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ONCOGENE-TRANSFORMED GRANULOSA CELLS AS A MODEL SYSTEM FOR THE STUDY OF STEROIDOGENIC PROCESSES

A. AMSTERDAM,[1]* I. HANUKOGLU,[1] B. S. SUH,[1] I. KEREN-TAL,[1] D. PLEHN-DUJOWICH,[1] R. SPRENGEL,[2] H. RENNERT[3] and J. F. STRAUSS III[3]

[1]Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel, [2]Center for Molecular Biology, University of Heidelberg, D-6900 Heidelberg, Germany and [3]Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

Summary—Highly steroidogenic granulosa cell lines were established by transfection of primary granulosa cells from preovulatory follicles with SV40 DNA and Ha-ras oncogene. Progesterone production in these cells was enhanced to levels comparable to normal steroidogenic cells, by prolonged (> 12 h) stimulation with 8-Br-cAMP, forskolin and cholera toxin, which elevate intracellular cAMP. The steroidogenic capacity of individual lines correlated with the expression of the ras oncogene product (p21) and the morphology of the cells. Formation of the steroid hormones was associated with *de novo* synthesis of the mitochondrial cytochrome *P*450scc system proteins. Since cholesterol import into mitochondria is essential for steroidogenesis, the expression of the peripheral benzodiazepine receptor (PBR) and the sterol carrier protein 2 was characterized in these cells. The induction of the expression of the genes coding for both proteins appeared to be mediated, at least in part, by cAMP. Stimulation of the PBR by specific agonists enhanced progesterone production in these cells. The phorbol ester 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) dramatically suppressed the cAMP-induced steroidogenesis, in spite of enhanced intracellular cAMP levels, suggesting that TPA can modify the effects of cAMP. cAMP stimulation suppressed growth of transformed cells concomitantly with induction of steroidogenesis. The transformed cells lacked receptors for the native stimulants, the gonadotropic hormones. After transfection of the cells with a lutropin (LH) receptor expression plasmid, the LH and hCG response was reconstituted. In these newly established cell lines gonadotropins were able to stimulate the formation of cAMP and progesterone in a dose-dependent manner with an ED_{50} characteristic of the native receptor. High doses caused desensitization to gonadotropins as observed in normal cells. These newly established oncogene-transformed granulosa cell lines can serve as a useful model to study inducible steroidogenesis and the effect of oncogene expression on this process.

INTRODUCTION

Granulosa cells of the ovary synthesize and secrete steroid hormones, such as estrogens and progesterone, which are crucial for the control of the reproductive cycles and pregnancy [1–7]. The steroid output of these cells is regulated by pituitary glycoprotein hormones, gonadotropins, which interact with specific receptor molecules on the cell membrane [8]. The receptors for both lutropin (LH) and follicle stimulating hormone (FSH) are members of the G protein-coupled receptor family with M_r 93 K and 75 K, respectively [9–12]. The interaction of the gonadotropins with their receptors leads to the activation of adenylate cyclase, stimulation of steroidogenesis and the induction of steroidogenic enzymes [1–7]. However, the physiological mechanism of steroidogenic enzyme induction may be explained by the actions of cAMP alone [13] and additional signal transduction mechanisms may be involved in gonadotropin action, e.g. inositol phosphate formation, calcium mobilization [14–16], modulation of potassium and chloride channels [17, 18] and tyrosine kinase activation [19, 20].

The study of the mechanisms of steroidogenesis is difficult in primary granulosa cells and this could be facilitated with the establishment of immortalized granulosa cells lines. Primary granulosa cells lines cease to divide upon prolonged stimulation with gonadotropins [1, 4]. Moreover, granulosa cells in ovarian follicles are heterogeneous in their gonadotropic

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*To whom correspondence should be addressed.

receptor content [21] and steroidogenic capacity [22–24], which vary according to the maturation stage of the follicles they derive from and their localization in the follicle. Thus, it is difficult to correlate the biochemical characteristic of the entire population with the function of the individual cells. These problems would be circumvented in monoclonal cell lines derived from a single cell, since the biochemical characteristics measured on the entire cell population would be representative of individual cells.

This review summarizes our recent studies on the establishment and characterization of immortalized granulosa cell lines by cotransfection of primary cells with SV40 DNA and Ha-ras oncogene [25–29]. The initial cell lines developed showed cAMP-mediated steroidogenic response but had no response to gonadotropins [25]. More recently we developed gonadotropin-responsive cells by additional transfection of the primary granulosa cells with an LH receptor expression plasmid [9, 29]. These new lines express the LH receptor constitutively, and can be stimulated by hCG to produce progesterone and its metabolite 20α-hydroxy-4-pregnen-3-one to a level comparable to that of gonadotropin-stimulated primary cells [29]. In the transfected, as in normal granulosa cells, this stimulation is attributable to the induction of expression of the steroidogenic enzymes, especially mitochondrial cytochrome *P*450scc, which catalyzes the first and rate-limiting step in steroid hormone biosynthesis [27]. Thus, the newly established steroidogenic cell lines can serve as a useful model for studying the cellular and the molecular mechanisms involved in gonadotropin-stimulated steroidogenesis and the effect of oncogenes on this process.

ESTABLISHMENT OF GRANULOSA CELL LINES USING ONCOGENES

Based on earlier observations for the establishment of cell lines, we initially tested SV40 DNA in combination with a series of oncogenes to transform rat granulosa cells. The first series of steroidogenic cell lines were obtained by cotransfection of primary granulosa cells with SV40 and Ha-ras oncogenes, using granulosa cells from prenatal follicles of rats treated with diethylstilbestrol [25]. Although these cells showed cAMP-inducible steroidogenesis, the induced progesterone secretion by these cells was low. Transfection with SV40 DNA alone also yielded cell lines but these very low steroidogenic capacity [25, 26]. Similarly, other granulosa cell lines established with SV40 DNA alone, showed low or no steroidogenic activity [30, 31].

The second series of cell lines we established were again developed by cotransfection of primary granulosa cells with SV40 DNA and Ha-ras oncogene, but this time using granulosa cells from preovulatory follicles of PMSG-treated immature rats [26]. These new cell lines secreted progesterone and 20α-hydroxy-4-pregnen-3-one after prolonged stimulation (>12 h) with forskolin, or cholera toxin at levels similar to primary cultures of granulosa cells. Forskolin increased intracellular cAMP levels within minutes, and induced rounding of the cells (Fig. 1). While increased cAMP induced steroidogenesis, it suppressed the growth of cells as determined by [^{3}H]thymidine incorporation into DNA [26]. Progesterone production induced by 8-bromo-cAMP, forskolin and cholera toxin was inhibited by the phorbol ester TPA even though TPA enhanced cAMP accumulation in response to forskolin or cholera toxin. The β-adrenergic agonist, isoproterenol, stimulated both cAMP accumulation and steroid secretion. LH, FSH, prostaglandins E1 and E2 and prolactin showed no effect on progesterone production [26].

To establish cell lines with gonadotropin responsiveness, granulosa cells from preovulatory follicles were transfected with an LH receptor expression plasmid, together with SV40 DNA and the Ha-ras oncogene [29]. LH receptor expression plasmid was prepared by inserting the complete coding region of LH receptor cDNA into an SV40 early promoter based eukaryotic expression vector [9]. Cell lines obtained after this triple transfection synthesized cAMP in a dose-dependent manner in response to hCG. Steroidogenesis was stimulated by hCG with an ED_{50} of about 100 pM, which is within the physiological range. The number of hCG receptor sites per cell after numerous passages and several freezing and thawing cycles was 2×10^4, with $K_d = 180$ pM. Stimulation with hCG induced pronounced morphological and biochemical changes [29]. These findings open the possibility of expressing selectively mutated receptor molecules in steroidogenic granulosa cells, to analyze their structure and function.

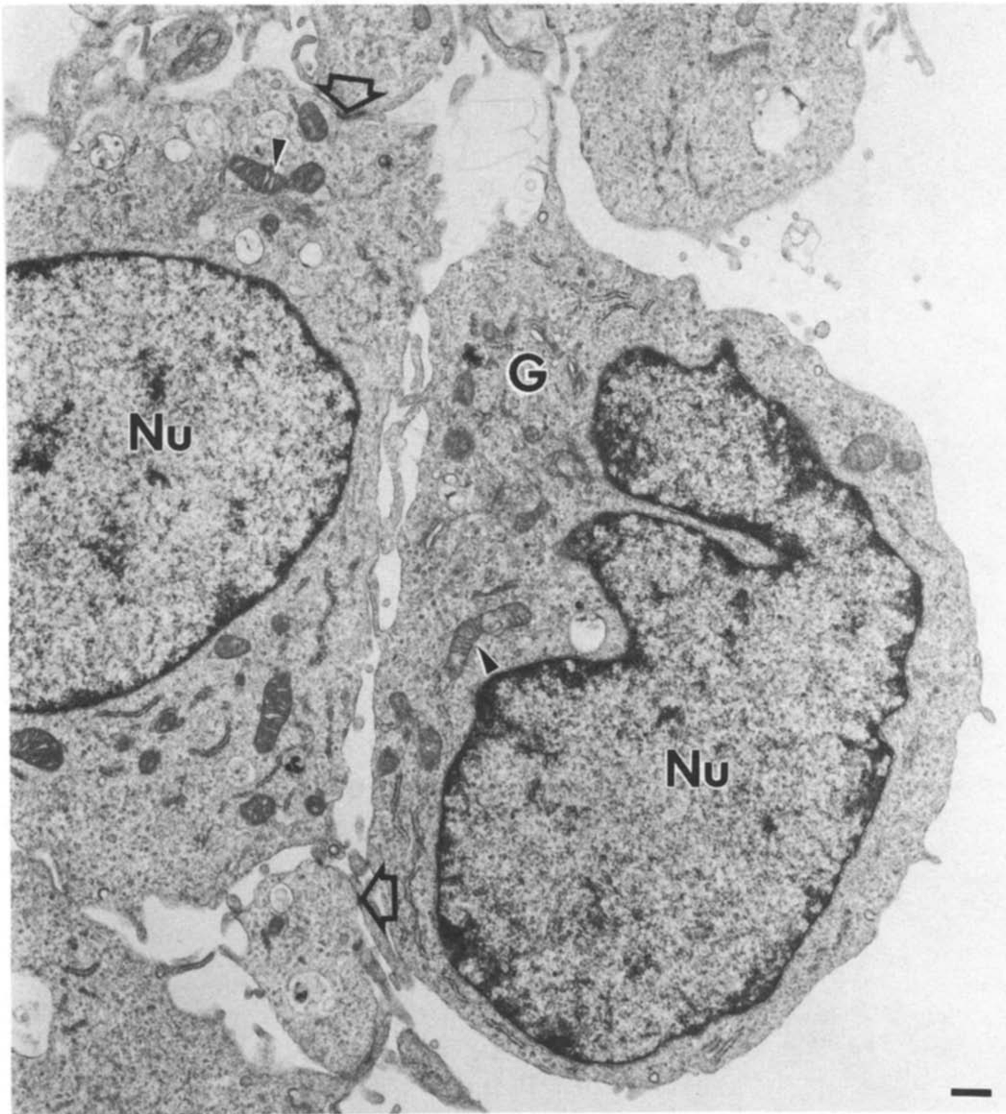

Fig. 1. Ultrastructure of SV40 DNA and Ha-ras oncogene transformed cells after stimulation with 1 mM 8-Br-cAMP for 48 h. The rounded cells show large nuclei (Nu), and small junctional elements between neighboring cells (wide open arrows). Lamellar mitochondria are scattered throughout the cytoplasm (arrow heads). The Golgi apparatus is well developed (G); bar = 0.5 μm.

MECHANISM OF INDUCTION OF STEROIDOGENESIS

In non-stimulated transformed cells the steroidogenic activity is very low and progesterone secretion becomes evident only after 12–24 h of stimulation, reaching maximal levels (> 100-fold of control) at 48 h [26, 27]. Immunoblot (Western) analysis of total protein from transformed cells revealed that progesterone synthesis closely follows the induction of the mitochondrial cytochrome *P*450scc system, which catalyzes the first step in steroid hormone biosynthesis [27]. The *P*450scc electron transport proteins, adrenodoxin and adrendoxin reductase, were also induced but adrenodoxin showed a faster induction. Immunofluorescence of the cells (Fig. 2) and electron microscopic examination using the immunogold technique (Fig. 3) showed that the *P*450scc system proteins were induced in all cells and incorporated into all mitochondria uniformly [27]. The uniformity of the response of the cells provided further evidence for the homogeneity of the cell line.

The results noted above indicate that the induction of progesterone synthesis is a result of induction of steroidogenic enzymes, and not

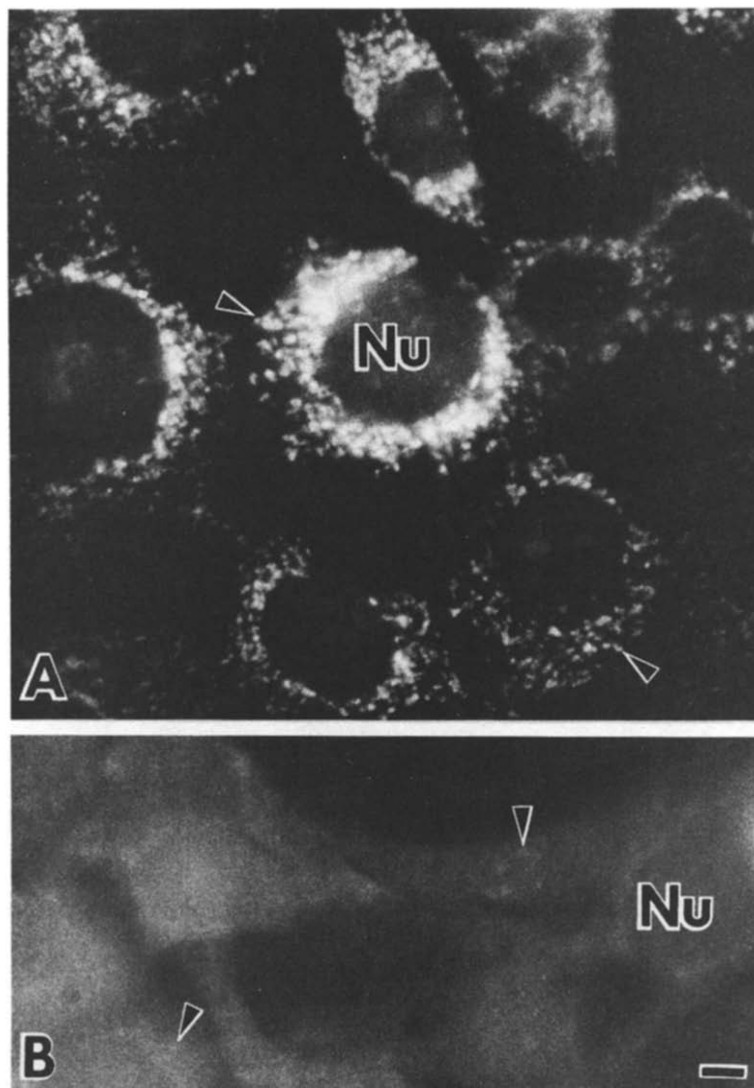

Fig. 2. Induction of adrenodoxin in SV40 and Ha-ras-transformed PO-GRS1 granulosa cell line by stimulation with 8-Br-cAMP. The cells were fixed and reacted first with an anti-adrenodoxin antibody and then fluorescein-labeled second antibody and visualized by fluorescence microscopy as described [27]. (A) Cells stimulated for 48 h with 1 mM 8-Br-cAMP show bright fluorescent mitochondria in the entire cytoplasm of all cells in the field, leaving the nuclei unstained. (B) Cells not treated with cAMP show a very faint staining in some mitochondria. The exposure time in (B) is twice that of (A); Nu, nucleus; bar = 2 μm.

activation of enzymes that are constantly expressed in the transformed cells. The stimulation of progesterone synthesis in normal granulosa cells also follows the same mechanism [3, 5]. The observed time courses of induction of the *P*450scc system proteins are similar to those observed in other steroidogenic cells [20]. After ovulation, granulosa and theca cells differentiate into corpus luteum which secretes progesterone in large quantities [1–7]. This *in vivo* process involves a great increase in the levels of the steroidogenic enzymes [3, 6, 32]. Thus, the induction of the steroidogenic enzymes in the transformed cells apparently reflects the physiological process of granulosa cell differentiation into luteal cells.

EXPRESSION OF PUTATIVE CHOLESTEROL CARRIER PROTEINS

When steroidogenic enzymes are at induced state, steroidogenesis is limited by the rate of its first and obligatory step, *P*450scc catalyzed conversion of cholesterol into pregnenolone [3]. Trophic hormones stimulate steroidogenesis by activating the intracellular transfer of

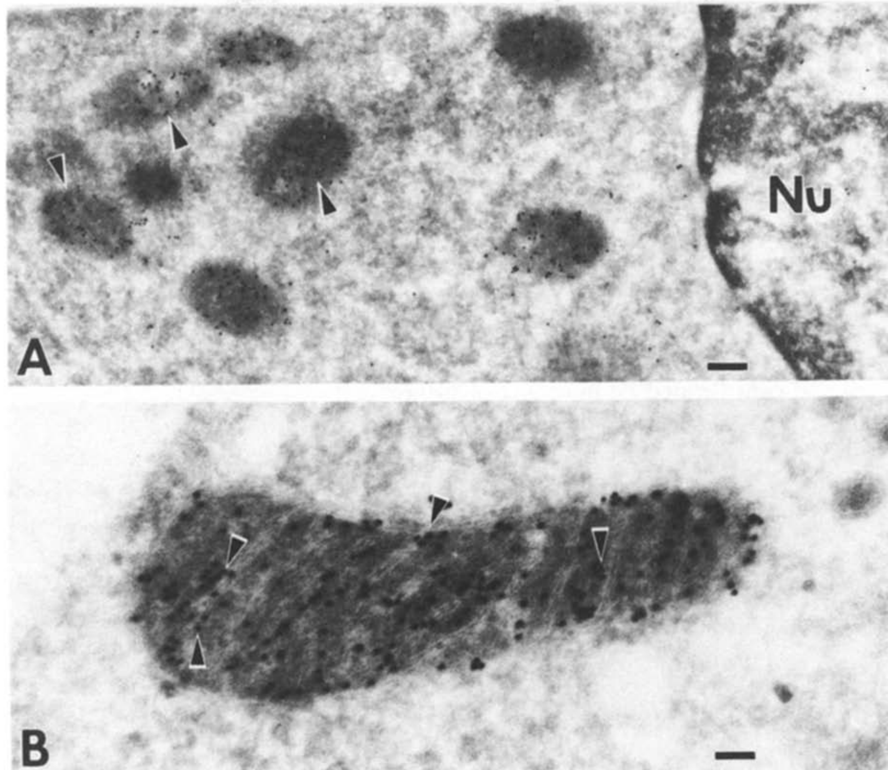

Fig. 3. Mitochondrial localization of adrenodoxin in SV40 and Ha-ras transformed cells by the cryo-immunogold technique. Cells were stimulated for 48 h with 1 mM 8-Br-cAMP. Ultrathin cryosections were stained with anti-adrenodoxin antibody and second antibody labeled with 15 nm gold particles as described [27]. (A) Part of a cell showing selective and uniform labeling in mitochondria in close association with the inner membrane, the cristae. Modest labeling in the cytoplasm is associated with polyribosomes; Nu, nucleus; bar = 0.3 μm. (B) A highly developed mitochondrion at higher magnification. Gold particles are mainly associated with mitochondrial cristae (arrow heads); bar = 0.1 μm. The negative staining of the mitochondrial membrane is due to the elimination of osmium tetraoxide from the fixation solution.

cholesterol from lipid droplets into the inner mitochondrial membrane where *P*450scc is located [33]. This process apparently involves specific carrier proteins that bind cholesterol [33]. We examined the expression of two of these putative carrier proteins in the transformed cell lines: (1) sterol carrier protein 2 (SCP2; also named non-specific lipid-transfer protein); and (2) peripheral benzodiazepine receptor [33–35].

We examined the distribution of SCP2 gene expression in the rat ovary and the role of gonadotropins and cAMP in the regulation of SCP2 mRNA levels [36]. *In situ* hybridization revealed that the most steroidogenically active ovarian compartments (e.g. corpora lutea and theca cells) contain significant amounts of SCP2 mRNA, whereas granulosa cells have modest levels. Gonadotropins increased the ovarian content of SCP2 mRNA along with *P*450scc mRNA. Similarly, stimulation of the transformed granulosa cells with 8-Br-cAMP also increased SCP2 mRNA and protein and *P*450scc mRNA, while actin mRNA levels remained unaffected. The cAMP stimulation of SCP2 mRNA accumulation was completely inhibited by actinomycin D and cycloheximide. The cAMP analog also increased SCP2 mRNA levels in a non-steroid hormone producing SV40 transformed rat granulosa cell line (GS-8). Thus, SCP2 gene expression in the ovary is correlated with the state of differentiation of the granulosa cells. The actions of gonadotropins on SCP2 gene expression may be mediated by a cAMP-dependent mechanism requiring RNA and protein synthesis. SCP2 gene expression is not obligatorily coupled to steroidogenic activity, as cAMP analogs can increase SCP2 mRNA in a line of transformed ovarian granulosa cells incapable of synthesizing steroid hormones [36].

The peripheral benzodiazepine receptor has been localized in the adrenal cortex [37–39], MA-10 Leydig tumor cells [40] and in both

normal and cancerous ovarian cells [41, 42]. It appears to be located mainly in the mitochondrial outer membrane [37]. Currently, it is not clear whether different types of ovarian cells express different classes of the receptor and whether these receptors perform similar functions. The mitochondria of oncogene transformed granulosa cells contain a high density of peripheral benzodiazepine receptor [43]. The number of mitochondria-associated receptors is increased by cAMP, and is much higher than in cells not derived from steroidogenic tissue. In both normal and transformed granulosa cells, a benzodiazepine agonist clearly elevates progesterone production [43]. These data are consistent with a role of the peripheral benzodiazepine receptor in ovarian steroidogenesis.

THE ROLE OF ras PROTEIN EXPRESSION IN INDUCIBLE STEROIDOGENESIS

As noted above, only cells transfected with the Ha-ras oncogene and SV40 DNA retained their steroidogenic capacity [26]. Expression of p21 in individual cotransfected lines correlated with their steroidogenic capacity (Fig. 4). In general, lines that showed more characteristic epithelioid morphology expressed higher amounts of the oncogene product p21 and also showed higher steroidogenic potential [44]. Both primary granulosa cells and luteinized cells express significant amounts of p21 (25, 26, 44]. Thus, the expression of the ras oncogene may be important for the expression of the steroidogenic enzymes under cAMP stimulation. However, we did not find any modulation of p21 expression upon cAMP stimulation [44]. In contrast, expression of p21 coded by the protooncogene could be modulated since it was modestly enhanced in luteinized cells compared to immature granulosa cells [28].

Cellular and viral oncogenes are defined by their ability to elicit neoplastic transformation [45–48]. Ras oncogenes are one of the most prevalent oncogenes in human [49–51] and carcinogen-induced animal tumors [52, 53]. Oncogenes have been also implicated in the control of cellular proliferation and differentiation [54–58]. The ras genes in the mammalian genome [59–61], Ha-ras-1, K-ras-2 and N-ras, encode proteins that bind guanine nucleotides [62], have GTPase activity [63, 64], associate with the plasma membrane [65, 66] and are homologous to G-proteins [67, 68]. These properties suggest that ras proteins participate in signal transduction across the cell membrane [69, 70].

The mechanism by which expression of the ras oncogene product p21 affects differentiation into a steroidogenic state is not yet clear and several possibilities may be considered: (i) it participates in a signal transduction pathway leading to differentiation; (ii) it antagonizes the anti-differentiating effect of SV40, since in cotransfected granulosa cells an isoform of the T antigen is not expressed in contrast to cells

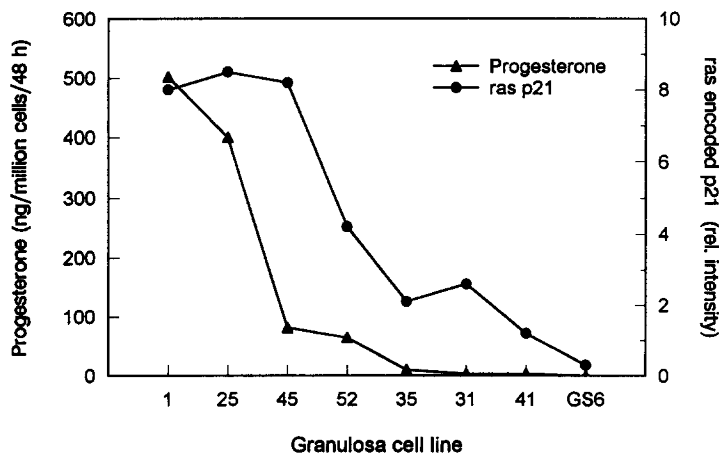

Fig. 4. Correlation of progesterone synthesis and Ha-ras oncogene product (p21) expression in different granulosa cells lines. All the cell lines except GS6 were established by cotransfection of granulosa cells with SV40 and Ha-ras oncogene as described [26]. GS6 was established by transfection of granulosa cells with SV40 DNA alone [26]. Progesterone was measured after 48 h incubation with 1 mM 8-Br-cAMP [26]. To measure the oncogene product p21 cells were labeled with [^{35}S]methionine. The radioactive proteins from each culture were immunoprecipitated with a monoclonal anti-ras antibody, electrophoresed on polyacrylamide gels and then quantitated by densitometric scanning of the specific band as described [26, 44].

transfected with SV40 alone [25, 26]; or (iii) it affects the expression of other cellular elements, such as cytoskeletal proteins, essential for the development of steroidogenesis in the cells [71].

As the expression of the Ha-ras oncogene may be involved in inducible steroidogenesis in immortalized granulosa cell lines, the product of the proto-oncogene may be implicated in this process in normal cells [28]. Investigating the role of non-mutated p21 in normal cells may illuminate the role of proto-oncogenes [55] and suppressor genes [46, 48] in the luteinization of normal granulosa cells.

CROSS-TALK BETWEEN DIFFERENT SIGNALS WHICH AFFECT GROWTH AND DIFFERENTIATION

Substances that elevate intracellular cAMP (cAMP analogs, forskolin and cholera toxin) suppress the growth of the transformed cells while stimulating their progesterone production via induction of the *P*450scc [26, 27]. The treatment of the cells with the glucocorticoid dexamethasone (DEX) enhanced their growth but did not stimulate steroidogenesis [72]. However, when DEX was added together with forskolin the effects of cAMP on suppression of cell growth and induction of steroidogenesis were enhanced. A similar synergistic effect of cAMP and DEX was also observed in primary rat granulosa cells from preovulatory follicles, suggesting that transfection with Ha-ras does not impede this effect. The stimulatory effect of cAMP and DEX on steroidogenesis was associated with *de novo* synthesis of the steroidogenic enzymes as revealed by immunocytochemistry and Western blots using antibodies to adrenodoxin. The cells treated with cAMP and DEX also showed a much higher incidence of gap junctions than cells treated with cAMP or DEX alone. Stimulation of progesterone production by cAMP was suppressed by the phorbol ester TPA. DEX blocked the inhibitory effect of TPA on steroidogenesis [72].

The TPA- and cAMP-responsive pathways have been shown to be coupled via interaction of the transcription factor AP-1 with CRE-BF1 [73] and CRE-BP2 [74]. A protein–protein interaction between AP-1 and the glucocorticoid receptor is also responsible for the cross-talk between the glucocorticoid-responsive and TPA-responsive pathways [75–81]. Based on these observations, our results may similarly reflect a cross-talk between cAMP-generated signals and DEX–glucocorticoid receptor interactions.

INVOLVEMENT OF THE CYTOSKELETON IN THE STEROIDOGENIC RESPONSE

Studies of the dynamic biochemical and morphological events occurring during steroidogenesis in granulosa and other steroidogenic cells suggest that the organization and expression of the actin-cytoskeleton may play a major role in the transduction of endocrine and paracrine steroidogenic signals, and in the coordination between the organelles involved in this process [1, 82, 83].

Since primary granulosa cells undergo a dramatic change in organization and expression of the cytoskeleton during differentiation, we examined the cytoskeleton of SV40 compared to SV40 and Ha-ras transfected cells which show considerably different morphologies [25, 26]. In ultrathin sections, the SV40-transformed cells showed a well developed network of thin filaments, very often parallel to the cell surface. In contrast, the SV40 and Ha-ras contransfected cells showed a poor network of thin filaments [26]. In 2–D gels of metabolically labeled cell extracts, actin expression was lower in the cotransfected cells, while tropomyosin isoforms 2 and 3, which show the highest affinities to actin among all isoforms of tropomyosin, were completely absent [71]. Upon cAMP stimulation, which induces rounding of the cells, no further down regulation of the actin cytoskeleton was observed. Thus, the lower expression of actin and actin binding proteins is associated with the development of steroidogenic capacity, in normal and also in transformed cells [1, 82, 84]. However, since changes in tropomyosin isoforms were observed in the transformed cells, the isoforms 2 and 3 may be the most important isoforms to modulate the organization of the actin cytoskeleton in these cells. The role of the ras oncogene on cytoskeleton organization and expression is not yet understood. However, since expression of ras and related proteins could modulate cell shape in yeast [85] and other cell types, this effect may be one of the important signals for differentiation.

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