主論文の要旨

Establishment of a Human Nonluteinized Granulosa Cell Line that Transitions from the Gonadotropin-Independent to the Gonadotropin-Dependent Status

（ゴナドトロピン非依存期からゴナドトロピン依存期に渡るヒト非黄体化顆粒膜細胞株の樹立）

名古屋大学大学院医学系研究科　健康社会医学専攻
発育・加齢医学講座　産婦人科学分野
（指導：吉川 史隆　教授）
巴雅蘇拉

Introduction

The ovaries are complex and precisely-regulated endocrine organs that are responsible for the production of the sex steroids and fertilizable ova. A greater understanding of the mechanisms of folliculogenesis and steroidogenesis may lead to improvements in the success rates of assisted reproductive technologies or the development of novel treatments for reproductive disorders. A follicle consists of the oocyte itself and two kinds of somatic cells surrounding the oocyte, the granulosa cells and the theca cells, which play main roles in steroidogenesis. Therefore, the ovarian function can be considered to be dependent upon the follicle functions, in other words, steroidogenesis, oogenesis, folliculogenesis, atresia, and luteinization. To understand the mechanisms of the follicle function at the molecular level, materials which are suitable for *in vitro* studies are needed. In the present study, we established a new cell line derived from human non-luteinized granulosa cells, which underwent the transition from gonadotropin-independent to gonadotropin-dependent status.

Materials and Methods

We obtained granulosa cells from a 35-year-old female and immortalized them by lentivirus-mediated transfer of several genes so as to establish a human non-luteinized granulosa cell line (HGrC1). We subsequently characterized HGrC1 and investigated its steroidogenic performance. This study was approved by the Ethics Committee of Nagoya University School of Medicine.

Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen). The tTA-advanced segment from the pTet-Off Advanced plasmid (Clontech) was recombined with a lentiviral vector, CSII-CMV-RfA, by the LR reaction (Invitrogen) to generate CSII-CMV-TetOff. The elongation factor 1 alpha promoter (EF) in CSII-EF-MCS was replaced with a tet-responsive promoter (TRE-Tight) from pTRE-Tight (Clontech) followed by a modified RfA fragment (Invitrogen) to produce a tet-responsive lentivirus vector, CSII-TRE-Tight-RfA. The entry vectors containing cDNAs for hTERT, human cyclinD1, human mutant CDK4 ($CDK4^{R24C}$: an inhibitor resistant form of CDK4), and HPV16 E6E7 were recombined with CSII-TRE-Tight-RfA by the LR reaction (Invitrogen) to generate CSII-TRE-Tight-hTERT, -CyclinD1, -CDK4R24C, and -16E6E7. The production and infection of recombinant lentiviruses were performed as described previously. The primary cells in a 6-well dish were infected with CSII-CMV-TetOff, CSII-TRE-Tight-hTERT, CSII-TRE-Tight-CyclinD1 and CSII-TRE-Tight-CDK4R24C lentiviruses at the multiplicity of infection of more than 5 on the third day. Growing cells were split into 12 wells of a 24-well plate on day 7, and subsequently infected with CSII-TRE-Tight-16E6E7. On day 13, when the primary culture without transgenes contained only a few colonies consisting of fibroblastic cells, many healthy colonies consisting of small round-shaped cells emerged in these cultures and were passaged into larger dishes. The cells were named HGrC1 and then were further characterized.

Results

HGrC1 expressed enzymes related to steroidogenesis such as StAR, CYP11A, aromatase and gonadotropin receptors. Stimulation with FSH increased the mRNA levels of aromatase, which consequently induced the aromatization of androstenedione to estradiol. Activin A increased the mRNA levels of the FSHR, which were synergistically upregulated with FSH stimulation. HGrC1 also expressed a series of ligands and receptors belonging to the TGF-β superfamily. A Western blotting analysis showed that BMP-4 and -15 phosphorylated Smad 1/5/8, while BMP-6, -7, -15, GDF-9 and AMH phosphorylated Smad 2/3.

Discussion

For humans, the granulosa cells available for *in vitro* studies were derived from patients undergoing IVF. Although the cells obtained from IVF are useful to some extent, there exist some limitations to the experiments that can be performed using these cells. First, the cells obtained from IVF patients are fully luteinized or in the luteinization process due to the administration of hCG prior to oocyte retrieval. Although the effect of hCG might be eliminated by means of culture in medium without hCG, hCG has a major influence on the differentiation of granulosa cells into luteinized cells, which includes effects on significant molecules related to intracellular signaling pathways and cell cycle regulation. Second, each follicle contains only a limited number of granulosa cells. Therefore, granulosa cells are usually pooled from several follicles, which might contain granulosa cells of different profiles. In addition, the limited number of cells makes it impossible to repeat experiments, making it necessary to perform experiments in cells from different patients. Another source of granulosa cells used for *in vitro* studies has been experimental animals. However, it should be noted that there may be species-specific differences in the granulosa cell function. Taken together, the use of homogenous granulosa cells derived from human ovarian follicles, which are not atretic or luteinized, is highly desired to improve research on steroidogenesis and folliculogenesis.

Accumulating evidences suggests that the TGF-β superfamily members expressed by ovarian somatic cells and oocytes is important for the intraovarian control mechanisms, including the recruitment and progression of follicles. We herein demonstrated that Smad 1/5/8 and/or 2/3 were phosphorylated with BMP-4, -5, -7, -9, -15, GDF-9 and AMH in the HGrC1 cells. These results indicate that HGrC1 may possess the characteristics of granulosa cells belonging to follicles in the early stage. HGrC1 might also be capable of displaying the growth transition from a gonadotropin-independent status to gonadotropin-dependent one.

In conclusion, we established an immortalized human granulosa cell line, which shows FSH-responsive aromatase activity, inducible FSHR expression and responsiveness to the TGF-β superfamily. Further *in vitro* studies using this cell line would be helpful to reveal the intrafollicular mechanisms of the human ovary, including the recruitment, progression and atresia of follicles.