INTRODUCTION

Follicle-stimulating hormone (FSH) is the specific central glycoprotein hormone of mammalian reproduction, and is necessary for sex maturation at puberty during the fertile phase (Simoni and Nieschlag, 1995). The FSH receptor (FSHR) is a member of the seven-transmembrane domain or G protein-coupled receptor (GPCR) superfamily of cell surface receptors (Kim et al., 2018). FSHR is mainly synthesized in granulosa and Sertoli cells, and moves to the membrane surface. Constitutively activating mutations in the FSHR gene were found to localize in an essential region of the transmembrane domain, which is highly conserved among FSHRs, and within FSHRs across different species (Simoni et al., 1997). Previous work by others has shown that transmembrane domains V and VI of the male FSHR bearing a D567G mutation maintain constitutive activity in such a constrained state that there is no appreciable effect on basal cAMP levels (Gromoll et al., 1996). Furthermore, a Ile160Thr mutation of the FSHR extracellular domain was reported to drastically reduce binding of iodinated human FSH in COS-7 cells. This resulted in a decrease of cAMP to 10% of the maximum value observed in wild type cells in response to exogenous FSH binding (Beau et al., 1998). Similarly, a cell line harboring Arg573Cys mutation in exon 10, which encodes the third loop, produced only about 30% of wild type cAMP levels (Tapanaine et al., 1997).

A novel FSH receptor mutation was identified in a patient with primary ovarian failure and infertility by whole...
exome sequencing (Bramble et al., 2016). A novel homozygous mutation, C175T, was detected in a Chinese woman with primary ovarian insufficiency (Liu et al., 2017). The polymorphism genotypes of FSHR G919A in premature ovarian failure of Iranian women may be associated with diminished ovarian reserve (Ghezelayagh et al., 2018). Two female siblings of Indian descent were diagnosed with primary ovarian failure and hypergonadotropic hypogonadism associated with mutations of the FSH receptor (T1253G; G1255A) (Katari et al., 2015).

Recent work from our group has focused on elucidating the function and role of glycoprotein hormones and their receptors in the Japanese eel (Kim et al., 2016a,b; Kim et al., 2018; 2019; Byambaragchaa et al., 2018a,b). To determine the mechanisms underlying eel reproduction, we investigated the activity of eelFSHR containing a mutation in the transmembrane VI domain. To our knowledge, this is the first report of constitutive activation of the eelFSHR (D540G) mutation and its effects on basic cAMP response.

**MATERIALS AND METHODS**

**Materials**

The mammalian expression vector pCDNA3, FreeStyle CHO-S suspension cells, and Lipofectamine-2000 were bought from Invitrogen Corporation (Carlsbad, CA, USA). The cloning vector pGEMTeasy was bought from Promega (Madison, WI, USA). cAMP HTRF assay kit was purchased from Cisbio (Codolet, France). Primary antibody coating of 96-well plate was conducted with 5A11 monoclonal antibody as previously reported (Kim et al., 2016). Polymerase chain reaction (PCR) reagents were from Takara (Shiga, Japan). The QIAprep-Spin plasmid kit was purchased from Qiagen. Inc. (Hilden, Germany). Fetal bovine serum was from Hyclone laboratories (Logan, UT, USA). Centriplus Centrifugal Filter Devices were purchased from Amicon Bio separations (Billerica, MA, USA). All other reagents used were from Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemicals (Osaka, Japan).

**Site-directed mutagenesis and vector construction**

To introduce eel FSHR point mutations to generate a constitutive mutant, an overlap extension PCR strategy was used as previously reported (Min et al., 2004). Two different sets of PCRs were performed. The newly synthesized full-length PCR products were eluted and cloned into an pGEMTeasy vector and transformed into DH5α competent cells. Plasmids were isolated and sequenced (Genotech, Korea). cDNAs encoding both wild type and mutant eel FSHR were digested with Eco RI and Xho I, and ligated into pcDNA3 as previously reported (Min et al., 2004). Plasmids were then purified and confirmed by restriction digestion analysis to ensure that the correct mutant PCR production had been inserted (designated as pcDNA3-eel FSHR-D540G).

**Transient transfection and production of rec-eelFSH protein**

Recombinant eel FSH protein was produced in CHO-S cells using the liposome transfection method as previously described (Byambaragchaa et al., 2018). Supernatants were collected and frozen at -80°C. The samples were concentrated by freeze-drying and mixed with PBS. The concentration of recombinant-protein was analyzed by enzyme-linked immunosorbent assay (ELISA) as previously reported (Byambaragchaa et al., 2018).

**cAMP assay**

Measurement of cAMP accumulation in CHO-K1 cells expressing wild type and mutant eel FSHR receptors was performed using cAMP Dynamic 2 competitive immunoassay kits (Cisbio Bioassays, Codolet, France) as described previously (Lee et al., 2017). These method uses a competitive immunoassay between native cAMP produced by cells and cAMP labeled with the dye d2. The specific signal-Delta F (energy transfer) is inversely proportional to the concentration of cAMP in the standard or sample. Results are calculated from the 665 nm/620 nm ratio and expressed as Delta F % (cAMP inhibition). cAMP concentrations were calculated from Delta F% values using GraphPad Prism software (GraphPad, Inc., La Jolla, CA, USA).

**Data analysis**

The Multalin interface–multiple sequence alignment tool was used for sequence analysis and comparisons: GraphPad Prism 6.0 (GraphPad, Inc, CA) and Grafit 5.0 (Eri-thiacus Software Limited, Surrey, UK) was used for cAMP EC50 value and stimulation curve analysis. Each curve was drawn using data from at least three independent experiments.
RESULTS

The mutation (D540G) is located at the C-terminal region of the third intracytoplasmic loop (IC) amino acid 540 of the receptor, and work by others has shown its involvement in a case of pseudoprecocious puberty (Laue et al., 1995) (Fig. 1). This region of the receptor is highly conserved among glycoprotein hormone receptors. CHO-K1 cells transiently transfected with the mutant receptor produced cAMP at basal levels that were consistently higher than those produced by the wild type receptor in the absence of eel FSH agonist. When 2.5 μg of plasmid DNA encoding the wild type receptor was transfected into cells, cAMP levels were increased in a dose-dependent manner. In contrast, transfection of the constitutively active mutant receptor increased cAMP levels to values much higher than those of cells expressing the wild type receptor, even in the absence of FSH stimulation. cAMP levels in cells expressing the D540G mutation reached

| eelFSH receptors | cAMP responses |
|-----------------|----------------|
|                 | Basal (nM/10⁴ cells) | EC₅₀ (ng) | Rmax (nM/10⁴ cells) |
| eelFSHR-WT (2.5 ug) | 1.0 ± 0.3 (1-fold) | 538.5 (100%) | 40.3 ± 2.8 (100%) |
| eelFSHR-D540G (2.5 ug) | 13.6 ± 0.5 (13.1-fold) | 559.5 (15%) | 27.6 ± 4.3 (68.5%) |
| eelFSHR-WT (5 ug) | 1.0 ± 0.2 (19.1-fold) | 543.8 (100%) | 61.3 ± 4.7 (100%) |
| eelFSHR-D540G (5 ug) | 19.6 ± 2.3 (19.6-fold) | 685.0 (20%) | 50.8 ± 3.5 (82.9%) |

Values are the means ± SEM of triplicate experiments. EC₅₀ values were determined from the concentration-response curves from in vitro bioassays.
concentrations 13.1-times higher than wild type. However, the maximal cAMP level was 68.5% relative to wild type (Fig. 2). Next, we analyzed the induction of cAMP production using 5 µg plasmid DNA for transfection. In these experiments, cAMP level were increased to an even greater extent (19.6-fold) in the absence of recombinant-eel FSH than that by the wild type cells. Maximal cAMP levels were also increased in the receptor cells transfected with 5 µg plasmid DNA, but approximately 83% compared to that in wild type (Table 1).

DISCUSSION

We characterized one constitutive activating mutation, D540G, in the eel FSHR, which has been previously reported in FSHR (Fig. 1). The molecular function of the constitutively activating D540G FSHR mutation is still unknown. To the best of our knowledge, this is the first report in which constitutively activating eel FSH receptor results in highly increased cAMP production. We reported that rat FSHR indicated highly basal cAMP level, even in the absence of a ligand (Min et al., 1996). Some studies suggest that gonadotropic hormones, particularly FSH, influences the development of ovarian tumors (Fragoso et al., 1998; Bas et al., 2009). These results indicate that tumors originating from granulosa cells contain FSHRs and that tumorigenesis is affected by FSH and by activation of FSHR-mediated signaling pathways (Stouffer et al., 1984; Nakano et al., 1989; Fragoso et al., 1998). Activating mutation in the FSHR was firstly described in the hypophysectomized 28-year-old male that was unexpectedly fertile (Gromoll et al., 1995b). Screening of the FSHR gene led to the characterization of a point mutation in exon 10, D567G, in the third transmembrane loop of the receptor protein. Basal cAMP levels were increased in COS-7 cells harboring this mutation, rising from 1.5- to 3-fold when compared to cells expressing wild type receptor (Gromoll et al., 1996). Other studies reported that the corresponding D to G mutation has occurred in the thyroid stimulating hormone receptor and luteinizing hormone receptor (Laue et al., 1995; Tonacchera et al., 1996). Inactivating homozygous A189V missense mutation in the FSHR was identified in about one-third of all Finnish XX ovarian failure (XXOF) patients (Aittomaki et al., 1995; Layman and McDonough, 2000). cAMP production was drastically reduced in immortalized MSC-1 cells in response to recombinant FSH. Another inactivating mutation of the FSHR gene was found at position 191 (N191I) in a healthy fertile woman and completely abolished the increase of cAMP production in response to FSH (Gromoll et al., 1995). These findings demonstrate that the number of functional receptor expressed on the cell surface is drastically reduced in these mutants, and that signal transduction is impaired as a result.

In the present study, we identified a novel constitutively activating mutant receptor, FSHR D540G, that resulted in highly increased cAMP production in the absence of eel FSH stimulation. Transfection of 5 µg of D540G mutant receptor resulted in a 19.6-fold increase in basal cAMP production in the absence of agonist when compared to cells expressing the wild type receptor. Future studies will provide insight into cell specific elements that are involved in gonadotropin receptor signal transduction during eel maturation.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

This research was financially supported by the Korean Research Foundation program (2018R1A2B6007794), Republic of Korea.

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