***In vivo* inhibition of keratinocyte growth factor receptor expression by estrogen and antagonism by progesterone in the mouse mammary gland**

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Abstract

Mammary gland development is regulated by complex interactions among mammogenic hormones and locally derived paracrine growth factors. In epithelial tissues, keratinocyte growth factor (KGF or FGF-7) originates in the stroma while its receptor (KGFR or FGFR2-IIIb) is present only in the epithelium. Previous work showed that estrogen but not progesterone could stimulate the synthesis of KGF in mammary stroma *in vivo*. The effects of 17 β -estradiol and progesterone on KGFR expression *in vivo* were examined in these studies. Peripubertal and mature virgin mice received subcutaneous injections of hormone in sesame oil after which KGFR mRNA levels were assayed by ribonuclease protection analysis of mammary gland RNA. Estradiol treatment caused a dose- and time-dependent decrease in KGFR mRNA level in mice from both age groups while stimulating ductal

growth after 7 days of treatment. Inhibition of KGFR expression was near maximal at an estradiol dose of 2 μ g after 1 day of treatment. Progesterone injection increased KGFR mRNA levels but this effect correlated with the stimulation of ductal growth. However, when progesterone was co-administered with estradiol, KGFR mRNA levels were maintained in the absence of any effect on ductal growth. Thus, estradiol inhibited KGFR mRNA only when elevated unopposed by progesterone. These data show that KGFR expression is determined by the ratio of estradiol and progesterone and suggests a mechanism through which these hormones can co-operate to optimize their growth-promoting effects. Consequences of hormone imbalance are also implicated.

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Introduction

Estrogen, progesterone, and prolactin are the primary regulators of *in vivo* mammary gland development while growth factors can be considered as mediators or modifiers of hormone action (Nandi *et al.* 1984, Imagawa *et al.* 1994b, Dickson & Lippman 1995). Of the principal mammogenic ovarian steroids, 17 β -estradiol is unique in being able to directly target stroma and epithelium, both of which contain estradiol receptors (Haslam & Nummy 1992, Couse *et al.* 1997), while progesterone receptors are found only in mammary epithelium (Shyamala *et al.* 1997). However, most of the epithelial cells stimulated to proliferate by estradiol *in vivo* do not contain estradiol receptors (Daniel *et al.* 1987, Zeps *et al.* 1998, Saji *et al.* 2000), suggesting that estradiol stimulates proliferation through indirect mechanisms including the induction of local growth factors or their receptors. In support of this possibility, recent studies examining tissue recombinants from estradiol receptor- α knock-out and normal mice suggest that estradiol regulation of epithelial proliferation

in the mammary gland is a paracrine event mediated by estradiol receptor-positive stromal cells (Cunha *et al.* 1997, Cooke *et al.* 1998). Estradiol can also indirectly affect mammary gland development by elevating prolactin and progesterone levels and inducing progesterone receptors in mammary epithelium (Imagawa *et al.* 1985, Haslam 1988, Bocchinfuso *et al.* 2000).

Estradiol or progesterone regulation of the synthesis of growth factors and their receptors in the mammary gland is largely unexplored. There is evidence that estradiol stimulation of ductal growth is mediated by an increase in epithelial epidermal growth factor (EGF) receptors and EGF levels (Haslam *et al.* 1992, Ankrapp *et al.* 1998). Other evidence shows that the expression of growth factors can change during different stages of mammary gland development raising the possibility of hormonal regulation (Pedchenko & Imagawa 2000b). We have begun to delineate the interactions of hormones with growth factors in the mammary gland by examining the hormonal regulation of the synthesis of keratinocyte growth factor (KGF or FGF-7) and its receptor, the KGF

receptor (KGFR or FGFR2-IIIb). KGF is a stromal growth factor that is expressed in intact or parenchyma-free mammary glands while the receptor is expressed only in mammary epithelium (Finch *et al.* 1995, Pedchenko & Imagawa 2000b). Progesterone and prolactin are mammaryogenic hormones that are directly mitogenic towards mouse mammary epithelial cells in primary culture (Imagawa *et al.* 1985) and can synergize with KGF in stimulating proliferation (Pedchenko & Imagawa 1998). Estradiol, in contrast, does not stimulate proliferation *in vitro* alone or in synergism with growth factors (Richards *et al.* 1988). In addition, progesterone can stabilize KGFR mRNA *in vitro* and via this mechanism may potentiate KGF mitogenesis (Pedchenko & Imagawa 1998).

Estradiol administration to intact or ovariectomized mice *in vivo* caused an elevation in the level of KGF mRNA and protein in mammary stroma (Pedchenko & Imagawa 2000a). Progesterone administration had no effect on KGF synthesis. These *in vivo* and *in vitro* findings suggested that progesterone and prolactin modulate the sensitivity of the mammary epithelium to KGF while estradiol targets the stromal compartment to upregulate KGF synthesis. In this study, the effect of estradiol on KGFR expression *in vivo* was examined in the absence and presence of progesterone. Surprisingly, estradiol inhibited KGFR expression but only in the absence of progesterone supplementation. Thus, the estradiol/progesterone ratio is critical in determining the steady-state level of KGFR.

Materials and Methods

Chemicals

17 β -Estradiol and progesterone were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hormones were first dissolved in ethanol and then mixed with 9 vol. sesame oil.

Animals

Ovary intact BALB/CAnNCr mice (Charles River) were used at 4 or 10 weeks of age. For some experiments, mice were ovariectomized at 3 and/or 8 weeks of age prior to hormone injection at 4 or 10 weeks of age. Mice were maintained under controlled temperature and lighting conditions, and were given food and water *ad libitum*. All animals were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all procedures described here were approved by the University of Kansas Medical Center animal care and use committee.

In all experiments three animals per treatment group were used. Hormonally treated mice received daily subcutaneous injections of steroid hormones in 10% ethanol/sesame oil (50 μ l) for 1–7 days as indicated; control animals

received vehicle only. At termination, the no. 4 inguinal mammary glands (with lymph nodes removed) were collected for RNA extraction or whole mount analysis of gland morphology by fixation in methacorn followed by defatting in acetone, and staining with iron–hematoxylin.

Ribonuclease protection assay

For ribonuclease protection assay (RPA) a 148 bp PCR fragment corresponding to exon K (bp 1270–1417) of the mouse bek gene (Miki *et al.* 1992) was cloned into pGEM3Zf(–) transcription vector (Promega, Madison, WI, USA). After linearization with HindIII, antisense transcripts were synthesized with T7 RNA polymerase using the MAXIscript *in vitro* transcription kit from Ambion (Austin, TX, USA) and 50 μ Ci of a 32 P-UTP (800 Ci/mmol; DuPont NEN, Wilmington, DE, USA). Full-length probe was recovered after electrophoresis of transcription products on a 5% polyacrylamide/7 M urea/Tris/borate/EDTA (TBE) gel. A mouse β -actin probe with protected fragment size of 245 nucleotides (Ambion) was labeled to 200 times lower specific activity than the KGFR probe by the addition of 100 mM UTP to the transcription reaction, and gel purified as described above. It was used as an internal control to monitor RNA amount and gel loading.

Total RNA was isolated from combined inguinal (no. 4) mammary glands (with lymph nodes removed) by homogenization in TRIzol reagent (Life Technologies, Grand Island, NY, USA). For some experiments, DNA was also recovered from TRIzol extracts of tissues according to the manufacturer's instructions and quantitated by spectrophotometry. RNase protection assays were performed using 20 μ g total RNA, 80 000 c.p.m. labeled KGFR probe and 20 000 c.p.m. of appropriate β -actin probe using RPA II Kit (Ambion). After hybridization overnight at 45 °C and RNase digestion, protected fragments were resolved on 5% polyacrylamide/7 M urea/TBE gel and exposed to autoradiography film at –70 °C with an intensifying screen. Band intensity was quantified using a Molecular Dynamics Personal Densitometer SI with ImageQuant 4.2 software (Molecular Dynamics, Sunnyvale, CA, USA). Appropriate negative control samples contained yeast tRNA instead of mouse RNA. Positive controls were not treated with RNase and confirmed that probes were used in about 100-fold excess to respective target mRNAs. Dividing signals from their protected fragments to β -actin bands normalized all data for KGFR mRNA expression.

Statistical analysis

Single comparisons were performed using Student's two-tailed *t*-test; for multiple comparison between various treatments groups, data were analyzed by one-way

Figure 1 Effect of 17β -estradiol on expression of KGFR mRNA in mammary glands of 5- and 11-week-old BALB/c mice. Starting from 4 or 10 weeks of age mice were injected daily with $20\text{ }\mu\text{g}$ estradiol (+E) or vehicle (V). After 7 days, the level of KGFR mRNA in the mammary glands was analyzed by RNase protection assay as described in Materials and Methods. Y- and Y+ represent probe incubated with yeast RNA in the absence and presence of RNase digestion. In Y- the lower and higher molecular weight bands are, respectively, full-length KGFR and actin probes. Film exposure time was adjusted to visualize the weak KGFR band in the 5-week, estrogen-treated group. The expected actin and KGFR protected fragments used for quantification are labeled on the figure.

ANOVA followed by the Student–Newman–Keuls test. A minimum of $P < 0.05$ was considered to be significantly different.

Results

Effect of 17β -estradiol treatment on KGFR expression

The effect of estradiol on KGFR expression in mammary parenchyma was compared in peripubertal (4 weeks old) and mature mice (10 weeks old). These groups were chosen to compare the effect of estradiol on KGFR expression during a period of active ductal elongation and branching (peripubertal) with essentially growth-quiescent glands (mature) which fill the fat pad.

To assess the effect of estradiol on the level of KGFR mRNA, peripubertal mice or mature virgin mice were injected with estradiol ($20\text{ }\mu\text{g}$) daily for 7 days before termination for the analysis of KGFR expression at, respectively, 5 and 11 weeks. Estradiol inhibited KGFR expression as shown in Fig. 1, a representative ribonuclease

Table 1 Effect of 17β -estradiol on the level of KGFR mRNA in mammary glands from ovary-intact and ovariectomized mice treated for 7 days prior to termination at 5 or 11 weeks of age. Numbers represent means $\pm$ S.E.M. from three independent experiments ($n=9$) for ovary-intact animals and means $\pm$ S.D. for ovariectomized animals ($n=3$)

	Age at termination	Vehicle	+ Estradiol
Ovary intact	5 weeks	1.34 ± 0.18	0.18 ± 0.02
	11 weeks	4.04 ± 0.64	0.46 ± 0.04
Ovariectomized	5 weeks ^a	0.54 ± 0.10	0.14 ± 0.02
	11 weeks ^a	0.4 ± 0.13	0.15 ± 0.05
	11 weeks ^b	0.85 ± 0.16	0.32 ± 0.15

Mice were ovariectomized when 3^a or 8^b weeks old. Estradiol was injected subcutaneously at a dose of $20\text{ }\mu\text{g/day}$ for all experiments except ^b at $2\text{ }\mu\text{g/day}$. Data were calculated as a ratio of KGFR to β -actin bands in each sample and expressed in arbitrary units. Significant differences between vehicle control and estradiol-treated animals were observed for each age group ($P < 0.001$, by paired Student's *t*-test).

protection analysis. A summary of data (Table 1) shows that estradiol inhibited KGFR expression in ovary-intact peripubertal and pubertal mice by approximately 85%. KGFR expression was higher in vehicle-treated mature mice as opposed to peripubertal mice due to more extensive ductal development giving a higher proportion of KGFR-expressing epithelial cells in these glands. Dose/response studies (Fig. 2) indicated that estradiol inhibition was observed at a 100-fold lower dose ($0.2\text{ }\mu\text{g}$) and near maximum at $2\text{ }\mu\text{g}$. The response was also rapid with the maximum inhibitory effect observed after 1 day, the shortest time-period examined (Table 2). This inhibitory effect of estradiol on KGFR mRNA levels coincided with estradiol stimulation of ductal growth in peripubertal mice. Figure 3A shows that ductal growth in control animals had only partially filled the mammary fat pad extending just beyond the lymph node in contrast to estradiol-treated animals (Fig. 3C) in which ducts fill the fat pad with the absence of end buds signifying the completion of ductal elongation. For mature mice with growth-quiescent mammary glands, a much less pronounced effect of estradiol on ductal growth was observed compared with peripubertal mice. Examination of DNA and RNA content of mammary glands from mature mice showed that estradiol treatment on average caused a slightly higher but statistically insignificant increase in DNA content but a significant increase in the level of total RNA (Fig. 4). Only ductal growth was observed in these animals.

The inhibitory effect of estradiol ($20\text{ }\mu\text{g}$) treatment on KGFR expression was also observed in 5- and 11-week-old mice ovariectomized at 3 weeks of age (Table 1) and in 11-week-old mice ovariectomized at 8 weeks of age treated with a lower dose of estradiol ($2\text{ }\mu\text{g/day}$). Ovariectomy caused mammary gland regression, resulting

Figure 2 Effect of different doses of 17β-estradiol on KGFR mRNA level in mammary glands of 5-week-old BALB/c mice. Four-week-old mice were injected daily with the indicated doses of estradiol (E in µg) for 7 days. KGFR mRNA level was analyzed as described in Fig. 1. Values are expressed as a percentage of vehicle-treated controls (–v7d). Bars represent means ± S.D. (n=6) from two experiments. Significant differences between groups are indicated by different letters (P<0.05, by Student–Newman–Keuls test).

in a reduction in KGFR mRNA levels (Table 1), and a reduced inhibitory effect of estradiol due most likely to the already depressed level of KGFR mRNA. Further studies employed ovary-intact mice in order to examine the effect of estradiol and progesterone treatment on non-regressed glands.

Effect of progesterone treatment on KGFR expression

Progesterone (400 µg) was administered to 4-week-old mice daily for 7 days and mammary glands collected for KGFR mRNA analysis. Treatment with this dose of

Table 2 Time-course effect of 17β-estradiol on the level of KGFR mRNA in mammary glands of 5-week-old BALB/c mice. Data (means ± S.D. (n=3)) are shown as the ratio of KGFR to β-actin bands in each sample and expressed in arbitrary units

Days of treatment	Vehicle	Estradiol
1	0.17 ± 0.06	0.06 ± 0.03*
3	0.17 ± 0.08	0.07 ± 0.02*
7	0.29 ± 0.08	0.10 ± 0.04*

Four-week-old mice were injected daily with vehicle or estradiol (20 µg). After 1, 3 or 7 days of hormone treatment mammary gland KGFR mRNA levels were determined.
*P<0.01 compared with vehicle control (unpaired Student’s t-test).

progesterone increased the level of KGFR mRNA about twofold (Table 3, Series 1). Figure 3B shows that progesterone treatment caused extensive ductal growth that filled the fat pad (note that end buds are not present) with increased side branching. Thus, this response correlated with the stimulation of ductal growth and the number of KGFR-expressing cells. In support of this view, neither a shorter period of stimulation (1 day (Table 3, Series 3)) nor treatment for 7 days with a lower dose of progesterone (Table 3, Series 2) affected KGFR expression or mammary gland morphology (not shown). To examine further the correlation between the effect of progesterone on the level of KGFR mRNA with the induction of ductal growth, the effect of progesterone treatment on recoverable total RNA and DNA was estimated. (Initially keratin 8 or 19 expression was also co-monitored to normalize for relative epithelial cell content of the mammary glands but since expression was found to be modulated by hormones, direct measurement of DNA content was used instead.) Figure 5 shows that only the highest dose of progesterone (400 µg) significantly increased mammary gland RNA and DNA content. Thus, the dose effect of progesterone on KGFR mRNA levels correlated with the stimulation of ductal growth. However, a subtle positive effect of progesterone on KGFR mRNA content per cell cannot be excluded and may occur, since progesterone has been shown to stabilize KGFR mRNA levels in mouse mammary epithelial cells *in vitro* (Pedchenko & Imagawa 1998).

Effect of co-administration of estradiol and progesterone on KGFR expression

Since estradiol and progesterone act in concert to stimulate mammary gland development, these two hormones were co-injected to examine their combined effect on KGFR expression. Estradiol and progesterone alone or together were injected for 7 days in mice of 4 and 10 weeks. The level of KGFR mRNA in 4-week-old animals receiving estradiol plus progesterone was similar to the control group, i.e. lower than the progesterone-treated group and higher than the estradiol-treated group (Table 4). Thus estradiol inhibition of KGFR expression (85%) was not observed when accompanied by progesterone. Compensatory stimulation of ductal growth induced by progesterone in the presence of estradiol does not explain this effect since the estradiol plus progesterone or estradiol treatment groups exhibited similar degrees of ductal growth due to the relatively low dose of estradiol (2 µg) (Fig. 3C, D). A threefold increase in parenchymal growth would have to occur in order to account for the effect of estradiol plus progesterone on the level of KGFR mRNA compared with estradiol alone (Table 4). (We have previously shown that estradiol at a higher dose of 20 µg per injection in combination with progesterone can cause lobuloalveolar development (Pedchenko & Imagawa 2000a) which could cause a multifold increase in DNA

Figure 3 Effect of progesterone and 17 β -estradiol on mammary gland morphology of 5-week-old BALB/c mice. Four-week-old female BALB/c mice were injected daily for 7 days with vehicle, progesterone (400 μ g), estradiol (2 μ g) or estradiol plus progesterone (2+400 μ g). Mammary gland whole mounts of the no. 4 inguinal mammary fat pad were prepared from (A) control, (B) progesterone-treated, (C) 17 β -estradiol-treated and (D) estradiol plus progesterone-treated mice. Arrows point to regions of increased ductal side branching.

content.) This effect of progesterone appears to be different from the effect of progesterone noted above, in that it causes an elevation in the level of *KGFR* mRNA that is independent of the stimulation of ductal growth.

Figure 4 Effect of hormones on mammary gland DNA and RNA content in 10-week-old mice. Ten-week-old mice were injected daily for 7 days with vehicle (V) or estradiol-17 β (E; 2 μ g), progesterone (P; 400 μ g), or both hormones combined (E+P). At the time of RNA extraction from the no. 4 mammary glands, DNA was also recovered according to the manufacturer's instructions. The means $\pm$ S.D. of DNA and RNA recovered per mammary gland are plotted ($n=3$). Significant differences between vehicle and hormone-treated groups are indicated by asterisks ($P<0.05$, by Student–Newman–Keuls test). There was no significant difference among the hormone-treated groups.

The trend towards estradiol plus progesterone counter-regulation continued in the 10-week-old mice, although progesterone appeared to be less stimulatory to the level of *KGFR* mRNA, $\sim 40\%$ versus $\sim 100\%$ increase in 4-week-old mice (Table 4). In addition, the effect of estradiol plus progesterone tended to be lower than the vehicle control group but higher than the estradiol-treated group (in all four experiments) although, due to intragroup variability, this difference was not statistically significant using the Student–Newman–Keuls multiple comparison test. Examination of mammary gland DNA and RNA content showed that progesterone or estradiol plus progesterone treatment of mature 10-week-old mice caused an equivalent small significant increase in RNA content (Fig. 4) but DNA content in only the estradiol plus progesterone treatment group was significantly different from vehicle controls. There was no significant difference in DNA content among the hormone-treated groups. Because control glands already exhibit full ductal growth that fills the fat pad, a relatively weaker effect on ductal growth was observed in mature mice compared with the growing mammary glands of peripubertal week-old mice. In addition and most importantly, we see that estradiol plus progesterone treatment does not significantly affect DNA content compared with estradiol treatment alone even though *KGF* mRNA levels are increased $\sim 100\%$ (Table 4).

To investigate further the effect of estradiol on the progesterone-induced increase in *KGFR* mRNA, progesterone (400 μ g) was injected with varying doses of

Table 3 Effect of progesterone on the level of KGFR mRNA in mammary glands from peripubertal mice

	Days of treatment	Vehicle	Progesterone	
			40 µg/day	400 µg/day
Series 1	7	100 ± 16.9	—	195 ± 28.8*
Series 2	7	100 ± 9.8	112.7 ± 11.3	174.9 ± 17.6*
Series 3	1	100 ± 7.3	—	81.5 ± 11.4
	7	100 ± 18.9	—	244 ± 27.3*

Ovary-intact mice of 4 weeks of age were given daily s.c. injections of progesterone for the indicated times and doses. Data for each experimental series are expressed as a percentage of vehicle-treated control animals. For Series 1, means ± S.D. (n=15) from five experiments are given. *Significant difference from vehicle control, *P*<0.01 by paired Student's *t*-test. For Series 2, means ± S.E.M. of three experiments (n=9) are plotted. *Significant difference from vehicle control, *P*<0.05 by ANOVA and Student–Newman–Keuls test. For Series 3, means ± S.E.M. of three experiments (n=9) are plotted. *Significant difference from vehicle control, *P*<0.01 by paired Student's *t*-test.

estradiol (0.2–20 µg) daily for 7 days into 4-week-old mice. Increasing concentrations of estradiol caused a dose-dependent decrease in the progesterone-induced elevation in KGFR mRNA level after 7 days of treatment (Fig. 6). Compared with the effect of estradiol alone (Fig. 2), the inhibitory effect of estradiol is reduced in the presence of progesterone (40% at 2 µg estradiol compared with 70% for estradiol alone). Therefore, when progesterone and estradiol are co-administered, estradiol inhibition is

attenuated by progesterone resulting in the maintenance of KGFR mRNA at a higher level than in the presence of estradiol only.

Discussion

The stimulatory effect of estradiol on ductal growth and/or branching in peripubertal and mature mice may be due to local (Daniel *et al.* 1987, Silberstein *et al.* 1994) and systemic effects. Previous *in vivo* work (Imagawa *et al.* 1990) and more recent studies using mice deleted in the genes for the progesterone receptor (Humphreys *et al.* 1997) or estrogen receptor-α (Bocchinfuso *et al.* 2000) show that estradiol alone does not stimulate ductal growth or side branching which is stimulated primarily by progesterone (Atwood *et al.* 2000). Most data indicate that estradiol acts indirectly since proliferating cells are largely devoid of estrogen receptors (Haslam 1988, Zeps *et al.*

Figure 5 Effect of progesterone on mammary gland DNA and RNA content. Five-week-old mice were injected daily for 7 days with vehicle (V) or progesterone (P; 4, 40, 400 µg). At the time of RNA extraction from the no. 4 mammary glands, DNA was also recovered according to the manufacturer's instructions. The means ± S.D. of DNA and RNA recovered per mammary gland are plotted (n=3). Significant differences between vehicle and hormone-treated groups are indicated by asterisks (*P*<0.05, by Student–Newman–Keuls test).

Table 4 Effect of co-administration of estradiol (E) and progesterone (P) on KGFR mRNA levels in mammary glands of 5-week-old and 11-week-old BALB/c mice. Values indicate means ± S.E.M. from three (5-week, n=9) and four (11-week, n=12) experiments

	KGFR mRNA levels	
	5 week	11 week
Vehicle	100 ± 22.4 ^a	100 ± 10.2 ^a
Estradiol	28.8 ± 12.8 ^b	31.4 ± 4.6 ^b
Progesterone	176.6 ± 65.6 ^c	145.7 ± 25 ^c
E+P	109.4 ± 39.0 ^a	62 ± 4.8 ^{a,b}

Mice, 4- or 10-weeks old, were injected for 7 days with vehicle, 17β-estradiol (2 µg), progesterone (400 µg) or E+P before termination. Mammary gland KGFR mRNA levels are normalized to the vehicle control group (100%). Significant differences between groups are indicated by different letters; *P*<0.05, by Student–Newman–Keuls test.

Figure 6 Effect of co-administration of 17 β -estradiol and progesterone on *KGFR* mRNA level in mammary glands of 5-week-old BALB/c mice. Four-week-old mice were injected daily for 7 days with the indicated doses of estradiol (E; 0.2, 2, 20 μ g) and 400 μ g progesterone (P400). The level of *KGFR* mRNA expression is normalized to the vehicle control group (100%). Bars represent means $\pm$ S.E.M. from three experiments ($n=9$). Significant differences between progesterone only and other hormone-treated groups are indicated by asterisks ($P<0.05$, by Student–Newman–Keuls test).

1998, Saji *et al.* 2000, Warner *et al.* 2000) and estradiol is unable to directly affect proliferation or *KGFR* expression in cultured mammary epithelial cells (Pedchenko & Imagawa 1998). Because estradiol may target the stroma to indirectly stimulate mammary epithelial cell proliferation (Cunha *et al.* 1997, 2000), we hypothesized that KGF is a potential estradiol-regulated stromal growth factor (Pedchenko & Imagawa 2000a). We have shown previously that estradiol treatment *in vivo* causes an elevation in KGF mRNA and protein in mammary stroma (Pedchenko & Imagawa 2000a). This raised the possibility that estradiol could affect ductal growth by upregulating the level of a stromal morphogen shown to be mitogenic towards mammary epithelium in primary culture (Imagawa *et al.* 1994a, Pedchenko & Imagawa 1998) and *in vivo* (Ulich *et al.* 1994). However, we find that raising the level of estradiol alone *in vivo* profoundly inhibits *KGFR* expression. This effect of estradiol appears to be on *KGFR* transcription since it is relatively rapid and occurs in the absence of any equivalent reduction in epithelial cell number. Nuclear run-off assays may reveal direct transcriptional repression. For the peripubertal animals used in this study, this estradiol response is most likely not significantly affected by endogenous ovarian hormones since circulating estradiol and progesterone levels are very low at this age (Atwood *et al.* 2000). In addition, control glands

exhibited limited ductal elongation (approximately 50% or less of the fat pad contained mammary parenchyma) by 5 weeks, and the response was also observed in ovariectomized mice. In mature, cycling animals the effect of exogenous estradiol occurs on a background of circulating ovarian-derived estradiol and progesterone (which is reflected in the vehicle-treated control animals). Nonetheless, in these animals a similar inhibitory effect of estradiol on *KGFR* expression was observed in these two age groups.

The ability of estradiol to stimulate ductal growth in 4-week-old mice while inhibiting *KGFR* expression suggests that estradiol can induce *KGFR*-independent ductal growth in peripubertal mice. This conclusion assumes that *KGFR* protein declines as well which is expected since the half-life of membrane receptors is relatively short. We have unsuccessfully attempted to examine the level of KGF binding to membrane preparations from mammary glands of vehicle and estradiol-treated mice. Binding assays have been inconclusive due to high background binding, probably reflecting the extensive binding of KGF to heparin in cell-associated glycosaminoglycans. Studies examining the effect of a dominant negative *KGFR* in a transgenic mouse model also showed that ductal growth could occur but that alveolar development was inhibited (Jackson *et al.* 1997b).

In contrast to the effect of estradiol, progesterone injection into peripubertal mice caused a significant (two-fold) increase in the level of *KGFR* mRNA resulting predominantly from an increase in *KGFR*-expressing epithelial cells, reflecting the stimulation of ductal growth. This interpretation is based upon the long time-course needed to observe the effect and correlation with a progesterone dose that stimulated ductal branching and an increase in DNA content of the gland. Also, in mature mice, the effect of progesterone on *KGFR* expression was reduced compared with peripubertal mice, probably due to the relatively smaller effect of progesterone alone on ductal growth in the quiescent fat pad.

Previously we obtained *in vitro* observations showing that progesterone, but not estradiol or prolactin, increased *KGFR* mRNA stability (Pedchenko & Imagawa 1998). Do the present data suggest that progesterone can also elevate *KGFR* mRNA levels *in vivo* by this or other mechanisms? This appears to be the case as revealed by the response to the injection of progesterone and estradiol combined. When both estradiol and progesterone were elevated, *KGFR* mRNA did not decrease. Because this hormonal combination, at the tested doses and time-course, did not further stimulate ductal growth, we interpret this effect as an inhibition of the estradiol response rather than the result of a compensatory increase in the number of *KGFR*-expressing cells. Thus, while progesterone alone does not seem to stimulate *KGFR* transcription, it can stabilize *KGFR* mRNA or possibly inhibit the effect of estradiol on *KGFR* transcription

through another mechanism. In a similar vein, direct or indirect antagonism of estradiol action by progesterone has been described for estradiol induction of mammary gland progesterone receptors during pregnancy (Shyamala *et al.* 1990, 1997).

When estradiol is delivered in the absence of progesterone it suppresses KGFR synthesis. This effect is a demonstration of principle but its physiological relevance is more complex. It is clear that the synthesis of the KGFR is dependent upon the ratio of estradiol and progesterone. Variation in the level of KGFR mRNA during different stages of mammary gland development could reflect this (Pedchenko & Imagawa 2000b). KGFR expression is highest at 2–3 weeks of age and decreases through the peripubertal period when animals begin cycling. It rises again in mature animals when progesterone exposure increases. KGFR expression in extracts of total mammary gland RNA is low during pregnancy which likely reflects lower expression in alveolar cells that is independent of the hormonal milieu of pregnancy (Pedchenko & Imagawa 2000b) and consistent with a vastly reduced mitogenic response of alveolar cells to KGF *in vitro* (W Imagawa & V K Pedchenko, unpublished observations). We can speculate that when estradiol and progesterone are both elevated (as during pregnancy), the level of KGFR in ductal epithelium is maintained while KGF synthesis is stimulated. These combined effects of estradiol and progesterone support the second phase of pregnancy-stimulated ductal proliferation which is necessary to support full alveolar development. This could explain why alveolar development, which is stimulated by estradiol in combination with progesterone, is blocked when KGFR function is attenuated by expression of dominant negative receptors (Jackson *et al.* 1997a,b). Others have discussed a role for progesterone in the pregnancy phase of ductal growth (Atwood *et al.* 2000). This interaction between estradiol and progesterone may also have implications for preneoplastic progression and tumorigenesis. If estradiol and progesterone interactions can become unbalanced upon the loss of estradiol or progesterone receptor expression or alterations in their signaling pathways, estradiol or progesterone dominance could then shift the balance of growth control to an altered state of abnormal growth factor or growth factor receptor function.

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