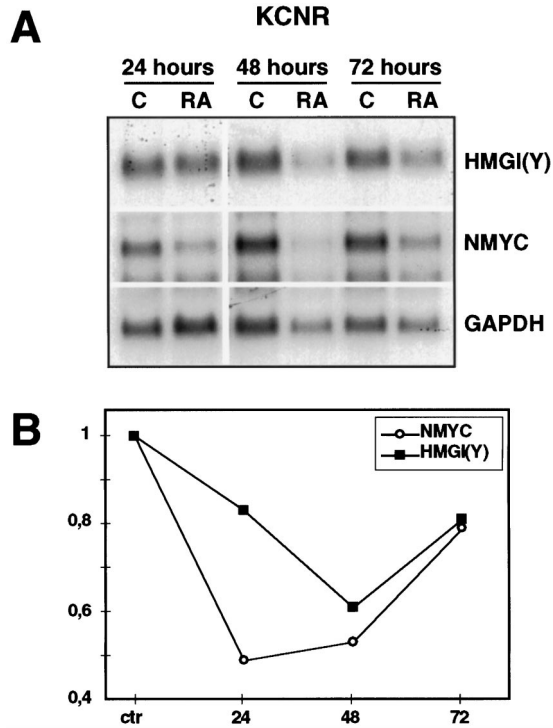


Fig. 5. NMYC inhibition by RA precedes HMGI(Y) down-regulation. *A*, KCNR cells were treated with control solvent or RA (1 μ M) for the indicated time. Then RNA was extracted and *HMGI(Y)* and *NMYC* expression was analyzed by Northern blot. Glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*) hybridization of the same blot is shown as a loading control. *B*, the relative expression of *HMGI(Y)* and *NMYC* genes was normalized for glyceraldehyde 3-phosphate-dehydrogenase expression after the densitometric analysis of the films, and the values are reported as arbitrary units.

genes in-frame with the GFP DNA sequence and transfected those constructs into the SY5Y cells. Through a period of selection with G418, we established pools of stable transfectants expressing the HMGI-GFP or HMGI-C-GFP chimeric proteins or the GFP protein

alone. Exogenous HMGI-GFP or HMGI-C-GFP chimeric transcript could easily be distinguished by the endogenous *HMGI(Y)* and *HMGI-C* because of their smaller sizes, as measured in a Northern blot (Fig. 7A). As expected, no endogenous *HMGI-C* transcript was found in parental and transfected cell lines, whereas the HMGI-C-GFP exogenous chimeric transcript was easily detectable in the corresponding pool (Fig. 7A). Similarly, the HMGI-GFP chimeric transcript was revealed in HMGI-GFP-transfected pools below the endogenous *HMGI(Y)*, which was expressed in both parental and transfected cells (Fig. 7A). At the protein level, the expression of the chimeras was detectable as a fluorescent signal at the observation of living cells at the fluorescent microscope. Although the GFP protein spread diffusely into the cells, the HMGI-GFP and HMGI-C-GFP chimeric proteins properly localized to the nucleus as expected for the HMGI proteins (Fig. 7B). Therefore, we examined the effects of RA on the different pools by [3 H]dThd incorporation and cell cycle fluorescence-activated cell sorting analysis. As expected, SY5Y cells showed a strong reduction in the incorporation of [3 H]dThd in the presence of increasing concentration of RA (ranging from 10^{-9} to 5×10^{-6} M), which accounted for 30% of the incorporation of control cells at 5×10^{-6} M RA (Fig. 7C). Under the same conditions, GFP and HMGI-GFP pools presented a similar response to RA. In contrast, RA only poorly affected [3 H]dThd incorporation in HMGI-C-GFP pool, which still showed 80 and 75% incorporation at 10^{-6} and 5×10^{-6} M RA, respectively (Fig. 7C). We also examined the cell cycle profile of the different pools and the results of one such experiments is summarized in Table 1. The three pools showed a cell cycle profile almost identical to that of wild-type SY5Y cells, indicating that neither the selection procedure nor the transfected constructs affected cell growth. As we found previously, under RA treatment, wild-type SY5Y cells accumulated in the G_0/G_1 phase of the cell cycle, determining a profound alteration of the ratio between the percentage of cells in the growth fraction ($S + G_2-M$) compared to the G_0/G_1 fraction (Table 1). Similar results were obtained with the GFP pool. Although we observed a slight reduction in the number of cells that accumulated in G_0/G_1 in the

Fig. 6. Differentiation and HMGI(Y) decrease induced by RA are abrogated in SK-N-BE cells overexpressing NMYC. *A*, SK-N-BE control clone 2N (*a* and *b*) and the NMYC overexpressing clone 9N (*c* and *d*) were treated with control solvent (*a* and *c*) or RA (10 μ M; *b* and *d*) for 15 days and photographed to record morphological differentiation. *B*, total RNA was extracted from parental SK-N-BE and from clones 2N and 9N, and *HMGI(Y)* and *NMYC* expression was assessed by Northern blot. Glyceraldehyde 3-phosphate-dehydrogenase hybridization to the same blot is shown as a loading control. *C*, the relative expression of *HMGI(Y)* and *NMYC* genes in parental SK-N-BE (■), clone 2 (▨), and clone 9 (□) with or without treatment with RA was normalized for glyceraldehyde 3-phosphate-dehydrogenase expression after the densitometric analysis of the films, and the values are expressed in arbitrary units.

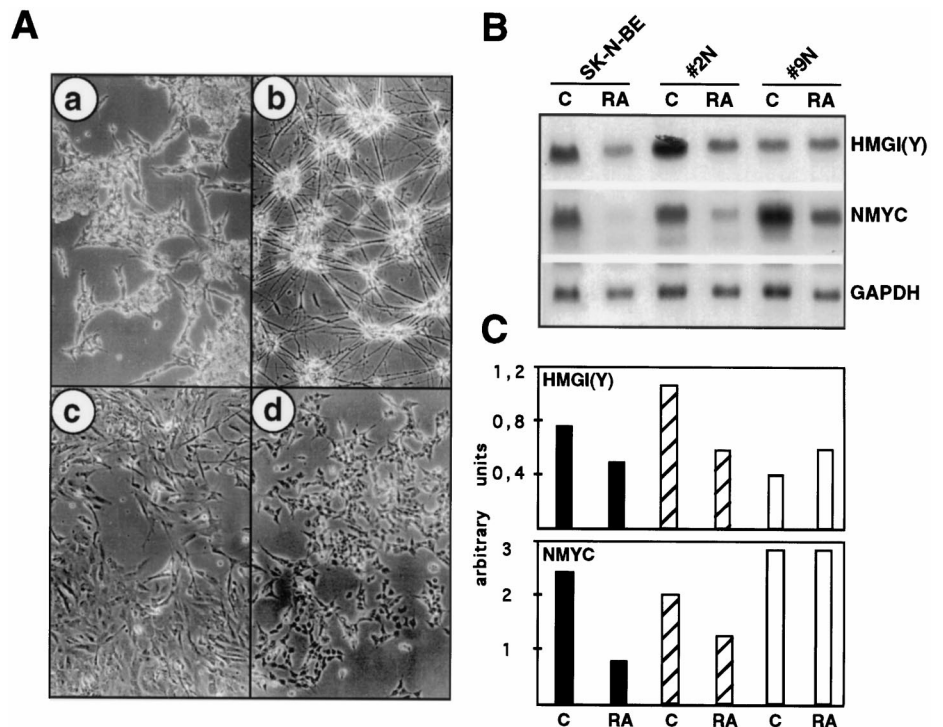
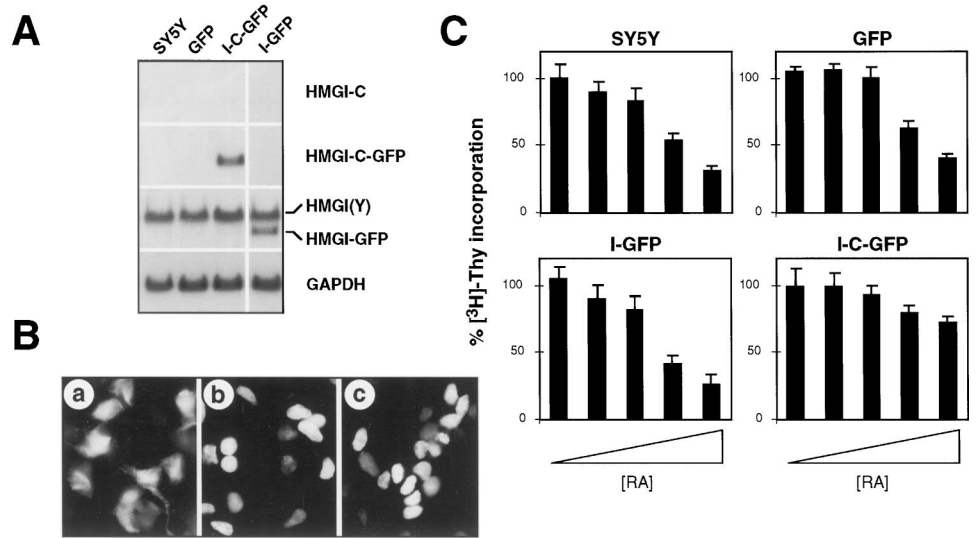


Fig. 7. Expression of the wild-type and GFP fusion proteins in the transfected pools and their effects on proliferation. A, total RNA was extracted from wild-type SY5Y cells, the GFP-transfected pool (*GFP*), the HMGI-C-GFP-transfected pool (*I-C-GFP*), and the HMGI-GFP-transfected pool (*I-GFP*) and analyzed by Northern blot for the expression of endogenous and exogenous *HMGI(Y)* and *HMGI-C* transcripts. B, fluorescent microscope microphotographs showing the diffuse intracellular localization of the GFP protein (a), compared to the nuclear localization of the HMGI-GFP (b) and HMGI-C-GFP (c) proteins. C, quadruplicate cultures of parental SY5Y cells, GFP pool, HMGI-GFP pool, or HMGI-C-GFP pool, plated at 10^4 cells/well, were grown for 4 days in the presence of either solvent or RA (ranging between 10^{-9} and 5×10^{-6} M) in 96-well plates. After an overnight pulse with [3 H]dThd, cells were harvested and counted for incorporated radioactivity. Columns, mean percentage (quadruplicate cultures) of [3 H]dThd incorporation compared to solvent-treated cells; bars, SD. These results are representative of at least three separate experiments.



HMGI-GFP pool, this effects was neither significant nor consistent. In contrast, the cell cycle profile of the HMGI-C-GFP pool was very similar in control and RA-treated cells, indicating that those cells became resistant to the antiproliferative effects of RA (Table 1). Interestingly, we did not observe significant alteration of the morphological response to RA in any cell line (data not shown). Altogether these results indicate that, although the expression of exogenous HMGI(Y) does not prevent a full response of SY5Y cells to RA, the expression of exogenous HMGI-C strongly reduces its effect on cell growth, thus counteracting at least one of the major biological effects of RA in sensitive NB cells.

DISCUSSION

The physiological pattern of expression of the ancillary transcription factors HMGI-C and HMGI(Y) suggests their possible role in mammalian development and tissue differentiation (13, 17). HMGI-C is expressed in most tissues during mouse embryogenesis, and its expression declines throughout development and essentially disappears before birth (13). HMGI(Y) expression also declines throughout development, but it remains detectable in several tissues even during adult life (17). The deregulated expression of HMGI-C and HMGI(Y) has been recently linked to the development of both benign and malignant neoplasias of mesenchymal and epithelial origin (4, 21–28, 30–33), raising the possibility that these proteins participate in tumorigenesis in several cell contexts. The expression of HMGI(Y) in the central and peripheral nervous system (17) and our recent observation that *HMGI-C* is regulated during the neurotypic differentiation induced by EGF in the neural crest-derived TC-1S cell line⁴ prompted

Table 1 Effects of the exogenous expression of *HMGI* and *HMGI-C* on the ability of RA to inhibit cell cycle progression in SY5Y cells

Fluorescence-activated cell sorting analysis of propidium iodide-stained wild-type or transfectant SY5Y cells, cultured for 4 days in the absence or presence of RA, was performed as described in "Materials and Methods." The results are representative of three different experiments.

Cell lines	CTR				RA			
	% G ₁	% S	% G ₂ -M	r ^a	% G ₁	% S	% G ₂ -M	r ^a
SY5Y	50.1	47.7	2.2	1.004	81.7	17.2	1.1	4.4645
GFP pool	47.2	49.4	3.4	0.8939	80.5	16.4	3.1	4.1282
HMGI-GFP pool	54.2	40.3	5.5	1.1834	75.7	20.1	4.1	3.1281
HMGI-C-GFP pool	46.5	47.6	5.9	0.8692	50.3	43.7	6	1.0121

^a r = % G₁/S + G₂-M.

us to thoroughly characterize the expression of *HMGI-C* and *HMGI(Y)* genes in neural cells and tumor tissues. Here, we focused on the analysis of the expression of the *HMGI(Y)* and *HMGI-C* transcripts in a number of NB cell lines and tumor specimens as well as in normal tissue deriving from embryonal and adult adrenal gland, from which NB frequently arises.

Among the NB cell lines analyzed, three RA-sensitive cell lines (KCNR, SY5Y, and SK-N-BE) showed undetectable levels of *HMGI-C* mRNA. On the contrary, we detected high levels of two *HMGI-C* transcripts in the three RA-resistant cell lines (SH-EP, SK-N-AS, and SK-N-SH). We revealed that one of these messages has a different size compared to the two previously described messages (4), indicating that *HMGI-C* gene gives origin to at least three different transcripts that may display alternative patterns of expression. The *HMGI-C* gene is also expressed in some of the *ex vivo* NBs, with no significant correlation with tumor grade in this small series of tumor specimens. Interestingly, although we observed *HMGI-C* expression in the embryonal adrenals, we did not detect it in normal adult adrenal tissue, suggesting that *HMGI-C* expression might be a feature of some neoplastic or undifferentiated rather than normally differentiated neural crest derivatives. In addition, we found high *HMGI-C* expression only in some NB cell lines resistant to RA, which might indicate that its expression is associated to a biological phenotype that is less prone to differentiation. NB is an aggressive and often lethal neoplasia especially in children who are ≥ 1 year old. *NMYC* amplification and chromosome 1p deletion are genetic lesions that show important prognostic value and may be relevant to the tumor biology (58, 59). Although the *p73* gene, a *p53* homologue, was recently mapped at the 1p region frequently deleted in NB cell lines (60), its role as a tumor suppressor gene is controversial. In any case, it is possible that other genetic lesion may contribute to the development and/or to the progression of NB tumors. Our data indicate that misexpression of the *HMGI-C* gene may represent one of such alterations. Indeed, we have shown that the expression of *HMGI-C* in RA-sensitive SY5Y cells impairs their responsiveness to growth arrest-inducing agents such as RA, thus suggesting that *HMGI-C* expressing tumors might be less sensitive to endogenous or therapeutic growth-inhibitory substances. A growing amount of evidence supports a role for HMGI-C in tumorigenesis, including its frequent rearrangement in benign tumors of mesenchymal origin (4, 21–26), the deregulated expression of a wild-type HMGI-C in benign and

malignant neoplasia due to genomic amplification (28) or deletion of gene expression control regions (42), and the ability of chimeric and truncated forms of HMGI-C to transform NIH3T3 fibroblasts (29). A possible mechanism for its activity in cell transformation comes from the observation that the alteration of the AP-1 complexes induced in oncogene-transformed cells is prevented by antisense constructs to the *HMGI-C* (61). This suggests that HMGI-C is able to induce compositional changes and functional activation of the AP-1 complex, resulting in an increased growth-promoting activity (61). It is well known that several members of the AP-1 complex are able to interfere with the transcriptional and biological activities of RA receptors (62, 63). Therefore, it is also possible that the exogenous expression of HMGI-C in NB cells may induce AP-1 activation and compositional changes responsible for the antagonistic activity against the RA receptor-mediated transcriptional response related to growth inhibition. A detailed characterization of the effects of exogenous HMGI-C expression in NB cells and the extensive analysis of its expression in larger series of NB samples will definitely help elucidate the role of HMGI-C in NB tumor biology.

The other member of the HMGI family, HMGI(Y), contributes to tumorigenesis in several tissues. In fact, increased expression of HMGI(Y) correlates with invasiveness, metastatic potential, and stage progression in several experimental and human tumors of epithelial origin (30–34, 64). In contrast, the results presented here show that *HMGI(Y)* is expressed at comparable levels in all of the NB cell lines studied. Our RT-PCR analysis confirmed the presence of the *HMGI(Y)* transcript in adult and embryonic adrenal tissue as well as in 100% of the NB specimens. Although the level of this analysis could be improved by using more quantitative PCR methodologies and by extending the number of the samples analyzed, our results indicate the absence of significant variations in the expression of the *HMGI(Y)* gene among the different stages of the NB tumors, thus ruling out a primary role of this protein in NB tumorigenesis. However, despite the widespread expression of *HMGI(Y)* in the NB tumors *ex vivo*, a correlation exists between *HMGI(Y)* expression and the biological behavior of NB cells in response to administration of differentiating drugs. This may be an interesting finding, in that, although NB cells and RA treatment have been largely used as a model to study neural cell differentiation, a number of NB cell lines are only partially sensitive to RA and show limited or no growth inhibition and differentiation. The reason for this variability is still unclear because most NB cell lines express a similar pattern of RA receptors and are capable of transducing RA-mediated signals. In fact, *HMGI(Y)* mRNA expression is inhibited by RA (and also by TPA; data not shown) in NB cell lines that are sensitive to its growth-inhibitory and differentiating effects. In contrast, the steady-state level of *HMGI(Y)* mRNA is consistently increased in cells that fail to differentiate and growth arrest upon RA treatment. Because the increase in the steady-state level of *HMGI(Y)* in response to RA does not require ongoing protein synthesis and is reduced by the inhibitor of RNA polymerases DRB, we expect that RA directly regulates *HMGI(Y)* transcription, although, from our experiments, additional effects on mRNA stability cannot be ruled out. The decrease of *HMGI(Y)* message induced by RA is associated with growth inhibition and neuronal differentiation of NB cells, whereas its up-regulation is associated with a RA-resistant phenotype. The observation that SK-N-BE cells, in which growth inhibition and neuronal differentiation are prevented by the expression of the exogenous NMYC, up-regulate *HMGI(Y)* gene expression in response to RA further supports this hypothesis. However, exogenous expression of HMGI in SY5Y cells did not alter their response to RA as far as growth inhibition and differentiation is concerned. This is consistent with more recent data showing that there is no significant decrease of the HMGI and HMGY proteins

within the 4 days of RA treatment necessary for the growth inhibition of KCNR and SY5Y, probably due to the long half-life of the proteins.⁵ Therefore, it appears that, although repression of *HMGI(Y)* mRNA expression by RA marks RA-sensitive cell lines and its up-regulation RA-resistant cell lines, its decrease should be considered more a consequence than a cause of RA-induced growth arrest. Whether this response has any causal link to additional long-term responses to RA remains to be investigated.

In conclusion, we have shown that the *HMGI-C* gene is misexpressed in a subset of NB tumors *ex vivo* and in NB cell lines resistant to RA and is responsible for RA resistance because its exogenous expression converted RA-sensitive into RA-resistant cells. On the other hand, the expression the *HMGI(Y)* gene, which is constitutive in all NB cell lines and tumors, is repressed by RA only in those cells capable of neuronal differentiation. Therefore, we suggest that the basal level of expression of the *HMGI-C* gene and *HMGI(Y)* responsiveness to RA might predict the effect of this agent on NB cell lines and possibly also on NB tumors *in vivo*. We also speculate that *HMGI-C* and *HMGI(Y)* genes may participate in differentiation- and growth-related tumor progression events of neuroectodermal derivatives.

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REFERENCES

- Grosschedl, R., Giese, K., and Pagel, J. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet.*, 10: 94–100, 1994.
- Johnson, K. R., Lehn, D. A., and Reeves, R. Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y. *Mol. Cell. Biol.*, 9: 2114–2123, 1989.
- Friedmann, M., Holth, L. T., Zoghbi, H. Y., and Reeves, R. Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene. *Nucleic Acids Res.*, 21: 4259–4267, 1993.
- Schoenmakers, E. F., Wanschura, S., Mols, R., Bullerdiek, J., Van den Berghe, H., and Van de Ven, W. J. Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours. *Nat. Genet.*, 10: 436–444, 1995.
- Ishwad, C. S., Shriver, M. D., Lassigne, D. M., and Ferrell, R. E. The high mobility group I-C gene (HMGI-C): polymorphism and genetic localization. *Hum. Genet.*, 99: 103–105, 1997.
- Solomon, M. J., Strauss, F., and Varshavsky, A. A mammalian high mobility group protein recognizes any stretch of six A-T base pairs in duplex DNA. *Proc. Natl. Acad. Sci. USA*, 83: 1276–1280, 1986.
- Chuvpilo, S., Schomburg, C., Gerwig, R., Heinfling, A., Reeves, R., Grummt, F., and Serfling, E. Multiple closely-linked NFAT/octamer and HMG I(Y) binding sites are part of the interleukin-4 promoter. *Nucleic Acids Res.*, 21: 5694–5704, 1993.
- Allander, S. V., Durham, S. K., Scheimann, A. O., Wasserman, R. M., Suwanichkul, A., and Powell, D. R. Hepatic nuclear factor 3 and high mobility group I/Y proteins bind the insulin response element of the insulin-like growth factor-binding protein-1 promoter. *Endocrinology*, 138: 4291–4300, 1997.
- Thanos, D., and Maniatis, T. The high mobility group protein HMG I(Y) is required for NF- κ B-dependent virus induction of the human IFN- β gene. *Cell*, 71: 777–789, 1992.
- Thanos, D. Mechanisms of transcriptional synergism of eukaryotic genes. The interferon- β paradigm. *Hypertension*, 27: 1025–1029, 1996.
- Fashena, S. J., Reeves, R., and Ruddle, N. H. A poly(dA-dT) upstream activating sequence binds high-mobility group I protein and contributes to lymphotoxin (tumor necrosis factor- β) gene regulation. *Mol. Cell. Biol.*, 12: 894–903, 1992.
- Yang-Yen, H. F., and Rothblum, L. I. Purification and characterization of a high-mobility-group-like DNA-binding protein that stimulates rRNA synthesis *in vitro*. *Mol. Cell. Biol.*, 8: 3406–3414, 1988.
- Zhou, X., Benson, K. F., Ashar, H. R., and Chada, K. Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. *Nature (Lond.)*, 376: 771–774, 1995.
- Manfioletti, G., Giancotti, V., Bandiera, A., Buratti, E., Sautiere, P., Cary, P., Crane-Robinson, C., Coles, B., and Goodwin, G. H. cDNA cloning of the HMGI-C phosphoprotein, a nuclear protein associated with neoplastic and undifferentiated phenotypes. *Nucleic Acids Res.*, 19: 6793–6797, 1991.

⁵ L. Di Marcotullio, G. Giannini, and V. Giancotti, unpublished data.

15. Rogalla, P., Drechsler, K., Frey, G., Hennig, Y., Helmke, B., Bonk, U., and Bullerdiek, J. HMGI-C expression patterns in human tissues. Implications for the genesis of frequent mesenchymal tumors. *Am. J. Pathol.*, *149*: 775-779, 1996.

16. Zhou, X., Benson, K. F., Przybylski, K., Liu, J., Hou, Y., Cherath, L., and Chada, K. Genomic structure and expression of the murine Hmgi-c gene. *Nucleic Acids Res.*, *24*: 4071-4077, 1996.

17. Chiappetta, G., Avantiaggiato, V., Visconti, R., Fedele, M., Battista, S., Trapasso, F., Merciai, B. M., Fidanza, V., Giancotti, V., Santoro, M., Simeone, A., and Fusco, A. High level expression of the HMGI(Y) gene during embryonic development. *Oncogene*, *13*: 2439-2446, 1996.

18. Lanahan, A., Williams, J. B., Sanders, L. K., and Nathans, D. Growth factor-induced delayed early response genes. *Mol. Cell. Biol.*, *12*: 3919-3929, 1992.

19. Giancotti, V., Pani, B., D'Andrea, P., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., Philp, R., Crane-Robinson, C., Nicolas, R. H., Wright, C. A., and Goodwin, G. H. Elevated levels of a specific class of nuclear phosphoproteins in cells transformed with *v-ras* and *v-mos* oncogenes and by cotransfection with *c-myc* and polyoma middle T genes. *EMBO J.*, *6*: 1981-1987, 1987.

20. Berlingieri, M. T., Manfioletti, G., Santoro, M., Bandiera, A., Visconti, R., Giancotti, V., and Fusco, A. Inhibition of HMGI-C protein synthesis suppresses retrovirally induced neoplastic transformation of rat thyroid cells. *Mol. Cell. Biol.*, *15*: 1545-1553, 1995.

21. Kazmierczak, B., Hennig, Y., Wanschura, S., Rogalla, P., Bartnitzke, S., Van de Ven, W., and Bullerdiek, J. Description of a novel fusion transcript between HMGI-C, a gene encoding for a member of the high mobility group proteins, and the mitochondrial aldehyde dehydrogenase gene. *Cancer Res.*, *55*: 6038-6039, 1995.

22. Staats, B., Bonk, U., Wanschura, S., Hanisch, P., Schoenmakers, E. F., Van de Ven, W. J., Bartnitzke, S., and Bullerdiek, J. A fibroadenoma with a t(4;12) (q27;q15) affecting the HMGI-C gene, a member of the high mobility group protein gene family. *Breast Cancer Res. Treat.*, *38*: 299-303, 1996.

23. Geurts, J. M., Schoenmakers, E. F., and Van de Ven, W. J. Molecular characterization of a complex chromosomal rearrangement in a pleomorphic salivary gland adenoma involving the 3'-UTR of HMGIC. *Cancer Genet. Cytogenet.*, *95*: 198-205, 1997.

24. Geurts, J. M., Schoenmakers, E. F., Roijer, E., Stenman, G., and Van de Ven, W. J. Expression of reciprocal hybrid transcripts of HMGIC and FHIT in a pleomorphic adenoma of the parotid gland. *Cancer Res.*, *57*: 13-17, 1997.

25. Bol, S., Wanschura, S., Thode, B., Deichert, U., Van de Ven, W. J., Bartnitzke, S., and Bullerdiek, J. An endometrial polyp with a rearrangement of HMGI-C underlying a complex cytogenetic rearrangement involving chromosomes 2 and 12. *Cancer Genet. Cytogenet.*, *90*: 88-90, 1996.

26. Kazmierczak, B., Pohnke, Y., and Bullerdiek, J. Fusion transcripts between the HMGIC gene and RTVL-H-related sequences in mesenchymal tumors without cytogenetic aberrations. *Genomics*, *38*: 223-226, 1996.

27. Ashar, H. R., Fejzo, M. S., Tkachenko, A., Zhou, X., Fletcher, J. A., Weremowicz, S., Morton, C. C., and Chada, K. Disruption of the architectural factor HMGI-C: DNA-binding AT hook motifs fused in lipomas to distinct transcriptional regulatory domains. *Cell*, *82*: 57-65, 1995.

28. Berner, J. M., Meza-Zepeda, L. A., Kools, P. F., Forus, A., Schoenmakers, E. F., Van de Ven, W. J., Fodstad, O., and Myklebost, O. HMGI-C, the gene for an architectural transcription factor, is amplified and rearranged in a subset of human sarcomas. *Oncogene*, *14*: 2935-2941, 1997.

29. Fedele, M., Berlingieri, M. T., Scala, S., Chiariotti, L., Viglietto, G., Rippel, V., Bullerdiek, J., Santoro, M., and Fusco, A. Truncated and chimeric HMGI-C genes induce neoplastic transformation of NIH3T3 murine fibroblasts. *Oncogene*, *17*: 413-418, 1998.

30. Bussemakers, M. J., van de Ven, W. J., Debruyne, F. M., and Schalken, J. A. Identification of high mobility group protein I(Y) as potential progression marker for prostate cancer by differential hybridization analysis. *Cancer Res.*, *51*: 606-611, 1991.

31. Ram, T. G., Reeves, R., and Hosick, H. L. Elevated high mobility group-I(Y) gene expression is associated with progressive transformation of mouse mammary epithelial cells. *Cancer Res.*, *53*: 2655-2660, 1993.

32. Chiappetta, G., Bandiera, A., Berlingieri, M. T., Visconti, R., Manfioletti, G., Battista, S., Martinez-Tello, F. J., Santoro, M., Giancotti, V., and Fusco, A. The expression of the high mobility group HMGI (Y) proteins correlates with the malignant phenotype of human thyroid neoplasias. *Oncogene*, *10*: 1307-1314, 1995.

33. Fedele, M., Bandiera, A., Chiappetta, G., Battista, S., Viglietto, G., Manfioletti, G., Casamassimi, A., Santoro, M., Giancotti, V., and Fusco, A. Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins. *Cancer Res.*, *56*: 1896-1901, 1996.

34. Chiappetta, G., Tallini, G., De Biasio, M. C., Manfioletti, G., Martinez-Tello, F. J., Pentimalli, F., de Nigris, F., Mastro, A., Botti, G., Fedele, M., Berger, N., Santoro, M., Giancotti, V., and Fusco, A. Detection of high mobility group I HMGI(Y) protein in the diagnosis of thyroid tumors: HMGI(Y) expression represents a potential diagnostic indicator of carcinoma. *Cancer Res.*, *58*: 4193-4198, 1998.

35. Williams, A. J., Powell, W. L., Collins, T., and Morton, C. C. HMGI(Y) expression in human uterine leiomyomata. Involvement of another high-mobility group architectural factor in a benign neoplasm. *Am. J. Pathol.*, *150*: 911-918, 1997.

36. Kazmierczak, B., Wanschura, S., Rommel, B., Bartnitzke, S., and Bullerdiek, J. Ten pulmonary chondroid hamartomas with chromosome 6p21 breakpoints within the HMGI(Y) gene or its immediate surroundings. *J. Natl. Cancer Inst. (Bethesda)*, *88*: 1234-1236, 1996.

37. Xiao, S., Lux, M. L., Reeves, R., Hudson, T. J., and Fletcher, J. A. HMGI(Y) activation by chromosome 6p21 rearrangements in multilineage mesenchymal cells from pulmonary hamartoma. *Am. J. Pathol.*, *150*: 901-910, 1997.

38. Dal Cin, P., Wanschura, S., Christiaens, M. R., Van den Bergh, I., Moerman, P., Polito, P., Kazmierczak, B., Bullerdiek, J., and Van den Bergh, H. Hamartoma of the breast with involvement of 6p21 and rearrangement of HMGIY. *Genes Chromosomes Cancer*, *20*: 90-92, 1997.

39. Kottickal, L. V., Sarada, B., Ashar, H., Chada, K., and Nagarajan, L. Preferential expression of HMGI-C isoforms lacking the acidic carboxy terminal in human leukemia. *Biochem. Biophys. Res. Commun.*, *242*: 452-456, 1998.

40. Screpanti, I., Scarpa, S., Meco, D., Bellavia, D., Stuppia, L., Frati, L., Modesti, A., and Gulino, A. Epidermal growth factor promotes a neural phenotype in thymic epithelial cells and enhances neuropoietic cytokine expression. *J. Cell Biol.*, *130*: 183-192, 1995.

41. Screpanti, I., Meco, D., Scarpa, S., Morrone, S., Frati, L., Gulino, A., and Modesti, A. Neuroendulatory loop mediated by nerve growth factor and interleukin 6 in thymic stromal cell cultures. *Proc. Natl. Acad. Sci. USA*, *89*: 3209-3212, 1992.

42. Tkachenko, A., Ashar, H. R., Meloni, A. M., Sandberg, A. A., and Chada, K. K. Misexpression of disrupted HMGI architectural factors activates alternative pathways of tumorigenesis. *Cancer Res.*, *57*: 2276-2280, 1997.

43. Albemayor, E., and Sidell, N. Human neuroblastoma cell lines as model for the *in vitro* study of neoplastic and neuronal cell differentiation. *Environ. Health Perspect.*, *80*: 3-15, 1989.

44. Bolande, R. P. Spontaneous regression and cytodifferentiation of cancer in early life: the oncogenic grace period. *Suev. Synth. Pathol. Res.*, *4*: 296-311, 1985.

45. Thiele, C. J. Biology of pediatric peripheral neurocutaneous tumors. *Cancer Metastasis Rev.*, *10*: 311-319, 1991.

46. Finklestein, J. Z., Krailo, M. D., Lenarsky, C., Ladisch, S., Blair, G. K., Reynolds, C. P., Sitarz, A. L., and Hammond, G. D. 13-*cis*-retinoic acid (NSC 122758) in the treatment of children with metastatic neuroblastoma unresponsive to conventional chemotherapy: report from the Childrens Cancer Study Group. *Med. Pediatr. Oncol.*, *20*: 307-311, 1992.

47. Peverali, F. A., Orioli, D., Toton, L., Ciana, P., Bunone, G., Negri, M., and Della-Valle, G. Retinoic acid-induced growth arrest and differentiation of neuroblastoma cells are counteracted by *N-myc* and enhanced by *max* overexpression. *Oncogene*, *12*: 457-462, 1996.

48. Giannini, G., Dawson, M. I., Zhang, X., and Thiele, C. J. Activation of three distinct RXR/RAR heterodimers induces growth arrest and differentiation of neuroblastoma cells. *J. Biol. Chem.*, *272*: 26693-26701, 1997.

49. Patel, U. A., Bandiera, A., Manfioletti, G., Giancotti, V., Chau, K. Y., and Crane-Robinson, C. Expression and cDNA cloning of human HMGI-C phosphoprotein. *Biochem. Biophys. Res. Commun.*, *201*: 63-70, 1994.

50. Johnson, K. R., Disney, J. E., Wyatt, C. R., and Reeves, R. Expression of mRNAs encoding mammalian chromosomal proteins HMGI and HMGI-Y during cellular proliferation. *Exp. Cell Res.*, *187*: 69-76, 1990.

51. Chau, K. Y., Patel, U. A., Lee, K. L., Lam, H. Y., and Crane-Robinson, C. The gene for the human architectural transcription factor HMGI-C consists of five exons each coding for a distinct functional element. *Nucleic Acids Res.*, *23*: 4262-4266, 1995.

52. Reynolds, C. P., Kane, D. J., Einhorn, P. A., Matthay, K. K., Crouse, V. L., Wilbur, J. R., Shurin, S. B., and Seeger, R. C. Response of neuroblastoma to retinoic acid *in vitro* and *in vivo*. *Prog. Clin. Biol. Res.*, *366*: 203-211, 1991.

53. Kaplan, D. R., Matsumoto, K., Lucarelli, E., and Thiele, C. J. Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. *Neuron*, *11*: 321-331, 1993.

54. Gaetano, G., Matsumoto, K., and Thiele, C. J. Retinoic acid resistant neuroblastoma cells and expression of insulin-like growth factor-II. *Prog. Clin. Biol. Res.*, *366*: 165-172, 1991.

55. Matsumoto, K., Gaetano, C., Daughaday, W. H., and Thiele, C. J. Retinoic acid regulates insulin-like growth factor II expression in a neuroblastoma cell line. *Endocrinology*, *130*: 3669-3676, 1992.

56. Matsumoto, K., Lucarelli, E., Minniti, C., Gaetano, C., and Thiele, C. J. Signals transduced via insulin-like growth factor I receptor (IGF₁R) mediate resistance to retinoic acid-induced cell growth arrest in a human neuroblastoma cell line. *Cell Death Differ.*, *1*: 49-58, 1994.

57. Thiele, C. J., Reynolds, C. P., and Israel, M. A. Decreased expression of *N-myc* precedes retinoic acid induced morphological differentiation of human neuroblastoma. *Nature (Lond.)*, *313*: 404-406, 1985.

58. Seeger, R. C., Brodeur, G. M., Sather, H., Dalton, A., Siegel, S. E., Wong, K. Y., and Hammond, D. Association of multiple copies of the *N-myc* oncogene with rapid progression of neuroblastomas. *N. Engl. J. Med.*, *313*: 1111-1116, 1985.

59. Caron, H., van Sluis, P., de Kraker, J., Bokkerink, J., Egeler, M., Laureys, G., Slater, R., Westerveld, A., Voute, P. A., and Versteeg, R. Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N. Engl. J. Med.*, *334*: 225-230, 1996.

60. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalou, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell*, *90*: 809-819, 1997.

61. Vallone, D., Battista, S., Pierantoni, G. M., Fedele, M., Casalino, L., Santoro, M., Viglietto, G., Fusco, A., and Verde, P. Neoplastic transformation of rat thyroid cells requires the *junB* and *fra-1* gene induction which is dependent on the HMGI-C gene product. *EMBO J.*, *16*: 5310-5321, 1997.

62. Schule, R., Umesono, K., Mangelsdorf, D. J., Bolado, J., Pike, J. W., and Evans, R. M. Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. *Cell*, *61*: 497-504, 1990.

63. Schule, R., and Evans, R. M. Cross-coupling of signal transduction pathways: zinc finger meets leucine zipper. *Trends Genet.*, *7*: 377-381, 1991.

64. Tamimi, Y., van der Poel, H. G., Denyn, M. M., Umbas, R., Karthaus, H. F., Debruyne, F. M., and Schalken, J. A. Increased expression of high mobility group protein I(Y) in high grade prostatic cancer determined by *in situ* hybridization. *Cancer Res.*, *53*: 5512-5516, 1993.