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ANTISENSE DNA-TARGETING PROTEIN KINASE A-RIA SUBUNIT: A NOVEL APPROACH TO CANCER TREATMENT

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1. ABSTRACT

Enhanced expression of the RIa subunit of cAMP-dependent protein kinase type I (PKA-I) has been shown during carcinogenesis, in human cancer cell lines and in primary tumors. We demonstrate that the sequence-specific inhibition of RIa gene expression by antisense oligonucleotides results in the differentiation of leukemia cells and growth arrest of cancer cells of epithelial origin and tumors in mice. The loss of RI by the antisense results in rapid increase in the half-life of the competitor molecule, RII protein, via its stabilization in a holoenzyme complex (PKA-II) that insures depletion of PKA-I and sustained inhibition of tumor growth. RI antisense, which restrains tumor cell growth by turning on the signals for blockade of tumor cell survival, namely blockade of the tyrosine kinase signaling, cell cycle deregulation and apoptosis, provides a single gene-targeting approach to treatment of cancer.

2. INTRODUCTION

The primary mediator of cAMP action in mammalian cells is cAMP-dependent protein kinase (PKA) (1). There are two isoforms of PKA, type I (PKA-I) and type II (PKA-II), which are distinguished by their cAMP-binding regulatory (R) subunits, RI versus RII, as the kinase isozymes share an identical catalytic (C) subunit (2). Four different regulatory subunits, RI, RIß, RIIa, and RIIb have been identified (3,4). Three distinct C subunits, Ca, Cb, and Cg have also been found (5–8); however, preferential coexpression of any one of these C subunits with either RI or RII subunit has not been found (7,8).

The two general classes of R subunits, RI and RII, contain two tandem cAMP-binding domains at the carboxyl terminus in which region the amino acids sequences are highly conserved. The RI and RII differ significantly in the amino terminus at a proteolytically sensitive hinge region that occupies the peptide substrate binding site of the C subunit in the holoenzyme complex (9). RI and RII also differ in molecular weight, isoelectric point, and immunological characteristics. Other differences among the types of R subunits, such as K_d for cAMP, subcellular localization, expression in development, expression in cell cycle, and expression in transformation and differentiation, suggest the possibility of differential control isozyme activities under varying physiological conditions (2, 10, 11). Thus, specific roles of the different isoforms of the R subunit, apart from inhibition of the C subunit, are implicated in the regulatory function of cAMP.

This review describes how modulation of each R subunit of PKA influences the cAMP growth regulatory action, and shows that such approach provides opportunities to unravel the mechanisms of PKA-regulation of cell growth and differentiation, and contributes toward discovery of innovative therapeutic tools for treatment of cancer.

3. cAMP-DEPENDENT PROTEIN KINASE ISOZYMES IN MALIGNANCY

A correlation between the changing ratio of PKA-I and PKA-II in ontogenic development and differentiation processes has been shown (12,13). Therefore, it is logical to ask if there are in fact any changes or abnormalities in the protein kinase of neoplastic cells.

Comparison of steady state levels of RI and RII between tumor and normal tissue in view of the possibility

of changes in those levels with the degree of anaplasticity is difficult. In spite of these difficulties, an interesting correlation has been made regarding differential expression of type I and type II PKA subunits and their mRNAs with neoplastic transformation and tumor growth. The ratio of PKA-I/PKA-II in renal cell carcinomas was about twice that in renal cortex, although the total soluble PKA activity was similar in both tissues (14). In surgical specimens of Wilms' tumor, the ratio of PKA-I/PKA-II was twice that in normal kidney, and the RI/RII ratio was 0.79 for normal and 2.95 for tumor ($p<0.01$) (15). In a study of pituitary tumors of the rat, RII was found at lower levels in nuclei isolated from tumors than in normal tissue (16). In the neoplastically transformed BT5C glioma cell line the ratio of type I/type II PKA was significantly higher (1.2) than the normal fetal brain cells (0.5), but the R and C subunits of protein kinase were expressed to a similar degree in both cell lines. Normal and malignant osteoblasts differ also in their isozyme response to hormones, with a relative predominance of type I activation in malignant cells and of type II in normal cells (17). The majority of primary human breast and colon carcinomas examined showed an enhanced expression of RI and a higher ratio of PKA-I/PKA-II than normal counterparts (18-21). Importantly, the relative overexpression of the RIa subunit of PKA was associated with poor prognosis in patients with breast cancer (22). It was shown by quantitative polymerase chain reaction (PCR) determination that the RIa mRNA expression is higher in serous ovarian tumors than in mucinous, endometroid, or clear cell ovarian tumors (23). The levels of RIa mRNA were elevated in ovarian cancer cell lines, which are highly tumorigenic, while RIa mRNA was expressed at lower levels in the nontumorigenic ovarian cancer cell lines and minimally expressed in the placental mRNA control.

Increased expression of RI has been shown to be associated with both chemical and viral carcinogenesis and oncogene-induced cell transformation. The initiation stage of the dimethylbenz (a) anthracene–induced mammary carcinogenesis in rats (24), and the incidence of gastric adenocarcinoma in rats by N-methyl-N´-nitrosoguanidine and the trophic action of gastrin on gastric carcinoma production (25) were correlated with a sharp increase in RI and type I PKA activity. Gharett *et al.* (26) and Wehner *et al.* (27) compared the PKA isozymes in normal 3T3 cells with SV40-transformed, spontaneously transformed, and methylcholanthrene-transformed cell counterparts. While normal cells contained only the type II isozyme, transformed cells contained an equivalent amount of total kinase activity, but these cells contained both type I and type II activities and an increased amount of RI. Ledinko and Chan (28) also observed a 3- to 6-fold increase in RI and the type I isozyme in rat 3Y1 cells transformed by human adenovirus type 12 compared to untransformed 3Y1 cells. Little or no change occurred in the type II isozyme following transformation. Marked increase in RI expression with decrease in RII expression was detected in Ha-MuSV–transformed NIH/3T3 clone 13-3B-4 cells (29,30), in TGF--transformed or v-Ki-ras oncogene–transformed rat kidney (NRK) cells (31), in TGF-–induced transformation of mouse mammary epithelial cells (32), and in point-mutated c-Ha-ras and c-erbB-2 protooncogene–transformed human mammary epithelial cell line MCF-10A HE (33).

These results suggest that RI may act as a mediator of various mitogenic stimuli and thus represents a potential target for the pharmacological control of cell proliferation.

4. RETROVIRAL VECTOR-MEDIATED OVEREXPRESSION OF PKA R AND C SUBUNITS

Our study demonstrated the effect of each R subunit isoform and C subunit of PKA on the proliferation of cancer cells. Through retroviral-vector-mediated infection, we have overexpressed the human RIa, RIIa, RIIb, RIIb-P (a mutant of human RIIß lacking the autophosphorylation site at RC interaction site, ^{114}SerAla), and Ca subunits of PKA in LS-174T human colon carcinoma cells (34). The mRNA expression as determined by Northern blot analysis showed an enhanced expression of the infected PKA subunit genes. However, two dimensional gel analysis demonstrated that the subunit expression at the protein level did not parallel mRNA expression. The RIa and RIIa proteins, which are abundantly expressed in parental cells, did not further increase in the infectants even though the mRNA levels increased. The novel 53-kDa species of RIIß, the human RIIß protein, which was not detected in parental cells, markedly increased in the RIIß and RIIß-P infectants. Importantly, RIa levels were markedly reduced in RIIa, RIIß, and RIIß-P infectants, while RIIa levels were not decreased in RIa infectants. These observations suggest that RI and RII expression are under post-transcriptional control that favors the expression of RII over that of RI.

Overexpression of the RII but not the RI subunit resulted in a striking shift in the PKA isozyme distribution. In RIIa infectants, PKA-I levels were markedly reduced and PKA-II levels increased even though RIIa protein levels were not increased. The PKA-II increase was associated with the presence of multiple kinase species, which may be the phosphorylated and unphosphorylated and cAMP-bound and cAMP-unbound form of PKA-II (2). In RIIß and RIIß-P infectants, PKA-I levels were almost completely eliminated, and different species of PKA-II that were not detected in either RIIa infectants or parental cells increased. In contrast, RIa infectants showed no apparent changes in the levels of either PKA-I or PKA-II. A small increase in PKA-I levels with no change in PKA-II levels was observed in Ca infectants.

These data suggest that the R and C subunits are in equilibrium between PKA-I and PKA-II and that PKA-II formation is highly favored. This preferential formation of PKA-II is not limited to LS-174T cells; it was previously observed in ras-transformed NIH3T3 cells and in AtT20 pituitary cells (3). We conclude that RIIa and C associate preferentially in LS-174T cells and that PKA-I is formed only if C subunit is present in excess over RIIa. The excess RIa not associated with C subunit may be degraded; therefore the increase in RIa or PKA-I cannot occur in RIa infectants even though RIa mRNA was increased.

We compared growth and transformed properties of the PKA subunit gene infectants with parental non-infectant control cells. We used the following criteria: growth in monolayer culture, thymidine incorporation into DNA, anchorage-independent growth, growth in serum-free medium, and cell morphology. We found that according to all these criteria, cells overexpressing RIa, RIIa, Ca, RIIß-P, and parental control cells behaved as transformed cells. In contrast, the RIIß cells exhibited restrained growth in monolayer culture, slower DNA synthesis, inability to grow in serum-free media, limited capacity to form colonies in soft agar, and change in morphology compared with parental cells (34). Thus, RIIß cells had the properties of untransformed rather than transformed cells.

RIIß overexpression markedly reduced RIa and PKA-I isozyme levels; however, the RIa/PKA-I downregulation alone may not account for the induction of untransformed-cell properties in LS-174T cells, since both RII cells and the mutant RIIß-P cells efficiently downregulated RIa/PKA-I but failed to show untransformed-cell properties. Otten and McKnight reported (35) that overexpression of RIIa, which sharply reduced both RIa and RIIß in ras-transformed NIH3T3 R3T3 cells, did not lead to reversion of the transformed phenotype. A decrease in RIa without an increase in RIIß has also been correlated with progression of disease in carcinogen-induced lung tumors (36). These results suggest that suppression of RIa alone, without a compensatory increase in RIIß, is not enough to induce phenotypic reversion/differentiation in cells such as HL-60, ras-transformed NIH3T3, and LS-174T cells. Overexpression of RIIß also induced reverse transformation in Ki-*ras*-transformed NIH3T3 DT cells (37) and SK-N-SH human neuroblastoma cells (38) and induced differentiation in HL-60 leukemia cells (39).

That the autophosphorylation-site mutant RIIß-P (human RIIß $Ser^{114}Ala$) failed to mimic the effects of RIIß (34) suggests the functional importance of the autophosphorylation site in RIIß in restraining transformation.

We therefore constructed a mutant of RIa at the pseudophosphorylation-site replacing human RIa Ala99 with Ser (RIa-P), and examined the effect of RIa-P on the growth of MCF-7 breast cancer cells. The mutant RIa-P overexpressing cells depleted PKA-II and showed growth inhibition with apoptotic nuclei (40). The results indicate that RIa-P having autophosphorylation site behaves like RIIß and thus blocks the functional RIa/PKA-I resulting in growth inhibition.

It also has been shown that RIa plays a role in cell proliferation by regulating cell cycle progression. The RIa retroviral vector-infected FRTL5 rat thyroid cells, human mammary MCF-10A cells, and Chinese hamster ovary cells demonstrated independence from hormone/serum requirement for cell proliferation and exhibited cell cycle distribution similar to that of parental cells growing in the presence of hormone/serum (41,42).

These studies suggest that abnormal expression of R-subunit isoforms of PKA is involved in neoplastic transformation and that induction of RIIß/PKA-IIß can restore growth control in transformed cells.

5. RIa ANTISENSE OLIGONUCLEOTIDE INHIBITION OF CANCER CELL GROWTH *IN VITRO*

The possibility that the RI cAMP receptor is a positive regulator essential for cancer cell growth was explored with the use of antisense strategy. The experimental data provided the first direct evidence that the RIa receptor is a positive effector of cancer cell growth.

The two general classes of PKA R subunits, RI and RII, have conserved amino acid sequences at the carboxyl terminus but differ significantly at the amino terminus (9). A synthetic RI antisense oligodeoxynucleotide corresponding to the N-terminus (the first 21 bases [codons 1–7]) of the human RIa was constructed (43).

The RIa antisense of unmodified oligodeoxynucleotide (15–30 μM) produced growth inhibition in human cancer cells of epithelial origin, including breast (MCF-7), colon (LS-174T), and gastric (TMK-1) carcinoma and neuroblastoma (SK-N-SH) cells (44), as well as HL-60 leukemia cells (45), with no sign of cytotoxicity.

Treatment with RIa antisense phosphorothioate oligodeoxynucleotide (6μM) brought about a marked reduction in RIa level with an increase in RIIß level as compared with untreated control cells (44,45), whereas RIIß antisense (43) (directed to 1–7 codons of human RIIß) phosphorothioate oligomer brought about converse effects, namely, an increase in RIa level and a decrease in RIIß level (43,44). Thus, RIa and RIIß antisense oligodeoxynucleotides each exerted a specific effect on their respective target mRNAs, and their targeting of either isoform of R subunit expression resulted in compensatory enhancement of the expression of the other form of R subunit.

It has been shown that mixed backbone oligonucleotides (MBOs) such as phosphorothioate oligodeoxynucleotide (PS-oligo) containing segments of 2'-O-methyloligoribonucleotides (2'-O-meRNA) or methylphosphonate oligodeoxynucleotides (me-PDNA) are more resistant to nucleases and form more stable duplexes with RNA than the parental PS-oligo (46,47). Such MBOs have been shown to have improved antisense activity over PS-oligonucleotides (47,48). We used MBO RIa antisense directed against the human RIa NH2-terminus 8–13 codons (49). Within a few days the antisense treatment brought about growth arrest with no sign of cytotoxicity in human breast cancer cells (MCF-7 and MDA-MB-231) in monolayer culture, and inhibited colony formation in soft agar (50,51).

6. RI ANTISENSE INDUCES SUSTAINED INHIBITION OF TUMOR GROWTH *IN VIVO*

The sequence-specific inhibition of RI gene expression results in the inhibition of *in vivo* tumor growth (52). A single subcutaneous (s.c.) injection into nude mice bearing LS-174T human colon carcinoma with RI antisense to 8-13 codons of human RI) resulted in an almost complete suppression of tumor growth for 7 days. There was no apparent sign of systemic toxicity. Even after 14 days, tumor growth was significantly inhibited in the antisense-treated animals. In contrast, tumors in untreated, saline-treated, or control antisense-treated animals showed continued growth.

The RI levels in tumors from the antisense-treated animals were within 24 hours and remained at low levels (10-20% of that in tumors from saline-treated animals) for up to 2-3 days (52). Specific targeting of RI by the antisense is evident since RII levels remained unchanged. At 5-7 days post-antisense treatment, the RI levels in tumors were elevated to levels similar to those in tumors from saline-injected animals. At day 3 after antisense treatment, tumors that contained unreduced amounts of RI contained a new species of R, RII along with a reduced amount of RII. The increase in RII expression was also found in tumors that contained decreased levels of RI without reduction in RII content. RII appeared 24 hours to 3 days post-antisense treatment but was not detected in control tumors (saline or control antisense-treated). Thus, the antisense-targeted suppression of RI brought about a compensatory increase in RII levels.

Upon antisense treatment, the level of RI, after its initial suppression for a few days, subsequently increased in tumors. In cells, RI can exist either in its subunit form or in the form of the PKA-I holoenzyme. As the RI subunit can act as a cAMP sink, RI in the holoenzyme complex may be of functional importance. We therefore examined the RI antisense effect on the PKA isozyme distribution in tumors. The antisense treatment completely eliminated PKA-I, the RI-containing holoenzyme and the RI subunit, from tumors within 24 hours (52). Importantly, this downregulation of PKA-I lasted for up to 57 days post-antisense treatment even when the RI levels increased and reached the levels of control tumors. This indicates that the RI that increased subsequently to its initial suppression after antisense treatment was present mostly in its subunit form rather than in its holoenzyme form, PKA-I.

Concomitant with the suppression of PKA-I, the antisense brought about changes in the PKA-II profile of tumors. PKA-II in antisense-treated tumors contained PKA-II (RII containing PKA-II). These data indicate that the C subunits of PKA in tumors are in equilibrium between PKA-I and PKA-II and that downregulation of RI by the antisense results in PKA-I downregulation with concomitant upregulation of PKA-II through the increase in RII. Thus, RI antisense-inhibition of tumor growths *in vivo* accompanied the compensatory increase in RII protein and PKA-II holoenzyme (RII-containing PKA), and persistent downregulation of PKA-I.

7. INCREASE IN RII HALF-LIFE UNDERLIES RI ANTISENSE MECHANISM OF ACTION

Examination of RII mRNA levels and rate of RII protein synthesis in the control and antisense-treated LS-174T colon cancer cells revealed that the mechanism of RII compensation demonstrated in the RI antisense-treated cells does not involve transcriptional or translational control. This implies that the increased RII protein observed in the antisense-treated tumor cells must be due to stabilization of the protein.

Pulse-chase experiments were performed to determine the half-life of RII protein in control and antisense-treated LS-174T cells (53). The half-life of RII in control cells was approximately 2.0 hr as measured by immunoprecipitation of ^{35}S-labeled RII protein from cell extracts after a cold chase with unlabeled methionine. In contrast, the half-life of RII protein in antisense-treated cells was 11 hr. This represents a 5.5- fold increase in the half-life of the RII protein upon treatment with RI antisense. Importantly, the half-life of RII protein did not change and the half-lives of RI and C decreased 24% and 18%, respectively, in the RI treated cells. A 3-fold increase in the RII half-life was also observed in LNCap prostate cancer cells upon treatment with RI antisense (53). The antisense treatment results in sequence-specific reduction of RI and growth arrest in LNCap cells. Thus, modulation of RII turnover rate may represent an important biological mechanism underlying the RI antisense mechanism of action.

It has been shown that the R and C subunits are stabilized against proteolysis when assembled as a holoenzyme. Stabilization of RI through binding to the C subunit has been demonstrated in S49 mouse lymphoma cells (54). Kin cells, which lack detectable C subunit, show a 10-fold increase in the turnover rate of RI protein and a significant decrease in steady-state RI levels when compared with wild type S49 cells (54, 55). However, when wild type S49 cells are treated with agents that raise cAMP and separate the R and C subunits, the RI protein is destabilized to the same extent observed in Kin cells. The C subunit is also exposed to degradative pathways when released from the holoenzyme complex. In NIH3T3 cells overexpressing C, the RI was also increased without changing the rate of RI synthesis or the level of RI mRNA. Pulse-chase experiments revealed a 4-fold increase in the half-life of RI protein as it becomes incorporated into the holoenzyme (56). Moreover, null mutations in the RI or RII gene in mice result in an increase in the level of RI protein in tissues that normally express the isoforms of the R subunit (56). This increase in RI protein occurred without increases in the RI mRNA level and the rate of RI protein synthesis, and concomitant with the increase in RI, there was a new appearance of PKA-I that contained RI (56). These studies demonstrate the cell's capacity to maintain cAMP-mediated control of C subunit activity and the important role played in this process by RI.

In the RI antisense-induced inhibition of cancer cell growth, we demonstrate the important role played in this process by RII. Cancer cells are characterized to

contain a greater amount of RI/PKA-I than RII/PKA-II as compared to their normal counterparts (13). HL-60 leukemia cells mainly exhibit RI/PKA-I. Upon treatment with 8-Cl-cAMP, that selectively downregulates PKA-I, RII was induced and cells were growth arrested (39). Overexpression of RII in an expression vector in Ki-*ras*-transformed NIH3T3 cells results in reversion of the phenotype to the untransformed fibroblasts (37). LS-174T colon carcinoma and LNCap prostate carcinoma cells mainly express RI, RII and C subunits of PKA, and RII subunit at an undetectable level (52). The loss of RI by the antisense would result in increased concentration of free "C" subunit. The RII rapidly responds to this perturbation through protein stabilization in holoenzyme complex. RII in the holoenzyme complex (PKA-II) is stabilized exhibiting an increased half-life. Thus, the loss of RI by the antisense results in an increase in RII and PKA-II, the RII containing PKA holoenzyme. Through this biochemical adaptation, the antisense-treated cancer cells change the ratio of PKA-I/PKA-II to that of normal cells and stop growing. The compensatory stabilization of RII protein may represent an important biochemical mechanism of RI antisense that ensures depletion of PKA-I leading to sustained inhibition of tumor cell growth.

8. RIa ANTISENSE REVERSION OF MULTIDRUG RESISTANT (MDR) PHENOTYPE

Increased expression of RIa has been observed in MDR cancer cell lines as compared to non-MDR parental cell lines (57). It was found that the enhanced expression of RIa mRNA in MDR cells parallels an increase in Ca subunit mRNA, indicating that RIa is the major form of R subunit composing the protein kinase A in the MDR cell lines examined.

Human colon carcinoma HCT-15 is one of the cell lines with the highest expression of MDR protein and is extremely resistant to conventional cytotoxic drugs (58, 59). We examined the effect of RIa antisense MBOs, PS-oligo containing segments of 2'-O-methyl-oligo RNA, and methylphosphonate oligo DNA. Daily treatment with RIa antisense 0.01 μM inhibited the growth of HCT-15 cells in monolayer culture (49). This treatment brought about a marked reduction in RIa expression at both the mRNA and protein levels (49). It was demonstrated that the sequence-specific inhibition of RIa gene expression results in inhibition *in vivo* HCT-15 tumor growth. Daily injections of RIa antisense at 5 mg/kg, i.p., 3 times per week for 3 weeks into nude mice bearing HCT-15 colon carcinoma resulted in 60–70% reduction of tumor growth. There was no apparent sign of animal toxicity. In contrast, tumors in untreated, saline-treated, or control antisense-treated animals showed continued growth.

The potency of antisense oligonucleotides in growth inhibition paralleled their efficacy in downregulating RIa protein levels in tumors. The most potent growth inhibitor, me-PDNA-PS-oligo antisense, almost completely eliminated the RIa protein from tumors (49). The downregulation of RIa was due to the sequence-specific effect of antisense, since the 4-base-mismatched control oligo could not mimic the effect of antisense. Interestingly, concomitant with the suppression of RIa expression was a switch in RII isoform expression; RIIß, which is not expressed in untreated and mismatched oligo-treated tumors, appeared along with the downregulation of RIIa. Thus, RIa antisense triggered the induction of RIIß protein, which has been related to cell differentiation (60–63).

9. RIa ANTISENSE INDUCTION OF CELL CYCLE DEREGULATION AND DIFFERENTIATION

The growth inhibition induced by RIa antisense accompanied changes in cell cycle phase distribution. LS-174T cells in culture exhibited a 50% growth inhibition upon daily treatment with RIa antisense of MBO, me-PDNA-PS oligo, or 2'-O-meRNA-PS-oligo, at 0.01 μM concentration for 6 days. Single treatment with MBO RIa antisense (5 μM, day 5) produced a similar degree of growth inhibition as did daily treatment (0.01 μM). The antisense inhibition of cell growth correlated with the suppression of RIa mRNA expression (49). Flow cytometric analysis demonstrated that the antisense treatment within 18 hours provoked an increase in the population of cells in the S phase of the cell cycle (49). The 4-base-mismatched control oligo, which exhibited effect on cell growth and RIa mRNA expression, also had no effect on cell cycle phase distribution.

A single-dose (20 μM) treatment of HL-60 cells with RIa antisense PS-oligo resulted in a 50% growth inhibition by day 8 (49). Flow cytometric analysis demonstrated that the antisense treatment resulted in an increase in the population of cells in the S phase of the cell cycle (49). Control oligo treatment had no effect on cell growth or cell cycle phase distribution. The results indicate that the antisense induced an inappropriate entry of cells into the S phase.

On the basis of these results, which implicate the possible role of RIa/PKA-I in the regulation of S-phase cell entry, we examined the effects of RIa antisense treatment on cyclin E levels because cyclin E/CDK2 complex is essential for the initiation of DNA replication (64). The biparametric assay of cyclin E and DNA content demonstrated that in untreated control cells, cyclin E levels were raised during the G1 phase, reached a peak level in G1/S transition, and sharply dropped in the S and G2M phases, whereas in RIa antisense-treated cells, cyclin E was induced to its peak level throughout the S and G2M phases (49). Thus, in RIa antisense-treated cells, the increase of cyclin E expression was delayed, occurring at the S and G2M phases as if cells were locked in the G1/S transition phase.

These results show that suppression of RIa expression by the antisense caused deregulation of the cell cycle at two critical points, one at the entry into the S phase and the other at the G2M phase, resulting in accumulation of cells in the S phase, which may lead to apoptosis/differentiation. In fact, RIa antisense treatment of HL-60 cells caused decreased expression of

myelomonocytic antigens, such as CD11b, CD11c, and CD14, and increased expression of the granulocytic antigen CD15, over 10-fold that of untreated control cells, indicating a granulocytic differentiation (49).

These data together with the finding (49) that RIa expression sharply increases in centrifugally elutriated HL-60 cells enriched in the G1/early S and late S/early G2M phases suggest an important role for RIa at two critical points in the cell cycle, one at the G1/S border and the other at the G2M phase.

10. RIa ANTISENSE INDUCES GROWTH ARREST AND APOPTOSIS

The mechanism of action of RIa antisense on the growth inhibition of MCF-7 hormone-dependent breast cancer cells was explored using RIa antisense PS-oligo containing a segment of 2'-O-methyl RNA (51). A single-dose treatment (0.1, 1, 5, and 10 μM) or daily treatment (0.01 and 0.1 μM) with RIa antisense produced growth inhibition in a time- and concentration-dependent manner. The growth inhibition accompanied changes in cell morphology. The antisense-treated cells exhibited an elongated and flat phenotype had an increased cytoplasm-to-nucleus ratio, and grew sparingly to form much smaller cell clusters than the untreated control cells. RIa antisense also induced apoptosis as evidenced by fragmentation or condensed chromatin.

These effects of RIa antisense on the biological parameters were clearly correlated with the sequence-specific inhibition of RIa protein expression. RIa levels in untreated control cells increased with cell culture time, peaking at day 3. Within 24 hours, the RIa antisense treatment (5 μM, single treatment) reduced the RIa level. On day 5, RIa antisense almost completely eliminated RIa protein, whereas the mismatched control oligo was incapable of reducing the RIa protein level throughout the experimental period. These results show that RIa antisense exhibited its time-dependent effect on shutting off the RIa protein, whereas the mismatched control oligo could not mimic the antisense effect of RIa downregulation. Moreover, RIa antisense, but not the mismatched control oligo, induced RIIß protein expression. RIIa, the isoform of RIIß, did not change its level throughout the culture time in the untreated control, antisense, or control oligo-treated cells.

In MDA-MB-231 hormone-independent breast cancer cells, RIa antisense similarly induced growth inhibition, changes in cell morphology, and apoptosis (50).

These results demonstrate that the RIa antisense, which efficiently depletes the growth stimulatory RIa, induces growth inhibition/apoptosis in breast cancer cells.

11. RIa ANTISENSE INDUCES BLOCKADE OF THE TYROSINE KINASE SIGNALING PATHWAY

Expression of the RIa subunit of protein kinase A type I is increased in human cancers in which an autocrine pathway for transforming growth factor alpha–epidermal growth factor receptor (EGFR) is activated (31–33). We investigated the effect of sequence-specific inhibition of RIa gene expression in OVCAR8 ovarian cancer cells because the overexpression of EGFR and c-erbB-2 plays a role in the development of ovarian cancers (65, 66). Treatment of OVCAR8 cells with RIa antisense phosphorothioate oligodeoxynucleotide containing a segment of 2'-O-methyl RNA resulted in reduced RIa expression, decreased EGFR, c-erbB-2, and c-erB-3, but not c-erbB-4, expression, and inhibited cell growth (67). A 4-base-mismatched control oligo had no effect on RIa, EGFR, c-erbB-2, and c-erbB-3 expression, or cell growth. In addition, in OVCAR8 cells overexpressing human EGFR cDNA in the 3' to 5' orientation, RIa expression was also suppressed and cell growth was inhibited (67). Sense EGFR cDNA transfection had no effect on cell growth or RIa expression. These results show that RIa antisense, which depletes RIa expression, leads to the blockade of both the serine-threonine and the tyrosine kinase signaling pathways in ovarian cancer cells.

12. PERSPECTIVE

Understanding the molecular mechanisms underlying the signal transduction pathways in higher organisms is crucial in addressing such longstanding questions in cell biology as what causes cells to begin and to stop dividing and how many disturbances in those growth control mechanisms give rise to cancer.

The role of cAMP as both positive and negative signal in mammalian cell proliferation has long been known. These dual functions of cAMP in eukaryotic cell growth appear to be governed by the predominance of either one of the two types of cAMP-dependent protein kinase (PKA), type I and type II present in the cell. The type I and type II PKA are distinguished by their cAMP-binding regulatory subunits, RI versus RII, as these kinase isozymes share an identical C subunit.

Modulation of the R subunit expression by the use of antisense strategy and retroviral vector-mediated gene transfer technology has provided direct evidence that two isoforms of cAMP receptor proteins, the RI and RII regulatory subunits of the PKA have opposite roles in cell growth and differentiation. RI is a growth-stimulatory protein, whereas RII is a growth-inhibitory and differentiation-inducing protein.

During the growth and differentiation of normal cells, the expression of the RI and RII is maintained in a strict balance. Deviation from such a balance is seen during cell transformation, in primary human tumors, and in cancer cell lines. Restoration of the normal balance of the RI/RII ratio via the site-selective cAMP analog 8-Cl-cAMP (13, 68), by RI antisense oligodeoxynucleotide, or via retroviral vector mediated RII overexpression directs cancer cells to stop growing and to differentiate rather than proliferate.

Evidence presented shows that the RI antisense oligonucleotide produces potent growth inhibition in a

variety of cancers of epithelial cell origin as well as in leukemia cells supporting its therapeutic application toward a broad spectrum of cancer. Importantly, the RI antisense is also a potent antitumor agent for multidrug-resistant tumors.

Future clinical application of RI antisense oligonucleotide may include its combination with the PKA regulator, 8-Cl-cAMP, differentiation agents, such as retinoic acid, monoclonal antibodies targeting growth factor receptors, and conventional chemotherapeutic agents. Importantly, it has been demonstrated that RI antisense does not interfere with but shows cooperative/synergistic growth inhibitory effect when combined with cytotoxic drugs (69).

That the biologic effects of antisense outlast antisense survival is an important feature of RI antisense. This is because RI antisense checks itself for its single gene-targeting mechanism of action, namely, the elimination of RI by increasing the competitive molecule RII, the growth-inhibitory and differentiation-inducing protein. The RII rapidly responds to loss of RI through protein stabilization in the holoenzyme complex, PKA-II (RII-containing holoenzyme). The RII in the holoenzyme complex is stabilized exhibiting an increased half-life, and thus remains in the cell for a longer time. This compensatory stabilization of RII protein may represent an important biochemical mechanism of RI antisense that ensures depletion of PKA-I leading to sustained inhibition of tumor cell growth. By this unique mechanism of action, RI antisense promotes the blockade of cancer growth inducing apoptosis/differentiation, the terminal end point of cancer cell survival.

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14. REFERENCES

1. Krebs, E.G. & J.A. Beavo: Phosphorylation-dephosphorylation of enzymes. *Ann Rev Biochem* 48, 923–939 (1979)

2. Bebbe S.J., J.D. Corbin: Cyclic nucleotide-dependent protein kinases. In: Boyer P.D., E.G. Krebs, editors. The Enzymes: control by phosphorylation. Part A, Bol 17. New York: Academic Press, 43–111 (1986)

3. McKnight G.S., C.H. Clegg, M.D. Uhler, J.C. Chrivia, G.G. Cadd, L.A. Correll, & A.D. Otten: Analysis of the cAMP-dependent protein kinase system using molecular genetic approaches. *Recent Prog Horm Res* 44, 307–355 (1988)

4. Levy F.O., O. Oyen, M. Sandberg, K. Taskem, W. Eskild, V. Hansson, & T. Jahnsen: Molecular cloning, complementary deoxyribonucleic acid structure, and predicted full-length amino acid sequence of the hormone-inducible regulatory subunit of 3',5'-cycl8ic adenosine monophosphate-dependent protein kinase from human testis. *Mol Endocrinol* 2, 1364–1373 (1988)

5. Uhler M.D., D.F. Carmichael, D.C. Lee, J.C. Chrivia, E.G. Krebs, & G.S. McKnight: Isolation of cDNA clones coding for the catalytic subunit of mouse cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 83, 1300–1304 (1986)

6. Uhler M.D., J.C. Chrivia, & G.S. McKnight: Evidence for a second isoform of the catalytic subunit of cAMP-dependent protein kinase. *J Biol Chem* 261, 15360–15363 (1986)

7. Showers M.O. & R.A. Maurer: A cloned bovine cDNA encodes an alternate form of the catalytic subunit of cAMP-dependent protein kinase. *J Biol Chem* 261, 16288–16291 (1986)

8. Beebe S.J., O. Oyen, M. Sandberg, A. Froysa, V. Hansson, & T. Jahnsen: Molecular cloning of a unique tissue-specific protein kinase © from human testis—representing a third isoform for the catalytic subunit of the cAMP-dependent protein kinase. *Mol Endocrinol* 4, 465–475 (1990)

9. Taylor S.S., J. Bubis, J. Toner-Webb, L.D. Saraswat, E.A. First, J.A. Buechler, D.R. Knighton, & J. Sowadski: cAMP-dependent protein kinase: prototype for a family of enzymes. *FASEB J* 2, 2677–2685 (1988)

10. Cho-Chung Y.S., T. Clair, G. Tortora, & H. Yokozaki: Role of site-selective cAMP analogs in the control and reversal of malignancy. *Pharmac Ther* 50, 1–33 (1991)

11. Cho-Chung Y.S., S. Pepe, T. Clair, A. Budillon, & M. Nesterova: cAMP-dependent protein kinase: role in normal and malignant growth. *Critical Reviews in Oncology/Hematology* 21, 33–61 (1995)

12. Lohmann S.M. & U. Walter: Regulation of the cellular and subcellular concentrations and distribution of cyclic nucleotide-dependent protein kinases. In: Greengard P. & G.A. Robison, editors. Advances in cyclic nucleotide and protein phosphorylation research. Vol 18. New York: Raven, 63–117 (1984)

13. Cho-Chung Y.S.: Role of cyclic AMP receptor proteins in growth, differentiation, and suppression of malignancy: new approaches to therapy [Perspectives in cancer research]. *Cancer Res* 50, 7093–7100 (1990)

14. Fossberg T.M., S.O. Døskeland, P.M. Ueland: Protein kinases in human renal cell carcinoma and renal cortex. A comparison of isozyme distribution and of responsiveness to adenosine 3',5'-cyclin monophosphate. *Arch Biochem Biophys* 189, 372–381 (1978)

15. Nakajima F., S. Imashuku, J. Wilman, J.E. Champion, & A.A. Green: Distribution and properties of type I and type II binding proteins in the cyclic adenosine 3',5'-monophosphate-dependent protein kinase system in Wilms' tumor. *Cancer Res* 44, 5182–5187 (1984)

16. Piroli G., L.S. Weisenberg, C. Grillo, & A.F. DeNicola: Subcellular distribution of cyclic adenosine 3', 5'-monophosphate-binding protein and estrogen receptors in control pituitaries and estrogen-induced pituitary tumors. *J Natl Cancer Inst* 82, 596-601 (1990)

17. Livesey S.A., B.E. Kemp, C.A. Re, N.C. Partridge, & T.J. Martin: Selective hormonal activation of cyclic AMP-dependent protein kinase isoenzymes in normal and malignant osteoblasts. *J Biol Chem* 257, 14983-14987 (1982)

18. Handschin J.S. & U. Eppenberger: Altered cellular ratio of type I and type II cyclic AMP-dependent protein kinase in human mammary tumors. *FEBS Lett* 106, 301–304 (1979)

19. Weber W., G. Schwoch, H. Schröder, & H. Hilz: Analysis of cAMP-dependent protein kinases by immunotitration: Multiple forms–Multiple functions? *Cold Spring Harbor Conf. Cell Prolif* 8, 125–140 (1981)

20. Watson D.M.S., R.A. Hawkins, N.J. Bundred, H.J. Stewart, & W.R. Miller: Tumor cyclic AMP binding proteins and endocrine responsiveness in patients with inoperable breast cancer. *Br J Cancer* 56, 141–142 (1987)

21. Bradbury A.W., D.C. Carter, W.R. Miller, Y.S. Cho-Chung, & T. Clair T: Protein kinase A (PKA) regulatory subunit expression in colorectal cancer and related mucosa. *Br J Cancer* 69, 738–742 (1994)

22. Miller W.R., M.J. Hulme, Y.S. Cho-Chung, & R.A. Elton: Types of cyclic AMP binding proteins in human breast cancers. *Eur J Cancer* 29A, 989-991 (1993)

23. McDaid H.M., M.T. Cairns, R.J. Atkinson, S. McAleer, D.P. Harkin, P. Gilmore, & P.G. Johnson: Increased expression of the RI subunit of the cAMP-dependent protein kinase A is associated with advanced stage ovarian cancer. *Br J Cancer* 79, 933–939 (1999)

24. Cho-Chung Y.S., T. Clair, & C. Shepheard: Anticarcinogenic effect on N6, O2'-dibutyryl cyclic adenosine 3',5'-monophosphate on 7,12-dimethylb3enz (a) anthracene mammary tumor induction in the rat and its relationship to cyclic adenosine 3',5'-monophosphate metabolism and protein kinase. *Cancer Res* 43, 2736–2740 (1983)

25. Yasui W. & E. Tahara: Effect of gastrin on gastric mucosal cyclic adenosine 3'. 5'-monophosphate–dependent protein kinase activity in rat stomach carcinogenesis induced by N-methyl-N´-nitrosoguanidine. *Cancer Res* 45, 4763–4767 (1985)

26. Gharrett A.J., A.M. Malkinson, & J.R. Sheppard: Cyclic AMP-dependent protein kinases from normal and SV40-transformed 3T3 cells. *Nature* 264, 673–675 (1976)

27. Wehner J.M., A.M. Malkinson, M.F. Wiser, & J.R. Sheppard: Cyclic AMP-dependent protein kinases from Balb 3T3 cells and other 3T3 derived lines. *J Cell Physiol* 108, 175-184 (1981)

28. Ledinko N., I-JAD Chan: Increase in type I cyclic adenosine3',5'-monophosphate-dependent protein kinase activity and specific accumulation of type I regulatory subunits in adenovirus type 12-transformed cells. *Cancer Res* 44, 2622–2627 (1984)

29. Tagliaferri P., T. Clair, M.E. Debortoli, & Y.S. Cho-Chung: Two classes of cAMP analogs synergistically inhibit p21 ras protein synthesis and phenotypic transformation of NIH/3T3 cells transfected with Ha-MuSV DNA. *Biochem Biophys Res Commun* 130, 1193–1200 (1985)

30. Clair T., S. Ally, P. Tagliaferri, R.K. Robins, & Y.S. Cho-Chung: Site-selective cAMP analogs induce nuclear translocation of the RIIß cAMP receptor protein in Ha-NuSV-transformed NIH/3T3 cells. *FEBS Lett* 224, 337–384 (1987)

31. Tortora G., F. Ciardiello, S. Ally, T. Clair, D.S. Salomon, & Y.S. Cho-Chung: Site-selective 8-chloroadenosine 3',5'-cyclic monophosphate inhibits transformation and transforming growth factor a production in Ki-ras-transformed rat fibroblasts. *FEBS Lett* 242, 363–367 (1989)

32. Ciardiello F., G. Tortora, N. Kim, T. Clair, S. Ally, D.S. Salomon DS, & Y.S. Cho-Chung: 8-Chloro-cAMP inhibits transforming growth factor a transformation of mammary epithelial cells by restoration of the normal mRNA patterns for cAMP-dependent protein kinase regulatory subunit isoforms which show disruption upon transformation. *J Biol Chem* 265, 1016–1020 (1990)

33. Ciardiello F., S. Pepe, C. Bianco, G. Baldassarre, A. Ruggiero, C. Bianco, M.P. Selvam, A.R. Bianco, & G. Tortora: Down-regulation of RIa subunit of cAMP-dependent protein kinase induces growth inhibition of human mammary epithelial cells transformed by c-Ha-ras and c-erbB-2 proto-oncogenes. *Int J Cancer* 53, 438–443 (1993)

34. Nestorova M., H. Yokozaki, E. McDuffie, & Y.S. Cho-Chung: Overexpression of RIß regulatory subunit of protein kinase A in human colon carcinoma cell induces growth arrest and phenotypic changes that are abolished by site-directed mutation of RIIß. *Eur J Biochem* 235, 486–494 (1996)

35. Otten A.D. & G.S. McKnight: Overexpression of the type II regulatory subunit of the cAMP-dependent protein kinase eliminates the type I holoenzyme in mouse cells. *J Biol Chem* 264, 20255–20260 (1989)

36. Lange-Carter C.A., T. Fossli, T. Jahnsen, & A.M. Malkinson: Decreased expression of the type I isozyme of cAMP-dependent protein kinase in tumor cell lines of lung epithelial origin. *J Biol Chem* 265, 7814–7818 (1990)

37. Budillon A., A. Cereseto, A. Kondrashin, M. Nesterova, G. Merlo, T. Clair, & Y.S. Cho-Chung: Point mutation of the autophosphorylation site or in the nuclear location signal causes protein kinase A RIIb regulatory subunit to lose its ability to revert transformed fibroblasts. *Proc Natl Acad Sci USA* 92, 10634–10638 (1995)

38. Kim S.N., G.R. Lee, Y.S. Cho-Chung, S.D. Park, & S.H. Hong: Overexpression of RIIß regulatory subunit of protein kinase A induces growth inhibition and reverse-transformation in SK-N-SH human neuroblastoma cells. *Int J Oncol* 8, 663–668 (1996)

39. Rohlff C., T. Clair, & Y.S. Cho-Chung: 8-Cl-cAMP induces truncation and downregulation of the RI subunit and upregulation of the RIIß subunit of cAMP-dependent protein kinase leading to type II holoenzyme-dependent growth inhibition and differentiation of HL-60 leukemia cells. *J Biol Chem* 268, 5774–5782 (1993)

40. Lee G.R., S.N. Kim, K. Noguchi, S.D. Park, S.H. Hong, & Y.S. Cho-Chung: $Ala^{99}ser$ mutation in RI regulatory subunit of protein kinase A causes reduced kinase activation by cAMP and arrest of hormone-dependent breast cancer cell growth. *Mol Cell Biochem* 195, 77–86 (1999)

41. Tortora G., S, Pepe, A.M. Cirafici, F. Ciardiello, A. Porcellini, T. Clair, G. Colletta, Y.S. Cho-Chung, & A.R. Bianco: Thyroid-stimulating hormone-regulated growth and cell cycle distribution of thyroid cells involve type I isozyme of cyclic AMP-dependent protein kinase. *Cell Growth Differ* 4, 359–365 (1993)

42. Tortora G., S. Pepe, C. Bianco, V. Damiano, A. Ruggiero, G. Baldassarre, C. Corbo, Y.S. Cho-Chung, A.R. Bianco, & F. Ciardiello: Differential effects of protein kinase A sub-units on Chinese-hamster-ovary cell cycle and proliferation. *Int J Cancer* 59, 712–716 (1994)

43. Tortora G., T. Clair, & Y.S. Cho-Chung: An antisense oligodeoxynucleotide targeted against the type RIIß regulatory subunit mRNA of protein kinase inhibits cAMP-induced differentiation in HL-60 leukemia cells without affecting phorbol ester effects. *Proc Natl Acad Sci USA* 87, 705–708 (1990)

44. Yokozaki H., A. Budillon, G. Tortora, S. Meissner, S.L. Beaucage, K. Miki, & Y.S. Cho-Chung: An antisense oligonucleotide that depletes RIa subunit of cyclic AMP-dependent protein kinase induces growth inhibition in human cancer cells. *Cancer Res* 53, 868–872 (1993)

45. Tortora G., H. Yokozaki, S. Pepe, T. Clair, & Y.S. Cho-Chung: Differentiation of HL-60 leukemia by type I regulatory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 88, 2011–2015 (1991)

46. Shibahara S., S. Mukai, H. Morisawa, H. Nakashima, S. Kobayashi, & N. Yamamoto: Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives. *Nucleic Acids Res* 17, 239–252 (1989)

47. Metelev V., J. Lisziewicz, & S. Agrawal: Study of antisense oligonucleotide phosphorothioates containing segments of oligodeoxynucleotides and 2'-O-methyloligoribonucleotides. *Bioorganic Med Chem Lett* 4, 2929–2934 (1994)

48. Monia B.P., E.A. Lesnik, C. Gonzalez, W.F. Lima, D. McGee, C.J. Guinosso, *et al.* Evaluation of 2'-modified oligonucleotides containing 2'-deoxygaps as antisense inhibitors of gene expression. *J Biol Chem* 268, 14514–14522 (1993)

49. Cho-Chung Y.S., M. Nesterova, A. Kondrashin, K. Noguchi, R. Srivastava, & S. Pepe: Antisense-protein kinase A: A single-gene-based therapeutic approach. *Antisense & Nucleic Acid Drug Development* 7, 217–223 (1997)

50. Srivastava R.K., A.R. Srivastava, Y.G. Park, S. Agrawal, & Y.S. Cho-Chung: Antisense depletion of RI subunit of protein kinase A induces apoptosis and growth arrest in human breast cancer cells. *Breast Cancer Res and Treat* 49, 97–107 (1998)

51. Srivastava R.K., A.R. Srivastava, P. Seth, S. Agrawal, & Y.S. Cho-Chung: Growth arrest and induction of apoptosis in breast cancer cells by antisense depletion of protein kinase A-RI subunit: p53-independent mechanism of action. *Mol Cell Biochem* 195, 25–36 (1999)

52. Nesterova M. & Y.S. Cho-Chung: A single-injection protein kinase A-directed antisense treatment to inhibit tumor growth. *Nature Medicine* 1, 528–533 (1995)

53. Cho-Chung Y.S., M. Nesterova, Y.G. Park, & Y.N. Lee: Protein kinase A-RI antisense: antitumor mechanism of action. *Antisense & Nucleic Acid Drug Development* In press (1999)

54. Steinberg R.A. & D.A. Agard: Turnover of regulatory subunit of cyclic AMP-dependent protein kinase in S49 mouse lymphoma cells. *J Biol Chem* 256, 10731–10734 (1981)

55. Orellana S.A. & G.S. McKnight: The S49 kin- cell line transcribes and translates a functional mRNA coding for the catalytic subunit of cAMP-dependent protein kinase. *J Biol Chem* 265, 3048–3053 (1990)

56. Amieux P.S., D.E. Cummings, K. Motamed, E.P. Brandon, L.A. Wailes, K. Le, R.L. Idzerda, & G.S. McKnight: Compensatory regulation of RI protein levels in protein kinase A mutant mice. *J Biol Chem* 272, 3993–3998 (1997)

57. Yokozaki H., A. Budillon, G.T. Clair, K. Kelley, K.H. Cowan, C. Rohlff, R.I. Glazer, & Y.S. Cho-Chung: 8-Chloroadenosine 3',5'-monophosphate as a novel modulator of multidrug resistance. *Int J Oncol* 3, 423–430 (1993)

58. Boyd M.R. & K.D. Paull: Some practical considerations and applications of the National Cancer Institute *in vitro* anticancer drug discovery screen. *Drug Dev Res* 34, 91–109 (1995)

59. Lee J.S., K. Paull, M. Alvarez, C. Hose, A. Monks, M. Grever, A.T. Fojo, & S.E. Bates: Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. *Mol Pharmacol* 46, 627–638 (1994)

60. Schwartz D.A. & C.S. Rubin: Identification and differential expression of two forms of regulatory subunits (RII) of cAMP-dependent kinase II in Friend erythroleukemic cells. *J Biol Chem* 260, 6296–6303 (1985)

61. Tagliaferri P., D. Katsaros, T. Clair, L. Neckers, R.K. Robins, & Y.S. Cho-Chung: Reverse transformation of Harvey murine sarcoma virus-transformed NIH/3T3 cells by site-selective cyclic AMP analogs. *J Biol Chem* 263, 409–416 (1988)

62. Tortora G., T. Clair, D. Katsaros, S. Ally, O. Colamonici, L.M. Neckers, P. Tagliaferri, T. Jahnsen, R.K. Robins, & Y.S. Cho-Chung: Induction of megakaryocytic differentiation and modulation of protein kinase gene expression by site-selective cAMP analogs in K-562 human leukemic cells. *Proc Natl Acad Sci USA* 86, 2849–2852 (1989)

63. Tortora G., A. Budillon, H. Yokozaki, T. Clair, S. Pepe, G. Merlo, C. Rohlff, & Y.S. Cho-Chung: Retroviral vector-mediated overexpression of the RIIß subunit of cAMP-dependent protein kinase induces differentiation in human leukemia cells and reverts the transformed phenotype of mouse fibroblasts. *Cell Growth Differ* 5, 753–759 (1994)

64. Gong J., F. Traganos, & Z. Darzynkiewicz: Expression of cyclins B and E in individual MOLT-4 cells and in stimulated human lymphocytes during their progression through the cell cycle. *Int J Oncol* 3, 1037-1042 (1993)

65. Lofts F.J. & W.J. Gullick: c-erbB2 amplification and overexpression in human tumors. In: Dickson RB, Lippman ME, editors. Genes, oncogenes, and hormones: advances in cellular and molecular biology of breast cancer. Boston: Kluwer Academic Publishers, 161–179 (1991)

66. Simpson B.J.B., H.A. Phillips, A.M. Lessells, S.P. Langdon, & W.R. Miller: c-erbB growth-factor-receptor proteins in ovarian tumours. *Int J Cancer (Pred Oncol)* 64, 202–206 (1995)

67. Alper O., N.F. Hacker, & Y.S. Cho-Chung: Protein kinase A-RI subunit-directed antisense inhibition of ovarian cancer cell growth: crosstalk with tyrosine kinase signaling pathway. *Oncogene,* In press (1999)

68. Cho-Chung Y.S., T. Clair, P. Tagliaferri, S. Ally, D. Katsaros, G. Tortora, L. Neckers, T.L. Avery, G.W. Crabtree, & R.K. Robins: Site-selective cyclic AMP analogs as new biological tools in growth control, differentiation, and proto-oncogene regulation. *Cancer Investigation* 7, 161–177 (1989)

69. Tortora G., R. Caputo, V. Damiano, R. Bianco, & S. Pepe *et al.*: Synergistic inhibition of human cancer cell growth by cytotoxic drugs and mixed backbone antisense oligonucleotide targeting protein kinase A. *Proc Natl Acad Sci USA* 94, 12586–12591 (1997)

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