

Cellular distribution of growth hormone-releasing hormone receptor in human reproductive system and breast and prostate cancers

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Summary. Growth hormone releasing hormone receptor (GHRH-R) mRNA and protein was first localized to the anterior pituitary gland, consequent with the action of its ligand on GH synthesis and release. Subsequent studies found GHRH-R also expressed in the hypothalamus and in systemic tissues including those of the reproductive system. In the present work, we studied the distribution of GHRH-R in human reproductive system of males and females by immunohistochemical method. GHRH-R immunostaining was localized in male reproductive system: Leydig cells, Sertoli and basal germ cells of the seminiferous tubules and prostate secretory cells. GHRH-R immunostaining was also demonstrated in the ovary: oocytes, follicular cells, granulosa, thecal and corpus luteum cells. Endometrial glands, placenta and normal mammary glands also showed GHRH-R immunostaining. Our results demonstrate the localization of GHRH-R in the reproductive system, which may mediate the direct action of GHRH in these tissues. Moreover, GHRH-R was demonstrated in prostate and breast carcinomas, opening a variety of possibilities for the use of GHRH antagonists in the treatment of prostatic and mammary tumors.

Key words: GHRH receptor, Reproductive system, Cancer, Human

Introduction

The hypothalamic peptide growth hormone releasing hormone (GHRH) stimulates GH gene transcription, synthesis and secretion by somatotropes. GHRH also acts on somatotrophic cells to stimulate their proliferation during development (Mayo et al., 2000; Zeitler and

Siriwardana, 2000). GHRH may have direct actions in fetal and placental development, reproduction and immune function as well. These direct effects may be achieved by hypothalamic or extrahypothalamic GHRH (Campbell and Scanes, 1992).

GHRH receptor (GHRH-R) belongs to the secretin/glucagon/vasoactive intestinal peptide B-III subfamily of G-protein coupled receptors (Laburthe et al., 1996; Mayo et al., 1996). In the pituitary gland, its expression was demonstrated in rat somatotropes (Morel et al., 1999). Nevertheless, GHRH-R mRNA has also been demonstrated in different extrapituitary rat tissues by RT-PCR (Guarcello et al., 1991; Bagnato et al., 1992; Matsubara et al., 1995; Takahashi et al., 1995; Mayo et al., 1996), pituitary and kidney (medulla and renal pelvis) showing the highest GHRH-R expression (Matsubara et al., 1995).

GHRH-R was also demonstrated in human anterior pituitary (Morel et al., 1999) and pituitary adenomas (Lopes et al., 1997; Matsuno et al., 2000). Although GHRH-R was localized in somatotropes, its expression seems not to be restricted to GH secreting pituitary tumors (Hashimoto et al., 1995; Tang et al., 1995; Lopes et al., 1997; Matsuno et al., 2000). Moreover, Fujinaka et al. (1996) demonstrated GHRH-R in human kidney but they did not find any expression in other tissues including liver, ovary or placenta.

Effects of GHRH on reproductive tissues under physiological conditions were described. (e.g. granulosa cell differentiation and oocyte maturation, testosterone production). GHRH acts on granulosa cells to promote differentiation and oocyte maturation (Moretti et al., 1990). On the other hand GHRH act in the modulation of spermatogonia maturation exerted by Sertoli cells (Fabbri et al., 1995). It also increases Leydig cell sensitivity to the gonadotropin stimulation of cAMP and testosterone production (Ciampini et al., 1992). On the other hand, GHRH antagonists inhibit the proliferation of various reproductive tumors including prostatic

(Jungwirth et al., 1997; Lamharzi et al., 1998; Rekasi et al., 2000b, 2001), ovarian (Kahan et al., 2000; Chatzistamou et al., 2001b,c), endometrial (Kahan et al., 2000) and breast carcinomas (Kahan et al., 2000; Chatzistamou et al., 2001a).

The aim of the present work was to study the localization of GHRH-R in human male (testis and prostate), and female (ovary and uterus) reproductive systems, as well as in mammary gland, by immunohistochemical methods using a specific antibody against GHRH-R. Immunoreactivity for GHRH-R was investigated in prostate and mammary carcinomas. Moreover, GHRH-R mRNA expression was also studied in uterus, mammary and prostate glands, prostate and mammary carcinomas.

Materials and methods

Normal human and tumor specimens were collected from the files of the Clinical University Hospital of Santiago de Compostela (Prof. J. Forteza). The local internal review board approved the collection and use of these specimens. Testis, prostate, breast, ovary and uterus (endometrium at the proliferative and secretory stage, and atrophic endometrium) (n=6 each) were collected from recent autopsies (less than 8 h postmortem) and healthy margins of surgical specimens (men aged 20-67 and women from newborn to 65 years old). Surgical samples of prostatic adenocarcinoma (n=6), and breast carcinomas: ductal carcinoma (*in situ* and invasive) and lobular carcinoma (n=6) were also collected. Moreover, adult rat (Wistar) ovaries were also obtained in order to study the expression of GHRH-R during the development of the ovarian follicles.

Tissues were immersion fixed in 10% buffered formalin for 24 h, dehydrated and embedded in paraffin. Sections 4 µm thick were collected on Histobond adhesion microslides (Marienfeld, Landa-Königshofen, Germany).

The GHRH-R polyclonal antibody was produced and tested as previously described (Boulanger et al., 1999) and recognizes the specific amino acid sequence 392-404 of the rat pituitary GHRH-R. This C-terminal intracellular region of the rat GHRH-R exhibits 85% sequence identity with the human GHRH-R. For immunohistochemical staining, sections were consecutively incubated with: 1) 8.85 mg/mL anti-GHRH-R (392-404) polyclonal antibody in 100 mM mono-di-phosphate buffered saline 150 mM NaCl, pH 7.4 (PBS) overnight at 4°C; 2) H₂O₂ 3% for 10 min; 3) horseradish peroxidase-conjugated dextran polymer system containing anti-rabbit secondary antibodies (Envision, Dako, Glostrup, Denmark) for 30 min; 4) 0.1% 3,3'-diaminobenzidine tetrahydrochloride (Dako) for 10 min. Between steps, the sections were washed twice for 10 min periods in PBS, and after step 4 with distilled water. Counterstaining was done with haematoxylin 30 s.

Controls included: 1) preadsorption of GHRH-R

antibody with the homologous antigen (peptide segments 392-404 of rat GHRH-R), 2) substitution of the primary anti-GHRH-R antibody with normal rabbit serum at the same concentration as the primary antibody; 3) alternate use of PBS in place of each essential step of the technique. Liver was used as negative tissue control.

Probes

To detect human GHRH-R mRNAs two 30 oligodeoxyribonucleotide probes were synthesized (Eurobio, Les Ulis, France). The probes were complementary to nucleotides 402-431 (GAG AAG TAA GAT TCC TCC TCA GCC AGC AGC), and 1305-1334 (GAT GAG GCA GCC TAG CAC ATA GAT GTC AGC) according to the reported GHRH-R complementary DNA sequence (NM 000823). The probes were 3' end-labeled with digoxigenin-11-dUTP (Roche, Meylan, France) and purified as previously described (Reecher et al., 2001).

In situ hybridization

Deparaffinized and rehydrated sections were digested with 3 mg/mL proteinase K (Roche) in a Tris (20 mmol/L)-CaCl₂ (2 mmol/L) buffer for 15 min at 37°C and postfixed in paraformaldehyde for 10 min. The slides were dehydrated in ethanol series and air-dried. Sections were then covered with hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 4X standard saline citrate (SSC) (1X SSC = 0.15 mol/L NaCl, 0.03 mol/L sodium citrate, pH 7.0), 1X Denhardt's solution (50X Denhardt's solution = 1% BSA, 1% Ficoll 400, 1% polyvinylpyrrolidone), 250 µg/mL yeast transfer RNA, and labels probes (30 pmol/mL of hybridization buffer). *In situ* hybridization was performed overnight at 40°C. Sections were washed twice in 2X SSC for 30 min at room temperature. Immunodetection of the hybrids was performed with alkaline phosphatase-conjugated anti-digoxigenin (Roche) and using NBT-BCIP as chromogen (Roche). No counterstain was performed. Controls of specificity of the *in situ* hybridization included: 1) hybridization with an excess of unlabeled probe at a ratio of 200:1; and 2) hybridization with a labelled oligonucleotide-sense probe.

Results

GHRH-R immunoreactivity was demonstrated in different cells of the human reproductive system (Table 1). Human testis (Fig. 1a) showed GHRH-R immunoreactivity in the interstitial Leydig and Sertoli cells. Immunoreactive cells of spermatogenic series were also observed in the base of seminiferous tubules. Controls led us to confirm the specificity of the primary antibody and the technique, i.e. no positive immunoreaction was observed in the negative controls (Fig. 1b) or in the negative tissue control (liver) (not

shown). In the normal prostate gland, secretory cells displayed a nuclear GHRH-R immunostaining (Fig. 1c). Prostatic carcinomas studied were positive for GHRH-R. Immunoreactivity was localized not only in the nuclei but also in the cytoplasm of tumor cells (Fig. 1d).

Human ovary showed GHRH-R immunostaining in the oocytes and follicular cells of primordial and primary follicles (Fig. 2a, b). Granulosa lutein and theca lutein cells of the corpus luteum were also intensely immunostained (Fig. 2c). Primordial, primary and secondary follicles of the rat ovary were positive for GHRH-R as well (Fig. 3a,b). Immunostaining was intense in the cytoplasm and nuclei of rat oocytes throughout all follicular development. As well as in the human ovary, GHRH-R immunoreactivity was intense in the rat corpus luteum (Fig. 3c).

GHRH-R immunoreactivity was demonstrated in the cytoplasm of uterine proliferative endometrial glands (Fig. 4a). A weak immunostaining was also found in the

nuclei of connective cells of the lamina propria and myometrium cells. Immunostaining was very weak in the secretory endometrium (not shown). Moreover, GHRH-R immunoreaction could be seen in glandular epithelia of gestational endometrium (Fig. 4b) and it was absent in atrophic endometrium (Fig. 4c).

First and third trimester placenta showed GHRH-R immunoreactivity in trophoblast; syncytiotrophoblast and cytotrophoblast, at the first trimester (Fig. 5a), and cytotrophoblast at the third trimester (Fig. 5b). Moreover, immunostained cells were observed in the stroma of the villi and endothelial cells of capillaries. Intense cytoplasmic labelling was shown in decidual cells with nuclear envelope reinforcement (Fig. 5c).

Normal human mammary glands showed GHRH-R labelling in ductal epithelium, myoepithelial cells and fibroblasts of lobular loose connective tissue. Positive reaction at the ductal epithelium was localized in the cytoplasm and nuclei (Fig. 6a). All breast carcinomas

Table 1. Summary of the GHRH-R immunohistochemical results, its cellular distribution and a relative intensity of immunoreaction. *In situ* hybridisation is compared with immunohistochemistry.

IMMUNOHISTOCHEMISTRY				IN SITU HYBRIDIZATION
Testis	Spermatogonia	C	+++	Spermatogonia
	Sertoli	C	+++	Sertoli
	Leydig	C	+++	Leydig
Prostate gland	Secretory cells	C/N	- / +++	Secretory cells
Prostate adenocarcinoma	Prostatic adenocarcinoma	C/N	++ / ++	Prostatic adenocarcinoma
Ovary	Oocytes (human)			Oocytes
	Primordial	C	+++	Primordial
	Primary	C	+++	Primary
	Corpus luteum			
	Granulosa lutein cells	C/N	+++ / +	
	Theca lutein cells	C/N	+++ / +	
Uterus	Endometrial glands:			Endometrial glands:
	Proliferative	C	+++	Proliferative
	Secretory	C	+	
	Atrophic		-	
	Gestational	C	+	
Placenta	Decidua	C/N	+++ / ++	Decidua
	Villi			Syncytiotrophoblast of
	First trimester			third trimester placenta
	Cytotrophoblast	C/N	++ / +++	
	Syncytiotrophoblast	C/N	++ / +++	
	Third trimester			
	Syncytiotrophoblast	C/N	++ / +	
Mammary gland	Secretory cells	C/N	+++ / +++	Secretory cells
	Myoepithelial cells	C	+	Myoepithelial cells
Mammary carcinomas	Lobular carcinoma	C/N	+++ / ++	Ductal carcinoma
	Ductal carcinoma	C/N	++ / +++	

C: cytoplasmic GHRH-R immunostaining; N: nuclear GHRH-R immunostaining.

showed strong GHRH-R immunostaining. No differences can be observed concerning the intensity of immunoreactivity between the different histological types of breast carcinomas (Fig. 6b,c). Some cases showed a cytoplasmic immunostaining pattern (Fig. 6b) but immunoreactivity, in other cases, was primarily nuclear (Fig. 6c). Fibroblasts and endothelial cells were positive in both normal tissues and tumors studied.

In order to ascertain the specificity of the immunohistochemical findings, *in situ* hybridization techniques were performed. The results were in good agreement with those found by immunohistochemical techniques. GHRH-R mRNA expression was observed in all tissues studied (some examples were shown in Fig. 7). GHRH-R mRNA was demonstrated in the cytoplasm of secretory cells of mammary (not shown) and prostatic glands (Fig. 7a). Uterine glands and fibroblasts of proliferating (Fig. 7b) and secretory (not shown) endometrium also showed the blue NBT deposit that indicates the GHRH-R expression. Prostatic and

mammary tumors, like normal tissue and as before the immunohistochemistry expressed GHRH-R mRNA, although the positive reaction was localized in the cytoplasm of prostatic and mammary carcinoma cells (Fig. 7c).

Discussion

GHRH-R have been previously demonstrated in different rat and human tissues by Matsubara et al. (1995) and Mayo et al. (1996) but to our knowledge this is the first demonstration of GHRH-R expression in human reproductive system by two complementary morphological techniques (immunohistochemical methods and *in situ* hybridization). GHRH-R immunostaining observed in testis was localized in Leydig cells, Sertoli cells and spermatogonia. Our results agree with previous data obtained in rats by Monts et al. (1996) since they demonstrated that GHRH-R were expressed in all testicular cell types (Leydig,

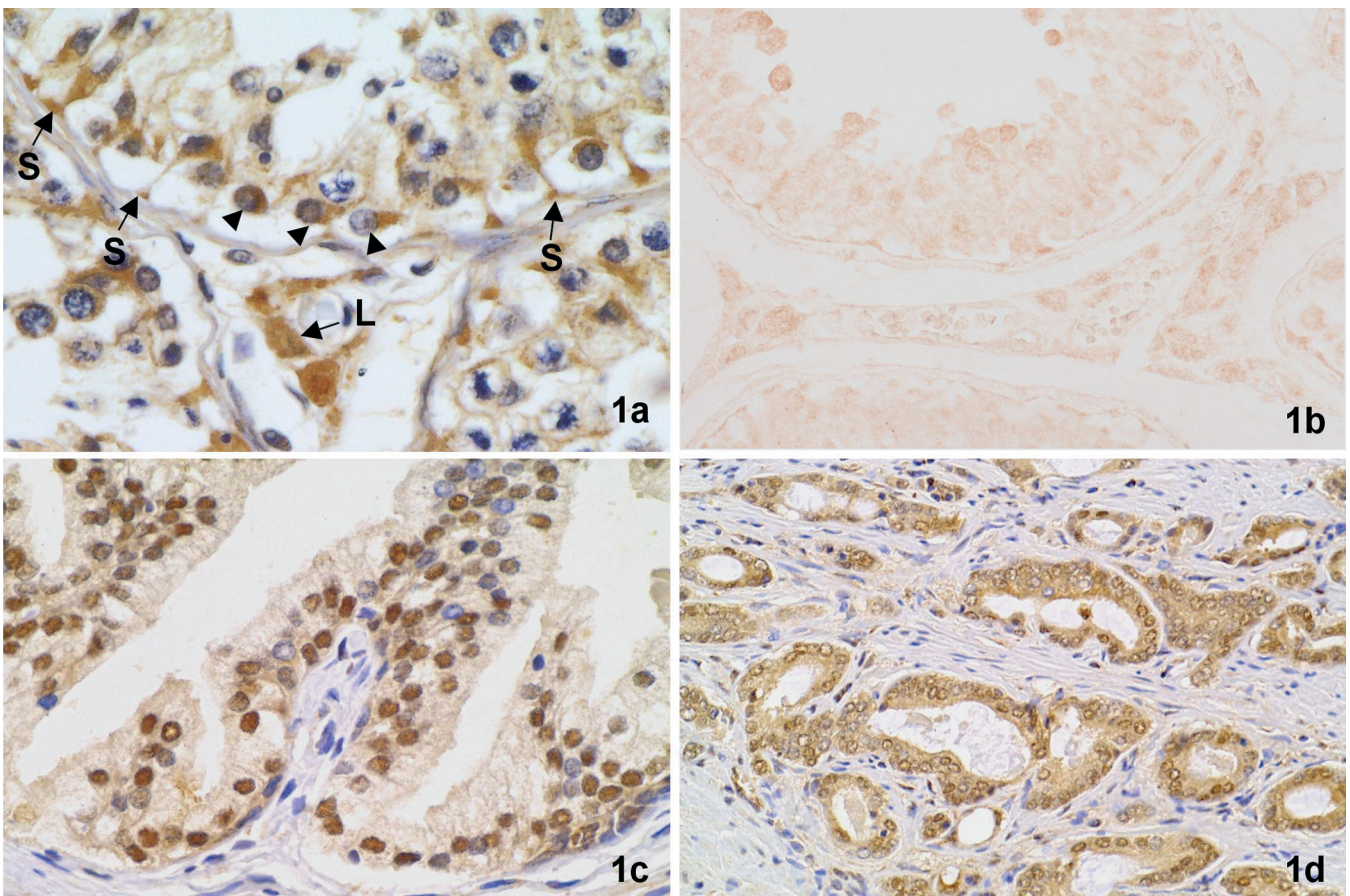


Fig. 1. a. Male reproductive system. Normal human testis (21 years old) immunostained for GHRH-R that shows the intense immunoreaction in the interstitial Leydig cell (L). In the seminiferous tubules Sertoli cells (S) are also immunostained as well as small round cells localized in the basal compartment (spermatogonia) (arrowheads). b. Preabsorption control in a human testis that shows no immunostaining. c. Normal human prostate gland immunostained for GHRH-R. Immunoreaction is mainly found in the nuclei of epithelial cells. d. GHRH-R immunoreactivity in an adenocarcinoma of the prostate. Note that immunohistochemical reaction is localized both in nuclei and cytoplasm of tumour cells. x 300

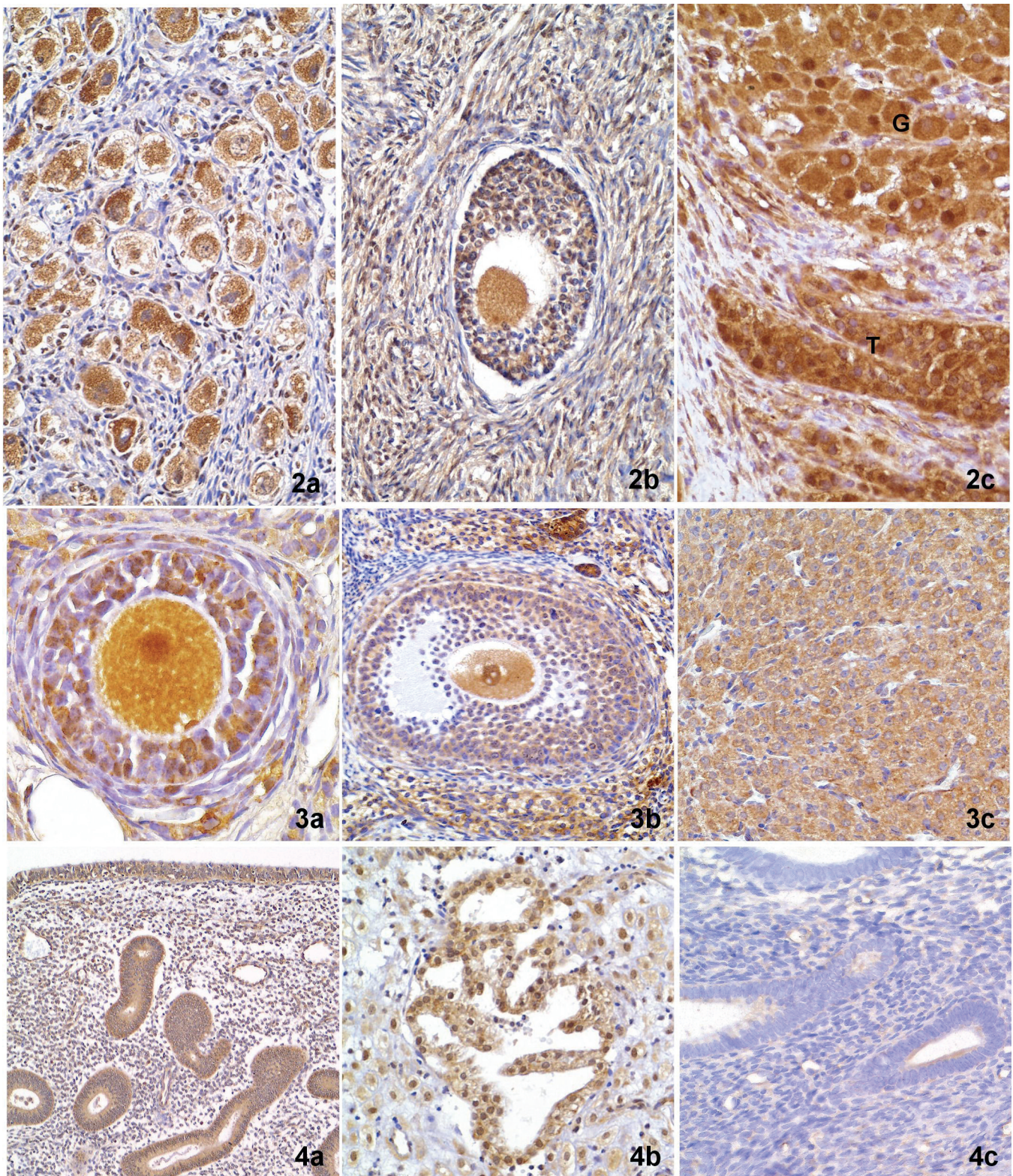


Fig. 2. Human ovary. **a.** Micrograph of a human ovary (from a newborn) that shows GHRH-R immunoreaction in the cytoplasm of oocytes and follicular cells of primordial follicles. Oocytes of primary follicles (**b**, 14 years old) as well as granulosa lutein (G) and theca lutein (T) cells of corpus luteum cells (**c**, 45 years old) also show GHRH-R immunoreactivity. In both cell types immunoreactivity was preferentially localized in the cytoplasm, although some nuclei show a positive reaction. x 300

Fig. 3. Rat ovary immunostained for GHRH-R. **a.** Multilaminar primary follicle. Note the intense immunoreactivity of the oocyte (cytoplasm and nuclei). Some follicular cells also present cytoplasmic immunoreactivity. **b.** Secondary follicle of a rat ovary displays intense immunostaining in the oocyte (nuclei and cytoplasm) as well as in the cytoplasm of granulosa and theca cells. **c.** Rat corpus luteum immunostained for GHRH-R. Note the intense cytoplasmic immunoreaction of the granulosa lutein cells. x 300

Fig. 4. Human uterus (**a**, **c** non pregnant, **b** pregnant human uterus). Proliferative endometrium (**a**) shows GHRH-R immunostaining in the endometrial glands and stromal cells. Human gestational endometrium (**b**) shows a moderate immunoreaction in the cells of endometrial glands. Atrophic endometrium (**c**, 65 years old) was not positive for GHRH-R. **a**, x 150; **b**, **c**, x 300

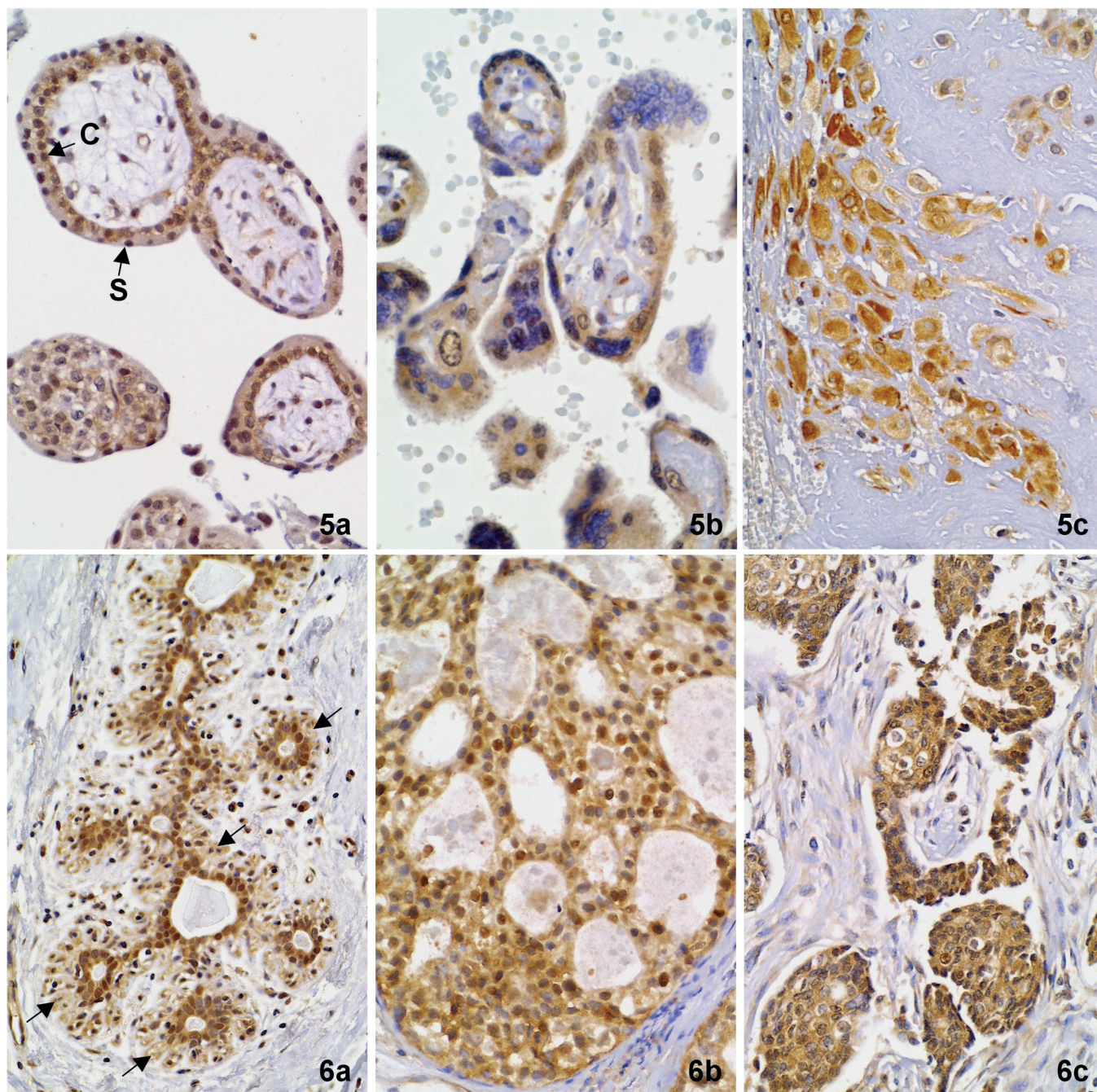


Fig. 5. Normal human placenta. GHRH-R immunostaining in the villi of a human first trimester placenta is localized both in syncytiotrophoblast and cytotrophoblast (a). Immunoreactivity is stronger in the nuclei than in the cytoplasm. Syncytiotrophoblast of the third trimester placenta is also immunoreactive (b). GHRH-R immunoreactivity is demonstrated in placental decidual cells. Note the intense cytoplasmic staining and the nuclear envelope reinforcement (c). a, b, x 300; c, x 150

Fig. 6. a. Normal human mammary gland. GHRH-R immunostaining is localized in the glandular epithelium, myoepithelial cells and lobular connective tissue. **b.** Ductal carcinoma *in situ* of breast. Note the intense GHRH-R immunoreaction of neoplastic cells that show cytoplasmic and nuclear pattern. **c.** Invasive ductal carcinoma of breast. GHRH-R immunostaining is mainly cytoplasmic. a, x 50; b, c, x 300

Sertoli and germ cells) using RT-PCR on cell fractioning and cultures. GHRH immunoreactivity and GHRH mRNA were also found in rat and human testis (Berry et al., 1992; Srivastava et al., 1993; Fabbri et al., 1995; Matsubara et al., 1995; Monts et al., 1996). GHRH might act through its receptor by a paracrine mechanism exerting a modulatory effect on the gonadotrophin action over the testosterone synthesis induction by Leydig cells (GHRH increases the sensitivity of Leydig cells to the LH/hCG stimulation of cAMP and testosterone production) and in the modulation of spermatogonia maturation exerted by Sertoli cells (Ciampani et al., 1992; Fabbri et al., 1995).

Secretory cells of human prostate showed intense nuclear GHRH-R immunoreactivity and cytoplasmic GHRH-R mRNA expression. Mayo et al. (1995) did not find GHRH-R mRNA in rat prostate using RT-PCR, but splicing variants of human GHRH-R were identified in normal prostate and in different human cancer cell lines including prostatic carcinoma by binding assays and nucleotide sequence analysis (Rekasi et al., 2000a,b). Co-expression of GHRH-R and GHRH was also demonstrated in different prostatic carcinoma cell lines suggesting that GHRH could have a local autocrine/paracrine role (Chopin and Herington, 2001). Moreover, GHRH antagonists inhibit *in vivo* and *in vitro* tumor cell proliferation probably reducing tumor production of IGF-II (Schally and Varga, 1999; Halmos et al., 2000; Kahan et al., 2000; Kineman, 2000).

Prostatic carcinoma showed GHRH-R immunostaining not only in the nuclei but also in the cytoplasm of tumour cells, showing a distinct immunostaining pattern from normal secretory prostatic cells.

Human and rat ovary showed GHRH-R immunoreactivity in the follicles throughout all stages of development. Oocytes, granulosa and thecal cells were immunostained. GHRH expression and synthesis was also reported in rat and human ovary (Brar et al., 1989; Bagnato et al., 1992; Matsubara et al., 1995). GHRH act on granulosa cells to promote maturation by amplifying the stimulatory action of FSH on cAMP production and granulosa cell differentiation (Moretti et al., 1990). Gonadotrophins promote the GHRH binding to granulosa cells and stimulate cAMP production in a dose-dependent manner (Bagnato et al., 1991). GHRH-R immunostaining in granulosa and theca cells suggest that GHRH action is mediated by its specific receptor. Moretti et al. (1990) and Mayo et al. (1996) demonstrated GHRH-R mRNA expression using RIA and RT-PCR in rats, although Matsubara et al. (1995) reported different results with RT-PCR and Southern blot hybridization. Such contradictory results may be due to the number of PCR cycles used in cDNA amplification as was discussed by Mayo et al. (1996) since the transcript is present at extremely low levels.

In the endometrial mucosa GHRH-R showed a differential expression related to menstrual cycle. GHRH-R immunoreactivity and *in situ* hybridization

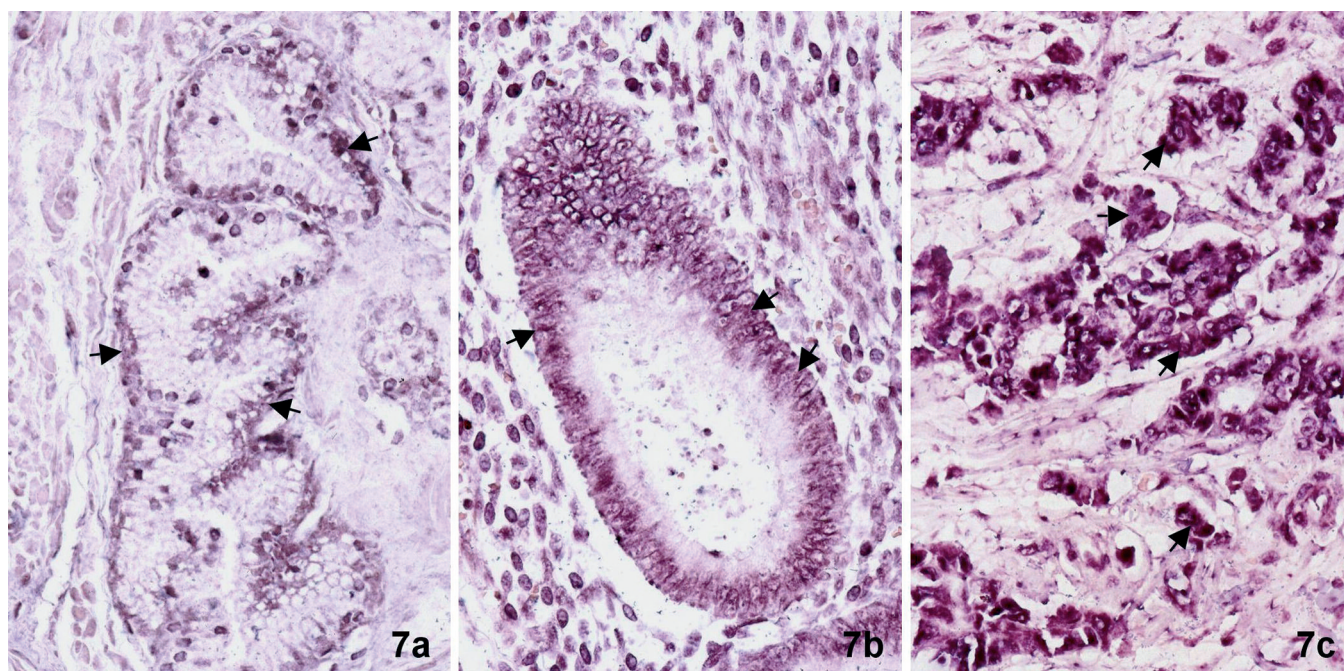


Fig. 7. *In situ* hybridization in human tissues. Prostatic glands (a), proliferative endometrial glands (b), and invasive ductal carcinoma (c) showed an intense signal (arrows). The labelling pattern is similar to that obtained by immunohistochemical techniques (compare with figures 1c, 4a, and 6c respectively). x 300

labelling was intense in the proliferative phase both in glands and stromal cells. Gestational endometrium and decidual cells also showed intense GHRH-R immunostaining, although it was nearly absent in atrophic endometrium. Our results in human uterus differ from those of Mayo et al. (1996) since they have not found GHRH-R expression in rat uterus with RT-PCR. Such discrepancies may be due to differences interspecies and/or different methods employed. GHRH was demonstrated in endometrial tumors of the endometrioid type (Chatzistamou et al., 2002). GHRH immunoreactivity was mainly localized in the cytoplasm although three cases also had strong nuclear immunoreaction. These authors also suggest a paracrine or autocrine role for GHRH in the development of the disease.

The demonstration of GHRH-R in human placenta is consistent with the findings of Mayo et al. (1996) in rat placentas where they have found GHRH-R mRNA using RT-PCR. On the other hand, Matsubara et al. (1995) and Fujinaka et al. (1996) did not detect GHRH-R mRNA in rat placenta. This may be due, as was discussed by Fujinaka et al. (1996), to the probe used that would not detect all the possible regions of GHRH-R.

GHRH-R was localized in human mammary gland (epithelial glandular cells and myofibroblasts, and in lobular stromal cells). We have also demonstrated using *in situ* RT-PCR the expression of the hGH in the epithelial cells and some fibroblasts of the mammary gland (Raccurt et al., 2002). In agreement with the results of Mol et al. (1995a,b), we observed a more prominent signal for hGH mRNA in the neoplastic epithelial cells than in normal mammary tissue. We hypothesized that locally produced hGH, per se, or increase in the local concentration of hGH may alter the proliferative behaviour of mammary epithelial cells. Although an anti-apoptotic effect of GH as was demonstrated by other groups in different cancer cell lines including carcinoma cell lines (Graichem et al., 2002). The role of autocrine produced hGH in mammary carcinoma was studied by stable transfection of the hGH gene or a translation deficient gene into MCF-7 cells (designated MCF-hGH and MCF-MUT respectively). Indeed, we observed a marked increase in MCF-hGH cell number in both free serum and serum containing media in comparison to MCF-MUT cells (Kaulsay et al., 1999). Interestingly the increase in cell number observed in the MCF-hGH cell line is achieved with extremely low concentrations of hGH of around 100 pM under the experimental conditions utilized. Conversely, GHRH antagonists inhibit the growth of human breast carcinoma cells and diminish the metastatic potential of cancer cells (Chatzistamou et al., 2001). Using RT-PCR these authors demonstrated the expression of mRNA for GHRH-R splice variant-1 in breast cancer cells. This effect could be due to inhibition of autocrine hGH production.

In the present study, the receptors for GHRH were found not only in the cytoplasm but also in the nuclei.

The cytoplasmic and nuclear GHRH-R immunostaining was not surprising because other membrane receptors showed similar immunostaining patterns. GH and PRL receptors show cytoplasmic and nuclear staining (Mertani et al., 1995, 1998; Lincoln et al., 1998; García-Caballero et al., 1996, 2000). An internalization process may explain GHRH-R immunoreactivity in the cytoplasm and nuclei. In fact, such internalization has been demonstrated for GHRH (Morel, 1994; Veyrat-Durebex et al., 2005). Internalization of human GHRH (1-29)NH₂ (Fluo-GHRH) is clathrin-dependent, while in the rat is caveolin-dependent. Upon internalisation GHRH is mainly directed to lysosomes. De novo synthesis and recycling maintain the optimal concentration of functional concentration at the plasma membrane level (Veyrat-Durebex et al., 2005). Moreover, GHRH-R was localized, by ultrastructural immunocytochemistry, in the secretory granules, cytosol and nucleus of somatotrope cells (Morel et al., 1999). Nevertheless, the GHRH-R role as a putative nuclear import signal for GHRH will deserve further investigation.

Antagonists of growth hormone-releasing hormone (GHRH) inhibit the proliferation of various human cancers (Schally et al., 2001), including prostatic (Jungwirth et al., 1997; Lamharzi et al., 1998; Rekasi et al., 2000b, 2001), ovarian (Kahan et al., 2000; Chatzistamou et al., 2001b,c), endometrial (Kahan et al., 2000) and breast carcinomas (Kahan et al., 2000; Chatzistamou et al., 2001a), *in vitro* and *in vivo*. Apparently GHRH antagonists act directly through specific binding sites expressed on tumor cells that differ from pituitary human GHRH-R (Rekasi et al., 2000a) and rat renal medulla (Boulanger et al., 2002). Similarly, GHRH antagonists inhibit the growth of UCI-107 ovarian cell carcinoma via a direct effect on the cancer cells (Chatzistamou et al., 2001b). Because of the structural homologies between the sequences of GHRH and VIP peptides and their receptors, analogs of GHRH usually bind to both GHRH-R and VIP receptors although with different affinities. Rekasi et al. (2000b, c) reported antagonists (e.g. JV-1-36) that have predominant GHRH antagonistic effect. JV-1-36 was demonstrated to significantly decrease the tumour growth, the IGF-II concentration in tumours, and the tumorigenicity of UCI-107 cells (Chatzistamou et al., 2001b). Moreover, different reports (Rekasi et al., 2000a; Halmos et al., 2002; Plonowski et al., 2002) have recently demonstrated that cell lines of human prostate cancer express splice variants 1 and 2 of GHRH-R. Among these truncated forms of GHRH-R, the splice variant 1 displays the greatest similarity to the pituitary GHRH-R and is predominantly detected in the human experimental cancer models, including prostate, and mammary carcinomas (Rekasi et al., 2000a; Halmos et al., 2002) as well as in primary endometrial carcinoma (Kiaris et al., 2003). Nevertheless, the GHRH-R localization using different techniques, not only immunohistochemistry but also *in situ* hybridisation,

with similar results supports the presence of the GHRH-R in peripheral tissues of the reproductive system.

In conclusion, we have shown by immunohistochemical and *in situ* hybridization the presence of GHRH-R in normal reproductive system and in carcinomas of prostate and breast. These results suggest a direct action of GHRH-R on these tissues and could raise the possibility of new therapeutic strategies in these tumours.

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References

- Bagnato A., Moretti C., Frajese G. and Catt K. (1991). Gonadotropin-induced expression of receptors for growth hormone releasing factor in cultured granulosa cells. *Endocrinology* 128, 2889-2894.
- Bagnato A., Moretti C., Ohnishi J., Frajese G. and Catt K.J. (1992). Expression of the growth hormone-releasing hormone gene and its peptide product in the rat ovary. *Endocrinology* 130, 1097-1102.
- Boulanger L., Andersen P.H. and Gaudreau P. (1999). Development of site-directed polyclonal antibody against the pituitary growth hormone-releasing hormone receptor and its use to estimate GHRH receptor concentration in normal and hypothyroid rats. *Neuroendocrinology* 70, 117-127.
- Boulanger L., Girard N., Strecko J. and Gaudreau P. (2002). Characterization of a growth hormone-releasing hormone binding site in the rat renal medulla. *Peptides* 23, 43-50.
- Brar A.K., Brinster R.L. and Frohman L.A. (1989). Immunohistochemical analysis of human growth hormone-releasing hormone gene expression in transgenic mice. *Endocrinology* 125, 801-809.
- Campbell R.M. and Scanes C.G. (1992). Evolution of the growth hormone-releasing factor (GRF) family of peptides. *Growth Regul.* 2, 175-191.
- Chatzistamou I., Schally A.V., Varga J.L., Groot K., Armatas P. and Halmos G. (2001a). Inhibition of growth and metastases of MDA-MB-435 human estrogen-independent breast cancers by an antagonist of growth hormone-releasing hormone. *Anticancer Drugs* 12, 761-768.
- Chatzistamou I., Schally A.V., Varga J.L., Groot K., Armatas P. and Bajo A.M. (2001b). Inhibition of growth and reduction in tumorigenicity of UCI-107 ovarian cancer by antagonists of growth hormone-releasing hormone and vasoactive intestinal peptide. *J. Cancer Res. Clin. Oncol.* 127, 645-652.
- Chatzistamou I., Schally A.V., Varga J.L., Groot K., Armatas P., Busto R. and Halmos G. (2001c). Antagonists of growth hormone-releasing hormone and somatostatin analog RC-160 inhibit the growth of the OV-1063 human epithelial ovarian cancer cell line xenografted into nude mice. *J. Clin. Endocrinol. Metab.* 86, 2144-2152.
- Chatzistamou I., Schally A.V., Pafiti A., Kiaris H. and Koutselini H. (2002). Expression of growth hormone-releasing hormone in human primary endometrial carcinomas. *Eur. J. Endocrinology* 147, 381-386.
- Chopin L.K. and Herington A.C. (2001). A potential autocrine pathway for growth hormone releasing hormone (GHRH) and its receptor in human prostate cancer cell lines. *Prostate* 49, 116-121.
- Ciampani T., Fabbri A., Isidori A. and Dufau M.L. (1992). Growth hormone-releasing hormone is produced by rat Leydig cell in culture and acts as a positive regulator of Leydig cell function. *Endocrinology* 131, 2785-2792.
- Fabbri A., Ciocca D.R., Ciampani T., Wang J. and Dufau M.L. (1995). Growth hormone-releasing hormone in testicular interstitial and germ cells: potential paracrine modulation of follicle-stimulating hormone action on Sertoli cell function. *Endocrinology* 136, 2303-2308.
- Fujinaka Y., Yokogoshi Y., Chen-Yu Z., Okura T., Kitagawa K. and Saito S. (1996). Tissue-specific molecular heterogeneity of human growth hormone-releasing hormone receptor protein. *FEBS Lett.* 394, 1-4.
- García-Caballero T., Morel G., Gallego R., Fraga M., Pintos E., Gago D., Vonderhaar B.K. and Beiras A. (1996). Cellular distribution of prolactin receptors in human digestive tissues. *J. Clin. Endocrinol. Metab.* 81, 1861-1866.
- García-Caballero T., Mertani H.C., Lambert A., Gallego R., Fraga M., Pintos E., Forteza J., Chevallier M., Lobie P., Vonderhaar B.K., Beiras A. and Morel G. (2000). Increased expression of growth hormone and prolactin receptors in hepatocellular carcinomas. *Endocrine* 12, 265-271.
- Graichem R., Liu D., Sun Y., Lee K.O. and Lobie P.E. (2002). Autocrine human growth hormone inhibits placental transforming growth factor- β gene transcription to prevent apoptosis and allow cycle progression of human mammary carcinoma cells. *J. Biol. Chem.* 277, 26662-26672.
- Guarcello V., Weigent D.A. and Blalock J.E. (1991). Growth hormone releasing hormone receptors on thymocytes and splenocytes from rats. *Cell Immunol.* 136, 291-302.
- Halmos G., Schally A.V., Varga J.L., Plonowski A., Rekasi Z. and Czompoly T. (2000). Human renal cell carcinoma expresses distinct binding sites for growth hormone-releasing hormone. *Proc. Natl. Acad. Sci. USA* 97, 10555-10560.
- Halmos G., Schally A.V., Czompoly T., Krupa M., Varga J.L. and Rekasi Z. (2002). Expression of growth hormone-releasing hormone and its receptor splice variants in human prostate cancer. *J. Clin. Endocrinol. Metab.* 8, 4707-4714.
- Hashimoto K., Koga M., Motomura T., Kasayama S., Kouhara H., Ohnishi T., Arita N., Hayakawa T., Sato B. and Kishimoto T. (1995). Identification of alternatively spliced messenger ribonucleic acid encoding truncated growth hormone-releasing hormone receptor in human pituitary adenomas. *J. Clin. Endocrinol. Metab.* 80, 2933-2939.
- Jungwirth A., Schally A.V., Pinski J., Halmos G., Groot K., Armatas P. and Vellido-Buenfil M. (1997). Inhibition of *in vivo* proliferation of androgen-independent prostate cancers by an antagonist of growth hormone-releasing hormone. *Br. J. Cancer* 75, 1585-1592.
- Kahan Z., Arencibia J.M., Csernus V.J., Groot K., Kineman R.D., Robinson W.R. and Schally A.V. (2000). Expression of growth hormone-releasing hormone (GHRH) messenger ribonucleic acid and the presence of biologically active GHRH in breast, endometrial, and ovarian cancers. *J. Clin. Endocrinol. Metab.* 84, 582-589.
- Kaulsay K.K., Mertani H.C., Tornell J., Morel G., Lee K.O. and Lobie P.E. (1999). Autocrine stimulation of human mammary carcinoma cell proliferation by human growth hormone. *Exp. Cell Res.* 250, 35-50.
- Kiaris H., Chatzistamou I., Schally A.V., Halmos G., Varga J.L., Koutselini H. and Kalofoutis A. (2003). Ligand-dependent and -independent effects of splice variant 1 of growth hormone-releasing hormone receptor. *Proc. Natl. Acad. Sci. USA* 100, 9512-9517.
- Kineman R.D. (2000). Antitumorigenic actions of growth hormone-

- releasing hormone antagonists. *Proc. Natl. Acad. Sci. USA* 97, 532-534.
- Laburthe M., Couvineau A., Gaudin P., Maoret J.J., Rouyer-Fessard C. and Nicole P. (1996). Receptors for VIP, PACAP, Secretin, GRF, Glucagon, GLP-1, and others members of their new family of G protein-linked receptors: structure-function relationship with special reference to the human VIP-1 receptor. *Ann. N.Y. Acad. Sci.* 26, 94-111.
- Lamharzi N., Schally A.V., Koppan M. and Groot K. (1998). Growth hormone-releasing hormone antagonist MZ-5-156 inhibits growth of DU-145 human androgen-independent prostate carcinoma in nude mice and suppresses the levels and mRNA expression of insulin-like growth factor II in tumors. *Proc. Natl. Acad. Sci. USA* 95, 8864-8868.
- Lincoln D., Sinowatz F., Temmin-Baker L., Baker H.I., Kölle S. and Waters M.J. (1998). Growth hormone receptor expression in the nucleus and cytoplasm of normal and neoplastic cells. *Histochem. Cell Biol.* 109, 141-159.
- Lopes M.B.S., Gaylinn B.D., Thorner M.O. and Stoler M.H. (1997). Growth hormone-releasing hormone receptor mRNA in acromegalic pituitary tumors. *Am. J. Pathol.* 150, 1885-1891.
- Matsubara S., Sato M., Mizobuchi M., Niimi M. and Takahara J. (1995). Differential gene expression of growth (GH) hormone-releasing hormone (GRH) and GRH receptor in various rat tissues. *Endocrinology* 136, 4147-4150.
- Matsuno A., Katami H., Nagashima T., Teramoto A., Osamura R.Y. and Kirino T (2000). Growth hormone-releasing hormone expression in pituitary somatotroph adenomas, studied by immunohistochemistry and *in situ* hybridization using catalyzed signal amplification system. *Human Pathol.* 31, 789-794.
- Mayo K.E., Miller T.L., DeAlmeida V., Zheng J. and Godfrey P.A. (1996). The growth-hormone-releasing-hormone receptor: signal transduction, gene expression, and physiological function in growth regulation. *Ann. NY Acad. Sci.* 26, 184-203.
- Mayo K.E., Miller T., DeAlmeida V., Godfrey P., Zheng J. and Cunha S.R. (2000). Regulation of the pituitary somatotroph cell by GHRH and its receptor. *Recent Prog. Horm. Res.* 55, 237-266.
- Mertani H.C., Pechoux C., Garcia-Caballero T., Waters M.J. and Morel G. (1995). Cellular localization of the growth hormone receptor/binding protein in the human anterior pituitary gland. *J. Clin. Endocrinol. Metab.* 80, 3361-3367.
- Mertani H.C., García-Caballero T., Lambert A., Gerard F., Palayer C.H., Boutin J.M., Vonderhaar B., Waters M., Lobie P. and Morel G. (1998). Cellular expression of growth hormone and prolactin receptors in human breast disorders. *Int. J. Cancer* 79, 202-211.
- Mol J.A., Henzen-Logmans S.C., Hageman P., Misdorp W., Blankenstein M.A. and Rijnberk A. (1995a). Expression of the gene encoding growth hormone in the human mammary gland. *J. Clin. Endocrinol. Metab.* 80, 3094-3096.
- Mol J.A., van Garderen E., Selman P.J., Wolfswinkel J., Rijnberk A. and Rutteman G.R. (1995b). Growth hormone mRNA in mammary gland tumors of dogs and cats. *J. Clin. Invest.* 95, 2028-2034.
- Monts B.S., Breyer P.R., Rothrock J.K. and Pescovitz O.H. (1996). Peptides of the growth hormone-releasing hormone family. Differential expression in rat testis. *Endocrine* 4, 73-78.
- Morel G (1994) Internalization and nuclear localization of peptide hormones. *Biochem. Pharmacol.* 47, 63-76.
- Morel G., Gallego R., Boulanger L., Pintos E., Garcia-Caballero T. and Gaudreau P. (1999). Restricted presence of the growth hormone-releasing hormone receptor to somatotropes in rat and human pituitaries. *Neuroendocrinology* 70, 128-136.
- Moretti C., Bagnato A., Solan N., Frajese G. and Catt K.J. (1990). Receptor-mediated actions of growth hormone releasing factor on granulosa cell differentiation. *Endocrinology* 127, 2117-2126.
- Plonowski A., Schally A.V., Busto R., Krupa M., Varga J.L. and Halmos G. (2002). Expression of growth hormone-releasing hormone (GHRH) and splice variants of GHRH receptors in human experimental prostate cancers. *Peptides* 23, 1127-1133.
- Raccurt M., Lobie P.E., Moudilou E., Garcia-Caballero T., Frappart L., Morel G. and Mertani H.C. (2002). High stromal and epithelial hGH gene expression is associated with proliferative disorders of the mammary gland. *J. Endocrinol.* 175, 307-318.
- Reecher S., Raccurt M., Lambert A., Lobie P.E., Mertani H.C. and Morel G. (2001). Prenatal and adult growth hormone gene expression in rat lymphoid organs. *J. Histochem. Cytochem.* 49, 347-354.
- Rekasi Z., Czompoly T., Schally A.V. and Halmos G. (2000a) Isolation and sequencing cDNAs for splice variants of growth hormone-releasing hormone receptor from human cancers. *Proc. Natl. Acad. Sci. USA* 97, 10561-10566.
- Rekasi Z., Varga J.L., Schally A.V., Halmos G., Armatis P., Groot K., Czompoly T. (2000b). Antagonists of growth hormone-releasing hormone and vasoactive intestinal peptide inhibit tumor proliferation by different mechanisms: evidence from *in vitro* studies on human prostatic and pancreatic cancers. *Endocrinology* 141, 2120-2118.
- Rekasi Z., Varga J.L., Schally A.V., Halmos G., Groot K. and Czompoly T. (2000c). Antagonistic actions of analogs related to growth hormone-releasing hormone (GHRH) on receptors for GHRH and vasoactive intestinal peptide on rat pituitary and pineal cells *in vitro*. *Proc. Natl. Acad. Sci. USA* 97, 1218-1223.
- Rekasi Z., Schally A.V., Plonowski A., Czompoly T., Csernus B. and Varga J.L. (2001). Regulation of prostate-specific antigen (PSA) gene expression and release in LNCaP prostate cancer by antagonists of growth hormone-releasing hormone and vasoactive intestinal peptide. *Prostate* 48, 188-199.
- Schally A.V. and Varga J.L. (1999). Antagonistic analogs of growth hormone-releasing hormone: new potential antitumor agents. *Trends Endocr. Metab.* 10, 383-394.
- Schally A.V., Comaru-Schally A.M., Nagy A., Kovacs M., Szepeshazi K., Plonowski A., Varga J.L. and Halmos G. (2001). Hypothalamic hormones and cancer. *Front. Neuroendocrinol.* 22, 248-291.
- Srivastava C.H., Breyer P.R., Rothrock J.K., Peredo M.J. and Pescovitz O.H. (1993). A new target for growth hormone releasing-hormone action in rat: the Sertoli cell. *Endocrinology* 133, 1478-1481.
- Takahashi T., Okimura Y., Yoshimura K., Shigeyoshi Y., Kaji H., Abe H. and Chihara K. (1995). Regional distribution of growth hormone-releasing hormone (GHRH) receptor mRNA in the rat brain. *Endocrinology* 136, 4721-4724.
- Tang J., Lagacé G., Castagné J. and Collu R. (1995). Identification of human growth hormone-releasing hormone receptor splicing variants. *J. Clin. Endocrinol. Metab.* 80, 2381-2387.
- Veyrat-Durebex C., Pomerleau L., Langlois D. and Gaudreau P. (2005). Internalization and trafficking of the human and rat growth hormone-releasing hormone receptor. *J. Cell Physiol.* 203, 335-344.
- Zeitler P. and Siriwardana G. (2000). Stimulation of mitogen-activated protein kinase pathway in rat somatotrophs by growth hormone-releasing hormone. *Endocrine* 12, 257-264.