Growth factors in ovarian function

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The studies of the effects of epidermal growth factor (EGF) and fibroblast growth factor (FGF) on bovine granulosa cells under chemically defined conditions *in vitro* first indicated that these growth factors have a role in stimulation of follicle cell proliferation (Gospodarowicz *et al.*, 1977). Even now, however, relatively few data in respect of the mitogenic action of these growth factors on follicle cells are available and most studies have concentrated on the role of growth factors in control of functional differentiation of follicle cells. This probably reflects the technical and interpretive difficulties associated with stimulation of follicle cell mitosis under defined conditions.

It is now evident that a range of growth factors influence not only proliferation but also functional differentiation of ovarian follicle cells. The growth factors of specific interest are the insulin-like growth factor-type I (IGF-I), transforming growth factor-type β (TGF- β), EGF and platelet-derived growth factor (PDGF).

Growth factors in the context of follicular development

Before ovulation is possible, the theca and granulosa cells of pre-antral and small antral follicles must undergo extensive proliferation and functional differentiation (Hsueh *et al.*, 1984). Based on relative numbers of receptors for pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH), granulosa cells of small antral follicles are thought to be primarily FSH-dependent. As follicular development proceeds, the number of LH receptors on granulosa cells is increased many fold and numbers of FSH receptors are reduced. Throughout development of the ovulatory follicle, theca cells respond to LH only (Richards *et al.*, 1976). Although aromatase activity is present in small antral follicles, oestrogen production at this stage of development is limited by an inability to produce the androgen substrate required for aromatization to oestrogen (Carson *et al.*, 1981). Growth beyond the small antral phase is therefore characterized by increased aromatase, androgen synthesis, and therefore follicular oestrogen production.

Functional differentiation of follicle cells during development is evident also in increased follicular production of inhibin, a follicular protein which inhibits selectively pituitary FSH secretion (Burger *et al.*, 1988).

Increased follicular secretion of oestradiol and inhibin is not only the hallmark of developing follicles, but also a regulator of pituitary gonadotrophin secretion. Together with changes in follicular sensitivity to pituitary gonadotrophins, follicular inhibin secretion is thought to mediate selection and dominance of the few ovulatory follicles. Factors which control follicular inhibin and oestradiol secretion and mitosis of follicle cells must therefore be considered important in regulation of follicular development.

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Growth factors, follicle cell differentiation and mitosis

Serum of calf fetuses and other biological preparations which have been used traditionally for maintenance of cell growth *in vitro* are thought to contain a variety of mitogens and growth factors. Studies of the effects of specific growth factors on ovarian cells must therefore be conducted in defined media in the absence of sera and other 'maintenance factors'.

Our studies of the effects of exogenous growth factors on follicle cells have been based on granulosa cells isolated from the ovaries of immature female rats treated with diethylstilboestrol *in vivo* (Richards, 1975). These cells were maintained in McCoy's 5A medium supplemented with glutamine (2 mM), non-essential amino acids and antibiotics. Exogenous growth factors were added as indicated below.

Aromatase activity and inhibin production were used as indicators of functional differentiation of granulosa cells after 48 h. Aromatase activity was measured radiometrically by conversion of [1,2-³H]testosterone to oestradiol-17 β and ³H₂O. In separate experiments, inhibin content of culture media was measured by pituitary cell bioassay (Zhang *et al.*, 1987a) or specific radioimmunoassay (McLachlan *et al.*, 1986).

IGF-I (somatomedin-C)

Addition of IGF-I (Sm-C) either alone or together with a constant dose of FSH (300 ng/ml) to rat granulosa cells resulted in dose-dependent increases in inhibin production over 48 h (Fig. 1). Although the dose of FSH used had previously been shown to give maximal stimulation of inhibin production, the response to FSH was further increased at all doses of IGF-I. This could possibly be due to enhancement of FSH-induced adenylate cyclase activity (Adashi *et al.*, 1986a), but the effects of FSH and IGF-I were not additive, which suggested that each agent acted through different cellular mechanisms (Zhang *et al.*, 1987b).

Whereas addition of pregnant mares' serum gonadotrophin (PMSG) to cultures resulted in significant increases in both aromatase activity and progesterone production, IGF-I at doses up to 60 ng/ml was without significant effect (Fig. 2). However, when IGF-I was added at a range of doses there was a dose-dependent increase in both steroidogenic activities above that observed in the presence of PMSG alone. These findings are consistent with results of similar studies reported by Adashi *et al.* (1985a).

Together with the ability of IGF-I to stimulate granulosa cell oxytocin secretion (Schams *et al.*, 1988), side-chain cleavage-P450 (Veldhuis *et al.*, 1986), low density lipoprotein and sterol metabolism (Veldhuis & Rogers, 1987; Veldhuis *et al.*, 1987), and FSH-induced adenylate cyclase activity (Adashi *et al.*, 1986a) and LH receptor induction (Adashi *et al.*, 1985b), these data establish that IGF-I is able to modulate basal and FSH-induced granulosa cell function.

The role of IGF-I in control of granulosa cell proliferation is not clear. Although IGF-I stimulated mitosis of cattle (Savion *et al.*, 1981) and pig (Baranao & Hammond, 1984a) granulosa cells, no effect on rat granulosa cell number was observed *in vitro* (Adashi *et al.*, 1984). IGF-I prevention of FSH-induced reduction in [³H]thymidine incorporation into rat granulosa DNA would, however, be consistent with a positive effect on rat granulosa cell mitosis (Adashi *et al.*, 1985c).

These effects of IGF-I are presumed to be mediated directly by specific receptors for IGF-I on granulosa cells. Baranao & Hammond (1984a) first demonstrated IGF-I binding to pig granulosa cells and IGF-I binding sites on rat granulosa cells were subsequently characterized (Davoren *et al.*, 1986; Adashi *et al.*, 1988a). Furthermore, IGF-I binding activity was increased by exposure of granulosa cells to FSH *in vitro* (Adashi *et al.*, 1986b) and *in vivo* (Adashi *et al.*, 1988b) through a cAMP-dependent mechanism (Adashi *et al.*, 1988c).

The physiology of IGF-I in follicular development is further complicated by observations that the granulosa cell itself produces IGF-I under the action of growth hormone (Davoren & Hsueh,

Fig. 1. Effect of IGF-I (Sm-C) on inhibin production by rat granulosa cells *in vitro*. Granulosa cells were isolated from diethylstilboestrol-treated immature rats and cultured for 48 h with IGF-I (Sm-C) alone or in combination with FSH (100 ng/ml). Values represent the mean $\pm$ s.e. of triplicate determinations. (Reproduced from Zhang *et al.* 1987b.)

1986), FSH and oestradiol-17 β (Hammond *et al.*, 1985; Hsu & Hammond, 1987) and that IGF-I is able to stimulate the thecal androgen production upon which follicular oestradiol synthesis is dependent (Hernandez *et al.*, 1988).

IGF-I secreted by granulosa cells therefore maximizes FSH-dependent differentiation by potentiating the trophic effects of FSH on aromatase and stimulation of thecal androgen synthesis. The physiological significance of this role for IGF-I is emphasized by the apparent ability of FSH to maintain responsiveness to IGF-I through increased IGF-I receptor activity.

Transforming growth factor-type β (TGF- β), inhibin and activin

As with IGF-I, TGF- β is seen also to have a positive effect on granulosa cell differentiation *in vitro*. FSH-dependent induction of granulosa receptors for both LH (Knecht *et al.*, 1986; Dodson & Schomberg, 1987) and EGF (Feng *et al.*, 1986) are potentiated by TGF- β *in vitro*. While TGF- β at concentrations up to 16 pM did not alter significantly basal aromatase or progesterone synthesis in rat granulosa cells, FSH-induced aromatase activity was enhanced in a dose-dependent manner (Hutchinson *et al.*, 1987; Fig. 3). In similar experiments, Ying *et al.* (1986) also reported an enhancement of FSH-induced aromatase in rat granulosa cells *in vitro*. The positive effect of TGF- β on granulosa cell differentiation was not limited to enhancement of FSH-induced activity; inhibin production was significantly greater than control (no exogenous hormones) when TGF- β was added to cultures of granulosa cells at a concentration of 1 ng/ml (Fig. 4). Addition of both FSH (1 ng/ml) and TGF- β (1 ng/ml) resulted in inhibin production which exceeded the sum of that

Fig. 2. Effect of IGF-I on basal and PMSG-induced aromatase activity and progesterone production by rat granulosa cells *in vitro*. Cells were cultured for 3 days with IGF-I in the presence or absence of PMSG (100 mU/ml). Values represent the mean $\pm$ s.d. of triplicate cultures. (Reproduced from Hutchinson *et al.*, 1987.)

produced in the presence of either hormone alone, suggesting that the effect of each hormone was mediated by distinct mechanisms (Zhang *et al.*, 1988).

Although TGF- β stimulates proliferation of mesenchymal cells, TGF- β inhibits mitosis of EGF-responsive epithelial cells (Moses *et al.*, 1985). Granulosa cells are of an epithelial cell type which responds to EGF with increased mitotic activity (Gospodarowicz *et al.*, 1977) and to TGF- β with reduced mitosis (Skinner *et al.*, 1987a). A preliminary report that expression of the TGF- β gene by ovarian tissue is stimulated up to 4-fold by FSH (Hernandez *et al.*, 1987) further suggests that ovarian TGF- β has a positive effect on follicular development by maximizing follicular responses to the trophic action of FSH and inhibition of EGF-induced mitosis.

The subunit which constitutes the homodimer which is TGF- β (Mason *et al.*, 1985) exhibits sufficient structural homology with the B-subunit of inhibin to indicate that TGF- β , inhibin and related dimers are products of the same family of genes (Tsonis & Sharpe, 1986, for review). As with TGF- β , inhibin and dimers of the inhibin B-subunit are also able to modulate granulosa and theca cell function *in vitro*.

While activin (the homodimer of the inhibin B-subunit) augments granulosa cell aromatase activity and inhibits FSH-induced progesterone synthesis (Hutchinson *et al.*, 1987), inhibin has no significant effect on basal or FSH-induced aromatase activity and progesterone synthesis at doses

Fig. 3. Effect of TGF- β on aromatase activity and progesterone production by rat granulosa cells *in vitro*. Cells were cultured for 3 days with TGF- β in the absence (open symbols) or presence of FSH (50 ng/ml; solid symbols). Values represent the mean $\pm$ s.d. of triplicate cultures. (Reproduced from Hutchinson *et al.*, 1987.)

up to 4 pM (Hutchinson *et al.*, 1987). Ying *et al.* (1986) report an inhibition of FSH-induced aromatase in the presence of inhibin at higher doses.

Basal androstenedione secretion by theca cells was not altered significantly by inhibin or activin *in vitro* (Hsueh *et al.*, 1987). Exogenous LH did result in a dose-dependent increase in androstenedione secretion and this was further enhanced by simultaneous addition of inhibin (1–30 ng/ml). Addition of activin resulted in a dose-dependent suppression of LH-induced androstenedione secretion (Hsueh *et al.*, 1987).

TGF- β , inhibin and activin are therefore products of the same gene family and have both positive and negative effects on FSH-dependent granulosa cell function and LH-dependent androstenedione production by theca cells.

Epidermal growth factor (EGF)

Whereas IGF-I and TGF- β enhance granulosa cell differentiation *in vitro*, EGF is generally inhibitory. EGF inhibits basal inhibin secretion as well as FSH-, 8-bromo-cAMP- and prostaglandin E₂-induced inhibin secretion by rat granulosa cells *in vitro* (Zhang *et al.*, 1987a) (Fig. 5).

Fig. 4. Effect of TGF- β and FSH on inhibin production by rat granulosa cells *in vitro*. Granulosa cells were isolated from diethylstilboestrol-treated immature rats and cultured for 1–4 days in medium alone (control) or in the presence of TGF- β (1 ng/ml), FSH (1 ng/ml) or TGF- β and FSH together. Values represent the mean $\pm$ s.e. for triplicate determinations. (Reproduced from Zhang *et al.*, 1988.)

This was consistent with earlier demonstrations of EGF-suppressed inhibin secretion by bovine granulosa cells maintained in high concentrations of fetal calf serum (Franchimont *et al.*, 1986) and rat granulosa cells (Bicsak *et al.*, 1986), although no effects on basal inhibin secretion were reported in the latter studies. These observations indicate that EGF inhibits granulosa cell function at a point subsequent to cAMP formation.

The inhibitory effect of EGF on granulosa cell function is further exemplified by inhibition of FSH-induced adenylate cyclase (Dodson & Schomberg, 1987), LH receptor activity (Mondschein & Schomberg, 1981; Knecht & Catt, 1983a; May *et al.*, 1987) and of FSH-induced aromatase activity (Hsueh *et al.*, 1981; May *et al.*, 1982). These effects are presumably mediated by specific granulosa cell receptors for EGF (Chabot *et al.*, 1986). High levels of EGF receptor activity on granulosa cells during pro-oestrus and oestrus (Mondschein & Schomberg, 1981) most probably reflect elevated concentrations of FSH in serum at these stages of the oestrous cycle as EGF receptor activity on granulosa cell is increased by exposure to FSH (Feng *et al.*, 1986).

EGF is able to act both directly and indirectly on granulosa cells to modulate differentiated function. EGF at concentrations of 0.1–100 ng/ml inhibited FSH and TGF- β -induced inhibin secretion. Most evident was complete blockade of the enhancement by TGF- β of FSH-induced inhibin productivity (Fig. 6).

Platelet-derived growth factor (PDGF)

Originally derived from human platelets PDGF was shown to be a potent mitogen for cells of mesenchymal origin (Deuel & Huang, 1983). Using endogenous ornithine decarboxylase activity as a measure of proliferative activity, the decarboxylase activity in pig granulosa cells could only be stimulated in the presence of a relatively crude platelet extract (Baranao & Hammond, 1984b).

Fig. 5. Effect of EGF on stimulated inhibin production *in vitro*. Granulosa cells were isolated from diethylstilboestrol-treated immature rats and cultured in medium alone (control), FSH, 8-bromo-cyclic AMP or PGE-2. Replicate cultures were established in the absence of EGF or increasing concentrations of exogenous EGF (1–100 ng/ml). Values represent the mean $\pm$ s.e. of triplicate determinations. (Reproduced from Zhang *et al.*, 1978a.)

Addition of purified PDGF to these cells was without effect, which implied either that PDGF did not stimulate granulosa cell ornithine decarboxylase activity, or that PDGF action required the presence of other unidentified components of platelet extracts. Purified PDGF was subsequently shown to enhance FSH-induced progesterone secretion by granulosa cells, adenylate cyclase activity (Knecht & Catt, 1983b) and LH receptor induction (Knecht & Catt, 1983b; Mondschein & Schomberg, 1984).

Possible relationship between mitosis and differentiation of granulosa cells

Although the effects of PDGF and IGF-I on granulosa cell mitosis are uncertain, TGF- β inhibits EGF-stimulated proliferation of bovine granulosa cells (Skinner *et al.*, 1987b). In contrast, EGF stimulates proliferation of granulosa cells *in vitro* (Gospodarowicz *et al.*, 1977; Gospodarowicz & Bialecki, 1979; Skinner *et al.*, 1987a). These observations, together with the respective positive and negative effects of TGF- β and EGF on granulosa cell differentiation, suggest an inverse relationship between mitosis and differentiation of granulosa cells (Fig. 7).

Granulosa cells proliferate in media containing sera but cannot be induced to undergo functional differentiation under these conditions. Differentiation of granulosa cells and maintenance of a differentiated state *in vitro* is possible only in chemically-defined media which do not support mitosis (Orly *et al.*, 1980; Savion *et al.*, 1981; Erickson, 1983; Epstein-Almog & Orly, 1985). The postulate that mitosis and differentiation of granulosa cells is related inversely (Chevalier *et al.*, 1981) is further supported by the observations that progesterone synthesis by

Fig. 6. Effect of EGF on inhibin secretion stimulated by FSH or TGF- β . Granulosa cells were isolated from diethylstilboestrol-treated immature rats cultured in the presence of FSH (1 ng/ml), TGF- β (1 ng/ml) and FSH together with TGF- β . Replicate cultures were established in the absence of exogenous EGF or increasing concentrations of EGF (0.1–100 ng/ml). Values represent the mean $\pm$ s.e. of triplicate cultures. (Reproduced from Zhang *et al.*, 1988.)

Growth factor	Mitosis	Differentiation
IGF-I	↓	↑
TGF- β	↓	↑
PDGF	?	↑
EGF	↑	↓

Fig. 7. Summary of the effects of insulin-like growth factor (IGF-I), transforming growth factor-type β (TGF- β), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) on granulosa cell function *in vitro*. $\uparrow$, Stimulation; $\downarrow$, inhibition; $?$, uncertain.

granulosa cells was inversely related to the spontaneous changes in mitotic activity which occurred over a 12-day culture (Epstein-Almog & Orly, 1985) and to stimulation of pig granulosa cells by theca-derived mitogens (Makris & Ryan, 1987; Skinner *et al.*, 1987b).

The apparent coincidence of FSH-induced differentiation with the period of maximal mitotic activity of granulosa cells in small and medium-sized antral follicles (Hirshfield & Midgley, 1978) does not necessarily argue against an inverse relationship between mitosis and differentiation in developing follicles. It is possible that individual granulosa cells enter asynchronously successive periods of mitosis and differentiation when de-differentiation of a cell is mandatory for each mitosis. As an increasing proportion of granulosa cells is prevented from re-entering successive periods of de-differentiation/mitosis, reduced mitotic activity would accompany ‘differentiation’ of the whole population of granulosa cells.

Thus both proliferation and differentiation would occur in an entire population of granulosa cells while an inverse relationship between mitosis and differentiation was maintained at the cellular

Fig. 8. Putative interaction of theca-derived growth factors with granulosa cells.

level. The antimitotic effect of TGF- β (see above) and inhibitors of mitosis present in the antral fluid of large sheep follicles (Carson *et al.*, 1988) would therefore be necessary adjuncts to trophic factors which induce differentiation of follicle cells.

These antimitotic effects are possibly exerted on granulosa cells directly by blocking the mitogenic effects of EGF and follicular oestradiol-17 β , or by inhibition of thecal EGF activity.

Theca cells as a source of ovarian growth factors

The demonstration that proliferation *in vitro* could be stimulated by addition of thecae to cultures of human granulosa cells (McNatty *et al.*, 1980) suggested that the mitogen present in steroid-free pig follicular fluid (Ledwitz-Rigby, 1980) might have been of thecal origin. Stimulation of granulosa cell mitosis by extracts of pig theca (Makris *et al.*, 1983) and culture media conditioned by rat thecae (Lobb *et al.*, 1988) and of sheep pituitary cells by media conditioned by chicken thecae (Tsonis *et al.*, 1988) confirmed the mitogenic action of thecae derived from several species. It is likely that this activity represents an EGF-like molecule identified in rat thecae (Skinner *et al.*, 1987a). On the basis of radioimmunoassay, radioreceptor and biological assay and electrophoretic mobility, rat thecae are also known to secrete TGF- β *in vitro* (Skinner *et al.*, 1987b).

As discussed above, while EGF inhibits differentiation and stimulates mitosis of granulosa cells, TGF- β stimulates differentiation and inhibits mitosis. Theca cells are therefore a source of two growth factors capable of modulating granulosa cell proliferation and differentiation (Fig. 8). As such it is likely that theca cells mediate the effect of trophic factors, possibly LH, on follicular development.

Conclusions

On the basis of changes in steroidogenic activity and secretion of specific proteins *in vitro*, IGF-I, TGF- β and related proteins, and PDGF enhance FSH-induced differentiation of granulosa cells *in vitro*. EGF is seen generally to be inhibitory of FSH-induced differentiation. For EGF and TGF- β ,

the effect of each growth factor on granulosa cell proliferation is the opposite of the effect of each on differentiation, suggesting an inverse relationship between differentiation and mitosis of granulosa cells.

Data indicate that the follicular theca cell is a source of EGF activity and of TGF- β . In that each of these growth factors has opposing effects on the granulosa cell, the theca most probably modulates granulosa cell mitosis and differentiation *in vivo*.

Proliferation and differentiation of granulosa cells are the essence of ovarian follicular development. Although these processes were thought originally to be controlled primarily by the pituitary gonadotrophins, evidence for direct involvement of intra-follicular growth factors in control of follicular development is overwhelming.

Follicular oestradiol, a major product of differentiated granulosa cells, a potent mitogen *in vivo* and yet devoid of any mitogenic action *in vitro*, represents a paradox in this discussion. Studies of the role of growth factors in ovarian function will lead to a more complete understanding of the control of follicular development by intra-follicular growth factors and, perhaps, the role of oestradiol in the growing follicle.

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