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Effect of Ovariectomy, Hypophysectomy and/or GnRH Analog (HRF) Administration on the Cell Proliferation of the MXT Mouse Hormone-Dependent Mammary Tumor

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Abstract—*The MXT tumor is an experimental mammary neoplasm which is maintained by serial transplantation using B6D2F1 mice, and which contains significant amounts of estrogen and progesterone receptors. The aim of the present study is to examine the effects of ovariectomy (OVX) or ovariectomy plus hypophysectomy (OVX-HX) on both the macroscopic growth and the cell proliferation of this tumor. This cell proliferation was evaluated by means of* in vivo *tritiated thymidine autoradiography. In addition, we investigated the effects of a GnRH analog (Gonadorelin: HRF, 5-oxo-Pro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-hydrochloride) on MTX tumor cell proliferation on 7 day-OVX and 5 day-HX (OVX-HX) mice. The uterine luminal epithelium was chosen to monitor the methodology.*

Our data clearly demonstrate that there is a delay in the growth of MXT tumors grafted into hypophysectomized animals and, to a lesser degree, ovariectomized animals. With respect to proliferation, castration induced a dramatic decrease of the thymidine labelling index (TLI) in the tissue used to monitor the methodology (the uterine luminal epithemium); in contrast, no cell proliferation was induced by hypophysectomy or HRF administration. In 4-week-old MXT tumors, ovariectomy also markedly decreased the TLI within a few days of its taking place. However hypophysectomy, performed on castrated animals, induced a significant and transient increase of cell proliferation in this neoplasm, an increase which lasted from the 2nd to the 5th day following the operation. The high basal level of MXT cell proliferation recorded in OVX-HX animals decreased dramatically after the administration of HRF between 12 and 48 h prior to the sacrifice of the animals. It is concluded that the HRF exerts a direct effect on the MXT tumor cells, and this HRF might be essential for their growth.

INTRODUCTION

THE MXT mammary tumor of the C57BL × DBA2f/F1 (B6D2F1) strain is a subcutaneously transplantable model initially developed by Watson *et al.* on an urethane-treated female mice carrying a pituitary isograft under the renal capsule [1]. This tumor contains significant amounts of estrogen (ER) and progesterone (PgR) receptors [2] and retains its sensitivity to estradiol (E_2) during the first 6–7 weeks of its growth [3]. Measured by the thymidine labelling index (TLI), its cell proliferation undergoes a decrease in 6-day-ovariectomized animals as compared to the intact ones, and this proliferation is significantly increased following either estradiol (E_2) [4] or progesterone [5] administration between 6 and 36 h prior to sacrifice. Histologically, some MXT tumor strains display a homogeneous pattern, whereas others are composed of different cell types [6]. In the present work we use two passages (T4 and T7) of the MXT BOG III strain composed of a homogeneous epithelial polygonal cell population; those strains are similar (histopathology, growth pattern, ER and PgR contents, etc) to those of the MXT BOG I T1-T10 strain previously described [6].

Like most ovarian-dependent experimental mam-

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mary tumors in rodents [7–15], the MXT model is also very sensitive to pituitary [16–18] and hypothalamic hormones [19]. The purpose of this work is to characterize further the effects of ovariectomy, hypophysectomy and the influence of a GnRH analog (HRF) on cell proliferation in MXT tumors borne by adult female mice. Uteri were used to monitor the methodology.

MATERIALS AND METHODS

1. *Chemicals*

We purchased [methyl-^{3}H]thymidine (sp. act.: 48 Ci/mmol) from Amersham Ltd (U.K.). Synthetic GnRH (gonadorelin: HRF; 5-oxo-Pro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-hydrochloride) was kindly provided by Dr. Segal of Ayerst (Belgium). All the solutions to be injected were prepared by means of appropriate dilution with sterile saline (NaCl, 9.0 g/l).

2. *Animals and tumor transplantation*

Nine- to 11-week-old mature female mice C57BL × DBA2f/F1 (B6D2F1 weighing 20–23 g) were purchased from IFFA-CREDO (Lyon, France). For the present experiment, standard breeding conditions were used. All the animals were kept in plastic cages in a room with controlled temperature (22 ± 1°C) and light exposure (from 7.00 am to 9.00 pm). An unlimited supply of food and water was provided.

The original MXT mouse mammary tumor was kindly provided by Dr. M. Schneider (Naturwissenschaftliche Fakultät IV, Universität Regensburg, F.R.G.) and tumors from this source were designated BOG III. The tumor was maintained in our laboratory by regular transplantations performed every 4 weeks on 8–10-week old female B6D2F1 mice. On each passage, several (approx. 10) tumors without visible areas of necrosis and measuring about 1 cm^3 were selected and dissected under sterile conditions. Fragments of each tumor were systematically taken for histological examination, and ER and PgR were assayed on a small tumor sample using the standard Dextran-coated charcoal method [20, 21]. The remaining tumor tissue was minced into 15 mm^3 pieces and two fragments were inoculated subcutaneously per mouse, one into each flank through a trochar (ga. 13). The present experiments were carried out on 4-week-old tumors of the 4th (T4) and 7th (T7) MXT BOG III transplant generations. The relevant transplants were performed in our laboratory.

3. *Surgical endocrine ablative procedures*

For growth or tritiated thymidine labelling experiments, tumors were inoculated as described above either into intact mice, or into animals which had undergone surgical castration (OVX) and/or hypophysectomy (OVX-HX, HX).

The removal of the ovaries was performed under tribromoethanol anesthesia by means of a bilateral and dorsal oophorectomy. Rising the procedure described by Lostroh and Jordan [22], hypophysectomy was performed by the transpharyngeal route under total anesthesia, under an i.p. injection of 0.25 ml per animal of avertin (amylic alcohol: 0.8 g/100 ml and tribromoethanol: 2 g/100 ml saline). Once the experiment had been carried on, the completeness of the ablation was checked by a study of serial sections of the sella turcica.

4. *Experimental schedule*

Macroscopic growth of tumors after oophorectomy and/or hypophysectomy. Twenty mice were randomly allocated into 4 groups (I, OVX, HX and OVX-HX) of 5 mice each. Seven days prior to the time of the tumoral grafts with the MXT BOG III T4 strain (day 0), the mice in groups OVX and OVX-HX were oophorectomized; 5 days prior to day 0, the mice in groups HX and OVX-HX were hypophysectomized. The animals from group I were left intact to serve as the control group.

The tumors were measured weekly for 9 weeks with calipers, and their size was expressed as 'an area' (mm^2) corresponding to the product of their two largest perpendicular diameters. The animals were also weighed at the same time.

Cell kinetic parameter evaluation. Effect of endocrine ablation on cell proliferation of uteri and MXT tumors. Ovariectomy. On day 0, 40 mice with one 4-week-old MXT BOG III T4 tumor on each flank were randomly split into 8 groups (01–08) of 5 mice each. The animals in group 01 were left intact to serve as the control group, whereas those in groups 02–08 were ovariectomized 2 (02), 4 (03), 6 (04), 8 (05), 10 (06), 12 (07) and 14 (08) days prior to their sacrifice (by cervical dislocation); this was performed on day 14 at 10.00 am.

One hour prior to their death, all the mice received an intraperitoneal (i.p.) injection of 0.1 ml saline containing 1 μCi of [^{3}H]TdR/g BW. Immediately after death, the uteri and MXT tumors were dissected, fixed and processed for autoradiography.

Hypophysectomy. On day 0 50 mice with one 4-week-old MXT BOG III T7 tumor on each flank were randomly split into 10 groups (H1–H10) of 5 mice each. The mice in group H1 were left intact to serve as the control group, whereas those in groups H2–H8, H9 and H10 were ovariectomized respectively 7, 10 and 14 days prior to their sacrifice. This operation was intended to suppress the possibility of mutual interference between endogeneous ovarian

hormones and hypothalamo-pituitary secretions on the TLI of the uterine luminal epithelium or on that of the MXT tumor.

Hypophysectomy was performed on the animals in groups H2–H10 1 (H2), 2 (H3), 3 (H4), 5 (H6), 6 (H7), 7 (H8), 10 (H9) and 14 (H10) days, respectively, prior to their sacrifice. All the animals were sacrificed on day 14 (after random allocation) at 10.00 am. The injection of tritiated thymidine, dissection and tissue removal were performed as described in the preceding paragraph.

Effect of GnRH analog (HRF) on cell proliferation in uteri and MXT tumors. Thirty-five mice with one 4-week-old MXT BOG III 17 tumor on each flank underwent surgical ovariectomy and hypophysectomy 7 and 5 days, respectively, prior to their sacrifice. They were then randomly split into 7 groups (G1–G7) of 5 animals each. Animals in each group received an i.p. injection of either a placebo (control group G1) or a solution containing HRF (groups G2 to G7), and were then sacrificed by cervical dislocation at various time intervals thereafter (see below). Group G1 received the vehicle only, i.e. an i.p. injection of 0.1 ml saline (NaCl 9 g/l), 24 h before being killed. Groups G2–G7 received an i.p. injection of 0.1 ml of a saline solution containing 25 μg HRF 6, 12, 24, 36, 48 or 72 h, respectively, prior to sacrifice (the HRF dose used in the present experiment was chosen according to Redding and Schally's protocol [19]). One hour prior to sacrifice, all the animals received an i.p. injection of 0.1 ml (25 μCi) [^{3}H]TdR. At the scheduled times all the animals in the same batch were killed by cervical dislocation. All the MXT tumors were subsequently dissected and processed for histological examination. Since they served as normal ovarian hormone target organs and checks for the methodology, uteri were systematically removed and processed similarily.

5. *Histological procedure and autoradiographic technique*

Immediately after death, the mammary tumors and uteri were processed for histology and autoradiography as previously described [3–5]. For the tumors, sections were systematically made through the center of the tissue and assembled 3 per slide. For the uteri, 4 transversal sections were assembled on a same slide with each section passing through the mid-portions of the horns. For each tumor and uterus, the TLI was assessed on two slides. Counts were made on a fixed number of cells taken from representative regions of the tissue. In the tumors, microscopic fields were randomly selected, 3 at the periphery and 3 in the center, giving an average of 3000 neoplastic cells analyzed per tumor. In the uteri, the TLI of the luminal epithelium was assessed on 300 cells. All the slides were identified by a code number alone. They were examined by the same investigator (Y. de L.), who did not know the corresponding experimental conditions.

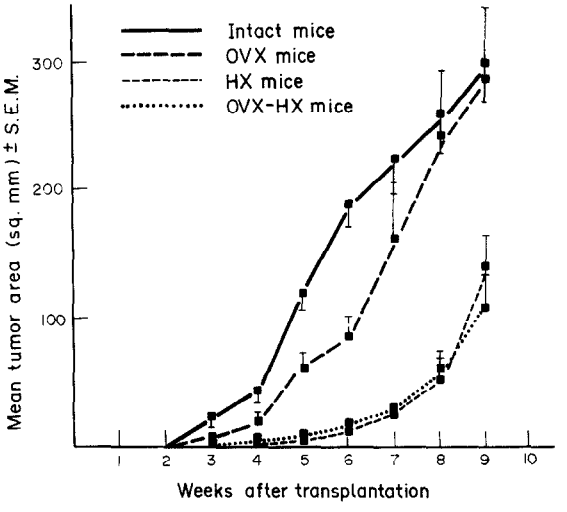

Fig. 1. Growth curves of MXT tumors (10 tumors per experimental condition) grafted in intact, ovariectomized (OVX), hypophysectomized (HX) or ovariectomized and hypophysectomized (OVX-HX) mice. Vertical bars indicate the standard error of the mean tumor size recorded at the time of the measurement. Statistical comparisons vs. corresponding control values: (1) I vs. OVX = P < 0.05 for the 4th week of tumor growth and P < 0.01 from the 5th to the 6th week; (2) I vs. HX = P < 0.001 from the 3rd to the 9th week; (3) HX vs. OVX = P < 0.001 from the 3rd to the 5th week and P < 0.01 from the 6th to the 9th week of tumor growth.

6. *ER and PgR characteristics of MXT tumors*

As hormone-dependent markers, ER and PgR were assayed on one pool of 10 MXT BOG III T4, and on another of 10 MXT BOG III T7 tumors; the tumors were of approximately equal size. Cytosolic ER and PgR concentrations were measured according to the standard Dextran-coated charcoal method previously described [20, 21]. The binding capacity of the tumor cytosolic fraction for [^{3}H]cstradiol and [^{3}H]ORG2058 was expressed in femtomols per mg of protein.

7. *Statistical analysis*

Results are given as mean ± S.E.M. The statistical comparisons of the data were performed by using the Fischer *F* test (one-way variance analysis).

RESULTS

1. *Growth of tumors*

Figure 1 shows the representative growth patterns of the MXT BOG III T4 tumor grafted into intact (I), ovariectomized (OVX), hypophysectomized (HX) and ovariectomized plus hypophysectomized (OVX-HX) animals.

The OVX operation exerted a weak but nevertheless significant slowing-down effect on subsequent tumor growth. Hypophysectomy dramatically decreased tumor growth either in the non-ovariectomized animals or in the OVX ones. In the hypophy-

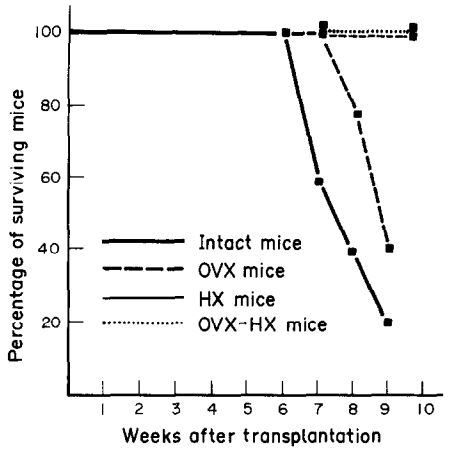

Fig. 2. Survival of intact, ovariectomized (OVX), hypophysectomized (HX) or ovariectomized and hypophysectomized (OVX-HX) mice bearing one MXT tumor on each flank (5 animals per experimental condition).

sectomized animals, the growth of the MXT tumor was identical in both the presence and absence of the ovaries. However, let us emphasize that, in semi-log coordinates, the tumor growth is identical in all groups from the 7th week to the end of the experimental schedule.

The corresponding survival rate in the tumor-bearing animals is given in Fig. 2 , and shows that death may occur in intact or OVX animals, but not in HX or OVX-HX ones.

2. *Effects of endocrine ablation and GnRH analog (HRF) administration on cell proliferation in uteri*

Effect of castration. The results are presented in Table 1. The 2 days following ovariectomy did not enhance significant effect on cell proliferation in the uterine luminal epithelium, whereas a longer period (4–14 days) led to a dramatic reduction in the TLI to a very low and constant basal level (approx. 20-fold).

Effect of castration and hypophysectomy. As shown in Table 1, hypophysectomy did not modify the very low basal level of cell proliferation in the uterine luminal epithelium of animals castrated between 7 and 14 days before sacrifice.

Effect of HRF administration. While in comparison with intact animals (see Table 1), the 7-day-castration and 5-day-hypophysectomy periods caused a marked decrease in the mean TLI value, HRF administration had no additional significant influence on the lowering effect already caused by OVX-HX in the uterine luminal epithelium.

3. *Effects of endocrine ablation and HRF administration on cell proliferation in MXT tumors*

Effect of castration. As shown in Table 2, cell proliferation was significantly lower in the OVX animals than in the intact ones. The TLI reached a minimal value on the 4th day following ovariectomy, and remained constant until the 14th day after this operation.

Effect of castration and hypophysectomy. Results are given in Table 2. One day after hypophysectomy, the mean TLI of the MXT tumors was significantly slowed down in comparison with that of the tumors of the intact animals. However, between 2 and 5 days after this operation, tumor cell proliferation increased and remained significantly higher than that of the control group. On the 6th day following hypophysectomy the TLI was already slowing down, and it reached a minimal value between the 10th and 14th day following hypophysectomy.

Effect of HRF administration. HRF and GnRH analog used in the present work brought about a very significant decrease in the cell proliferation in the MXT tumor; this took effect between the 12th and the 48th hour, with a minimal TLI value reached at the 24th hour. The control group consisted of animals ovariectomized and hypophysectomized 7 and 5 days before sacrifice, respectively (Table 3).

4. *ER and PgR content of MXT tumors*

The pooled tumor fragments of the 4th and 7th transplants which were used as sources of implants for experiments in which growth and TLI were studied contained an average of 40 and 35 fmoles ER/mg protein and 45 and 125 fmoles PgR/mg protein, respectively.

DISCUSSION

From the pioneering work of Huggins *et al.* [23–25] on experimental mammary tumors it has been firmly established that their growth can be stimulated by estrogen, progesterone, insulin, prolactin and other hormones [9, 26–34]. With regard to the MXT model, our group has already demonstrated the mitogenic effect exerted by estradiol [3, 4], progesterone [5] or prolactin [16, 17].

In the work reported on here, we further characterized the hormone sensitivity of the MXT model by studying the effects of GnRH analog (HRF) on MXT tumor cell proliferation in 7-day-ovariectomized and 5-day-hypophysectomized (OVX-HX) mice. The uterine luminal epithelium was chosen to monitor the methodology. This proliferative effect was approached by means of an *in vivo* [^{3}H]TdR labelling technique followed by autoradiography. An approach developed by Hughes *et al.* [35], the measurement of the thymidine labelling index (TLI) is a first class method of evaluating the cells proliferating in a tissue. This TLI represents the percentage of cells engaged in DNA synthesis.

With respect to the uterine luminal epithelium,

Table 1. *TLI recorded in the uterine luminal epithelium of mice* (5 *per experimental condition*) *after ovariectomy* (*OVX*) *or ovariectomy followed by hypophysectomy* (*OVX-HX*)

	OVX*			OVX-HX*	
Group	Number of days after OVX	TLI ± S.E.M. (%)†	Group	Number of days after HX	TLI ± S.E.M. (%)†
01	Control	30.1 ± 3.6	H1	Control	29.4 ± 2.9
02	2	22.1 ± 2.9	H2	1	1.8 ± 0.3 ***
03	4	3.6 ± 0.8 ***	H3	2	2.8 ± 0.8 ***
04	6	1.1 ± 0.4 ***	H4	3	1.9 ± 0.6 ***
05	8	1.0 ± 0.3 ***	H5	4	1.7 ± 0.4 ***
06	10	1.4 ± 0.6 ***	H6	5	2.1 ± 0.6 ***
07	12	1.5 ± 0.3 ***	H7	6	2.0 ± 0.7 ***
08	14	1.7 ± 0.4 ***	H8	7	1.7 ± 0.3 ***
			H9	10	1.7 ± 0.4 ***
			H10	14	1.6 ± 0.2 ***

*Ovariectomy (OVX) was performed 2–14 days (groups 02–08) prior to the sacrifice of the animals; hypophysectomy (HX) was performed 1–7 days prior to the sacrifice of 7-day-OVX mice (groups H2–H8), 10 days prior to the sacrifice of 10-day-OVX mice (group H9) and 14 days prior to the sacrifice of 14-day-OVX mice (group H10).

†Mean TLI values (± S.E.M.) of OVX or OVX-HX groups were statistically compared (Fischer *F* test) to the corresponding control (intact animals of groups 01 or H1) values (*** $P < 0.001$).

castration induced a marked decrease in cell proliferation, so confirming the well-known mitogenic effect of ovarian hormones on their most sensitive target organ [4, 36, 37]. The fact that hypophysectomy performed on the OVX animals did not further affect TLI is in good agreement with reports previously published by other authors [38–40]. Furthermore, HRF had no detectable effect on this tissue since his administration did not cause any cell proliferation.

The hormonal control of mammary tumorigenesis in rodents has been the subject of numerous investigations [31–34], and accumulated evidence has substantiated the key role of the hypothalamo-pituitary axis. Indeed, hypophysectomy and/or ovariectomy slowed down the growth of 3-methylcholanthrene [7], 7,12-dimethylbenz(a)anthracene [8–10], methyl-nitrosourea [11–13] and GR [14, 15] rodent mammary tumors. We made similar findings with respect to the MXT tumor since we found that hypophysectomy, and to a lesser degree ovariectomy, markedly slowed down the growth of this neoplasm. In this connection, we previously reported that PRL played an important role in MXT cell proliferation; CB-154, a potent suppressor of endogenous PRL secretion [41], significantly decreased this MXT growth [16, 18] whereas the administration of exogenous ovine PRL or the stimulation of endogenous PRL secretion by sulpiride [42] induced a mitogenic stimulation in this model which lasted from the 6th to the 48th hour after administration [16, 17]. The significant decrease in cell proliferation recorded in the MXT tumors in the 7-day-OVX and 1-day-HX animals might correspond to the radical suppression of the PRL mitogenic effect.

A totally new and interesting result concerns the increase in cell proliferation which lasted from the 2nd to the 5th day after the hypophysectomy performed on the OVX animals. This finding can be interpreted to mean that an unknown 'negative feedback', exerted by the hypothalamo-pituitary axis on MXT cell proliferation, might have been interrupted by the HX during this period of time. It is clear that further investigations are needed to understand why hypophysectomy induced a dramatic and transient mitogenic effect on the MXT mouse mammary tumors in OVX mice. Neverthe-

Table 2. TLI recorded in the MXT mouse mammary tumor (5 per experimental condition) after ovariectomy (OVX) of ovariectomy followed by hypophysectomy (OVH-HX)

OVX*			OVX-HX*		
Group	Number of days after OVX	TLI ± S.E.M. (%)	Group	Number of days after HX	TLI ± S.E.M. (%)
01	Control	14.2 ± 0.9	H1	Control	15.6 ± 0.8
02	2	8.8 ± 0.5	H2	1	10.6 ± 0.6 **
03	4	5.2 ± 0.4 ***	H3	2	15.1 ± 1.4
04	6	4.2 ± 0.6 ***	H4	3	19.3 ± 1.9 **
05	8	3.8 ± 0.8 ***	H5	4	19.9 ± 1.3 **
06	10	3.9 ± 0.7 ***	H6	5	19.8 ± 1.7 **
07	12	4.8 ± 0.8 ***	H7	6	17.2 ± 0.6
08	14	5.9 ± 0.6 ***	H8	7	14.2 ± 0.4
			H9	10	3.6 ± 0.4 ***
			H10	14	3.9 ± 0.5 ***

*,†See Table 1. ** $P < 0.01$ and *** $P < 0.001$ as compared to the control value (01 or H1).

Table 3. Effects of GnRH on the TLI recorded in the MXT tumor borne by 7-day-castrated and 5-day-hypophysectomized B6D2F1 mice (5 animal per experimental condition)

Groups* (hours after injections)	Mean TLI (%) ± S.E.M. in LE after vehicle or GnRH injections
G1 (control)	18.8 ± 0.8
G2 (6)	15.8 ± 1.2
G3 (12) †	5.9 ± 0.9 ***
G4 (24)	4.8 ± 0.8 ***
G5 (36)	6.8 ± 1.1 ***
G6 (48)	11.3 ± 1.0 **
G7 (72)	17.6 ± 0.9

*Animals of each group received an i.p. injection of either a placebo (0.1 ml saline: group G1) or of a solution containing 25 μg HRF (groups G2–G7) and were sacrificed 6–72 h later.
†Mean TLI values (± S.E.M.) of groups G2–G7 were statistically compared (Fischer F test) to the G1 group values (** $P < 0.01$; *** $P < 0.001$).

less, we used this OVX-HX model which shows a very high basal level of cell proliferation in order to study the effect of the administration of HRF to this MXT cell proliferation at different times before sacrifice.

It has previously been shown that GnRH and its various analogs have an inhibitory effect on mammary cancer growth [43–46]. To be more precise, after administering a GnRH analog to MXT bearing-mice, Redding and Schally [19] reported an antimitogenic effect of this hormone on the macroscopic growth of the tumor. Our results are in total agreement with those reported by these authors since we have shown that HRF induces an antimitogenic effect which lasts from the 12th to the 48th hour after administration. We can assume that this effect is direct because our experiments were carried out on castrated animals [47, 48]. A direct antimitogenic effect on GnRH on breast cancer has been reported by Miller *et al.* [49], who demonstrate that the growth of MCF-7 cells is inhibited by an agonist of this hormone.

All these observations lead us to the conclusions that hypophysectomy and, to a lesser degree, ovariectomy markedly slowed down the growth of our MXT mammary tumor. Hypophysectomy, performed on castrated animals, induced a significant and transient increase in cell proliferation in this neoplasm, dramatically decreasing after the administration of HRF, a GnRH analog. The direct

inhibitory effect of HRF on cell proliferation reported in the present work is of great interest. Indeed, this could partly explain the effect of hypophysectomy on human mammary cancer, where its action (direct or indirect) remains conjectural. And it is for this reason that we are now investigating this hypothalamic effect on mammary cancer cell proliferation in greater detail.

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