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Drug development for ovarian hyper-stimulation and anti-cancer treatment: blocking of gonadotropin signaling for epiregulin and amphiregulin biosynthesis

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Abstract

Gonadotropins play a crucial role in ovarian homeostasis and fertilization through the activation of the cAMP cascade. However, gonadotropin hyper-stimulation may be associated with higher risk for ovarian cancer development. It has been suggested, that high gonadotropin levels in peritoneal and ovarian cystic fluids of patients suffering from benign ovarian cysts, may lead to malignancy. Moreover, we have recently discovered that gonadotropin stimulation can activate the MAPK cascade in target cells. Using DNA microarray technology and RNA from human granulosa cells, we discovered that stimulation with saturating doses of gonadotropins dramatically elevates activity of genes coding for epiregulin and amphiregulin. These gene products can bind and activate the EGF receptor and ERBB4, which are associated with the development of various cancers such as ovarian, breast endometrial and other non-gynecological malignancies. Gonadotropin receptors are expressed not only in the gonads, but also in non-gonadal tissues and in cancer cells. The discovery that gonadotropins activate certain mitogenic signal transduction pathways, may serve as a guide for novel anti-cancer therapy by (1) specific interference at the receptor level to block the gonadotropic response, or arresting the receptor expression and (2) blocking downstream mitogenic signals generated by these hormones, like attenuation of the expression of epiregulin and amphiregulin that belong to the EGF family, using anti-sense and/or SiRNA techniques targeted to suppress their expression. Moreover, since amphiregulin and epiregulin act as mediators of luteinizing hormone (LH) action in the mammalian ovulatory follicles, regulation of the expression of these factors may open new possibilities in treatment of ovarian malfunction implicated with ovarian hyper-stimulation.

Keywords: EGF-like growth factors; Ovarian cancer; Ovulation; Gonadotropins

Gonadotropin hormones control the main functions of the human ovary. Prior to ovulation, luteinizing hormone (LH) triggers a cascade of events in the ovarian follicle including resumption of meiosis of the oocyte, cumulus expansion, rupture of the follicular wall and extrusion of the cumulus–oocyte mass (reviewed in [1,2]). However, due to the restricted expression of its receptor in the cumulus cells surrounding the oocytes and the oocyte itself, many LH effects are thought to be indirect [3,4]. Indeed, it was suggested that elevated intracellular cAMP levels subsequent to LH stimulation, could pass through gap junctions communicating between granulosa cells and between cumulus cells and the oocyte [3,5]. The binding site of rat and human gonadotropins has been characterized in normal and neoplastic ovarian tissue (reviewed in [6–9]) and the mRNA of LH/hCG receptor has been up regulated in human ovarian carcinoma [10]. Recently, LH was detected in peritoneal cavity and cystic fluid in ovarian cancer patients, demonstrating a correlation between hormone levels and degree of malignancy [11–15]. It was recently demonstrated that LH stimulation induces the transient and sequential expression of the epidermal growth factor (EGF) family members amphiregulin, epiregulin and betacellulin in mouse ovary [16]. Incubation of follicles with these growth factors recapitulates the morphological and biochemical events triggered by LH, including cumulus expansion and oocyte maturation [16]. Thus, these EGF-related growth factors can act in a paracrine manner to mediate LH signaling throughout the

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follicle. Taken together, these observations suggest, that high levels of gonadotropins may elevate the risk for specific cancers, such as ovarian and breast cancers [17–23] through the induction of amphiregulin and epiregulin expression.

In the present review we discuss the possibility that the effect of LH on the development of cancer involves the stimulation of genes of the EGF family growth factors, such as amphiregulin and epiregulin. These factors were highlighted by DNA microarray technique [24–26] to be up regulated in human follicular cells (granulosa) obtained from in vitro fertilization (IVF) patients [27]. A complementary approach of the LH dependent expression of the growth factors in the mouse ovarian follicle [16] is further discussed.

1. LH stimulates the expression of genes coding epiregulin and amphiregulin

It was recently demonstrated that epiregulin and amphiregulin expression is elevated in mouse ovulatory follicle following LH stimulation [16]. Moreover, expansion of the cumulus was shown to be exerted in vitro by these growth factors. However, it was neither clear whether this stimulation is mediated by cAMP nor how this signal is terminated. As can be seen in Table 1, forskolin (Fk), that can activate non-specifically the adenylate cyclase, and thus elevate cAMP intracellular levels (reviewed in [28]), was more potent than LH in elevation of expression of epiregulin and amphiregulin, suggesting for the first time that cAMP can serve as a second messenger for the up regulation of these growth factors. According to these observations we suggest a model for the role of LH in stimulating epiregulin and amphiregulin expression in control of ovulation in the mammalian follicle (Fig. 1).

We have screened by DNA microarrays ovarian gene profile activity of women treated with fertility hormones, which is in routine use in IVF protocols for the induction of ovulation [29,30]. To reveal gonadotropin-dependent genes, we isolated RNA from granulosa cells obtained from IVF treatment [24–27,31] subsequent to culturing them in monolayers to release them from desensitization to gonadotropic hormones, followed by in vitro stimulation with LH, FSH or Fk. Our results showed genes that have not been reported previously to be modulated by gonadotropins [22,32]. A significant number of these genes could be implicated with potential elevated risk for cancer development (see Table 1). Moreover, that may, at least in part, account for the potential of gonadotropin hormones to induce neoplastic transformation. These genes could be divided into three categories: (1) elevation of genes coding for oncogenes, (2) elevation of genes coding for growth

Table 1
Modulation of genes coding for growth factors in primary human granulosa cells, their receptors and associated proteins

Gene	Accession no.	Abbreviation	Fold change above control		
			LH	FSH	FK
Amphiregulin (schwannoma-derived growth factor)	NM_001657.1	AREG	286.2	41.8	859.0
Epiregulin (EREG)	NM_01432.1	EREG	60.3	14.9	66.7
Transforming growth factor-beta type III receptor	L07594	TGF-beta	27.1	3.6	1.3[a]
Transducer of ERBB2,1	AA675892	TOB1	7.40	2.3	2.3
v-erb-a avian erythroblastic leukemia viral oncogene homolog-like 4 (ERBB4)	NM_005235.1	ERBB4	4.5[b]	4.5[b]	10.0[b]
Aberrant (short) epidermal growth factor receptor	K03193.1	EGFR	1.1[a]	−1.1[a]	1.2[a]
Epidermal growth factor receptor (avian erythroblastic leukemia viral (v-erb-b) oncogene homolog) (EGFR)	NM_005228.1	EGFR	1.0[a]	1.1[a]	−1.5[a]
Latent transforming growth factor beta binding protein 2	NM_000428.1	LTBP2	−8.5	−5.7	−7.4
Growth arrest-specific 6	L13720	GAS6	−8.5	−5.7	−25.8
Epidermal growth factor receptor pathway substrate 8 (EPS8)	NM_04447.1	EPS8	−3.0	−3.0	−6.7
A disintegrin and metalloproteinase domain 12, ADAM12 (meltrin alpha)	NM_003474.2	ADAM12	−2.2	−1.6	−2.0
cDNA FLJ13733 fis, clone PLACE3000147, highly similar to *Homo sapiens* metalloproteinase with thrombospondin type 1 motifs ADAMTS1	AK023795.1	ADAMTS1	−1.8	−1.5	−5.0
Epidermal growth factor (beta-urogastrone) (EGF)	NM_01963.2	EGF	−1.2[a]	−1.5[a]	−1.6[a]

Primary granulosa cells were obtained from women undergoing IVF procedure. Granulosa cells were cultured for 7 days in hormone-free medium in order to release them from desensitization to gonadotropin hormones [27,31]. Cells were then stimulated with 3 IU/ml of human luteinizing hormone (LH), 3 IU/ml of human follicle stimulating hormone (FSH) or 50 mM of forskolin (Fk). RNA isolated from the cells of the different treatments was hybridized on U133 Affymetrix DNA arrays [25,32]. Data are mean of duplicate experiments. Deviation from the mean did not exceed 17%.

[a] Non-significant changes.

[b] Changes that could not be calculated because of extremely low expression in all treatments.

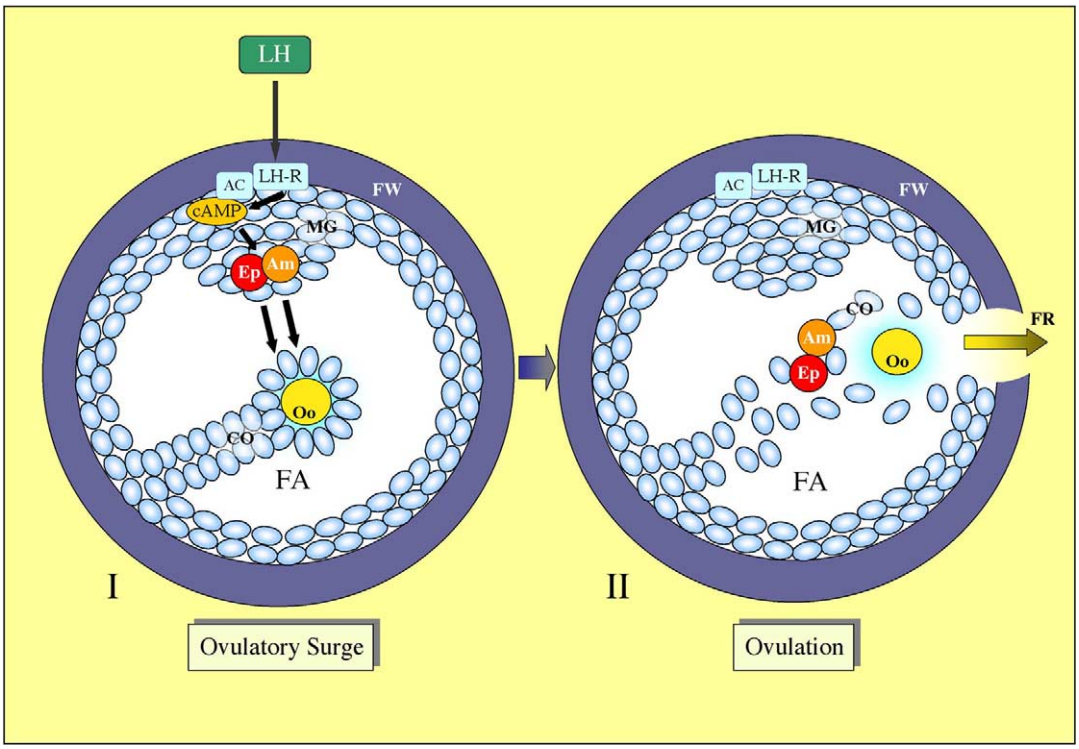

Fig. 1. Tentative role of LH in stimulating epiregulin and amphiregulin expression in control of ovulation in the mammalian follicle. The ovulatory hormone LH binds to its specific receptor (LH-R) and activates the hormone-sensitive adenylate cyclase (AC), leading to elevation of intracellular cAMP and de novo synthesis of epiregulin (Ep) and amphiregulin (Am) in the follicular cells of membrana granulosa (MG). These growth factors released to the follicular antrum (FA) can propagate the ovulatory signal [16], by stimulating the expansion of the cumulus ooforus (CO), resumption of meiosis and breakdown of the follicular wall (FW), to allow the extrusion of the cumulus–oocyte mass towards the fallopian tube.

factors and their receptors, and (3) decrease in genes coding for tumor suppressor activity (E. Rimon and A. Amsterdam, unpublished data).

There was a considerable elevation in genes coding for TGF-beta [33,34] and transducer of ERBB2,1 [35]. In addition, a considerable down-regulation was evidenced in the genes coding for enhancing-factor-2, in TGF-beta-binding protein-2, and in growth arrest specific factor-6 (Table 1 and ref. [36]). The modulation of these genes expression may enhance cell proliferation and cancer development. Most dramatically, elevated expression of epiregulin and amphiregulin was found after gonadotropin/cAMP stimulation (see Table 1).

Epiregulin and amphiregulin are growth factors of the epidermal growth factor (EGF) family [37]. Epiregulin has a mitotic activity in various primary cell types such as rat hepatocytes [38], as well as in various types of human tumor cell lines, the highest of which was observed in epithelial tumor cell lines [39]. It was, therefore, suggested that epiregulin is involved in the progression of carcinomas [39]. Similar to other EGF-type growth factors, epiregulin exerts its proliferative effects via the tyrosine kinase pathway through autophosphorylation of EGF receptors [40]. Moreover, involvement of epiregulin expression in tumorigenesis in vivo through activated Ki-RAS signaling pathway in human colon cancer cells has been recently suggested [41]. Furthermore, it was demonstrated that epiregulin was up regulated and stimulated growth of human pancreatic cancer cells [42]. In human breast carcinoma cell lines, epiregulin was involved in the stimulation of tyrosine phosphorylation of ERBB, mainly ERBB-4 and epidermal growth factor receptor (EGFR). It was, therefore, concluded that ERBB-4 and EGFR are receptors for epiregulin [43]. Amphiregulin and its receptor, EGFR, were expressed in a series of invasive ductal breast carcinoma specimen [44]. The presence of high levels of amphiregulin was confirmed by immunohistochemistry in 83 specimen (59 primary ovarian tumors and 24 extra-ovarian carcinomas) obtained from 68 ovarian carcinoma patients [45]. These data strongly implicates EGF-related peptides in the pathogenesis and outcome of human ovarian cancer. We discovered a significant reduction in gene activity of specific metalloproteinases of the ADAMS family (see Table 1), essential for activation of these growth factors [46]. Moreover, although there was no significant change in ERBB4 or EGFR expression, a significant down-regulation of epidermal growth factor receptor substrate 8 (EPS8), which is essential for the mitogenic signals from the phosphorylated EGF receptor [47,48]. This suggests that in the normal ovary there is a blockade of the growth factors potential to act as mitogens on the granulosa cells themselves, since expression of both

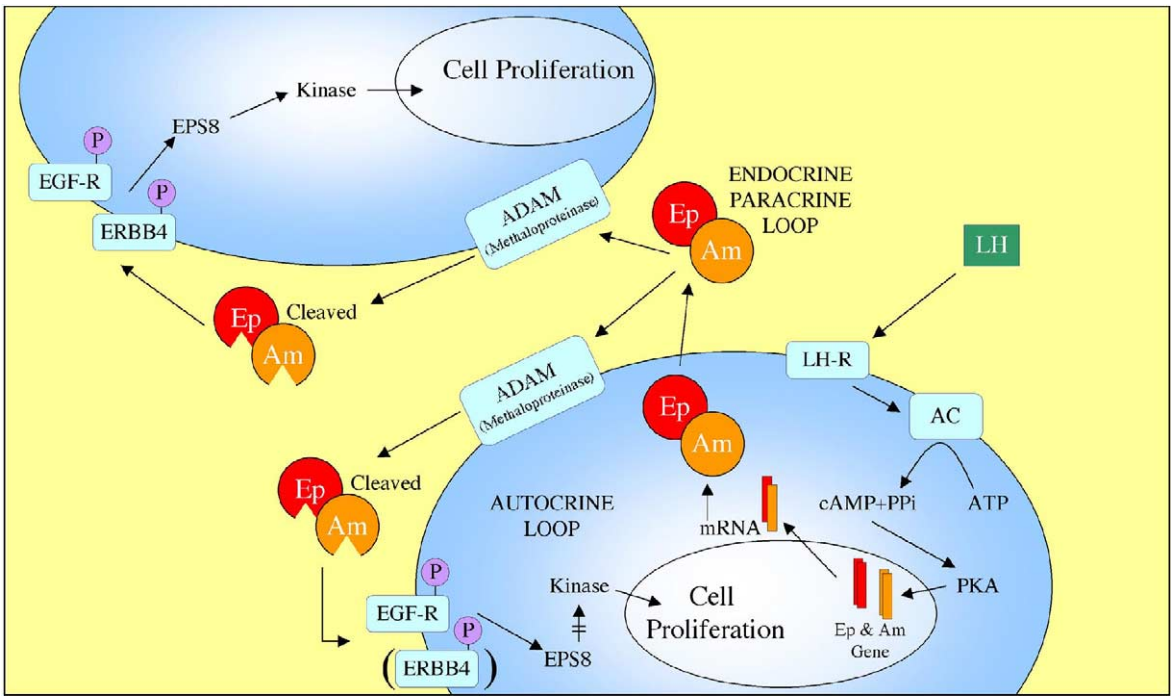

Fig. 2. Tentative involvement of LH in the development of ovarian and other cancers implicated in paracrine and endocrine action of epiregulin and amphiregulin. LH may trigger intracellular epiregulin (Ep) and amphiregulin (Am) formation in LH responssive granulosa cells or cancer cells through activation of adenylate cyclase (AC) and accumulation of cAMP. Upon secretion of Ep and Am, these growth factors can be activated by specific cleavage of extracellular metaloproteinases of the ADAM family associated with the membrane [46]. The activated growth factors can either bind and activate EGF receptor (EGF-R) or ERBB4 by phosphorylation (p) and activate a mitogenic pathway either in the same cell (autocrine loop) or adjuscent cells (paracrine loop), or distal cells and tissues (endocrine action). Most probably, the autocrine loop could not exist in normal granulosa cells because of down-regulation of ADAMs, marginal expression of ERBB4 and a dramatic down-regulation of EPS8.

ADAMS and EPS8, a signal transduction factor related to EGF stimulated cascade, is down-regulated. Attenuation of the genes coding for specific metalloproteinases ADAM12 and ADAMTS1 may suggest, that the possible enhanced formation of epiregulin and amphiregulin is not targeted to enhance granulosa cell proliferation through an autocrine loop, but rather may activate epithelial ovarian cells, accepted to be the origin of ovarian carcinoma (reviewed in [49,50]). A suggested model for a possible paracrine and endocrine effect of epiregulin and amphiregulin following LH stimulation of ovarian follicular cells is presented in Fig. 2.

2. Possible connection between LH production and carcinogenesis

Studies conducted over the past 15 years have demonstrated, that LH receptor is found not only in the gonads, but also in non-gonadal tissues [9,10,51], including gametes, early embryos/blastocysts oviduct, uterus, cervix, placenta, fetal membranes, umbilical cord, brain, spinal cord, neural retina, breast, adrenal, urinary bladder, bone cavernous sinus carotid rete, vascular complex, prostates, seminal vesicles, epididymis, sperm, ovarian epithelial cells and cancer cells (reviewed in [9]). These observations suggest that gonadotropins may modulate gene profile activity in non-gonadal target cells. It was found very recently, that excess of human chorionic gonadotropin (hCG) production in transgenic female mice, stimulates pituitary enlargement and subsequent progression to adenoma [52]. The mammary gland of these animals showed marked labuloalveolar development followed by mammary tumors, characteristic of adenocarcinoma. Molecular explanation for these phenomena has not yet been suggested.

In a detailed investigation [15], it was clearly indicated that significant concentrations of LH are found in peritoneal fluid and cyst fluids of women suffering from ovarian tumors (Fig. 3). Moreover, a significant correlation was evident between high levels of LH and the degree of malignancy (Fig. 3). However, the clinical value of serum LH levels for diagnosis and monitoring patients is still under debate [12,21,53]. On one hand, reduction of elevated serum gonadotropins by gonadotropin releasing hormone (GnRH) analogs in ovarian cancer patients did not prevent recurrence nor did it lead to growth restriction [54]. On the other hand, high levels of LH were consistently found in malignant effusions, such as ascites or ovarian cysts, compared with non-malignant ovarian tumor and ascites [14,15]. It seems that LH levels in peritoneal and cyst fluid are more relevant to the prognosis and the

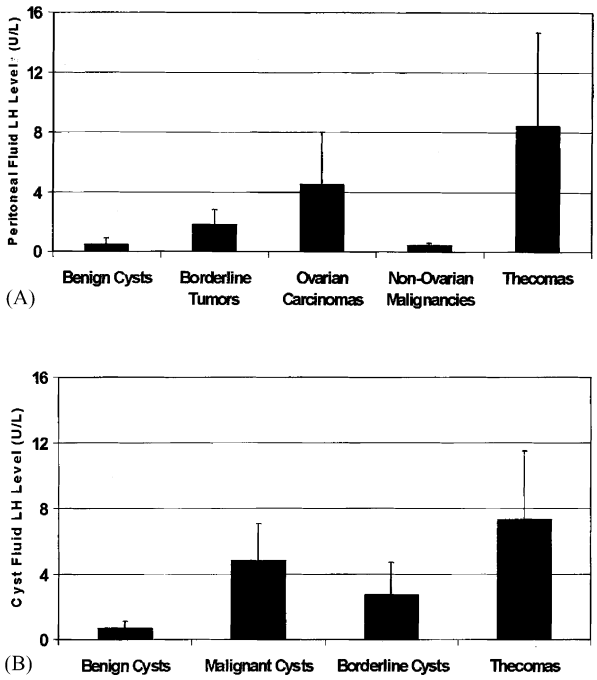

Fig. 3. Luteinizing hormone (LH) content in human ovarian cancer measured by radio-immuno-assay (RIA). (A) The mean + S.D. of peritoneal fluid LH levels in patients from different study groups. The LH levels were significantly lower in patients with benign ovarian cysts comparing to patients with borderline ovarian tumors, ovarian cancer and thecomas ($P < 0.005$). Peritoneal fluid LH levels in patients with non-ovarian malignancies were significantly lower than in patiens with ovarian cancer and with thecomas ($P < 0.04$). (B) The LH levels in the cyst fluids were significantly lower in benign ovarian cysts as compared to malignant ovarian cysts, borderline tumors and thecomas ($P < 0.01$). Modified from ref. [15] with permission.

propagation of the disease, rather than blood LH levels. This is a rational approach since the spreading of the malignant ovarian cancer originates in most cases from the surface of the ovarian epithelium, which faces the peritoneal cavity (reviewed in [49,50,55]). Since LH is produced and secreted by the cancerous tissue, it will be first released to the peritoneal cavity, rather than into the vascular system. Moreover, the presence of high levels of LH content in the cyst fluid of the cancerous tissue strongly suggest that LH is synthesized de novo in the cancerous cells. Our discovery, that epiregulin and amphregulin are synthesized in ovarian follicular cells, raises the possibility that LH could induce the formation of these EGF growth factors in the cancerous cells, which could then trigger further propagation of the cancerous cells.

This proposed mechanism could potentially suggest a novel strategy of treating specific cancers responsive to LH stimulation, by blocking the LH-mediated up-regulation of epiregulin and amphiregulin. This could be achieved, at least in principal, by blocking the LH receptor using specific antibodies [56–58] and/or the up regulation of the EGF growth factors by antisense probes [59] or SiRNA [60,61] administered directly to the peritoneal cavity, rescuing other organs not directly exposed to the peritoneal cavity.

3. Role of EGF-like growth factor in normal ovarian function compared to malignant transformation

What might be the difference in LH-induced epiregulin and amphregulin biosynthesis between normal versus transformed tissue? According to recent observation [16], EGF-like growth factors are essential for the successful ovulatory process. However, in the normal process of triggering ovulation there must be a secured mechanism, which could terminate the LH responsive signal in epiregulin and amphiregulin action. (1) In the normal ovary there is a process of desensitization to the hormone upon corpus luteum formation, in spite of elevation of LH receptor expression (reviewed in [32,62]). (2) Our data, obtained from DNA array, showed that upon luteinization of the human granulosa cells, down-regulation of specific metalloproteinases occurs. This is necessary for the specific cleavage of the growth factors, which allows the activation of EGFR or ERBB4 (see Fig. 2). (3) There is a marginal ERBB4 expression in normal granulosa cells, while the EGFR content does not dramatically change upon LH stimulation (see Table 1). There is, however, possible down-regulation of the EGFR signaling due to a clear down-regulation of the epidermal growth factor receptor substrate 8 (EPS8) [47,48]. This receptor substrate was recently found to be involved in Src mediated transformation and could generate redundancy in the Ras/Rac pathways [47].

It is tempting to suggest that in the malignant tissue, LH stimulation for the formation of epiregulin and amphiregulin is not down-regulated and, therefore, could continuously stimulate ovarian and other cancer cells expressing a functional LH receptor. The possibility that LH dependent signals are not down-regulated in malignant cells compared to normal tissue, is an important challenge for the future. Further understanding of this proposed mechanism may hopefully provide us with new tools to control the gonadotropin response in the formation of EGFs, both in ovarian malfunction, such as unexplained un-ovulation and neoplastic transformation, which leads to ovarian cancer. An alternative possibility, through which LH can trigger its mitogenic effect, is raised by the activation of the MAPK cascade by LH. Indeed, we have found, that phosphorylation of ERK1 and ERK2 occurs in primary human granulosa cells [63] and in RAS transformed rat granulosa cells [64,65] (see Fig. 4). However, this activation occurs within 5 min and therefore, does not seem to be mediated by de novo synthesis of epiregulin or amphregulin. Down-regulation of MAPK activation by LH in steroidogenic cells occurs within a couple of hours [66]. However, if the desensitization to

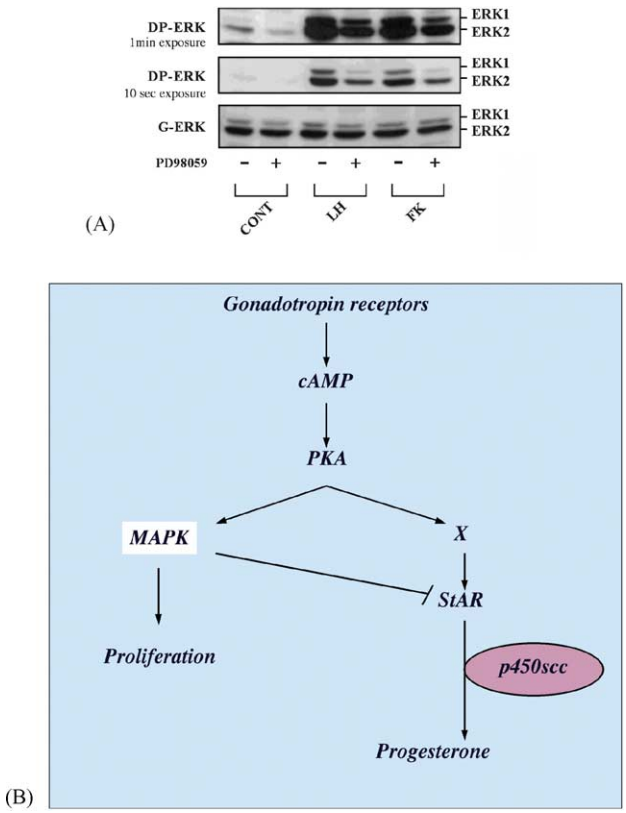

Fig. 4. Activation of steroidogenesis and proliferation by LH. (A) Human granulosa cells were serum-starved for 16 h and then stimulated with LH (3 IU/ml) or FK (50 μM) in the presence or absence of inhibitor of the MAPK cascade (ERK1 and ERK2 phosphorylation (PD98059) for 20 min. Cell lysates were subjected to immunoblotting with non-phosphorylated (DP)-ERK antibody (upper panel) or with anti-general ERK antibody (G-ERK; lower panel). The positions of ERK1 and ERK2 are indicated. Modified from ref. [64]. (B) Schematic representation of the signaling pathway controlling gonadotropin-induced differentiation and/or proliferation. Gonadotropins activate adenylate cyclase and PKA, which activate on one hand the steroidogenic pathway by up regulation of the steroidogenic acute regulatory (StAR) protein and cytochrome P450 side chain cleavage (P450scc), and on the other hand, it activates mitogenic pathway (MAPK) by phosphorylation of ERK1 and ERK2. Modified from ref. [65].

LH stimulation does not occur in ovarian cancer cells, it can contribute to the propagation of ovarian cancer development following LH dependent up-regulation of epiregulin and amphiregulin.

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