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Activation of human gonadotropin-releasing hormone receptor promotes down regulation of ARHGAP18 and regulates the cell invasion of MDA-MB-231 cells

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ABSTRACT

The Gonadotropin-Releasing Hormone Receptor (GnRHR) is expressed mainly in the gonadotrope membrane of the adenohypophysis and its natural ligand, the Gonadotropin-Releasing Hormone (GnRH), is produced in anterior hypothalamus. Furthermore, both molecules are also present in the membrane of cells derived from other reproductive tissues such as the breast, endometrium, ovary, and prostate, as well as in tumors derived from these tissues. The functions of GnRH receptor and its hormone in malignant cells have been related with the decrease of proliferation and the invasiveness of those tumors however, little is known about the molecules associated with the signaling pathways regulated by both molecules in malignant cells. To further analyze the potential mechanisms employed by the GnRHR/GnRH system to reduce the tumorigenesis of the highly invasive breast cancer cell line MDA-MB-231, we performed microarrays experiments to evaluated changes in genes expression and validate these modifications by functional assays. We show that activation of human GnRHR is able to diminish the expression and therefore functions of the Rho GTPase-Activating Protein 18 (ARHGAP18). Decrease of this GAP following GnRHR activation, correlates to the higher of cell adhesion and also with reduction of tumor cell invasion, supporting the notion that GnRHR triggers intracellular signaling pathways that acts through ARHGAP18. On the contrary, although a decline of cellular proliferation was observed during GnRHR activation in MDA-MB-231, this was independent of ARHGAP18 showing the complex system in which is involved the signaling pathways regulated by the GnRHR/GnRH system.

1. Introduction

The Gonadotropin-Releasing Hormone Receptor (GnRHR) is a G protein-coupled receptor mainly located in gonadotrope membrane of the adenohypophys and its natural ligand, the Gonadotropin-Releasing Hormone (GnRH), is a decapeptide synthesized in the hypothalamus (Ulloa-Aguirre et al., 2006). In addition, both molecules are also present in the membrane of cells derived from other reproductive tissues such as the breast, endometrium, ovary, and prostate, as well as in tumors derived from these tissues (Aguilar-Rojas et al., 2016). To date, the functions of this receptor and its hormone in malignant tissue have been related with processes that regulate cell proliferation and tumor invasiveness (Teng et al., 2015; Millar et al., 2004; Moretti et al., 2002;

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Kahán et al., 1999, 2000; Sharoni et al., 1989; Miller et al., 1985); however the signaling pathways involved in these processes are not well known. Nevertheless, the ability of the GnRHR/GnRH system to reduce cell tumor invasion and metastatic potential has been associated with actin cytoskeleton remodeling, high substrate adhesion, and hyper-elevated RhoA activity (Aguilar-Rojas et al., 2012; Dobkin-Bekman et al., 2009; Enomoto et al., 2006; Yates et al., 2005; Davidson et al., 2004).

The small GTPase RhoA, is a protein that belongs to the human Rho GTPase family (Etienne-Manneville and Hall, 2002). The function of this molecule is to regulate cell migration and adhesion by controlling the formation of actin stress fibers and the assembly of focal adhesion (Goicoechea et al., 2014; Ridley and Hall, 1992). Like other GTPases, RhoA is active when it is binding to GTP and inactive when it is binding to GDP. Accessory proteins regulate the conversion between active and inactive states. The inactive GDP-bound state is converted into an active GTP-bound state by Guanine nucleotide Exchange Factors (GEF). GTPase-Activating Proteins (GAP) delimits GTP hydrolysis and Guanine nucleotide Dissociation Inhibitors (GDI) are the proteins responsible for mantling the cytosolic pool of inactive GTPase (Csepanyi-Komi et al., 2013; García-Mata et al., 2011; Rossman et al., 2005).

Numerous GAPs control the action of RhoA in diverse cell types, but little is known about the molecules associated with this GTPase in cells undergoing malignant state. The GAP protein, Rho GTPase-Activating Protein 18 (ARHGAP18), has shown to be a negative regulator of RhoA through angiogenesis, but principally during cell invasion (Chang et al., 2014; Forbes et al., 2011; Stefansson et al., 2011; Hu et al., 2009; Hahn et al., 2005). Knockdown of ARHGAP18 in the breast tumor cells MDA-MB-231, promoted delay in cell migration by a substantial increase in cell adhesion produced by an increase in actin stress fibers and a great number of focal adhesions (Maeda et al., 2011). The changes induced by the suppression of ARHGAP18 activities were similar to those induced by high levels of active RhoA, demonstrating a tight correlation in the functions of both molecules and their importance in controlling tumor cell invasion.

To further analyze the potential mechanisms employed by the GnRHR/GnRH system to reduce the tumorigenesis of the highly invasive breast cancer cell line MDA-MB-231, we performed microarrays experiments to evaluated changes in genes expression and validate these modifications by functional assays. We hypothesized that activation of GnRH receptor in these cells would lead changes in the expression of genes associated with cell adhesion and invasion. In this sense, we found that GnRHR activation by the GnRH agonist (GnRHa) Buserelin, lead to downregulation of ARHGAP18 at transcriptional and translational levels. Decrease of this GAP following GnRHR activation, correlates to the higher of cell adhesion and also with reduction of tumor cell invasion, supporting the notion that GnRHR triggers intracellular signaling pathways that acts through ARHGAP18. On the contrary, although a decline of cellular proliferation was observed during GnRHR activation in MDA-MB-231, this was independent of ARHGAP18 showing the complex system in which is involved the signaling pathways regulated by the GnRHR/GnRH system. In conclusion, our data showed that changes in migration and invasion promoted by the activation of GnRHR in MDA-MB-231 correlates to the lack of ARHGAP18 but also suggest that the effects of GnRHR on cell proliferation could include signaling pathways independents of ARHGAP18.

2. Materials and methods

2.1. Constructions

The complete complementary human DNA (cDNA) of Wild-Type Gonadotropin Releasing Hormone Receptor (GnRHR-WT) (GeneBank access number L07949) was cloned in the expression vector pcDNA3.1 (Invitrogen, USA) at Kpn1 and Xba1 sites (New England BioLabs, USA). The overexpressed mutant form of GnRHR, lacking Lys191 (GnRHR-DesK) (Arora et al., 1999), was constructed by site-directed mutagenesis (Stratagene, USA). All constructions were verified by dye terminator cycle sequencing (Perkin Elmer, USA) and shown to encode the correct amino acid sequences.

2.2. Cell culture and transient transfection of MDA-MB231 cells

The highly invasive breast cancer cell line, MDA-MB-231, was obtained from the American Type Culture Collection (ATCC, USA) and cultivated in Leibovitz's medium supplemented with antibiotics and 10% Fetal Calf Serum (FCS) (Invitrogen) in a humidified chamber at 37 °C and at 5% CO_2.

GnRHR-WT and GnRHR-DesK constructions were transiently expressed in MDA-MB-231 cells (denominated in this work as, GnRHR-WT or GnRHR-DesK, respectively). Transfections were performed with 250,000 cells/well in 12-well culture plates (Costar, USA), with 1.0 μg DNA/well, employing the FuGENE HD transfection reagent and following the manufacturer's protocol (Roche Applied Science, Germany). Experiments were performed 24 h after transfection. Cells transfected with empty pcDNA3.1 vector were employed as negative controls.

2.3. Wound healing assay

MDA-MB-231 cells were seeded in Collagen type I from calf skin (10 mg/ml)-coated 12-well plates (Sigma-Aldrich, USA) and transfected with GnRHR-WT or GnRHR-DesK plasmids. Cells transfected with the empty vector, were employed as negative control. Twenty-four h post-transfection, the cells were starved overnight in free-serum medium. Then, a wound was made on the confluent cell monolayer by scratching it with a sterile 200-μl micropipette tip. Cells were allowed to migrate in free-serum media complemented with the agonist of GnRH (GnRHa) Buserelin, at 10^{-5} M for 48 h (Sigma-Aldrich). Images were capture at 0, 24, and 48 h using an ECLIPSE Ti inverted microscope (Nikon, Japan) with a 10X/0.25 NA objective (Nikon), attached a Clara E camera system (Andor, UK). The images were analyzed with the aid of Icy bioimage analysis software http://icy.bioimageanalysis.org (de Chaumont et al., 2012).

2.4. DNA microarray analysis

2.4.1. Printing of arrays

Change in gene expression evoked by the activation of GnRHR-WT and GnRHR-DesK were measured during cell migration employing the Human 70-mer oligo library V4.0.3 from OPERON Oligo Sets. This is a whole-genome oligonucleotide human microarray plate that contains 35,035 oligonucleotide probes, representing approximately 25,100 unique genes and 39,600 transcripts. The Human library was printed, prehybridized and fixed as previously was shown (Luna-Moreno et al., 2007). The experiments were performed at the Microarray Unit of the Cellular Physiology Institute of the National Autonomous University of Mexico (IFC-UNAM, México).

2.4.2. Total RNA isolation, probe preparation, and hybridization to arrays

In brief, MDA-MB-231 cells were seeded above Collagen type I and transfected as was described above. Twenty-four h later, GnRHR-WT and GnRHR-DesK cells were incubated with Buserelin for 24 h. Control cells were maintained in FCS-free medium for 24 h. Total RNA (RNAt) from each condition was extracted employing

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