The gonadotropin-releasing hormone system: Perspectives from reproduction to cancer (Review)

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Abstract. Recently, an increasing amount of evidence indicates that human gonadotropin-releasing hormone (hGnRH) and its receptor (hGnRHR) are important regulatory components not only to the reproduction process but also in the regulation of some cancer cell functions such as cell proliferation, in both hormone-dependent and -independent types of tumors. The hGnRHR is a naturally misfolded protein that is retained mostly in the endoplasmic reticulum; however, this mechanism can be overcome by treatment with several pharmacoperones, therefore, increasing the amount of receptors in the cell membrane. In addition, several reports indicate that the expression level of hGnRHR in tumor cells is even lower than in pituitary or gonadotrope cells. The signal transduction pathways activated by hGnRH in both gonadotrope and different cancer cell types are described in the present review. We also discuss how the rescue of misfolded receptors in tumor cells could be a promising strategy for cancer therapy.

Contents

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1. Introduction

Cancer remains among the leading causes of mortality worldwide with an increase of 7.5 million of deaths in 2008 to 8.2 million in 2012. Furthermore, 32.6 million people are living with cancer according to the latest GLOBOCAN estimated cancer incidence, mortality and prevalence (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx). Despite the advances in the diagnosis and treatment of human malignancy, it is necessary to find new improved strategies to treat cancer. The human gonadotropin-releasing hormone (hGnRH) is a key hormone in the regulation of reproduction. However, an increasing number of reports have been shown the participation of GnRH and its receptor, not only in the reproduction but also in the regulation of tumor cell behavior. The hGnRH has been shown effective in controlling cell growth and invasiveness in certain type of tumors, both *in vivo* and *in vitro*. Furthermore, in some cases it has been reported to possess anti-oncogenic activity. These characteristics make the hGnRH/hGnRH receptor (hGnRHR) an ideal model in the study of new approaches for cancer treatment. The present review will focus on the signal transduction pathways activated when the hormone binds its receptor, in gonadotrope cells and in several types of cancer and how the relatively new concept of hGnRHR overexpression by pharmacoperons could be a new therapeutic strategy for cancer treatment.

2. GnRH receptor signaling in the pituitary

The tissue distribution pattern of the hGnRH and its receptor is wide and diversified. Therefore, it is not surprising that the cell signaling pathways produced from the interaction of the receptor with its hormone largely depend on the cellular context in which this occurs (1). In pituitary gonadotropes, the interaction between hGnRHR with its ligand induces conformational changes in both, the receptor itself and the coupled G-protein. The G protein family belongs to the heterotrimeric GTPases that are integrated by three subunits: Gα, Gβ and Gγ. There is a wide range of G proteins, which have been subclassified based on their structural differences in the Gα subunit. However, in gonadotrope cells the hGnRHR can be coupled to Gαq/11, and according to some reports, to Gαs subunit (2). The structural change of the G protein modifies the affinity for GDP, and the guanine nucleotide exchange

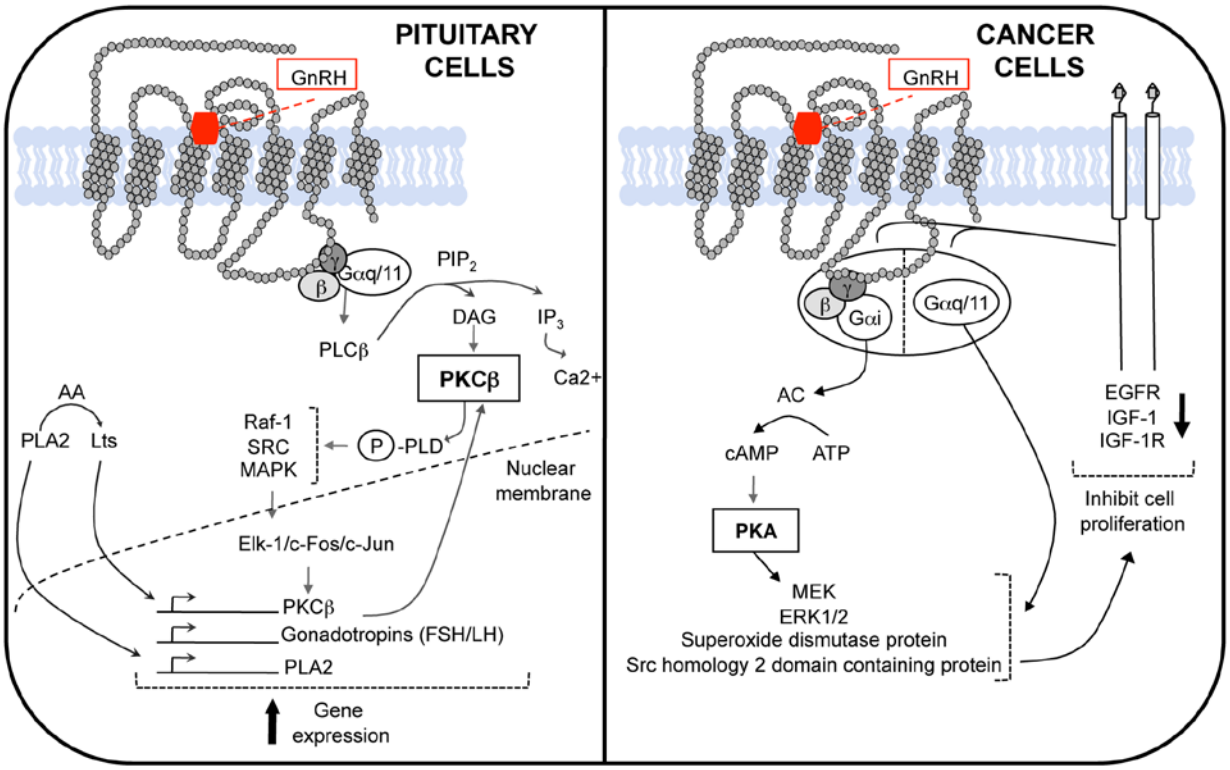

Figure 1. Signal transduction pathways activated by hGnRH. In pituitary cells (left panel) the hGnRHR once activated by its ligand is able to couple to Gαq/11 protein initiating the signal transduction pathway mediated by protein kinase C (PKC). This kinase is able to phosphorylate and activate another protein the phospholipase D (PLD) and the latest also phosphorylate the fibrosarcoma protein kinase 1 (Raf-1), protein tyrosine kinase SRC and some mitogen-activated protein kinases (MAPKs). The activation of Raf-1/MAPKs ends with the phosphorylation of transcription factors, such as Elk-1, c-Fos and c-Jun, which positively regulate the transcription of the gonadotropin α subunit gene, of the same PKC gene and the phospholipase A2 gene (PLA2). On the other hand in cancer cells (right panel) the receptor once activated by GnRH is able to couple both Gαi and Gαq/11 proteins depending on the cell background. When the receptor couples to Gαi it activates the adenylate cyclase (AC) that converts ATP in cyclic AMP (cAMP) that also in turn activate the protein kinase A (PKA). Once the PKA signal tranduction pathway is active the kinase phosphorylates and activated downstream kinases that directly or indirectly induce a decrease in epidermal growth factor receptor (EGFR), insulin-like growth factor I receptor (IGF-1R) and insulin-like growth factor 1 (IGF-1) that inhibit cell proliferation. In ovarian cancer, for instance, the hGnRHR also couples to Gαq/11 protein initiating the PKC signaling pathway resulting also in inhibition of cell proliferation.

factor facilitates the passive substitution of GDP for GTP. This nucleotide exchange causes the Gα subunit separation of the heterotrimer without affecting the association between the Gβ and Gγ subunits, which remain as one (3). Early studies on hGnRH signaling pathway in gonadotrophs, showed that only the Gαq/11 subunit could initiate an intracellular signaling cascade, through its main effector, the phospholipase Cβ (PLCβ) (4). However, further functional studies have shown that Gβγ dimer is also able to activate PLCβ; in fact it has been observed that in response to a sustained stimulation of hGnRH other effectors such as phospholipase A2 (PLA2) and D (PLD) can also be activated consecutively through both, Gα or Gβγ (Fig. 1) (5).

Phospholipase C uses phosphatidylinositol 4,5 bisphosphate (PIP2) as substrate and the enzymatic hydrolysis of this phospholipid produces the first wave of the second messengers, diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3) (2). The endoplasmic reticulum membrane contains IP3 receptors that can function as Ca^{2+} channels when activated by their specific ligand. The interaction of IP3 changes the structural conformation of its receptor, causing an increase in Ca^{2+} channel sensitivity with the consequent release of the cation into the cytosol. Subsequently, an increase in the concentration of Ca^{2+} activates the L-type voltage-sensitive calcium channels located in the cell membrane (6,7). Several studies have demonstrated the functional association between the accumulation of intracellular Ca^{2+} induced by hGnRH and the exocytosis of gonadotropins (8). In gonadotrope cells, the production of DAG and Ca^{2+} release into the cytosol are the main events in the synthesis and release of gonadotropins in response to hGnRH through the activation of protein kinase C (PKC) (9).

Due to the pleiotropic activity of the PKC, its activation is considered to be the central event in the activation of the hGnRH system. Studies performed in different cell systems have reported that two PKC isoforms α and βII have the ability to directly phosphorylate several residues of PLD and therefore activate the kinase (10,11). PLD hydrolyzes the phosphatidylcholine (PC) producing fosfatidilethanol (PET) and phosphatidic acid (PA), and both can act as second messengers increasing furthermore the signaling pathways initiated by hGnRH. PLD also rapidly activates fibrosarcoma protein kinase 1 (Raf-1), protein tyrosine kinase SRC and some mitogen-activated protein kinases (MAPKs) that are the immediate effectors in gonadotropes (10,12-14).

The direct or indirect activation of PKC upon via the Raf-1/MAPKs ends with the phosphorylation of transcription factors, such as Elk-1, c-Fos and c-Jun, which positively

regulate the transcription of the gonadotropin α subunit gene and the phospholipase A2 (PLA2) gene (2,15,16). In the gonadotrope cell line αT3-1, the PLA2 generates arachidonic acid (AA), which in turn is substrate for the lipoxygenase that converts AA into leukotrienes. These leukotrienes are also involved in the induction of gene expression of the gonadotropins α subunit as well as PKCβ (17,18). In turn all the signal transduction pathways induced by hGnRH and transduced by the axis hGnRHR/Gαq/11/PLC/PKC, culminate in the event of synthesis and release of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fig. 1) (19). The activation of a second G protein by the hGnRH stimulation in the gonadotrope remains controversial, some reports have indicated the activation of Gαs with the consequent activation of adenylate cyclase (AC) and the formation of the second messenger, cyclic adenosine monophosphate (cAMP), which in turn is the cofactor of the protein kinase A (PKA); however, further experiments are necessary to confirm these results (20).

Another component of these complex networks of signal transduction pathways is regulated by both frequency and amplitude of pulses of hypothalamic hGnRH secretion into the bloodstream depending on the physiological needs of the organism (21). It has been reported that when gonadotrope cells are exposed to a high frequency pulsation of hGnRH, the synthesis and release of gonadotropin α subunit and the β subunit of LH is induced. While hGnRH-low frequency pulsatility induces the synthesis and release of the β subunit of FSH (22).

3. Human GnRH receptor signaling in cancer cells

Prostate cancer. Prostate tumors and cell lines derived from these cancers express hGnRH, as well as the hGnRHR; in fact, almost 80% malignant prostate tumors present binding sites for hGnRH (23,24). Assays carried out in human prostate-cancer biopsy, shown that these binding sites were mediated by specific hGnRH receptors (25). Several studies have shown that the hGnRH/hGnRHR system promotes a decrease in cellular proliferation of malignant prostate tumors (26,27). Keeping this in mind, several research groups have been employing hGnRH as antineoplastic drugs for many years (28-33).

In tumor cell lines such as LNCaP (androgen-sensitive prostatic-cancer cell line), DU145 cells (a human androgen-independent cancer-cell line) and in PC-82 cells (an androgen-dependent cell line) specific binding sites by hGnRH have been shown (26,27,30). The complete mechanism by which hGnRHR is activated in prostate cancer is unknown; however, the identification of mRNA for hGnRH in LNCaP cells suggests a local paracrine/autocrine system in this tumor cells (34).

Many research groups have investigated the signaling pathway activated by the hGnRH/hGnRHR system to inhibit cell proliferation and ultimate demonstrate cross-talk between growth factor-receptors and hGnRHR. This interaction was observed in LNCaP and DU145 cells, where an association between hGnRHR and epidermal growth factor receptor (EGFR) was shown. In both cell lines the hGnRH stimulation abrogates epidermal growth factor (EGF)-induced transcription factor c-fos expression and reduces the number of EGF-binding sites in membrane. Furthermore, in DU145 cells, inhibition of the EGF receptor phosphorylation and reduction of EGF-binding sites in cell membrane after hGnRH treatment were demonstrated (35). Collectively, these results demonstrated that hGnRHR activation abrogates cell proliferation via EGFR signaling. On the other hand, an important inhibition of the mitogenic action of the insulin-like growth factor 1 (IGF-1) was also observed in DU145 cells after hGnRH-stimulation (36). The inhibitory effects of hGnRH in the proliferation of prostate cancer cells, appears to be mediated via coupling and activating the G protein Gαi, and not by Gαq/11 as observed in gonadotropes (37). In addition to the antiproliferative effect exhibited by the hGnRH/hGnRHR system in prostate cancer it also comprises direct induction of apoptotic signaling (38,39). hGnRH induces apoptosis in DU145 cells involving the activation of c-Jun N-terminal kinase (JNK) by a decrease of protein kinase B (PKB) activity. The activation of JNK could also be mediated by inhibition of the upstream activator of JNK, the mixed-lineage kinase 3 (MLK3) (38). However, details in the mechanisms by which hGnRH induced apoptosis remain to be determined.

Ovarian and endometrial cancer. Temporal and specific expression of hGnRH/hGnRHR has been shown in human ovary cells (40). Radioligand assay carried out in different cell lines and tumor biopsy specimens demonstrated high-affinity binding sites for ^{125}I-labeled hGnRH agonist ([D-Trp^6] LHRH) in 70% of primary ovarian cancers as well as in 83% of primary endometrial cancers (41,42). The elucidation of the cellular function of hGnRHR system in extra-pituitary tumor cells has been the goal of many researches. These reports have shown that the expression of both hormone and receptor are able to cause growth inhibition in malignant cells (42-45). At the same time, clinical data show that the expression of hGnRH/hGnRHR in epithelial malignant ovary tumors could be considered as a favorable prognostic factor (46).

Although the signaling pathways by which hGnRHR affects cell proliferation in ovarian cancer are still undetermined, it is clear that they are distinct from that in the anterior pituitary (Fig. 1). The specific intracellular signaling cascades that could be coupled to hGnRH in human ovary cancer are the activation of the PKC system. In the human endometrial cancer cell line HHUA, hGnRHR activation was able to downregulate the cellular proliferation via PKC and in human ovarian mucinous cystadenocarcinoma samples, hGnRH agonists also activate PKC protein (47,48). The PKC activation by hGnRHR, could be the link between receptor activation and MAPK kinase cascades to inhibit cell proliferation. In ovarian cancer cells SKOV-3 and OVCAR-3, a pronounced cell proliferation inhibition and activation of extracellular signal-regulated kinase (ERK)1/2 via Gαq/PKC, was demonstrated after hGnRH-stimulation (49).

On the other hand, the antiproliferative action of hGnRHR via ERK1/2 also has been reported as a PKC-independent process suggesting that hGnRHR signaling may vary by cell type (50). In ovarian carcinoma and endometrial carcinoma samples, the activation of the signaling pathways by hGnRHR could be associated with the coupling between the receptor and the Gi protein (51,52). The link between hGnRHR and Gi

could explain the differences in the signal transduction pathways activated by hGnRH receptors in malignant tumors and the anterior pituitary. Caov-3 cells shown growth inhibition in response to hGnRH, and also are able to activate several proteins such as adapter protein Src homology 2 domain containing (SHC), superoxide dismutase protein (SOD), MAP kinase kinase protein (MEK) and activation of ERK, via Gi coupling (50).

As mentioned above for prostate cancer, the effects of hGnRHR in mitogenic signaling pathways have also shown cross-talk between this receptor and growth factor receptors in ovary cancer cells. In endometrial (HEC-1A) and ovarian (EFO-21 and EFO-27) cancer cells, hGnRH receptor activation suppresses the phosphorylation of EGFR and inhibits the activation of MAP-kinase/ERK1/2 (53). Similar results were observed in DU-145 cells where hGnRH receptor activation abrogates EGFR-induced *c-fos* expression and reduces the concentration of EGF-binding sites, resulting in downregulation of cellular proliferation (35). In EFO-21 and EFO-27 cells the inhibition of the EGF receptor is mediated through the coupling between hGnRHR and Gi protein (54). The molecular mechanism for hGnRHR to inhibit the EGFR action may be mediated by direct interaction with intracellular mechanisms activated by EGF as well as by the decrease in the number of EGF receptors present on these cells. For example, in xenografts of OV-1063 cells, hGnRH agonists were able to significantly decrease tumor growth as well as the levels in membrane of EGFR and also its mRNA (55). Furthermore, chronic treatment with hGnRH, in these cells, was able to decrease the levels of insulin-like growth factor I receptor (IGF-1R) (44). A completely new mechanism of GnRH action in endometrial cancer cells has been recently described by Cho-Clark and colleagues (56) demonstrating the participation of a hGnRH metabolite, the GnRH(1-5), as an active component in the transactivation of EGFR via an orphan GPCR, the GPR101, in Ishikawa cells. Although cross-talk between hGnRHR and growth factor receptors has been clearly demonstrated, it seems to vary in different cellular contexts. Taken together these results show different layers in the complexity of the hGnRH/hGnRHR system regulation in cancer cells. Furthermore, a compound develop by Schally's group, the Dox-14-O-hemiglutarate conjugated to [D-Lys6] GnRH-I (AN-152, AEZS-108; Æterna Zentaris Inc., Quebec, QC, Canada) is currently in phase III clinical trial on ovarian and endometrial cancer due to its promising results in cancer treatment (57).

Breast cancer. Breast cancer is the most common diagnosed cancer and the main global cause of death in women. Approximately, from 75 to 80% of breast cancers are hormone-dependent expressing both estrogen and progesterone receptors (58,59). Approximately 15-20% of breast cancers overexpress the human epidermal growth factor receptor-2 (HER2), and about half of these tumors also express steroid hormone receptors. Unfortunately, 10-15% of breast cancers do not express estrogen or progesterone receptors, nor HER2. These so-called triple-negative breast cancers do not benefit from specific therapies that target these receptors and, therefore, have the worst outcome (59). hGnRH stimulates gonadotropin secretion from the hypothalamus and thereby controls gametogenesis and steroidogenesis in the gonads (60). hGnRH-stimulated gonadotropin secretion can be blocked with antagonists or with a sustained stimulation with agonists, causing the so-called 'medical castration' underlying the use of hGnRH analogs to treat hormone-dependent neoplasms (59,60). In addition to expressing the hGnRHR, the tumors also revealed the presence of hGnRH, indicating the existence of an autocrine/paracrine hGnRH/hGnRHR system that might regulate tumor growth and invasiveness (61). Approximately, 50-60% of human breast cancer expresses hGnRHR and it has been highly speculated that activation or inhibition of hGnRHR signaling may directly affect cell growth (62-66). Morgan and colleagues (66) demonstrated that hGnRH receptor was expressed in a wide range in 298 primary breast cancers, but most importantly, its expression was significantly higher in patients with triple-negative phenotype.

Initially, it was described that in tumor cells the hGnRHR was exclusively coupled to Gi and consequently inhibit the cAMP accumulation, this action presumably mediates the anti-proliferative effect in these cells (61,67,68). Some reports, however, indicated that for tumor cells the hGnRHR is able to activate several G-proteins in a specific cell background (69). The evidence so far indicates that the anti-proliferative response induced by hGnRHR activation results in apoptosis and G2/M arrest in the cell cycle. This process is mediated via a coordinated dynamic pattern of MAPK, cell cycle, apoptotic and cytoskeletal-related signaling (70).

4. Non-endocrine related cancers

As mentioned above, a broad variety of human cancers, but not normal tissue, express the hGnRH/hGnRHR system, thus, chronic administration of hGnRH agonists are widely and successfully used for the treatment of hormone-dependent tumors. However, study of the hGnRH system in non-hormone-dependent cancers has recently been initiated. For instance, the cutaneous melanoma is still the leading cause of skin cancer deaths in developed countries (71). Moretti and colleagues (72) demonstrated that hGnRHR were expressed in melanoma cells, and the stimulation with a hGnRH agonist had a significant inhibitory effect on tumor progression and neoangiogenesis, by interfering with the activity of growth factors. In multiple myeloma cells RPMI 8226, the use of an hGnRH agonist induces apoptosis and inhibits cell growth by increasing the expression of anti-oncogenes p21 and p53 (73). hGnRH as treatment in colon cancer has been initiated, and preliminary reports suggest an anti-angiogenic property of the hormone (74). As an innovative colon cancer treatment, Schreier and colleagues (75) designed a derivate bioconjugate between a chemotherapeutic agent and a hGnRH analog to target colon cancer cells. Their results demonstrated that the treatment with the bioconjugate changed the protein expression profile of multiple intracellular processes. The use of hGnRH analogs in endometrial cancer had variable responses, some patients respond to therapy but the overall response was suboptimal (76). In some cases, such as in human bladder cancer cells hGnRHR showed a tendency to form nanodomains; however, the effect of any hGnRH analog has not been used in this type of cancer (77).

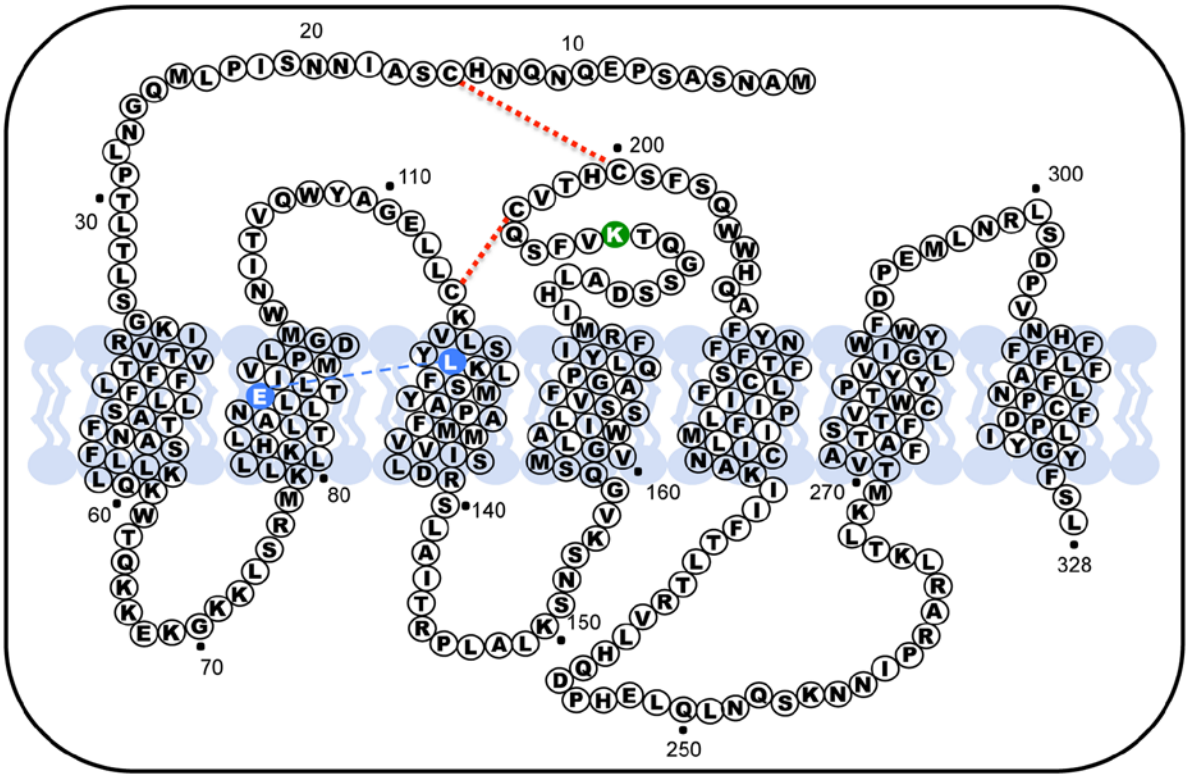

Figure 2. Graphic representation of the human GnRH receptor. Simplified representation of the hGnRHR showing the disulphide bridges between Cys14-Cys200 and Cys114-Cys196 (red lines), the extra amino acid found in primates and humans Lys191 (green circle), and the salt bridge between Glu90Lys121 (blue circles).

5. Rescue of misfolded hGnRH receptors

The hGnRHR in its natural form exists as a misfolded protein indicating that the cell intentionally synthesizes conformational misrouted receptors. This characteristic made us hypothesize that the decrease in protein membrane expression is the result of a complex evolutionary reproductive process (78). This hGnRHR that is normally functional become misrouted and then degraded by the cell quality control, therefore ceasing its activity. Among the G-protein coupled receptors, hGnRHR has several specific characteristics including the absence of a terminal carboxy-tail. Another peculiarity of the hGnRHR is the presence of a Lys residue in position 191 of the extracellular loop (EL) 2, in rodents GnRH receptors this orthologous amino acid is absent, making the last protein one residue smaller (327 amino acids). In rodent the lack of this residue confers the GnRH receptor an increased plasma membrane expression (79); therefore, the presence of Lys191 in humans limits the number of receptors exported from the endoplasmic reticulum to the membrane by interfering, primarily, with formation of the Cys14-Cys200 disulphide bridge (Fig. 2) (80). When the disulphide bridge forms, the hGnRHR is recognized by the cell as correctly folded and allows it to be exported to the plasma membrane. As a result, the presence of the Lys191 interferes with the probability of bridge formation and could explain the decrease in trafficking to the plasma membrane. In fact, the deletion of Lys191 from the hGnRHR increases the plasma membrane expression of the protein (81,82). Another structural feature that modulate trafficking of the receptor to the plasma membrane is the formation of a Glu90-Lys121 salt bridge that is essential for the transit through the quality control system of the cell, in fact the Glu90Lys mutant, which led to hypogonadotropic hypogonadism, was retained in the endoplasmic reticulum and represents a loss of function receptor (83,84). Further studies led to the demonstration that this particular mutation was fully rescue by pharmacoperone drugs by the formation of a surrogate bridge from residues Asp98 and Lys121 that can substitute for the original salt bridge that is broken in Glu90Lys mutant (84). Moreover, this human receptor mutation was able to be rescued by four different chemical classes of pharmacoperones that interact identically by creating the same surrogate bridge (85). In fact, pharmacoperones are able to rescue most of the hGnRH receptor mutants, even though mutations appear through the whole receptor sequence, further analysis indicated that these drugs might stabilize the relation between TMD2 and TMD3 domains on the hGnRHR allowing them to pass the quality control of the cell, and thus reaching the membrane. Therefore, it is clear that in human Lys191 is part of a complex motif that results in decreased efficiency in expression (80,82,86).

6. Conclusions

This overview shows that the hGnRH/hGnRHR system activates different signal transduction pathways in gonadotrope and tumor cells, coupling to different G-proteins depending on the cell context. Most interesting is the finding that this hormone/receptor system is present not only in reproductive tissue but also in tumor cells with various degrees of the expres-

sion. In some cases the expression of hGnRHR is related to cancer progression; for example, in ovarian carcinomas of early stages the presence of this receptor is higher when compared with advanced stages of carcinoma. Also in prostate cancer loss of hGnRHR expression with tumor progression has been demonstrated (87,88). Moreover, nanomolar concentrations of GnRH II antagonists induce apoptotic cell death in human endometrial, ovarian, and breast cancer *in vitro* and *in vivo*, via dose-dependent loss of mitochondrial membrane potential and activation of caspase-3 (89). According to the literature, in several tumor cell lines, the expression level of GnRHR is significantly lower than in pituitary or the gonadotropes (57). The demonstration that hGnRHR exists as a misfolded protein has raised the speculation that in tumor cells this compartmentalization and retention of the receptor in the ER function as a protective mechanism to avoid cell death induced by hGnRHR activation (88,90). In prostate cancer cultures it was demonstrated that IN3 enhances the GnRH agonist apoptotic effect by increasing the hGnRHR in the plasma membrane (88). The use of a careful pulsatile pharmacoperone therapy in a knock-in mouse expressing the hGnRHR mutant E90K is able to restore the mutation from ER retention to the plasma membrane. Also spermatogenetic proteins associated with steroid transport and synthesis, and androgen levels were restored with pharmacoperone administration (91). The hGnRHR plasma membrane increase by pharmacoperones, due to the trafficking of the receptor from the ER, may represent an important research area to evaluate the clinical use of IN3 in tumor cells. Given the clinical utility of hGnRH, further studies of pharmacoperones are necessary to characterize this compound as a step to a more effective and perhaps new therapeutic strategy for cancer patients.

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