Application of CRISPR /dCas9 Systems for Increasing the Expression of FSH Receptor in Human Granulosa Cells

by

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May 13, 2022

Application of CRISPR /dCas9 Systems for Increasing the Expression of FSH Receptor in Human Granulosa Cells

Koç University

Graduate School of Health Sciences

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Aylin Seren GÜLLER

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To science…

ABSTRACT

Application of CRISPR /dCas9 Systems for Increasing the Expression of FSH Receptor in Human Granulosa Cells

Aylin Seren GÜLLER
Master of Science in Reproductive Biology
May 13, 2022

FSH (follicle-stimulating hormone) is necessary for the ovary to produce oocytes and the production of steroid hormones. Follicular development is arrested when FSH is not present. The change from FSH-independent to FSH-dependent growth in granulosa cells depends on FSHR (follicle-stimulating hormone receptor). CRISPR activation (CRISPRa) is based on the ability of dCas9, a CRISPR protein variant that cannot act as an endonuclease, to connect to genes without altering the DNA. CRISPRa systems bind to DNA and recruit transcription factors to enhance gene expression. CRISPRa strategies use a variety of transcriptional activators, including fusion proteins and Cas system re-engineering. SunTag, SAM, and VPR have all shown significant improvements over the initial dCas9-VP64 technique; therefore, there are several options to choose from when activating genes in various cell lines.

The objective of this research has been established to determine if CRISPR dcas9 technology could be used to increase FSH receptor expression in HGRC1 cells (human granulosa cell line) and to investigate the possible effects of dCas9 application on intracellular pathways . The results have indicated that, the dcas9 system can activate FSHR, and increased FSH receptor activity affects several fundamental cellular events involving steroidogenic, life-sustaining, and cell death related signalling sub-pathways. After using the dcas9 system to activate FSHR in human granulosa cells, we have demonstrated a significant increase in the gene expression profile of FSHR, AKT, aromatase, MAPK8, MAPK1, ESR1, PGR, MTOR, p53, caspase 3. The examination of FSHR-related proteins has revealed that the protein levels of FSHR, AKT, aromatase, and p-38 MAPK have also increased. The functionality of dcas9 activated FSHR was tested using follitropin alpha (Gonal-f). It has also been shown that although the gene expression levels of FSHR, AKT, aromatase, MAPK8, p53, and caspase 3 increased on a time-

dependent manner, the increment in MAPK1, ESR1 (Estrogen Receptor1), PGR (Progesterone Receptor), and MTOR was temporary.

The results of our research led us to conclude that dcas9 technology can be used to increase FSHR at the gene and protein levels efficiently. Genetically engineered granulosa cell for increased FSHR can be used as in vitro models to investigate in vitro maturation (IVM), FSH hyper/hyposensitivity, polycystic ovary syndrome (PCOS) and cancer studies.

Key words: Follicle, FSHR, Granulosa Cells, CRISPR/dcas9

ÖZETÇE

İnsan Granüloza Hücrelerinde FSH Reseptörünün Ekspresyonunu Arttırmak için CRISPR /dCas9 Sistemlerinin Uygulanması

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FSH, ovaryumda folikülogenezin desteklenmesi ve steroid hormonlarının üretimi için gereklidir. FSH'nın eksikliğinde foliküler gelişim durur. Granüloza hücrelerinde FSH' dan bağımsız büyümeden FSH' ya bağımlı büyümeye geçiş FSH reseptörünün (FSHR) bu hücrelerdeki anlatımına bağlıdır. CRISPR aktivasyonu, endonükleaz görevi bulunmayan bir CRISPR protein varyantı olan dCas9' un DNA'yı değiştirmeden genlere bağlanma yeteneğine dayanır. CRISPR aktivasyon (CRISPRa) sistemleri DNA' ya bağlanır ve gen anlatımını geliştirmek için transkripsiyon faktörlerini kullanır. CRISPRa stratejileri, füzyon proteinleri ve Cas sisteminin yeniden mühendisliği dahil olmak üzere çeşitli transkripsiyonel aktivatörleri kullanır. SunTag, SAM ve VPR' nin tümü, ilk dCas9-VP64 tekniğine göre önemli gelişmeler göstermiştir; bu nedenle, çeşitli hücrelerdeki genleri aktive ederken seçilebilecek birkaç seçenek vardır.

Araştırmamızın ana hedefi, insan granüloza hücre hattında (HGRC) FSH reseptörünün anlatımını artırmak için CRISPR dcas9 teknolojisinin kullanılıp kullanılamayacağını belirlemek ve bu uygulamanın hücre içi yolaklardaki olası etkilerini incelemektir. dCas9 sisteminin FSHR' yi aktive edebildiğini ve artan FSH reseptör aktivitesinin steroidojenik, yaşamı sürdürme ve ölüm sinyalizasyonunda yer alan birkaç alt yolu etkilediği sonucuna ulaşılmıştır. İnsan granüloza hücrelerinde FSHR' yi etkinleştirmek için dcas9 sistemini kullandıktan sonra, FSHR, AKT, aromataz, MAPK8, MAPK1, ESR1, PGR, MTOR, p53, kaspaz 3' ün gen anlatımını önemli ölçüde arttığını gösterdik. FSHR ile ilgili proteinler incelendiğinde FSHR, AKT, aromataz ve p-38 MAPK' da protein seviyesinin yükseldiği de gözlendi. dcas9 ile aktive edilen FSHR' nin işlevselliği, follitropin alfa (Gonal-f) tedavisi kullanılarak test edildi. FSHR, AKT, aromataz, MAPK8, p53 ve kaspaz 3' ün gen

anlatım seviyesinin bütün zamanlara bağlı olarak artması gösterilmişken, ESR1, PGR ve MTOR' un gen anlatımının geçici olarak arttığı gösterilmiştir.
Araştırmamızda, dcas9 teknolojisinin gen ve protein seviyelerinde FSHR' yi etkili bir şekilde artırmak için kullanılabileceği ortaya konmuştur. Genom düzenleme teknikleri kullanılarak arttırılmış FSH reseptörü, in-vitro oosit maturasyonunda, kadınlarda sık görülen FSH duyarlılığı sorunlarının araştırılmasında özellikle, polikistik over sendromu (PCOS), overin aşırı ve yetersiz duyarlılığı, kanser hastalarındaki çalışmalarda yardımcı olabilir.

Anahtar Kelimeler: Folikül, FSHR, Granüloza Hücresi, CRISPR/dcas9

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ABBREVIATIONS

AKT	V-Akt Murine Thymoma Viral Oncogene Homolog
AMH	Anti-Mullerian Hormone
ARO	Aromatase
BMP	Bone Morphogenetic Protein
cAMP	Cyclic Adenosine Monophosphate
CASP3	Caspase3
CC	Cumulus Cell
COC	Cumulus-Oocyte Complex
CREB	Camp-Response Element Binding Protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	Clustered Regularly Interspaced Short Palindromic Repeats Activation
crRNA	CRISPR RNA
CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1
dCas9	Dead Cas9 Endonuclease
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone Sulfate
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate-Buffered Saline
E2	Estradiol
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPD	Eukaryotic Promoter Database
ERK	Extracellular Signal-Regulated Kinase
ESR1	Estrogen Receptor 1
FBS	Fetal Bovine Serum
FSH	Follicle-Stimulating Hormone
FSHR	Follicle-Stimulating Hormone Receptor
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDF	Growth Differentiation Factors
GFP	Green Fluorescent Protein
GnRH	Gonadotropin-Releasing Hormone
gRNA	Guide RNA
HCG	Human Chorionic Gonadotropin
HDL	High-Density Lipoprotein
HEK	Human Embryonic Kidney
HGF	Hepatocyte Growth Factor
HGRC	Human Non-Luteinized Granulosa Cell
HR	Homologous Recombination
IGF	Insulin-Like Growth Factor-I
IVF	In Vitro Fertilization
IVM	In Vitro Maturation

JNK	Jun N-Terminal Kinase
KGF	Keratinocyte Growth Factor
LB	Lysogeny Broth
LDL	Low-Density Lipoprotein
LH	Luteinizing Hormone
LHCGR	Luteinizing Hormone Receptor
LIF	Leukemia Inhibitory Factor
MAPK	Mitogen-Activated Protein Kinases
MGC	Mural Granulosa Cells
MOI	Multiplicity Of Infection
MPF	M-Phase Promoting Factor
MTOR	Mammalian Target Of Rapamycin
NHEJ	Non-Homologous End-joining
PAM	The Protospacer Adjacent Motif
PCOS	Polycystic Ovary Syndrome
PDE	Phosphodiesterase
PGC	Primordial Germ Cell
PGR	Progesterone Receptor
PKA	Protein Kinase A
SAM	Synergist Activation System
SDS–PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
sgRNA	Single-Guided RNA
SMAD	Signal Transduction Molecules
TDF	Theca Differentiation Factors
TGF	Transforming Growth Factor
TP53	Tumor Protein 53
tracrRNA	Trans-Activating Crispr RNA
TSS	Transcription Start Site
ZP	Zona Pellucida

Chapter 1:
INTRODUCTION

1.1.Ovary

The ovaries are a couple of almond-formed, pinkish-white organs, 3 cm long, 1.5 cm wide, and 1 cm thick. The ovary comprises a cortex and a medulla. The medulla is situated in the focal area of the ovary. It contains moderately massive, tangled veins, lymphatic vessels, and nerves. The cortex is situated in the fringe area of the ovary, encompassing the medulla[1] (Figure 1).

The two principal elements of the ovary are the development of gametes and steroid chemicals. The development of gametes in females is called oogenesis. [2]. Estrogens and progestogens, the two primary gatherings of steroid chemicals, are discharged by the ovaries. Estrogens support the development of internal and external genitalia and are involved in development of the female sex characteristics in adolescence. During pregnancy, progestogens prepare the internal genital organs, particularly the uterus.[3] The two chemicals assume a fundamental part in the period by setting up the uterus for implantation of the treated ovum. If implantation does not occur, the endometrium of the uterus is shed off, and the monthly cycle happens. [4]

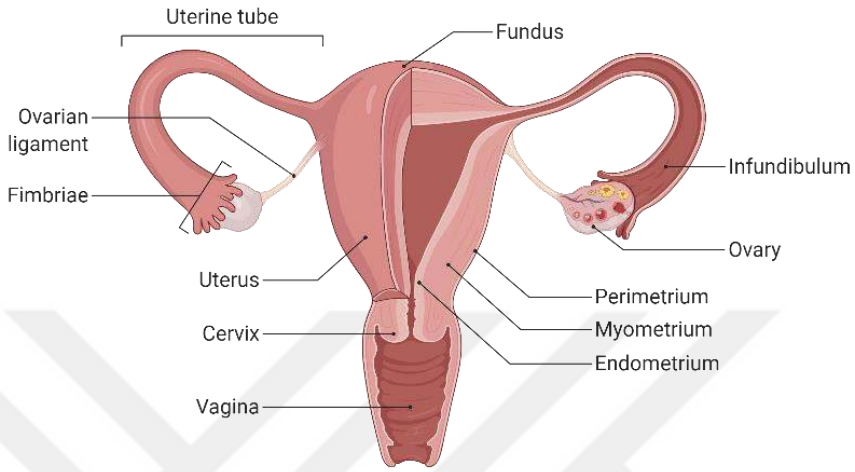

Figure 1. Female reproductive system. *The ovaries are a couple of almond-shaped, pinkish-white organs, 3 cm long, 1.5 cm wide, and 1 cm thick. (Generated via Biorender.com)*

1.1.1. Folliculogenesis

1.1.1.1. Follicle

The follicle is the functional unit of the ovary.[5] The follicle structure consists of an oocyte and surrounding granulosa and theca cells (Figure 2). During folliculogenesis, the growth and maturation of the oocyte within each follicle; proliferation and development of granulosa and theca cells occur in a highly coordinated manner.[6] This coordination indicates that the ovulating oocyte is ready for fertilization and subsequent embryonic development. Some morphological and molecular changes occur within the follicle structure to develop the follicle and ultimately for the successful completion of this developmental process.[6]

In order the immature oocyte to complete the meiosis, growth, and development; the granulosa cells also need the oocyte for their proliferation, differentiation, and function.

This two-way interaction continues throughout the development. Complex cell-cell interactions regulating the development of ovarian follicles occur with endocrine, autocrine, paracrine regulators, and intercellular connections. [7]

Each follicle contains one oocyte and is found in the cortical region of the ovary. The early stage of oogenesis occurs in fetal life and during fetal period, mitotic divisions increase the number of oogonia[8]. As the details will be overviewed later in this chapter, follicles undergo cyclic growth and maturation in small groups during puberty. A cyclical pattern of follicular maturation and ovulation go on with the menstrual cycle. Usually, only one oocyte reaches maturity and it is released from the ovary via ovulation with each menstrual cycle. Most of the oocytes present at birth are not fully mature and the primary oocytes that reside in primordial and primary follicles can undergo atresia. throughout reproductive life in females.[8]

The development of the human ovary begins 3-4 weeks after implantation. Important events such as germ cell migration, gonadal sex differentiation, mitotic divisions and atresia of germ cells, and follicular formation begin and get completed during fetal life. Events such as meiosis and follicular growth, and atresia in germ cells, which continue before puberty and throughout reproductive life, also begin in late fetal life. Although it is unknown which factors provide this migration in humans, it has been suggested that chemotactic and cell adhesion factors are of importance. Primordial germ cells increase their number by mitosis after reaching genital ridge. [9]

In the mouse, these cells divide rapidly under the influence of members of the transforming growth factor β (TGFβ) superfamily.[10] In human, by the 20th week of fetal life, there are approximately 7 million oogonia and between the third and fifth months, the oogonia forming primary oocytes start the first meiotic division and arrest in the diplotene stage of the first meiosis. This arrest takes place until puberty. As oogoia undergo apoptosis during fetal life, their number decreases to a couple of millions at the time of birth. At puberty, the presence of approximately 300,000 follicles or oocyte is defined. [10]

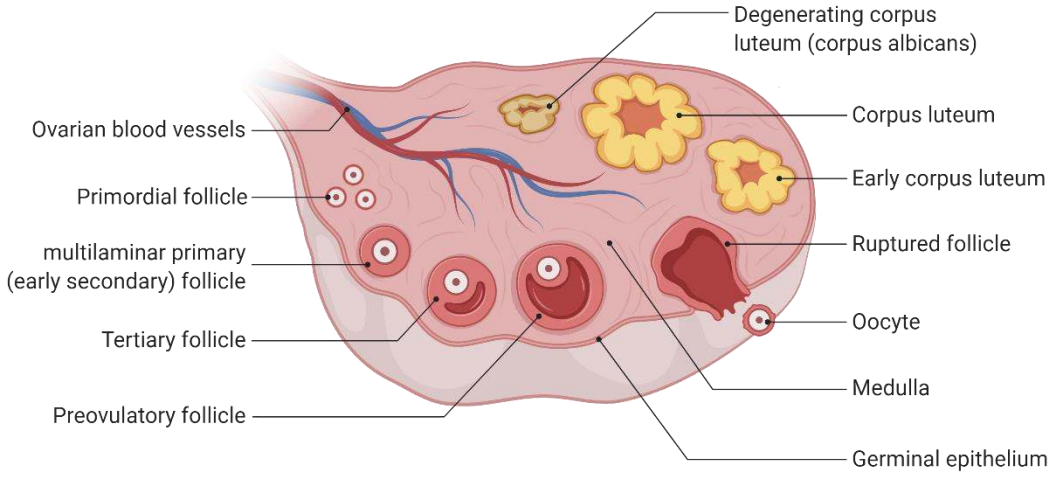

Figure 2. The general architecture of the ovary. *The ovary comprises a cortex and a medulla. The medulla is situated in the focal area of the ovary. The cortex is situated in the fringe area of the ovary, encompassing the medulla. The follicles are located in the cortical region. (Generated via Biorender.com)*

1.1.1.1.2. Primordial Follicle Development

Primordial follicle development in humans begins at 16 weeks and ends at postnatal six months. Primordial follicles representing the ovarian reserve consist of a diplotene oocyte surrounded by a single layer of flat pre-granulosa (follicular) cells. In oogonia, the beginning of mitosis and its surrounding with granulosa cells called follicle formation protects it from atresia. After 28 weeks, oogonia that have not entered meiosis is no longer observed in the ovary. Oogonia is also absent in the newborn ovary. Primordial follicles pass from the dormant phase to the primary stage with the onset of growth.[11]

1.1.1.1.3. The Primary Follicle

The nucleus of the pending oocyte has partially condensed prophase chromosomes and is highly enlarged. This structure, called the germinal vesicle, can protect DNA. In the initial stages of folliculogenesis, the oocyte enlarges, and the granulosa cells become cuboidal. The factors that initiate preantral follicle development by stimulating primary follicle formation are still not fully defined. Studies have shown that primordial follicles reach the primary stage with autocrine-paracrine interactions of signals originating from different compartments in the ovary (oocyte, granulosa and theca cells, stroma) rather than a single hormone or signal pathway. [12]

Moreover, FSH is not required to activate the primordial follicles because primordial follicles do not contain FSH receptors. During primordial to primary follicle transformation, squamous granulosa cells become cuboidal, and oocyte diameter increases. Some members of the transforming growth factor-beta (TGF-ß) family are involved in this activation. Bone morphogenetic proteins BMP-4 and BMP-7 (released from an ovarian stroma and theca cells); growth differentiation factor 9 (GDF-9) plays a role in this phenomenon. [12] Zona pellucida (ZP) is formed as a glycoprotein matrix, secreted between the oocyte and granulosa cells. FIGα transcription factor has been shown for the expression of three glycoproteins of ZP, namely ZP1, ZP2, and ZP3. In the absence of ZP3, which is one of the significant components of ZP, it was observed that folliculogenesis continued in mice, but these mice were infertile because oocytes adhered to the oviducts after ovulation. [13]

1.1.1.1.4. Preantral Follicle and Theca Cells

The beginning of follicular development causes morphological events such as the growth of the oocyte within the follicle, granulosa cell proliferation associated with the formation of the multilayer structure, and the formation of a thecal layer around the follicle outside the basement membrane. Until the early antral follicle stage, events are independent of gonadotropins and are regulated by intraovarian and intrafollicular mechanisms. Studies have shown that preantral follicle development usually continues in FSHβ knockout mouse models or hypogonadal mice. [14] However, the preantral stage in the follicle is considered to be the last gonadotropin-independent stage before transitioning to the early

antral stage. The transition from the preantral follicle to the early antral follicle stage is a critical stage in terms of dependence on gonadotropins and the fate of the follicle, which results in continued development or atresia. [14] (Figure 3)

Therefore, follicular development can be divided into three stages: i. primordial and primary follicle stages (gonadotropin-independent stage), ii. the transition from preantral to the early antral stage (gonadotropin-sensitive stage), iii. developmental stages that continue after the early antral stage. In the transitional phase, the growth of the follicle is mainly regulated by intraovarian regulators (growth factors, cytokines, and gonadal steroids), the presence of FSH can stimulate growth. [14,15]

Despite the controversial effects of FSH on preantral follicle development, it is now a well-known fact that some locally produced factors mediate follicle growth in this period. Again, activins, BMP-4 and BMP7, oocyte-derived GDF-9 and BMP-15 play a role in preantral and antral follicle development. Although the formation of the preantral follicle begins to occur gradually, the theca cell layer formation takes place in the transition to the early antral follicle, and this event plays an essential role in determining the fate of the follicle. With the regulation of the theca cell layer, important physiological events for early follicular development such as increased steroidogenic responses to gonadotropins, formation of the thecal layer, increased blood flow support including structural and ovarian regulators, and production of androgens for estrogen biosynthesis in granulosa cells occurs with the formation of this layer. [16] Theca cell layer formation occurs without luteinizing hormones (LH) because there are no LH receptors in stromal cells to differentiate into theca cells. After the granulosa cells of the follicle have two or more layers, theca differentiation factors (TDF) released from these cells stimulate the expression of LH receptors of theca precursor cells and the mRNAs of steroidogenic enzymes (CYP11A, 3β-HSD, and CYP17) required for androgen biosynthesis. The formation of the theca cell layer in the preantral follicle is an important physiological event for follicular development. Steroidogenic response to gonadotropins increases with the theca cell layer regulation. In addition, blood flow support from the theca cell layer provides ovarian regulators for follicle development. Production of aromatizable androgens for estrogen biosynthesis occurs in the theca cell layer. As the primary source of androgens in the ovary theca cells provide precursors for estrogen synthesis in granulosa cells. The theca cells interact with granulosa cells with the hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) they secrete. [17]

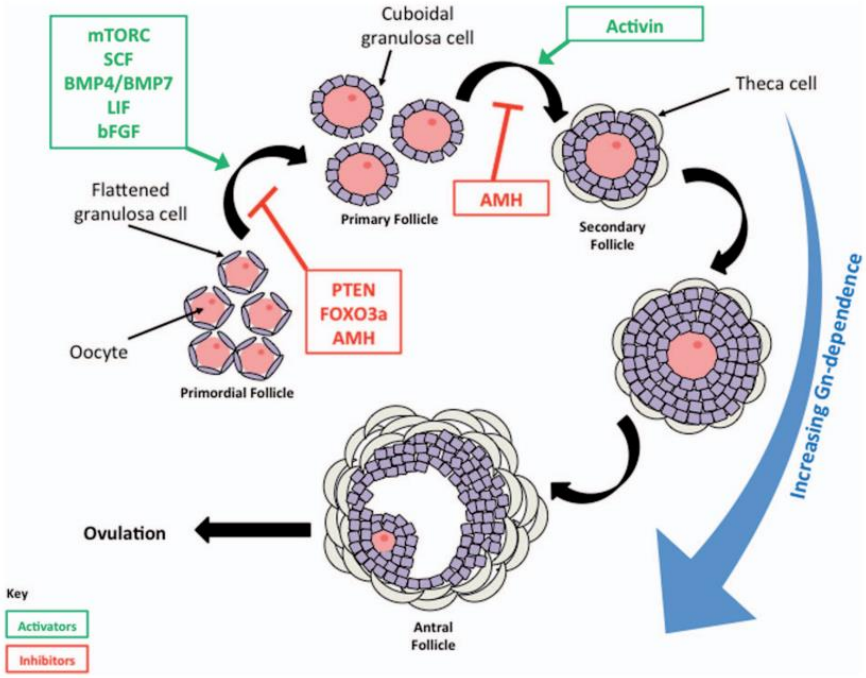

Figure 3. A summary of folliculogenesis. *Follicular development can be divided into three stages: i. primordial and primary follicle stages (gonadotropin-independent stage), ii. the transition from preantral to the early antral stage (gonadotropin-sensitive stage), iii. developmental stages that continue after the early antral stage. (Reprinted from[17])*

1.1.1.1.5. Granulosa Cell Differentiation

At the preantral follicle stage, the oocytes are surrounded by granulosa cells that have not yet differentiated. With the formation of a fluid-filled cavity called the antrum, the granulosa cells differentiate into two anatomically and functionally different types of cell lines. Mural granulosa cells (MGC) are located along the follicle wall and have steroidogenic activity; cumulus cells (CCs), on the other hand, are the cells that form the cumulus-oocyte complex (COC) structure, which is in contact with the oocyte with corrugated type connections and has transzonal extensions. Factors GDF-9 and BMP-15 are central regulators in MGC/CC differentiation. The oocyte, controls the differentiation and critical functions of granulosa cells and determines the ovulation rate. As a result of oocyte removal from the follicle, increased apoptosis was observed in cumulus cells, and this situation was reversible when these cells were exposed to factors secreted from the

oocyte. Granulosa and cumulus cells express TGFβ superfamily receptors and intercellular signal transduction molecules (SMAD). TGFβ superfamily and SMAD intracellular cascade activation plays a central role in signaling between oocyte and cumulus cells. [18,19]

1.1.1.1.6 Cumulus Expansion

The initiation of cumulus expansion depends on two signaling pathways. The first is stimulation with gonadotropins or epidermal growth factor (EGF)-like peptides; the other is related to the effect of the paracrine factors secreted from the oocyte. Factors that stimulate cumulus expansion by secretion from the oocyte enable the cumulus cells to synthesize extracellular matrix molecules by responding to gonadotropin / EGF signals. In addition to provide a structural support for follicle formation and maturation, the extracellular matrix, regulates the entry and exit of these signals into cells. It is also essential for the survival and proliferation of granulosa cells and in this way, it affects the fate of the follicle.[20]

1.1.1.1.7. Nuclear Maturation

The secondary messenger cyclic adenosine monophosphate (cAMP), which is produced by granulosa cells and transmitted to the oocyte via gap junctions, plays a role in the meiotic arrest of the oocyte, and this effect is provided by the phosphorylation of the regulatory protein M-phase promoting factor (MPF), which is responsible for the initiation of nuclear maturation. High cAMP concentration inhibits the continuation of meiosis. LH stimulation before ovulation causes a change in the structure of the corrugated connections between the oocyte and the surrounding cumulus cells, and therefore the level of cAMP in the oocyte decreases. MPF activity begins to increase rapidly, which leads to the destruction of the nuclear sheath and chromosome condensation. Before ovulation, the oocyte enters its final maturation stages, completing the first meiotic division and discarding its first polar body. The second meiosis begins and arrests at metaphase. Moreover, the oocyte remains at this stage until fertilized by the sperm. [20,21,22]

1.1.1.1.8. Antral Follicle and Luteinization

The transition from the gonadotropin-sensitive preantral stage to the early antral stage is followed by follicular developmental stages. The transition to the early antral stage is also the stage in which the fate of the follicle is determined whether the follicle will undergo apoptosis or not. GDF-9 prevents apoptosis of granulosa cells and follicular atresia at this stage, and the thecal factors secreted from theca cells also suppress apoptosis of granulosa cells. Apoptosis can occur at any stage of follicular development, but it is considered that, the transitional stage in humans that is the most sensitive stage to teratogenic signals. [22] Gonadotropin-releasing hormone (GnRH), produced and secreted from the hypothalamus in female, stimulates the release of heterodimeric glycoprotein hormones called as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary (Figure 4). FSH stimulates follicular maturation together with locally produced estradiol. Adenylyl cyclase is activated as a result of binding of FSH to its G-protein accompanying the FSH receptor expressed by granulosa cells, and this event results in an increase in cAMP and activation of protein kinase A. FSH stimulation and estradiol together activate cytochrome P450 aromatase, inhibin α and β subunits, and LH receptor expressions. LH concentrations stimulate maximum androgen production from theca cells. Activin and inhibin peptide hormones constitute feedback mechanisms that stimulate and inhibit FSH production and release in the pituitary. [22,23] Oocytes are also involved in regulating the inhibin-activin-follistatin system of granulosa cells. Inhibin B increases due to the culture of mural granulosa cells with oocytes. The preovulatory follicle ovulates the oocyte in response to LH secreted from the pituitary, and some functional and morphological events occur in the remaining structure. Granulosa cells differentiate into luteal cells that produce the progesterone necessary to maintain the pregnancy until placental development. Before ovulation, the oocyte inhibits follicular luteinization. Cumulus cells have deficient steroidogenic activity (progesterone production) compared to MGCs from the same follicle. [24]

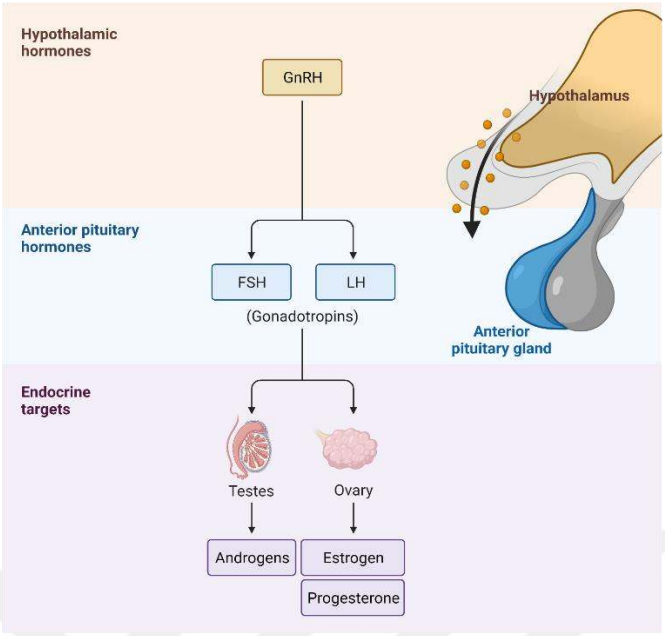

Figure 4. The gonadotropin secretion. *Gonadotropin-releasing hormone (GnRH), produced and secreted from the hypothalamus in female, stimulates the release of heterodimeric glycoprotein hormones called as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. (Generated via Biorender.com)*

1.1.1.1.9. Dominant Follicle Selection and the role of FSH

Follicles in the antral stage grow through the early, mid, and late antral stages. During this period, more growth in the antral cavity, increased oocyte diameter, proliferation in granulosa and theca cells, and increased vascularization in theca cells are observed. FSH is a critical determinant of follicle growth during this period. A cohort consisting of a certain number of antral follicles cyclically starts to grow further with the effect of FSH and compete for dominant follicle selection. [25] As the selected cohort grows, it is critical to modulate the steroidogenic activity and responses of antral follicles in this cohort to gonadotropins while inhibiting possible premature luteinization. In this way, certain follicles are made more sensitive to FSH, and the basis for the selection of dominant follicles is formed. [25] The available information shows that all these events are realized by some hormones produced locally at the autocrine-paracrine level. These hormones are Activin, BMP-6 from granulosa cells, GDF-9, and BMP-15 from the oocyte, and BMP-2,3b,4, and 7 arising from theca cells.[26,27] In human and animal models it has been shown

that increasing FSH receptor expression, aromatase activity, and inhibin production in granulosa cells; suppressing progesterone production, LH receptor expression, and androgen production in theca cells can increase the oocyte maturation in vitro. [27]

1.1.1.2. Gametogenesis

Gametogenesis is a process that involves the division and development of diploid or haploid precursor cells to create mature haploid gametes. [28] Males and females of sexually reproducing organisms have separate processes of gametogenesis: 1. Spermatogenesis, 2. Oogenesis.[28] Only oogenesis will be revised here, as it is related to the female reproductive system.

1.1.1.2.1. Oogenesis

By the third month of fetal development, the oogonia have started to mature into primary oocytes, the majority of which have already reached meiosis I prophase. The early and late phases of oogenesis coexist in the fetal ovary because oogenesis is not coordinated. Despite the fact that there are several million oocytes at birth, most of them degenerate, while the remainder persist in prophase I for decades. Only around 400 mature and ovulate throughout a woman's menstrual cycle.[31] Individual follicles continue to develop and mature once a woman achieves sexual maturity, and a few (on average one per month) are ovulated.[32] The oocyte quickly completes meiosis I just before ovulation, separating into two cells: one becomes the secondary oocyte, containing most of the cytoplasm and organelles, while the other becomes the first polar (Figure 5). Meiosis II commences quickly and progresses to the metaphase stage during ovulation, when it pauses until fertilization occurs, at which point it resumes.[32] As FSH is needed for follicular growth it has an indirect but important effect on the maturation of oocytes.

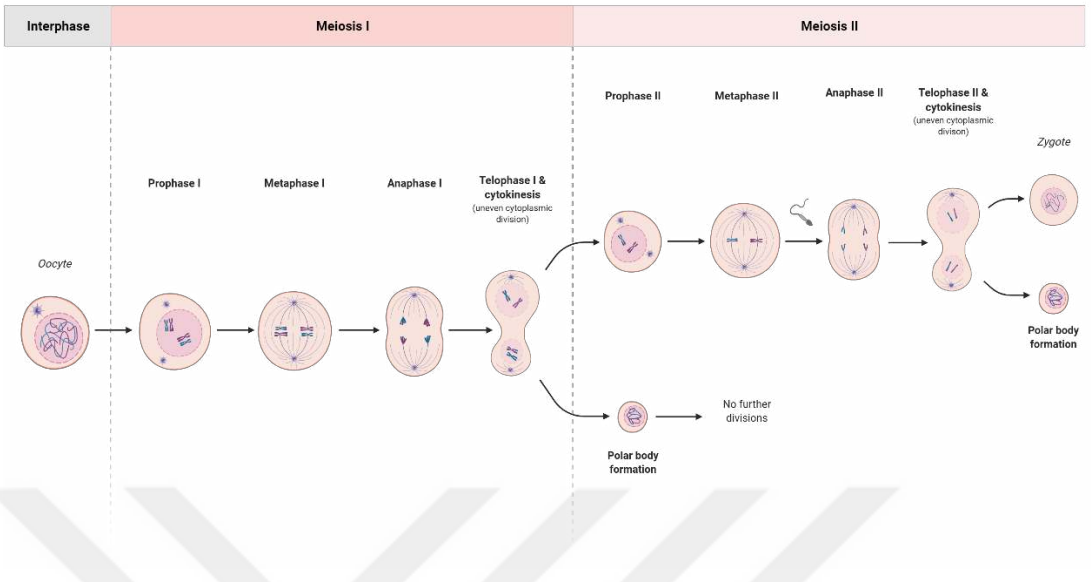

Figure 5. Oogenesis. [32] *The oocyte completes meiosis I just before ovulation, separating into two cells: one becomes the secondary oocyte, containing most of the cytoplasm and organelles, while the other becomes the first polar body. (Generated via Biorender.com)*

1.2.Steroidogenesis

Sex hormones are cholesterol-based steroid chemicals produced primarily in the testes, ovaries, and adrenal cortex. The biosynthesis pathways for male and female sex hormones are the same. The ultimate result of steroidogenesis of sex hormones is determined by the presence or absence of certain metabolizing enzymes in the cell.[33] Androgens (testosterone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androstenedione, and dihydrotestosterone), estrogens (estradiol, estriol, and estrone), and gestagens (progesterone and 17-hydroxyprogesterone) are among the sex steroids found in human blood. The Leydig cells in the testes create testosterone, the most important male hormone. Dihydrotestosterone (DHT) is a potent androgen that is produced in specific peripheral tissues by the enzyme 5-reductase (type 1 and type 2), which mediates some testosterone-induced effects.[33,34] The adrenal cortex secretes DHEA, DHEAS, and androstenedione in equal proportions in both sexes.[34,35] Aromatization of androgens, particularly those obtained from adrenal steroidogenesis, produces estrogens. Although the ovaries generate massive volumes of androgens, only a

small percentage of these are secreted into the bloodstream, with the remainder being aromatized to estradiol, the primary estrogen.[35,36] Aromatization of androgens is mediated by the enzyme aromatase. The primary progestogen, progesterone, is generated in theca and granulosa cells, the adrenal gland, and the testes.[35,36]

1.2.1. Two-cell, Two-gonadotropin Theory

LH receptors are found only on theca cells in preantral and antral follicles, while FSH receptors are found only on granulosa cells. The membrane of interstitial cells in the theca interna contains the LH receptor. With the LH stimulation, androgens are formed in the theca cells, and aromatization of the formed androgens into estrogens occurs in the granulosa cells. As with preantral granulosa cells, some granulosa cells in antral follicles convert specific amounts of androgens to more potent 5α androgens. In the granulosa cells of the large antral follicles, estrogens are mostly formed by aromatization of androgens. As the follicle grows, the theca cells begin to express LH receptors, p450scc, and 3β hydroxysteroid dehydrogenase. LH allows cholesterol to enter the mitochondria, the first step in steroid synthesis. [37] After luteinization and vascularization following the ovulation, granulosa cells can use HDL cholesterol with a system other than the LDL cholesterol pathway. As the follicle grows, the theca cells secrete p450c17, the rate-limiting enzyme that converts 21-carbons to androgens. This enzyme is absent in granulosa cells and depend on androgens from theca cells for estrogen production. Increased activity of the aromatization system indicates increased maturity of granulosa cells. The fact that P450C17 is only in theca cells and that P450 aromatase is only in granulosa cells are findings which strongly support the two-cell-two-gonadotropin theory for estrogen production. [38] (Figure 6)

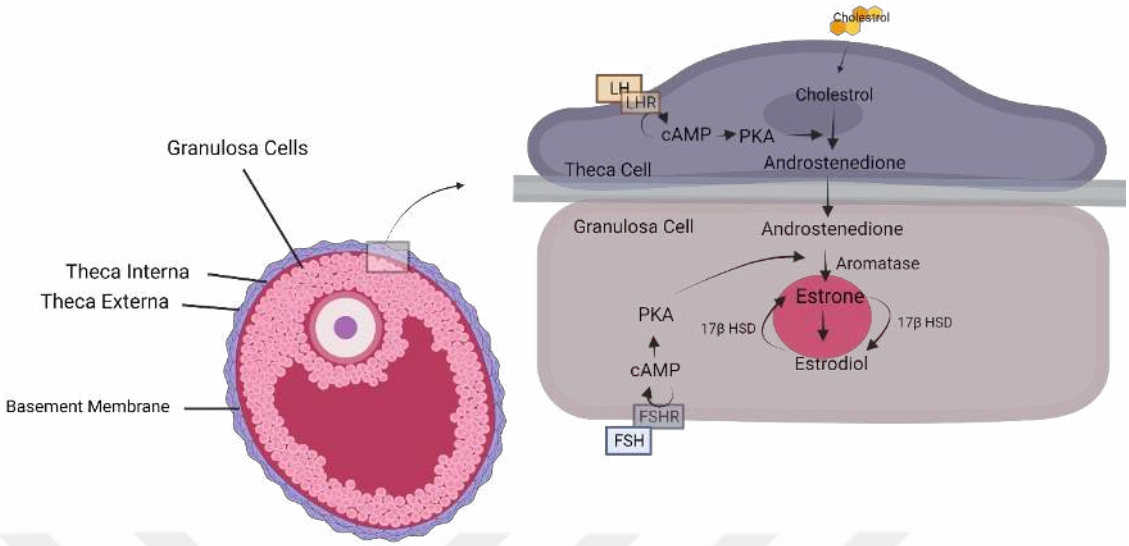

Figure 6. Two-cell, two-gonadotropin theory. *LH receptors are found only on theca cells in preantral and antral follicles, while FSH receptors are found only on granulosa cells until ovulation. (Generated via Biorender.com)*

1.3.FSH and FSH receptor (FSHR)

The hormone FSH is central to the human reproductive system, gonad development, and gamete production. It is used to treat hypogonadotropic hypogonadism and infertility in men and women. Although FSH, LH, and hCG have the same alpha subunit, they have different receptor-specific beta subunits. Circulating FSH stimulates follicle development in the ovaries by acting on FSH receptors on granulosa cells. FSH hormone plays a role in the growth of follicles, the increase in the production rate of granulosa cells, the aromatization of androgenic precursors, and the selection of the dominant follicle. FSH enhances the development of LH receptors in the granulosa cells of large antral follicles. As the follicle grows, it continues to secrete estradiol, so the remaining follicles undergo atresia. This single follicle matures and ovulates upon the LH surge. [39]

FSHR is expressed in the reproductive tissues and controls the reproductive activity in response to FSH release from the pituitary gland. In female, FSHR is found in the granulosa cells of the developing follicle and increases the division of granulosa cells in response to FSH and stimulates them to produce follicular steroid hormones. While FSHR

is found in excess in the developing follicles, it decreases rapidly in the atretic ones. FSH and LH are members of the glycoprotein hormone family. These are heterodimeric hormones containing a common α and β subunit that confers functional specificity on each hormone. These glycoprotein hormones act through G-protein coupled receptors on the target cell surface. [39,40]

FSHR belongs to the G protein pair receptor family. The protein structure of this receptors is characterized by a large extracellular region that concerns hormone-binding affinity and specificity, a seven-transmembrane region responsible for receptor activation and signal transduction, and a small carboxy-terminal intracellular region [41] (Figure 7). The human FSHR protein consists of 695 amino acids. The first 17 amino acids encode the hydrophobic signal peptide. Therefore, the mature protein consists of 678 amino acids. [42] The FSH receptor gene is located in the 2p21 chromosome region in humans. The FSH receptor gene consists of 10 exons and nine introns. Nine exons encode the extracellular region of the human receptor with a length between 69-251 bp. The 10th exon encodes the C-terminal part of the extracellular region, transmembrane, and intracellular regions.[43]

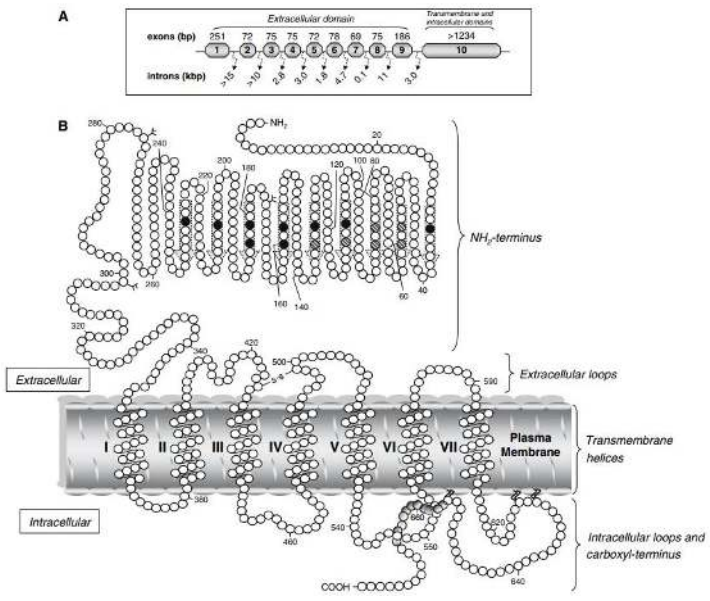

Figure 7. Structural organization of the human FSHR gene. *The FSHR is composed of a single polypeptide chain that threads back and forth across the lipid bilayer seven times, forming unique a-helical transmembrane domains (I–VII). (Reprinted from[42])*

1.3.1. Molecular Mechanisms Related to FSHR

The FSHR has been reported to associate with other membrane receptors on a functional and/or physical level, increasing the variety of FSH activity.[44] The FSHR might be a component of di/trimeric homomers. Heterodimerization of the FSHR with the luteinizing hormone (LH) receptor (LHCGR) appears to be important in controlling ovarian development and selection. Other types of receptors, like tyrosine kinase receptors, might play a role in modulating FSHR activity. Another of them is the insulin-like growth factor-1 receptor (IGF-1R), which seems to be required for FSH-induced granulosa cell differentiation through a signaling pathway that includes the thymoma viral oncogene homolog 3 (AKT3).[44] Similarly, ERK1/2 activation requires the activity of the epidermal growth factor receptor (EGFR) during granulosa cell development. An automated technique[45] was used to examine the relationship between the FSHR and EGFR signaling networks, revealing that EGFR-dependent signals may activate the ERK1/2 pathway through p38 mitogen-activated protein kinases (MAPK). [45,46] (Figure 8). While FSH is well recognized for supporting gamete development in males via Sertoli cell nurturing, it also exhibits steroidogenic action in ovarian granulosa cells. This is accomplished via the protein kinase A (PKA) pathway, activated by ATP conversion to cAMP by adenylyl cyclases, which are critical targets of the Gs protein component. Several decades ago, the relationship between cAMP and PKA was discovered. Phosphodiesterase (PDE) enzymes, which metabolize the second messenger into 5′AMP, negatively influence intracellular cAMP growth.[47] Within the cell, cAMP signaling is segregated both geographically and temporally. In Sertoli cells, cAMP binding to PKA increases the secretion of PKA catalytic subunits, promoting cellular proliferation by indirectly phosphorylating the extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK. pERK1/2 has been shown to have a role in both cAMP-dependent and -independent steroidogenesis. Suppression of pERK1/2 inhibits progesterone production, implying that cAMP-dependent ERK1/2 phosphorylation stimulates FSH-dependent steroidogenic signal. [44] Molecular processes governing steroidogenic stimuli in the Leydig cell may vary from those in FSH-responsive cells. Steroid hormones may be generated in Leydig cells through an EGFR-regulated pathway involving ERK1/2 and CREB signaling without cAMP recruitment.[44, 48] Treatment of theca cells with LH produced similar

findings, inducing differential, ERK1/2-dependent regulation of progesterone and androgen synthesis. [44,49] However, the function of ERK1/2 in influencing steroidogenesis is still unknown since it is an inhibitor, while other investigations have shown that MAPK activation promotes steroid synthesis.[49]

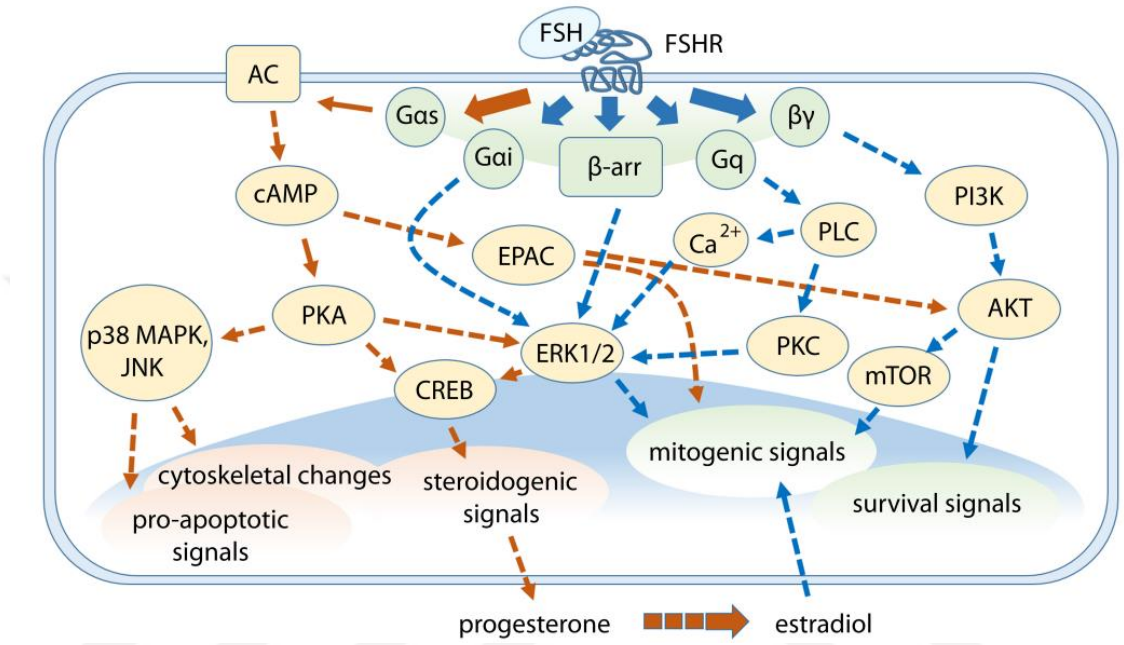

Figure 8. The cross-talk between FSH-dependent steroidogenic, life, and death signals. *FSH can promote cell survival and growth at the intracellular molecular level. (Reprinted from[44])*

Involvement of PKA in various signaling pathways implies that the kinase is a master regulator of various FSH-dependent cell activities, including those linked to steroidogenesis and cell differentiation. Intracellular signaling pathways controlled by PKA, on the other hand, do not overlap those regulated by FSH.[50] FSH activates the p38 MAPK pathway.[44,50]

Parts of the steroidogenic activity and the proteasome are compartmentalized into distinct organelles in gonadal cells, preventing cellular collapse before sufficient amounts of sex steroid hormones are generated. [51] This role is most likely enabled to restrict the number of follicles capable of ovulation and keep sex steroid production intact during the early stages of apoptosis. These issues reflect the link between intracellular signaling cascades regulating steroidogenic signals and pro-apoptotic stimuli, whose dominance is stage-

specific, is regulated by a complex intracellular network involving cAMP and activating the pro-apoptotic protein p53 and is regulated by several paracrine factors. In this situation, the relationship between cAMP/PKA and p38 MAPK activation may offer a biological basis for steroidogenic cell death. The function of p38 and Jun N-terminal kinase (JNK) in preovulatory granulosa cells of primates has been linked to apoptotic events, indicating that these enzymes may be involved in selecting the dominant follicle. Activation of pERK1/2 in the dominant follicle would offset this effect, validating this MAP kinase's anti-apoptotic and proliferative actions (Figure 9). Furthermore, ovarian granulosa cell death is connected to a reduction in ERK1/2 activity.[44,52]

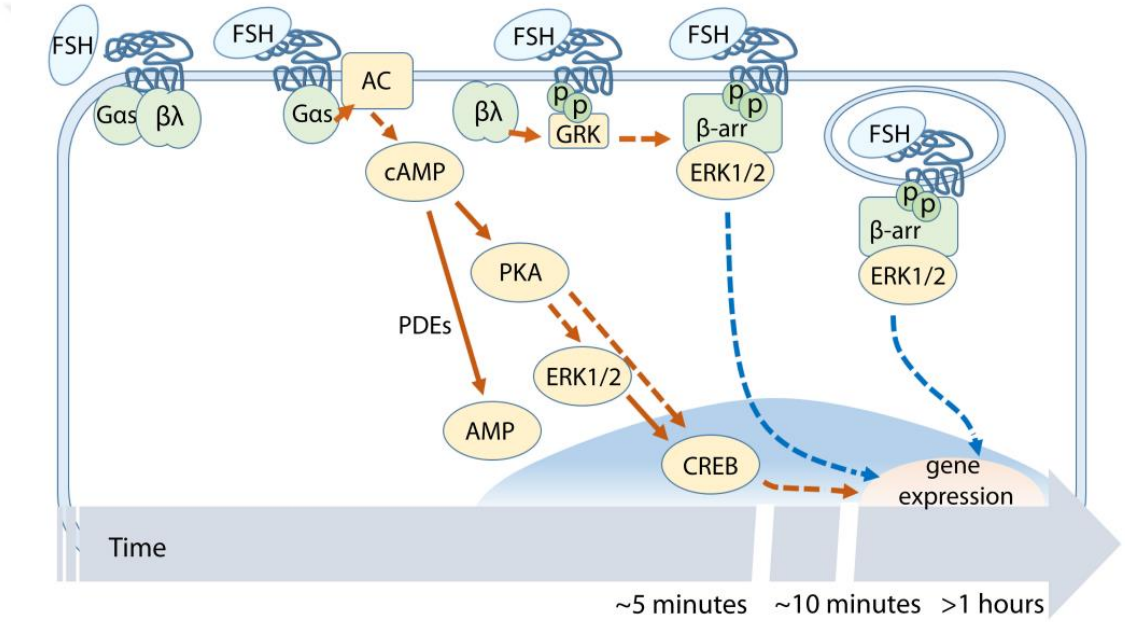

Figure 9. Temporal succession of FSH-dependent events. *The FSHR has associated with other membrane receptors on a functional and/or physical level, increasing the variety of FSH activity. (Reprinted from[44])*

FSH can promote cell survival and growth at the intracellular level. FSH stimulates Sertoli cell proliferation in the male gonads through the AKT and ERK1/2 pathways. The proliferative and anti-apoptotic roles of these signaling cascades are represented during folliculogenesis, oocyte maturation, and ovarian development. A fundamental goal in FSH functioning in the granulosa cell during the antral stage of folliculogenesis is the production of steroid hormones, primarily controlled by cAMP/PKA pathway activation.

Estrogens are the last products necessary for the normal growth of the dominant follicle at the expense of disturbing other follicles, causing them to become atretic. Follicular atresia is widely recognized to be caused by a decrease in FSH support.[52]

1.4. Clinical Approach: The need for enhanced FSH action

IVM (in vitro maturation) is a method for inducing immature oocytes obtained at various stages of embryonic development. Immature oocytes taken from various clinical sources have varying rates of developing into embryos and achieving live birth. [53] IVM may be used to treat individuals with polycystic ovary syndrome (PCOS), ovarian hyperresponsiveness, and hyporesponsiveness, as well as to help cancer patients maintain their fertility. [54] Healthy babies have been born with this method across the globe. Clinical IVM technology advancements are primarily focused on the IVM medium, as well as the culture environment and operating process optimization. With advancements in in vitro fertilization (IVF) efficiency and culture systems, a natural cycle or modest stimulation may now be more appropriate for women undergoing IVF procedures.[55] Combining natural cycle/mild stimulation IVF with IVM was offered as a novel therapeutic option. The combination of low stimulation IVF and IVM, in particular, is predicted to not only become a viable alternative to existing conventional therapies, but also a possible first-line therapeutic option.[55]

Gonadotropin may enhance cumulus cell growth and oocyte nucleus and cytoplasm maturation, enabling the production of embryos and blastocysts at the cleavage stage and playing a key role in follicular development. The cytoplasmic maturation of oocytes may be enhanced by adding follicle-stimulating hormone to the culture medium for oocyte maturation. [56] Some researchers suggest that the concentration of follicle-stimulating hormone affects its action. A rather high cleavage rate (79.1%) and blastocyst rate (16.1%) may be produced when the concentration is 5 g/mL. [57] Protein synthesis, oocyte metabolism, and oocyte maturation may all be improved by adding LH or human chorionic gonadotropin to the IVM culture media. [58] The concentration of estradiol (E2) in the human body rises as the number of follicles grows. Estradiol also helps oocytes retain their meiotic arrest and promotes cytoplasmic maturation. Nuclear maturation occurs quicker than cytoplasmic maturation in oocytes cultured in vitro. As a result, adding E2 to the culture media aids in the synchronization of nucleus and cytoplasm

growth in oocytes. [59] Enhanced FSH action is also necessary in all situations where hyporesponsiveness of granulosa cells takes place.

1.5.The CRISPR System

The CRISPR-Cas9 system is an RNA and protein-based system first discovered in prokaryotes such as bacteria and archaea to protect the cell against bacteriophage infections, invading plasmids, and foreign nucleic acids.[60] Short DNA sequences of invasive pathogen origin are stored in infected cells in the form of "spacers" in the CRISPR sequences of the host genome. In the CRISPR-Cas system, the copy of palindromic (recognition sequences are the same in both DNA strands) repeat clusters of DNA is called "trans-activating RNA (tracr RNA)," and the copy of "spacer" regions is called "CRISPR RNA (crRNA).[61] The CRISPR sequence is copied and transformed into small RNAs containing a single "spacer" known as CRISPR RNA (crRNA). Spacer sequences in these crRNAs binds to Cas proteins and the tracrRNA molecule to form effector complexes that recognize foreign DNA or RNA sequences by base pairing. Cas proteins are helicases that bind to RNA copied from DNA's palindromic repeat clusters or cut DNA paired with RNA spacers. TracrRNA and crRNA combine to form single-guided RNA (sgRNA) that can direct Cas9 to induce double-stranded DNA scission at adjacent protospacer motifs (PAM) regions.[62] Cas proteins, such as Cas9, cleave target nucleic acid to generate an adaptive immune response. Doing this makes it possible to make insertions or deletions into DNA from where it was cut [62,63] (Figure 10).

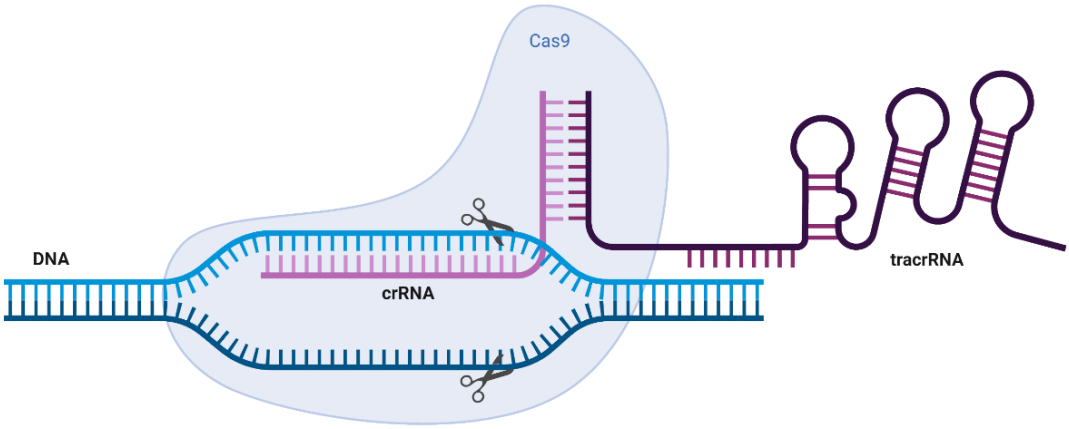

Figure 10. The CRISPR System. *Spacer sequences in these crRNAs binds to Cas proteins and the tracrRNA molecule to form effector complexes that recognize foreign DNA or RNA sequences by base pairing. (Generated via Biorender.com)*

There are many different Cas proteins involved in CRISPR systems. Cas1 and Cas2 proteins, found in all CRISPR-Cas systems, play a role in adaptation. Other Cas proteins are associated only with certain types of CRISPR-Cas systems. According to the structure of the CRISPR region and the content of Cas proteins, CRISPR-Cas systems are divided into three main classes Type I, II, and III. The Type II system is the most well-studied and most likely to be adapted to eukaryotic organisms.[64] The endonuclease that functions in the Type II CRISPR-Cas system is Cas9. It consists of SpCas9 (Streptococcus pyogenes Cas9) nuclease and guide RNA (gRNA) components. The system is based on Cas9 endonuclease protein creating double-strand breaks in the target sequence via gRNA. In this system; provided the specific sequence to be found, and by enabling the Cas9 protein to come to the region where this sequence was, it caused DNA breaks in the target sequence.[65]

The gRNAs used are approximately 20 nucleotides long. Thus, non-specific bindings are reduced to a small extent. For gRNAs to find the target sequence, the PAM (front spacer associated motif) sequence must be present in the target sequence[66] (Figure 11). The purpose of the PAM sequence is to ensure the formation of an RNA-DNA hybrid structure between the target sequence and the gRNA. The CRISPR/Cas9 system enabled the

formation of desired double chain breaks in the target region in the genome of mammals.[66,67]

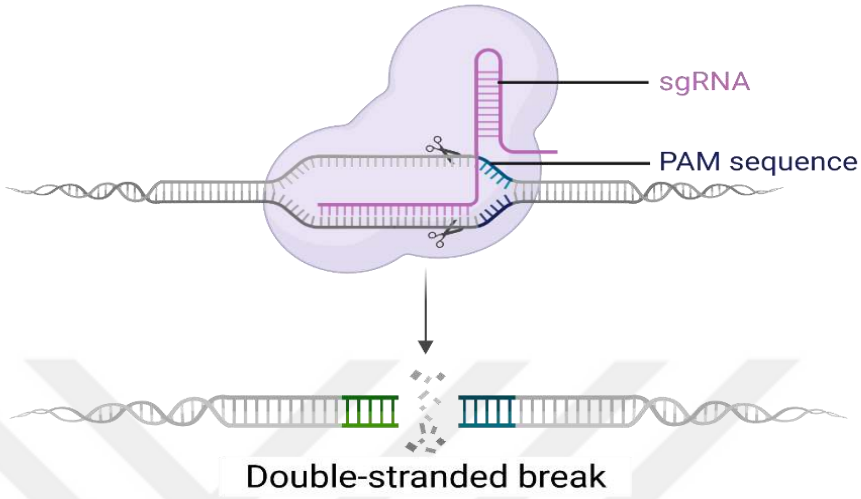

Figure 11. The CRISPR PAM system. *The purpose of the PAM sequence is to ensure the formation of an RNA-DNA hybrid structure between the target sequence and the gRNA. (Generated via Biorender.com)*

dsDNA breaks induced by nucleases are repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR). NHEJ is an error-prone process and often causes frameshift mutation at the target site resulting in genetic knockout or insertion or deletion of nucleotides resulting in the insertion of stop codons. Repair of double-stranded DNA breaks with HR depends on the presence of template DNA containing sequences specific to the targeted site. When dsDNA is a template DNA molecule with sequence homology in the environment of the breaks, DNA damage can be repaired by HR. This mechanism can be used to achieve precise gene modification or gene insertion. Nuclease-induced double-stranded DNA breaks at a specific site can be repaired either by non-homologous end-joining (NHEJ) or by homologous recombination (HR). Repair by NHEJ is always by insertion or deletion of arbitrary base pairs, resulting in disruption and silencing of the gene. If there is donor DNA in the medium, compatible arms are formed by cutting with the same nuclease simultaneously, and gene insertion is performed with

NHEJ. In the presence of donor DNA, gene modification or gene insertion by HR-mediated nucleotide substitutions occurs.[67]

The ability to quickly and precisely insert, extract, and even edit DNA sequences has attracted the scientific community's attention in a wide range of biotechnology fields such as medicine, energy, and even environmental studies. By programming nucleases with site-specific DNA binding domains, they can have enhanced performance, accelerated nuclease assembly, and significantly lower the cost of genome editing.[68]

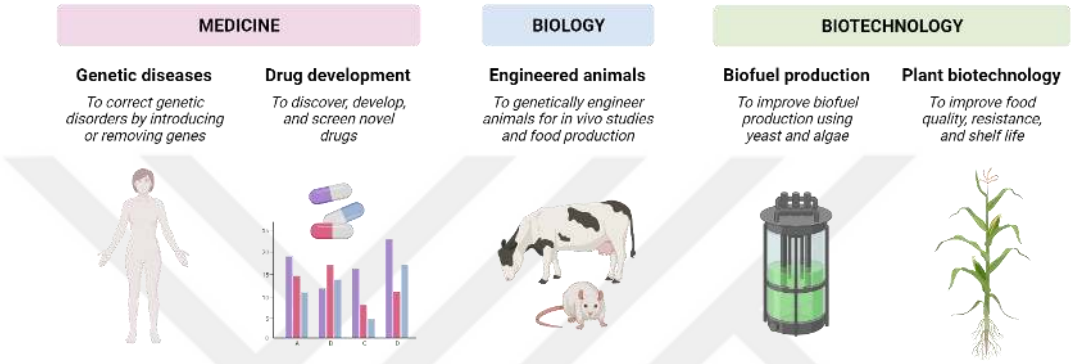

Figure 12. Application fields of CRISPR system. *The ability to quickly and precisely insert, extract, and even edit DNA sequences has attracted the scientific community's attention in a wide range of biotechnology fields such as medicine, energy, and even environmental studies. (Generated via Biorender.com)*

1.2.1. dCAS9

The altered nuclease domains of Cas9 from Streptococcus pyogenes (by introducing a H840A mutation into the HNH domain and a D10A mutation into the RuvC domain) in order to produce a nuclease-deficient "dCas9" protein (also called the dCas9 null mutant). Although this "blind" and "dead" variant of Cas9 is no longer capable of cleaving DNA, it can still target and bind DNA with the same accuracy when led by the sgRNA molecule. Instead of causing irreversible changes to the genome, dCas9 binding causes the target area to interfere with the transcription of the gene, resulting in the reversible silence or activation of the target gene. Researchers began inserting effectors repressor proteins and activator domains into dCas9.

CRISPR activation relies on dCas9, a CRISPR protein variation that lacks the capacity to serve as an endonuclease, to attach to genes without modifying the DNA.[69] CRISPRa

systems attach to DNA and recruit transcription factors to boost gene expression. The transcriptional activators used by CRISPRa techniques vary: some use fusion proteins, while others re-engineer Cas system components. SunTag, SAM, and VPR have all demonstrated considerable improvements over the original dCas9-VP64 approach, therefore there are a variety of alternatives to select from when activating genes in different cell lines [70](Figure 13).

dCas9 system may be employed for reversible gene activation, epigenetic regulation, and gene suppression, among other things. The targeted gene may be regulated by fusing numerous effectors (such as repressor proteins or activators) to dCas9, which is reversible, does not break DNA, and does not produce any deleterious mutations.[71]

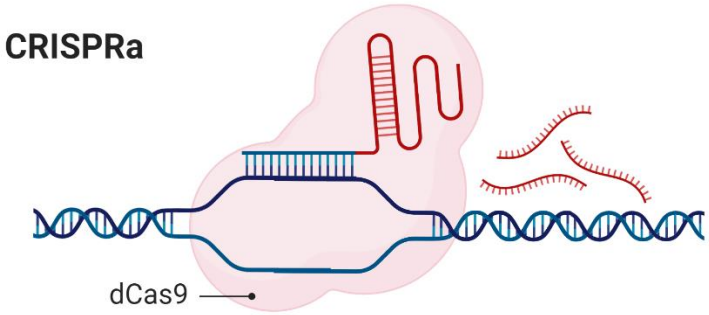

Figure 13. The CRISPR activation system. *CRISPR activation relies on dCas9, a CRISPR protein variation that lacks the capacity to serve as an endonuclease, to attach to genes without modifying the DNA. (Generated via Biorender.com)*

1.2.1.1. The SAM System

The system activator can use to enhance a synergetic effect. This modified gRNA has been designed for activation capacity. Fusion of MS2 proteins including p65 and human

heat shock factor 1 (HSF1) can fuse to activation in the system. This innovative dCas9-SAM system can consistently enhance gene expression by 10- to 1000-fold, depending on baseline expression.[72] SAM consistently demonstrates the greatest levels of gene activation when targeting single genes when compared to other CRISPR activators, making it a preferred technique for gene activation investigations. SAM, on the other hand, demonstrates activation levels similar to other prominent activation strategies when it comes to multiplex gene regulation (activating many genes at once) (VPR and SunTag)[73](Figure 14).

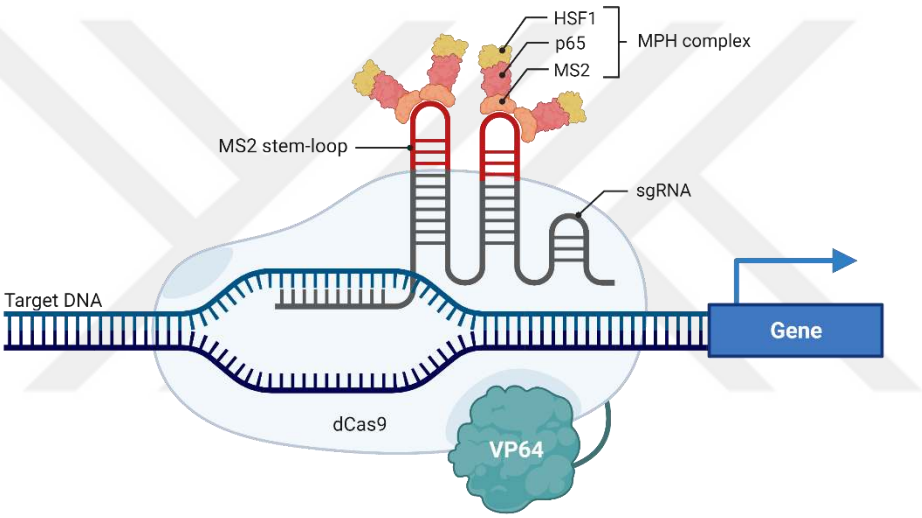

Figure 14. The CRISPR SAM system. *To enhance transcription, SAM employs specially modified sgRNAs. This is accomplished by engineering a dCas9/VP64 fusion protein that contains aptamers that bind to MS2 proteins. (Generated Biorender.com)*

Chapter 2:

MATERIALS & METHODS

2.1. Chemicals, Reagents, Media and Cell Lines and Instruments

2.1.1. Chemicals, Reagents, Media

Nuclease free water (Cat No: DW003L, Eco Tech biotechnology), T4 Ligase Buffer (Cat No: M0202S, New England Biolabs), T4PNK enzyme (Cat No: M0201S, New England Biolabs), qPCR (Thermal Cycler, Bio Rad T100), BsmBI (NEB), Antarctic Phosphatase (NEB), T4 Ligase buffer with ATP (10X) (Cat No: M0202S, New England Biolabs), LB (Cat No: 800-061CG, Multicell), NucleoSpinR isolation kit (Cat No: 740588.50, Mini, Cat No: 740412.10 Midi, Macherey-Nagel), Fugene (Cat No: E2692, Promega), LightCycler SYBR Green I Mastermix (Cat No: 04887352001), PBS (Cat No: L0615-500, Biowest), DMSO (Cat No: D2650- 100ml, Sigma), Puromycin (Cat No: P8833-100mg, Sigma), Hygromycin (Cat No: H-270-1), M-MLV Reverse Transcriptase (Cat No: 28025-013, Invitrogen), Paraformaldehyde (Cat No: 158127-500g, Sigma Aldrich), Triton-X100 (Cat No: T9284-100ml, Sigma), BSA (Cat No: A3311-50g, Sigma), Tween 20. (Cat No: P1379-500ml, Sigma), Glycreol (Cat No: G5516- 500ml Sigma), Laemli Buffer 2X (Cat No:S3401 Sigma), 2-mercaptoethanol (Cat No:M3148, Sigma Aldrich,), Poly-l-lysine coated slides (Cat No: J2800AMNZ, Thermo Scientific), GONAL-f 75 IU (MERCK), DMEM (Cat No: D6429-500ml, Sigma), FBS (Cat No: S181H-500, Biowest), Pen/Strep. (Cat No: L0022-100, Biowest), Trypsin-EDTA (Cat No: 325-542- EL MultiCell).

2.1.2. Cell Line and Cell Culture Conditions

HGrC1 (immortalized human granulosa cell line) cells were maintained in DMEM with 10% FBS and 1% PS. They were planted on 12-well culture plates and incubated in a 37°C incubator with a humidified 5% carbon dioxide.

2.1.3. Antibodies

Alexa Fluor 488 goat anti-mouse IgG(H+L) (Invitrogen (Carlsbad) Cat No: A11029), Alexa Fluor 488 goat anti-rabbit IgG(H+L) (Invitrogen (Carlsbad) Cat No:ab150077), Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Mouse mAb (Cell Signaling, Cat No:#9216), Akt (pan) (C67E7) Rabbit mAb (Cell Signaling, Cat No:#4691), Aromatase Polyclonal Antibody (Invitrogen, Cat No:PA1-21398), FSHR Polyclonal Antibody (Invitrogen, Cat No:PA5-99424).

2.1.4. Plasmids

Lenti sgRNA(MS2)_puro backbone (Plasmid #73795, Addgene), MS2-P65-HSF1_GFP (Plasmid #61423, Addgene), lenti dCAS-VP64_Blast (Plasmid #61425, Addgene).

2.1.5. Instruments

Micropipette (Eppendorf), 12-well plates (Sarstedt Cat No: 83.3921), Serological pipettes (5 ml, 10 ml, 25 ml and 50 ml, Greiner Bio-One, Austria), 6well plates (Sarstedt Cat No: 83.3920), 10 cm culture dish (Sarstedt Cat No: 83.3902), 35 mm culture dish.(Nest Cat No: 706001), Incubator, New Brunswick Galaxy 170S , Centrifugator (Thermo SL-40R), Shaker (Wisd WiseShake SHO2D), LightCycler 480 instrument (Roche, Switzerland) (LightCycler 480 II), chemiluminescence sensing system (Licor), -20°C and -80°C freezers (Beko, Turkey and Thermo Fisher Scientific Inc., MA, USA), Zeiss light microscope (Zeiss Primo Star), Leica dmi8/SP8 Confocal Microscope (Heidelberg, Germany)

2.2. CRISPR/dCas9

2.2.1. The Design of the CRISPR Vectors

The guide RNA for SAM-based CRISPR systems was built using published data. While designing the gRNA, we identified the human FSHR promoter sequence from the

literature. The EPD (Eukaryotic Promoter Database) website was utilized to identify the target gene's promoter region. The sequences for the targeted gene were chosen and imported into the SnapGene program. This platform makes it simple to get the necessary markers in the target gene sequence. This tool was used to select and mark the transcription start site (TSS). When creating gRNAs for activation systems, it is critical to keep the designed genes between 0 (TSS) and -200 base pairs in length. The SnapGene tool was used to determine the distance and closeness of the gRNA sequences discovered for FSHR in the genome engineering database to the TSS region. The remaining gRNAs between the target area were also analyzed using the CHOP-CHOP website (https://chopchop.cbu.uib.no/) for off-target binding rates. The gRNAs with the lowest self-complementary and off-target binding that were closest to the TSS were chosen. For the human FSHR gene, two separate gRNAs were created (Table 1, Figure 15).

Name	**Sequence (5' to 3')**
FSHR-1-f	CACCTGCCATTTTTGGCATAATTACA
FSHR-1-r	AAACACATTAATACGGTTTTTACCGT
FSHR-2-f	CACCAATGGTGAACAGCAAGGAGACT
FSHR-2-r	AAACAGTCTCCTTGCTGTTCACCATT

Table 1. *gRNAs designed for FSHR gene.*

2.2.2. Annealing

Annealing was performed on the gRNAs acquired separately from the manufacturer as top and bottom. The gRNAs were produced as lyophilized, and a stock solution was made at a concentration of 100M in nuclease-free water. A mixture was made using 1 µl top and 1 µl bottom gRNA from the stock, 1 µl T4 Ligase Buffer (T4 DNA Ligase buffer

containing 10 mM ATP), 0.5 µl T4PNK enzyme (T4PNK 3' phosphor minus), and 6.5 µl T4PNK enzyme, 1 µl nuclease free water. The PCR apparatus was set to 37 °C for half an hour, 95 °C for five minutes, and progressively cooled to 5 °C and 25 °C during a one-minute period. In the PCR instrument, the prepared mix was run. Following that, annealed gRNAs were kept at a temperature of -20 °C.

2.2.3. Digestion

To insert the gRNAs generated for dCas9 systems, the lenti sgRNA puro backbone was employed. Vector cutting enzyme and buffer were administered according to the manufacturer's procedure (www.addgene.org). 5 µg vector, 3 µl buffer , and 3 µl BsmBI enzyme was diluted in nuclease-free water to a final concentration of 30 µl. Due to the fact that the BsmBI enzyme operates at a temperature of 30 at 55 °C, the prepared mix was placed in the PCR apparatus for 16 hours before cutting. Following vector cleavage, the linearized vector's terminal segments were phosphorylated to avoid random self-coupling. 3 µl of Antarctic Phosphatase and 3 µl of buffer (10X) were added to the vector tube and incubated for 30 minutes at 37 °C.

2.2.4. Ligation

Ligation is the process by which gRNA is inserted into a cut and linearized vector. To begin, RNAs are diluted to a concentration of 1:200 in nuclease-free water. We generated a 50 ng vector, 1 µl gRNA, 1 µl T4 Ligase buffer with ATP (10X), and µl T4 DNA Ligase buffer. It was incubated for 16 hours at 16 °C. Tubes that had been ligated were kept at -20 °C.

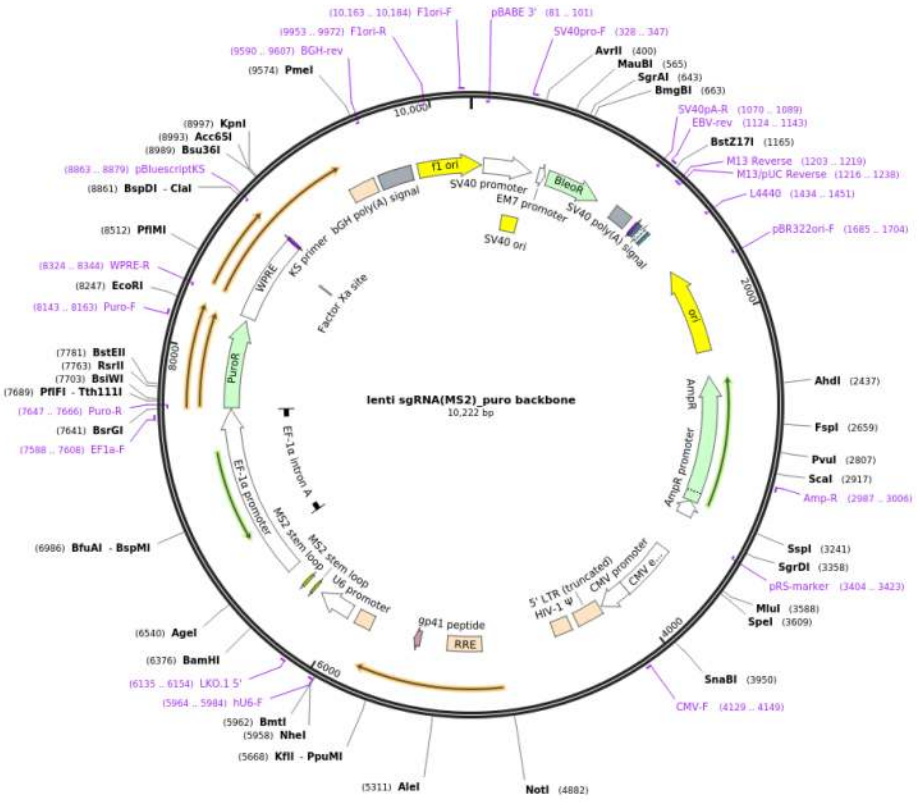

Figure 15. The Lenti sgRNA Puro backbone. *The plasmid used for this set of experiments has CMV promoter, puro resistant sequence, cut sites of restriction enzymes.*

2.2.5. Transformation

The technique of creating by introducing ligated products into bacteria is known as transformation. 5 µl of ligation product was mixed with 50 µl of STBL3 competent bacteria in the same tube. This mixture was cooled for 30 minutes before being placed in a water bath at 42 °C for 45 seconds. After that, 250 µl of antibiotic-free LB broth was added to the shaker and incubated for 1 hour at 37 °C at 225 rpm. After the incubation time was completed, it was spun at 3000 rpm for 5 minutes, and 250 µl of the supernatant was removed, with the remaining 50 µl inoculated on LB agars containing Ampicillin. It was incubated at 37°C for 24 hours.

2.2.6. Colony Formation

On LB agar plates, colonies were examined for formation. Colonies were separated from colony containing plates and incubated overnight in a shaker at 37 °C in 5ml LB (with Ampicillin).

2.2.7. Plasmid Isolation

The NucleoSpinR plasmid isolation kit (Macherey Nagel) was used to isolate the plasmids. For plasmid isolation, the instructions included in the kit were followed. Plasmids were isolated and kept at - 20°C.

2.2.8. Sanger Sequencing Analysis

To verify that the gRNAs were appropriately inserted into the vectors and replicated properly in the bacteria, a sample of the isolated plasmids was submitted to the sequencing. A mixture of 500 ng plasmid and 1 µl hU6 primer was created, and the company (Macrogen) sequence barcodes were glued to the Eppendorf tubes holding these mixtures. The provided findings were examined using Sanger sequencing.

2.2.9. Transfection of 293T Cells

The suitable FSHR plasmids were generated for transfection based on the sequencing analysis. 293T HEK cells were grown in DMEM media with 10% FBS and 1% Pen/Strep. When they reached roughly 80-90 percent confluency, they were passaged. 293T cells were plated of 300.000 cells per well onto 12-well plates for transfection. Following 24 hours, 293T cells were transfected with 1.5 µg total DNA and 4.5 µl Fugene, as specified by the manufacturer. By mass, gRNA, dCas9-effector, and activator plasmids were co-transfected in a 1:1:1 ratio. For gRNA, a system including dCas9-effectors (dCas9-VP64) and activators (MS2-p65-hsf1 GFP) was employed. Due to the GFP in the construct, the successfully transfected cells became GFP positive.

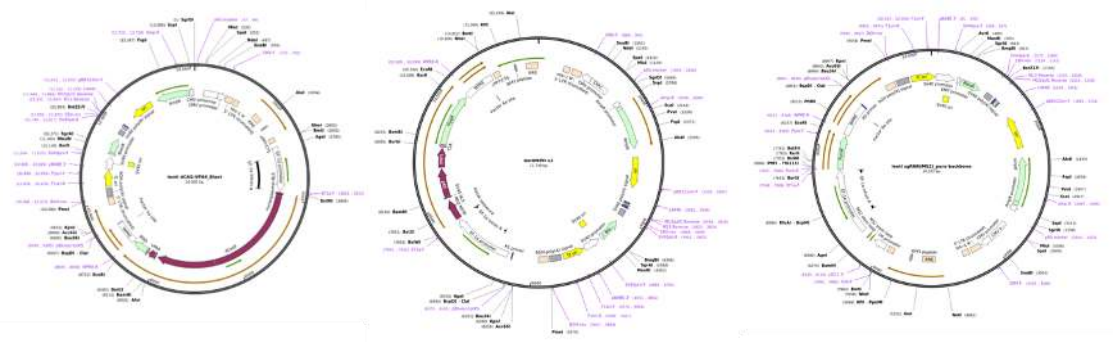

Figure 16. The construction of activation system. *The plasmid on the left side has dCAS9, VP64, Blast, second one MS2, P65, HSF1, Hygro and final one is the puro backbone for gRNA.*

2.2.10. MOI (Multiplicity of Infection) Calculation

Prior to transduction into HGRC1 cells, the MOI was used to estimate the number of viruses that may enter a cell and infect it. We used viruses to carry the dCas9-VP64, ms2-p65-Hsf1 and gRNA vectors. For each virus and gRNA, the MOI was determined. Each virus was given a separate 12-well growing plate containing 100,000 HGRC1 cells. There were three dosages used to assess virus concentrations: 10 µl, 20 µl, and 30 µl. Changing the medium and starting selection were done after 16 hours of transduction. Blasticidin antibiotic was supplied at a concentration of 2, 5 and 10 µg/ml to the dCas9-VP64 virus, and selection was accomplished in 7 days with 5 µg/ml. For 7-8 days, 50, 75 and 150 µg/ml hygromycin was used to select for the ms2-p65-Hsf1 virus and 75 µg/ml gave best result. Puromycin with 0.25, 0.5, 1 µg/ml was used to inhibit the selection of viruses with gRNAs and 0.25 µg/ml dose of puromycin accomplished after one week. When all of the cells in the antibiotic's negative control group have died, the selection process has come to end. The MOI was estimated after the cells had been extracted and counted using a hemocytometer. The results of this experiment were used to calculate the mean MOI for the viruses tested.

2.3. Gonal-f Treatment

0.33 µl of 75 IU/ml stock Gonal-f was prepared into 1 ml fresh medium as 25 IU/ml and added to each well. The medium was removed after 5 min, 15 min and 1 hour. After application of 0.05 % Trypsin-EDTA for 5 min, centrifugation was applied at 1500 rpm for 5 minutes. The supernatant was removed and the pellet was stored at -80 °C.

2.4. The measurement of Estradiol

1 ml of medium sample was collected from all experimental groups and taken to tubes. An electrochemiluminescence immunoassay was carried out in Clinical Biochemistry Laboratory of Koç University Hospital. This experiment has been repeated three times.

2.5. qPCR

Cell pellets were stored at -80 °C until RNA extraction. Nucleospin RNA kit was used to extract total RNA. M-MLV Reverse Transcriptase was used to make cDNA from 1000 ng of RNA, according to the protocol. In a LightCycler 480 equipment, qRT-PCR was performed in triplicate using LightCycler SYBR Green I Mastermix. 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Cq data were normalized to reference GAPDH gene expression, and relative expressions were estimated as fold changes to a reference sample using the ddCq technique.

GENES	FORWARD PRIMERS	REVERSE PRIMERS
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
MTOR	GCTTAGAGGACAGCGGGGAA	TGCAGGACGCTCACATTGCT
FSHR	TTGAACTGAGGTTTGTCCTCACCA	GGCCTCAGGGTTGATGTAGAGAGC
AKT	GCAGCATCGCTTCTTTGCCG	GCTGTCATCTTGGTCAGGTGG
MAPK8	GCAGAAGCTCCACCACCAAAGA	TGCACCTAAAGGAGAGGGCTG
MAPK1	TGGCAAGCACTACCTGGATCAG	GCAGAGACTGTAGGTAGTTTCGG
TP53	CTCCGGGGACACTTTGCGTT	TCTGACTGCGGCTCCTCCAT
CASP3	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA
ESR1	TCCAGCAGTAACGAGAAAGGA	AGCCAGAGGCATAGTCATTGC
PGR	GCCAGCCAGAGCCCACAATA	TGTGCTGCCCTTCCATTGCC
CYP19A1	GGTCACCACGTTTCTCTGCT	GCAAGCTCTCCTCATCAAACCA

Table 2. Primer List used for qPCR. *Glyceraldehyde 3-Phosphate Dehydrogenase, Mammalian Target of Rapamycin, Follicle-Stimulating Hormone Receptor, V-Akt Murine Thymoma Viral Oncogene Homolog, Mitogen-Activated Protein Kinases 8, Mitogen-Activated Protein Kinases 1, Protein53, Caspase 3, Estrogen Receptor 1 and Progesterone Receptor genes were evaluated, respectively.*

2.6. Western Blotting

After being placed in Laemmli buffer (SDS sample buffer with 2-mercaptoethanol), the total cell lysates were boiled at 95 °C for 15 minutes to extract the proteins. Proteins were extracted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred to polyvinylidene fluoride membranes after they had been separated by SDS–PAGE. First, membranes were blocked for an hour with DPBST (PBS with 0.1% Tween 20 containing 5% milk), followed by overnight incubation at four °C with antibodies specific for FSHR, aromatase, AKT, and Phospho-p38 MAPK (1:1000). Followed by three washes (10 minutes each) in DPBST, incubation occurs with Horse Radish Peroxidase (HRP)-conjugated Goat anti-Rabbit and anti-Mouse IgG (1:1000) for 1 hour at room temperature. Ultimately, the membranes were treated with an advanced chemiluminescence sensing system.

2.7. Immunofluorescent Staining

HGRC1 cell samples were fixed on coverslips in 4% paraformaldehyde for 20 minutes before rinsing with DBPS. Except for the FSHR antibody, permeabilization in 0.1% Triton-X-100 for 10 minutes at room temperature was utilized (due to the receptors are on the cell membrane). Following that, the blocking agent (Super Block) was used for 20 minutes of blocking. The cells were treated for 90 minutes at 37 °C with FSHR, aromatase, AKT, and Phospho-p38 MAPK antibodies (1:50). After rinsing in DPBS containing 1% Tween 20, cells were incubated for 90 minutes at 37 °C in different humidified chambers with Alexa Fluor 488 goat anti-mouse and anti-rabbit IgG(H+L). Following rinsing, cells were mounted on multiple slides for 10 minutes using a mounting solution made of 1:1 Glycerol: Hoechst 33342 (10g/ml). Finally, the HGRC1 cells were placed on slides and examined using a 405 nm and 488 nm diode lasers under a Leica dmi8/Sp8 (Heidelberg, Germany) confocal microscope.

CHAPTER 3: RESULTS

3.1. CRISPR/dCas9 Design and Sequences

Prior to the TSS, gRNA was developed. We have typically targeted the areas before TSS when creating gRNA since it is known that targeting the pre-TSS for activation and that targeting the 200-base fraction before TSS is more successful for dCas9 systems.

The plasmids into which the gRNAs were cloned underwent sequence analysis. Blue sections indicate the proper sequences. Two cloned plasmids were generated, one of them was successfully cloned. Following these findings, CRISPR experiments were performed (Figure 17).

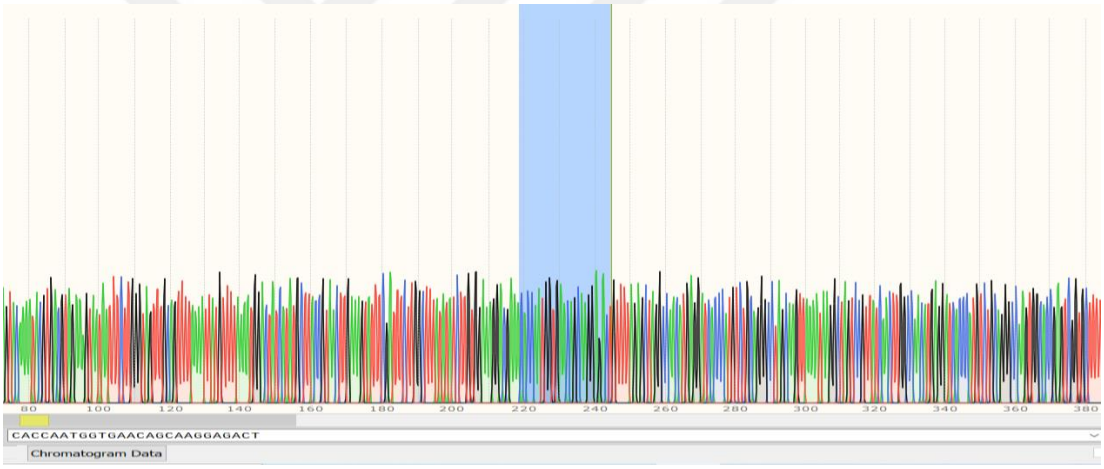

Figure 17. Result of Sanger sequence analysis. *One of the gRNAs was found to be inserted into plasmid.*

3.2. MOI

Each virus was provided with its 12-well plate containing 100,000 HGRC1 cells. Three doses were used to evaluate viral concentrations: 10 µl, 20 µl, and 30 µl. After 16 hours of transduction, the medium was changed, and selection was initiated. Blasticidin was supplied to the dCas9-VP64 virus at concentrations of 2, 5, and 10 µg/ml; selection was achieved in 7 days at a dosage of 5 µg/ml. 2 µg/ml was insufficient for selection, whereas 10 µg/ml caused cell death the next day. 50, 75, and 150 µg/ml of hygromycin were used for 7-8 days to select for the ms2-p65-Hsf1 virus; however, 75 µg/ml produced the best results. 50 µg/ml was not insufficient for selection, but 150 µg/ml caused cells to die the

next day. Puromycin concentrations of 0.25, 0.5, and 1 µg/ml were applied to the selection of viruses containing gRNAs, with the 0.25 µg/ml dosage being effective after one week. At above 0.5 µg/ml dosage, cells died the next day (Figure 18).

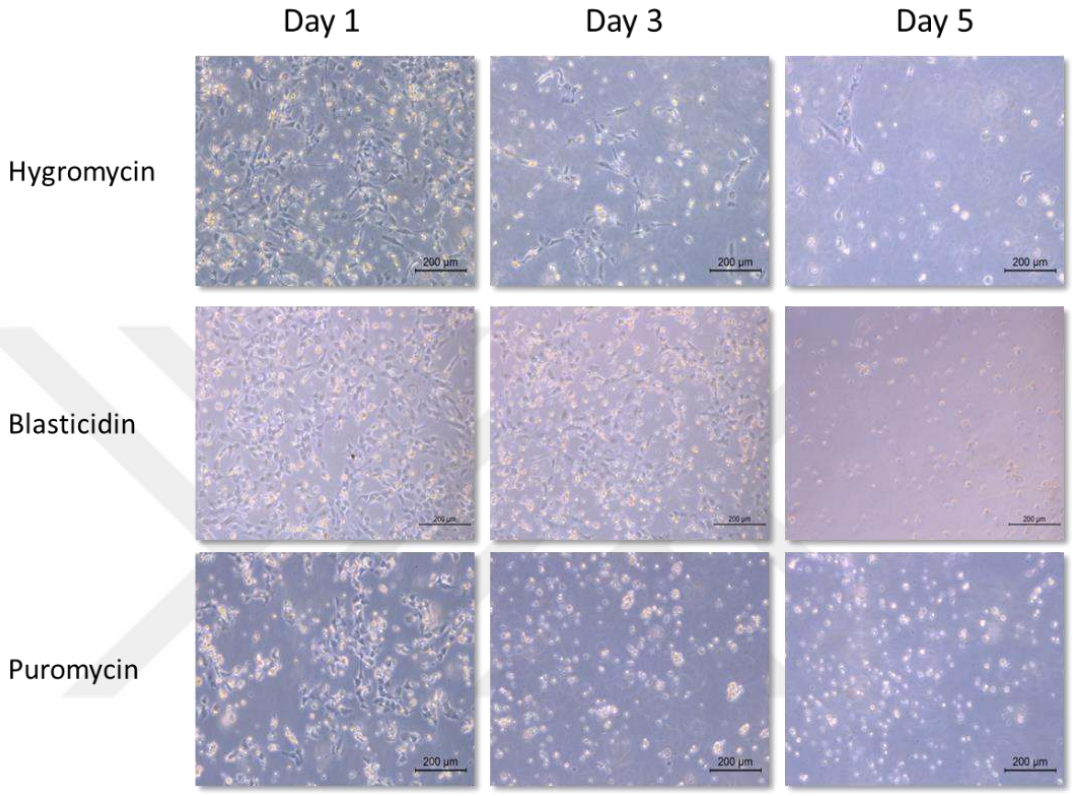

Figure 18. Selection of Hygromycin, Blasticidin and Puromycin applications. *75 µg/ml for Hygromycin, 5 µg/ml for Blasticidin, 0.25 µg/ml for Puromycin were found to be the optimum doses of selection.*

3.3. Gene Expression Analysis by qPCR

qPCR was used to assess the impact of CRISPR/dCas9 systems on gene expression. The NT (non-targeting) gRNA was created as a non-targeting gRNA sequence in the genome. The system plasmids did not show any non-specific effect. FSH treatment (Gonal-f) was applied to the control and CRISPR/dcas9 groups for 5 minutes, 15 minutes and 1 hour to evaluate the increase of the FSH receptor and its effect on the pathway.

FSHR expression was increased in dcas9 group compared to control and NT. FSHR expression in the dcas9 with Gonal-f treated groups increased at all time-depending

groups compared to the only Gonal-f treated groups. This result showed that the dcas9 system has worked successfully (Figure 19).

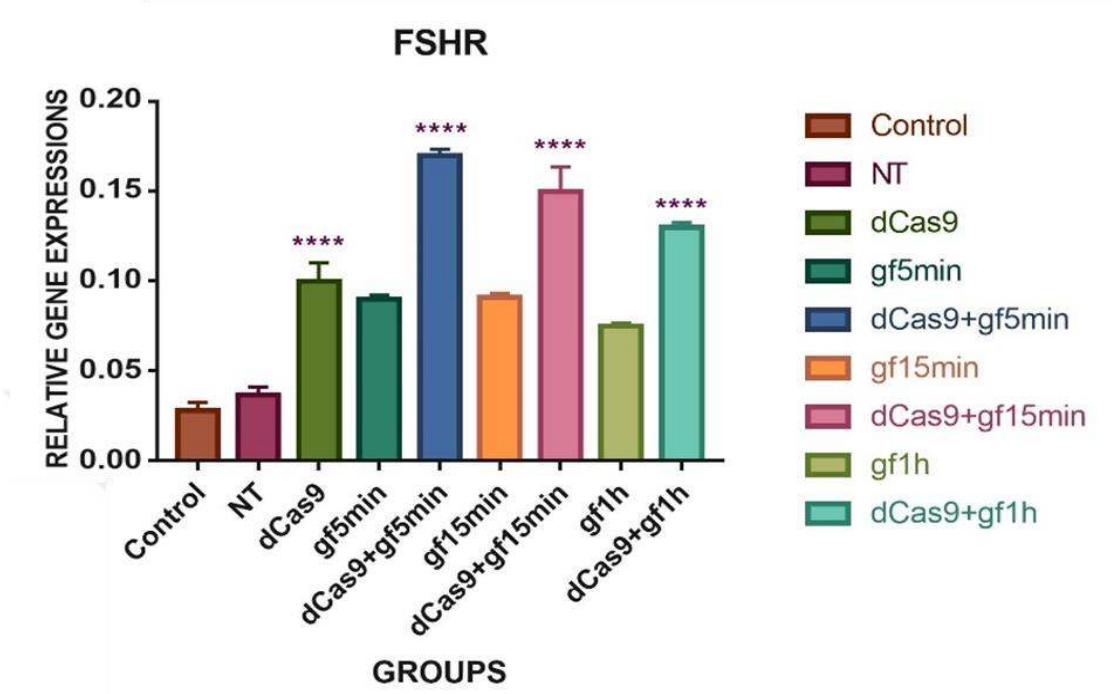

Figure 19. FSHR gene expression in the experimental groups. FSHR has been found to increase significantly in all groups that has dCas9 genome edition. *($p<0.05$, Decimal number format is 10^{-4} on Y axis)*

Aromatase expression increased significantly in the dcas9 group compared to the control and NT groups. The increase was significant in all time intervals in dcas9 with Gonal-f treatment groups (Figure 20).

When **MAPK1** gene expression was evaluated, a significant increase was observed in the dcas9 system group compared to control and NT. A significant increase was seen in the groups dcas9 with Gonal-f treatment in 5 minutes and 1 hour groups. In the treatment applied for 15 minutes, dcas9 with Gonal-f was almost the same with only the Gonal-f treatment (Figure 21).

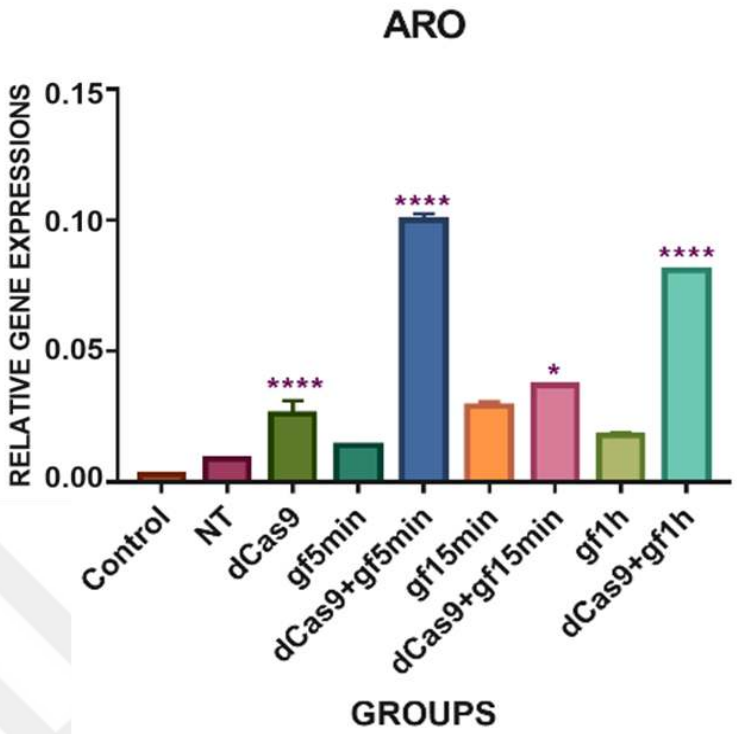

Figure 20. Aromatase gene expression in the experimental groups. Aromatase expression increased significantly in the dcas9 group compared to the control and NT groups. The increase was significant in all time intervals in dcas9 with Gonal-f treatment groups. *($p<0.05$, Decimal number format is 10^{-3} on Y axis)*

MAPK8 gene expression was evaluated and a significant increase was observed in the dcas9 system group compared to control and NT. A significant increase in the groups treated with dcas9 with Gonal-f treatment was shown in the all time-dependent treatment groups (Figure 22).

When the **ESR1** gene was evaluated for estrogen receptor, the dcas9 system showed a significant increase compared to the control and NT groups. While there was a significant increase in dcas9 with Gonal-f treatment at 5 and 15 minutes, the increase after 1 hour of application was almost the same as only Gonal-f treatment (Figure 23).

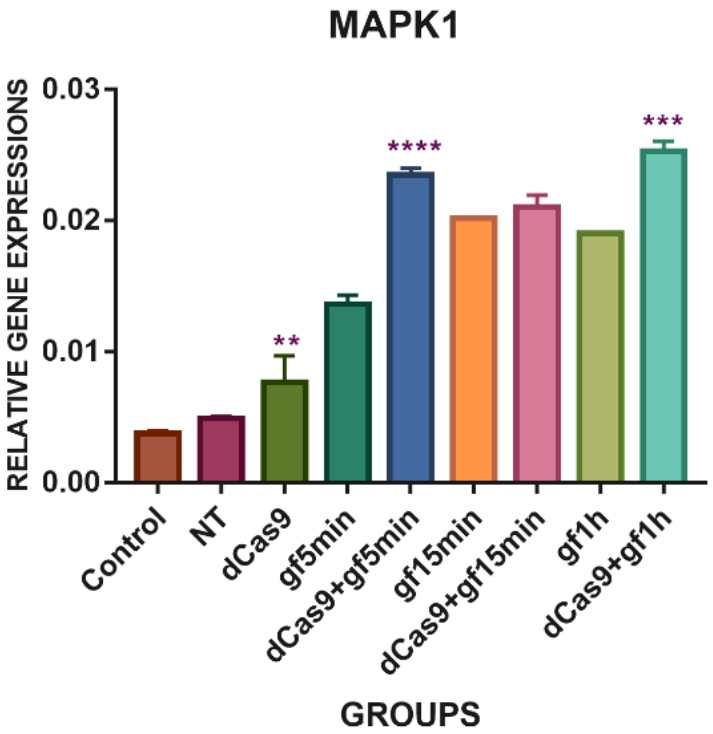

Figure 21. MAPK1 gene expression in the experimental groups. A significant increase was observed in the dcas9 system group compared to control and NT. A significant increase was seen in the groups dcas9 with Gonal-f treatment in 5 minutes and 1 hour groups. *($p<0.05$)*

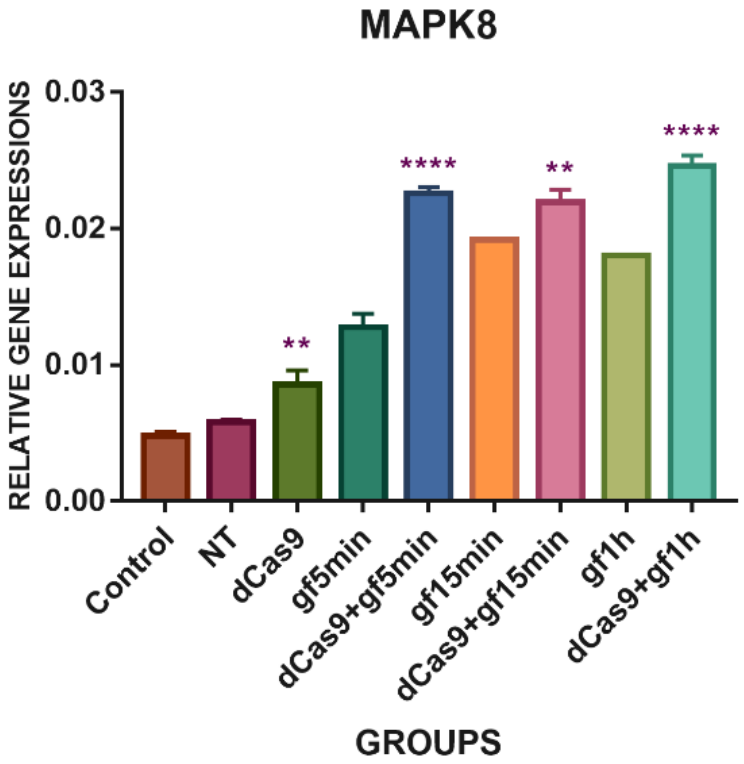

Figure 22. MAPK8 Gene expression in the experimental groups. A significant increase was observed in the dcas9 system group compared to control and NT. A significant increase in the groups treated with dcas9 with Gonal-f treatment was shown. *($p<0.05$)*

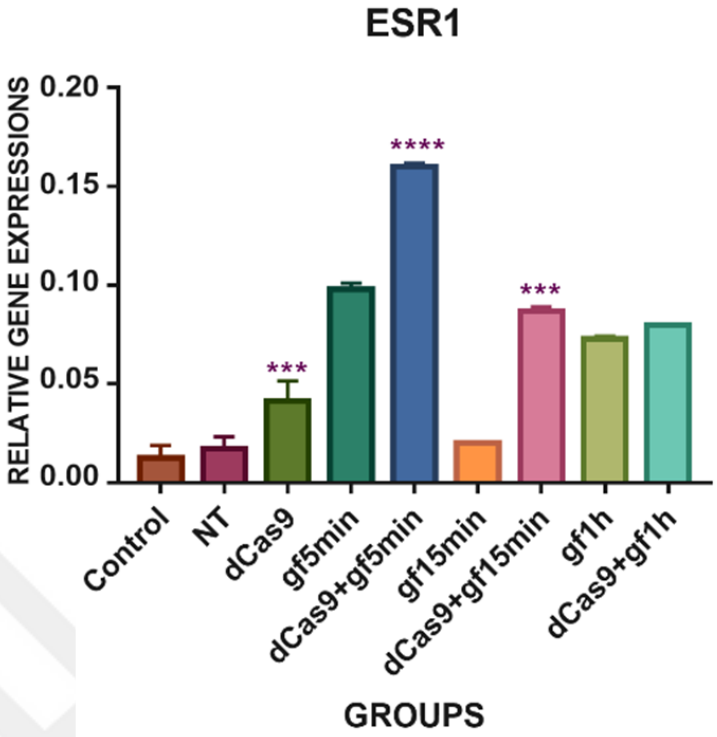

Figure 23. ESR1 gene expression in the experimental groups. The dcas9 system groups showed a significant increase compared to the control and NT groups. *($p<0.05$, Decimal number format is 10^{-4} on Y axis)*

When the **PGR** gene was analyzed, the dcas9 system showed a significant increase compared to the control and NT groups. After 5 minutes and 1 hour of Gonal-f treatment, there was a significant increase in dcas9; however, after 15 minutes of application, the increase was the same as only Gonal-f treatment was used (Figure 24).

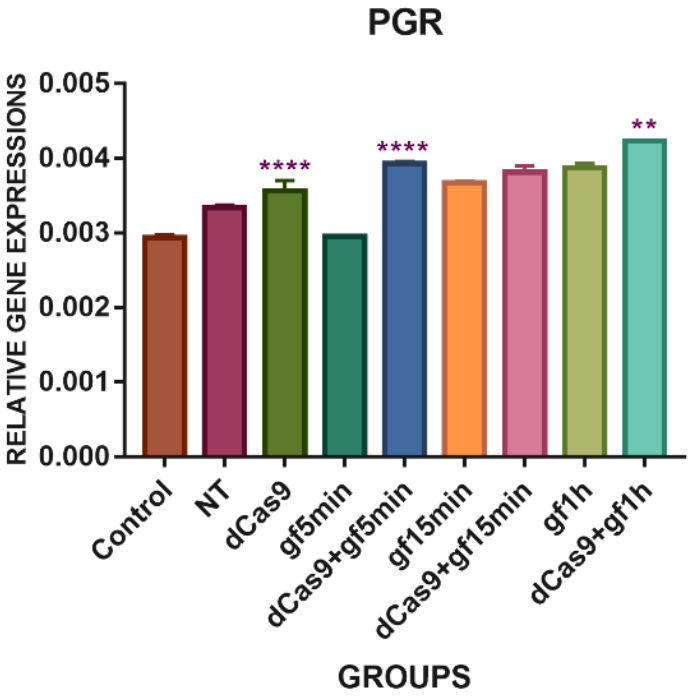

Figure 24. PGR gene expression in the experimental groups. The dcas9 system showed a significant increase compared to the control and NT groups. After 5 minutes and 1 hour of Gonal-f treatment, there was a significant increase in dcas9 group.

When **MTOR** gene expression of the dcas9 system was compared to the control and NT groups, a significant increase was detected. After 15 minutes and 1 hour of Gonal-f treatment, a significant rise was seen in the dcas9 group. dcas9 with Gonal-f group was similar to only Gonal-f treatment performed for 5 minutes (Figure 25).

A significant increase in **AKT** gene expression was detected in the dcas9 system groups compared to the control and NT groups. All time-dependent treatment groups treated with dcas9 and Gonal-f showed a significant increase (Figure 26).

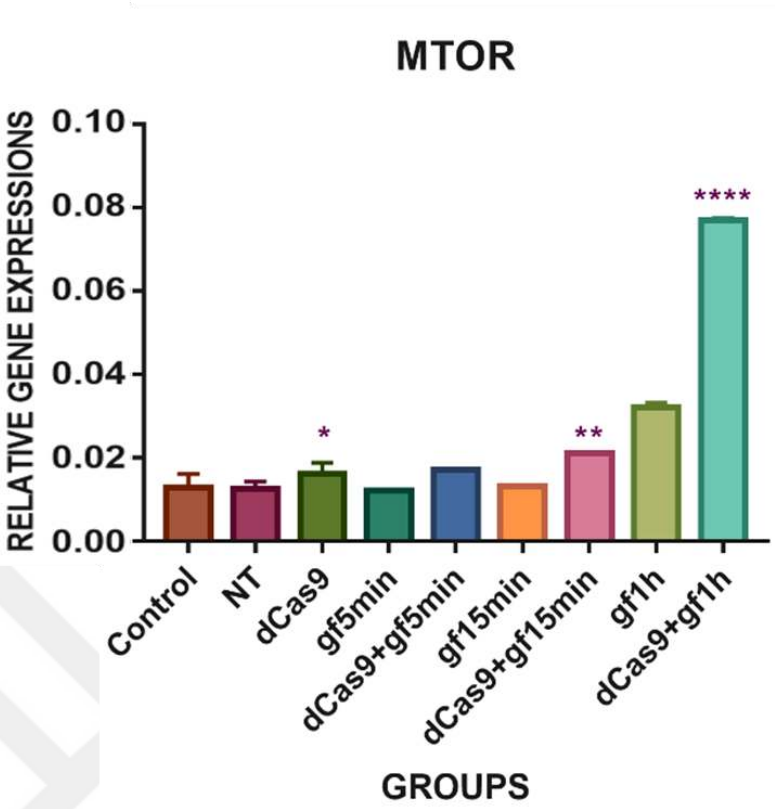

Figure 25. MTOR gene expression in the experimental groups. When dcas9 system groups were compared to the control and NT groups, a significant increase was detected. After 15 minutes and 1 hour of Gonal-f treatment, a significant rise was seen in the dcas9 group. *($p<0.05$, Decimal number format is 10^{-4} on Y axis)*

When the expression of the **P53** gene was measured, a significant increase was shown in the dcas9 system group compared to the control and NT groups. A significant increase was seen in all time-dependent treatments in the groups treated with dcas9 with Gonal-f treatments (Figure 27).

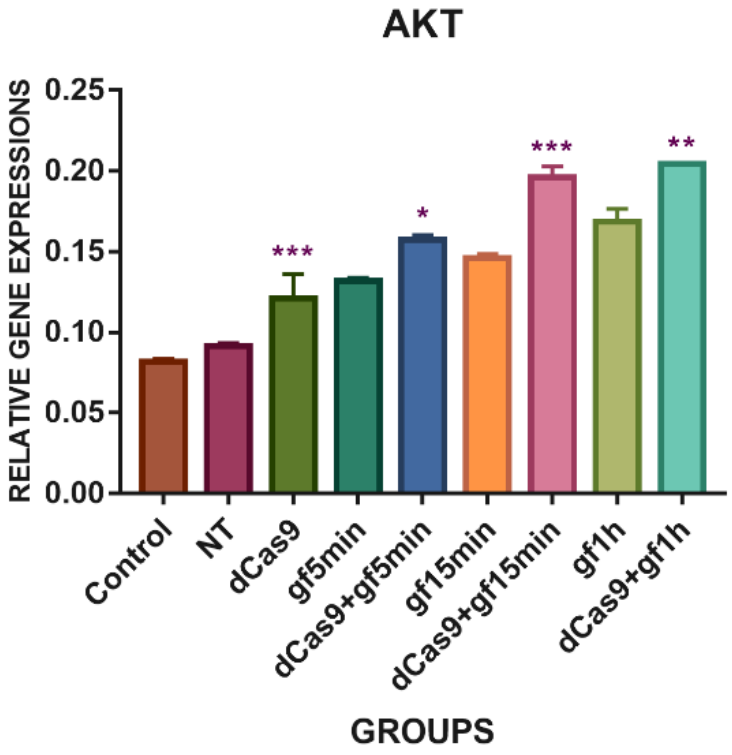

Figure 26. AKT gene expression in the experimental groups. A significant increase in **AKT** gene expression was detected in the dcas9 system groups compared to the control and NT groups.

When the expression of the **CASP3** gene was examined, it was found that the dcas9 system groups have shown a significant increase compared to the control and NT groups. In all time-dependent treatments, a significant increase was seen in the groups dcas9 with Gonal-f compared to treated with only Gonal-f (Figure 28).

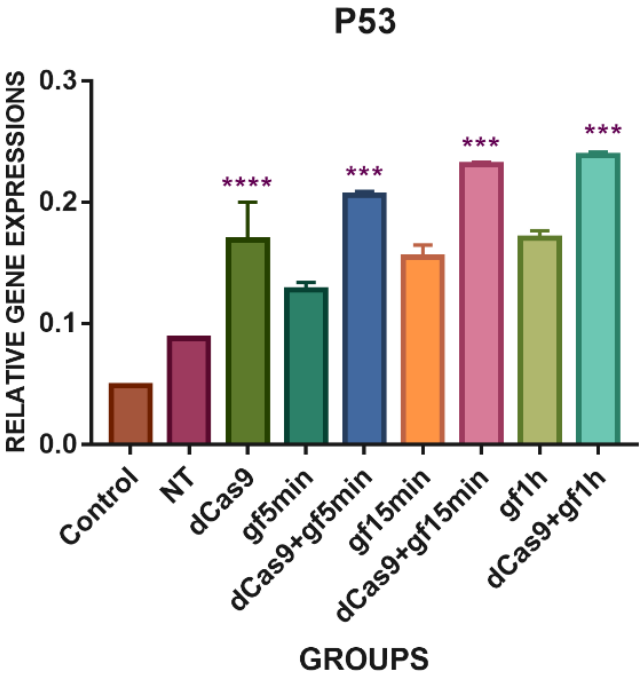

Figure 27. P53 gene expression in the experimental groups. A significant increase was shown in the dcas9 system groups compared to the control and NT groups.

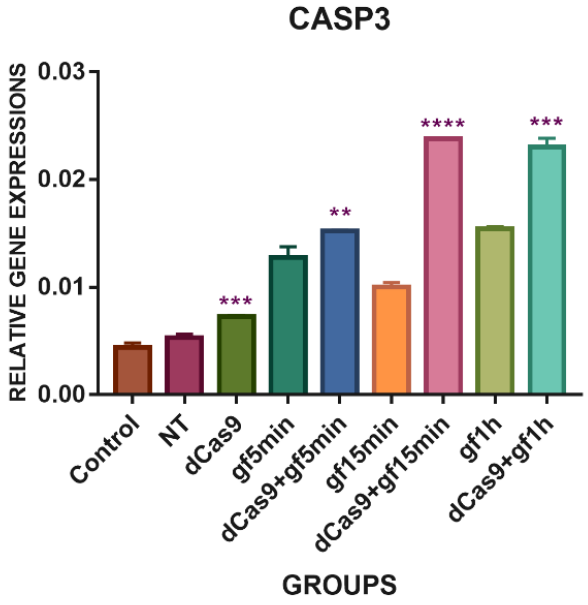

Figure 28. CASP3 gene expression in the experimental groups. The dcas9 system groups had a significant increase compared to the control and NT groups.

3.4. Protein Expression Analysis by Western Blotting

Western blotting was used to investigate the influence of the CRISPR/dcas9 system on protein expression of **P-38 MAPK, AROMATASE, AKT, FSHR** genes. GAPDH gene was used as a positive control in the western blot on all of the experimental groups. This set of experiments has been repeated twice.

When the **P-38 MAPK** protein was examined, it was found that the protein level has increased in the dcas9 group and the dcas9 groups treated with Gonal-f for 5 minutes when compared to the control group. After 15 minutes of Gonal-f treatment and 1 hour of Gonal-f treatment, the protein band in the dcas9 groups similar intensisty to the protein band in the control group.

Compared to the control group, **aromatase** protein is enhanced in the dcas9 and time-dependent dcas9 with Gonal-f treatment groups. In 15 minutes of treatment, it is observed that the increament level is less than the other groups.

Comparing the **AKT** protein levels in the dcas9 group and the dcas9 groups treated with Gonal-f for 5 minutes, it was shown that the AKT protein level increased in both of these groups when compared to the control group. The protein band in the dcas9 groups was less intense than the protein band in the control group after 15 minutes of Gonal-f treatment and 1 hour of Gonal-f treatment.

Comparing the dcas9 group with the dcas9 group treated with Gonal-f for 5 minutes, the **FSHR** protein level increased in the dcas9 group and the dcas9 group treated with Gonal-f for 5 minutes. After 15 minutes and 1 hour of Gonal-f treatment, the protein bands in the dcas9 groups were close to the control group in intensity (Figures 29, 30).

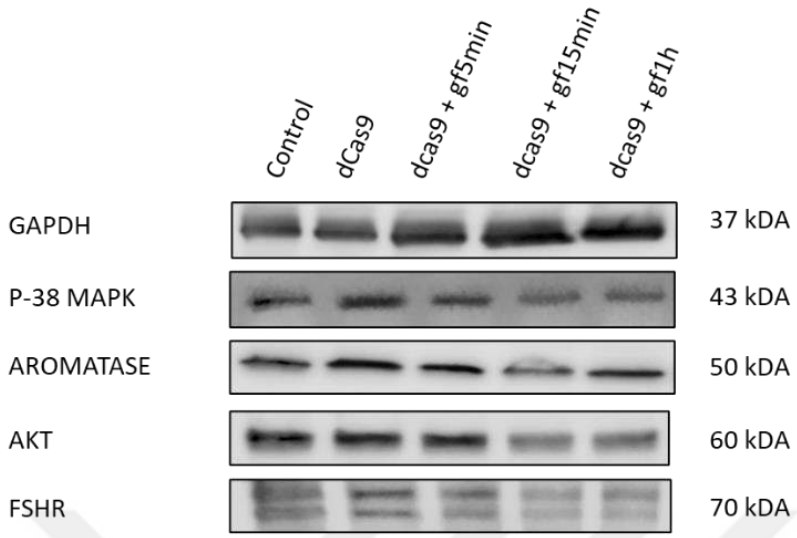

Figure 29. The First Repetition of Western Blotting. The explanation can be followed in the text.

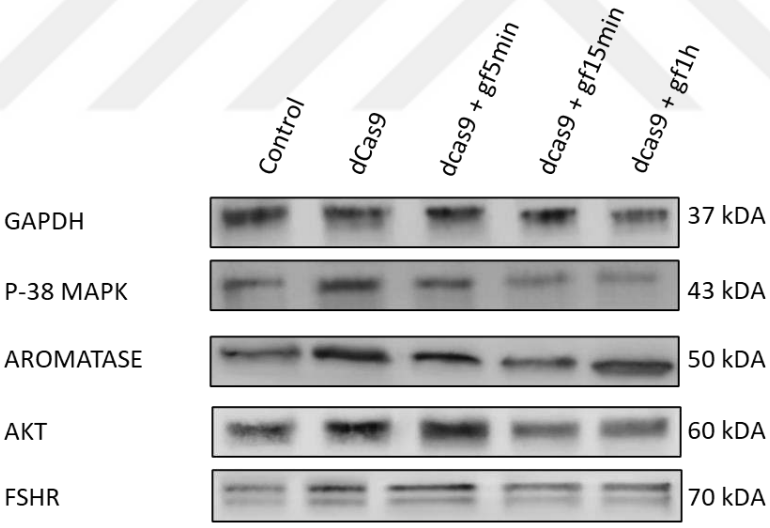

Figure 30. The Second Repetition of Western Blotting. The explanation can be followed in the text.

3.5. Estrodiol (E2) Levels

Estrodiol (E2) levels were foıund to be higher in the 5 minutes only Gonal-f treatment and dcas 9 with Gonal-f treatment, while the other groups were similar to the control. This experiment has been repeated three times for each tested group, however a statistically significant difference has not been observed, although 5min Gonal-f and dCas9+Gonal-f groups have showed a higher level of E2.

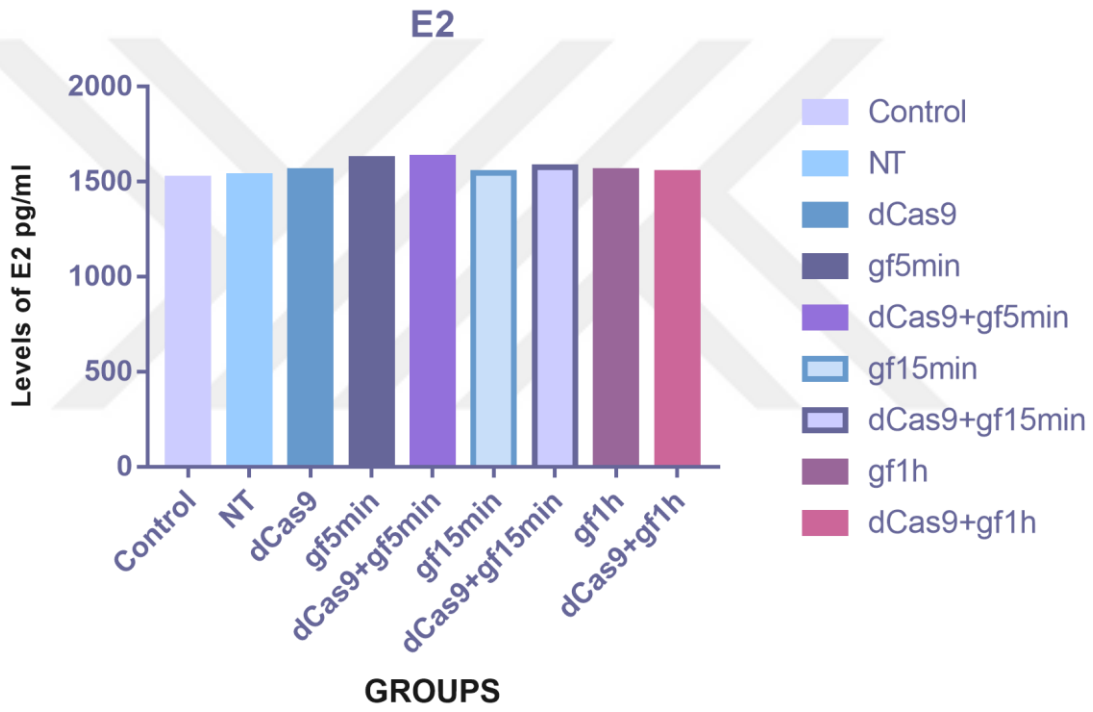

Figure 31. Levels of E2 in Experimental Groups. The explanation can be followed in the text.

3.5. Immunofluorescent Staining

In correlation with the qPCR and Western blotting findings, an immunofluorescent staining was performed for the significantly different groups FSHR, aromatase, AKT and MAPK. For this purpose, dCas9, dcas9+Gonal-f 5 min and control groups were selected for staining, as these groups show somewhat correlence in both gene expression and protein expression analysis. The expression of FSHR and aromatase has been found to increase in both dCas9 and dcas9+Gonal-f 5 min groups, showing slightly a more intense staining in the dcas9+Gonal-f 5 min (Figure 32). The expression of AKT and MAPK also was higher in both dCas9 and dcas9+Gonal-f 5 min compared to the control group, however the intensity of the staining did not show much correlation with the intense expression found in both qPCR and Western blotting analysis, a fact that also may be related to the capability of those primary antibodies working more efficiently via Western blotting (Figures 29-30, 32).

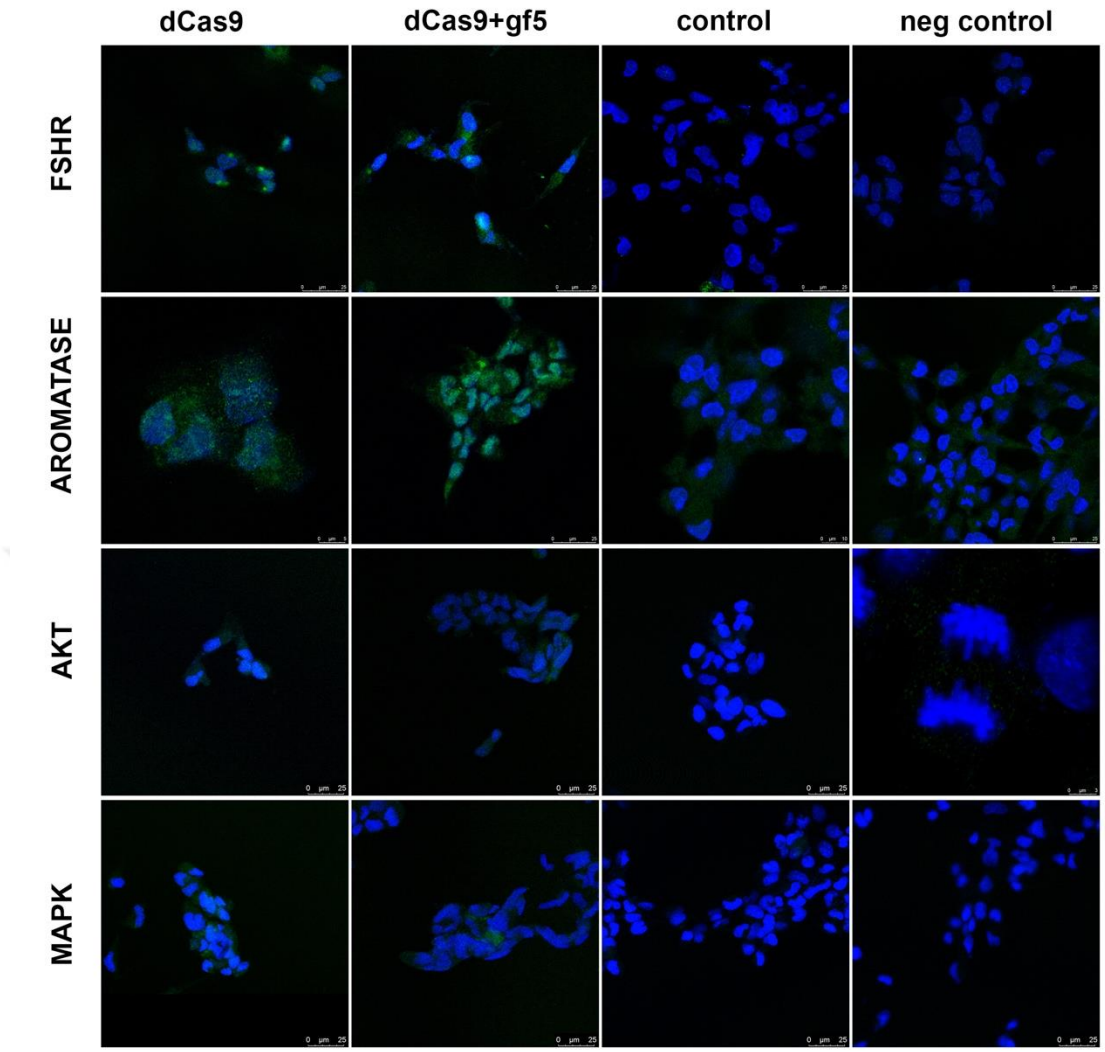

Figure 32. Immunofluorescent staining of FSHR, Aromatase, AKT and MAPK in HGRC1 cells. The expression of FSHR and aromatase has been found to increase in both dCas9 and dcas9+Gonal-f 5 min groups, showing slightly a more intense staining in the dcas9+Gonal-f 5 min. The expression of AKT and MAPK also was higher in both dCas9 and dcas9+Gonal-f 5 min compared to the control group, however the intensity of the staining was not satisfactory enough.

Chapter 4:

DISCUSSION

In the ovary, FSH is required for the generation of oocytes and steroid hormones that regulate sexual function. In the lack of FSH, follicular development is stopped, and also the female is infertile. Follicle growth develops on FSH dependence throughout late folliculogenesis, and FSH works primarily on granulosa cells through the FSHR[44,74] . As a result, the expression and activation of FSHR in granulosa cells have been found to be crucial for the change from FSH-independent to FSH-dependent growth.

The follicle-stimulating hormone (FSH) can also have a role in regulation of gametogenesis by acting on gonadal target cells. P38 mitogen-activated protein kinases (MAPK) are involved in transmitting death signals in steroidogenic cells through p53 and caspases.[75,76] It is expected that pro-apoptotic signals, necessary for controlling atresia of non-dominant ovarian follicles, should be temporary and balanced by mitogenic signals.

The modulation of steroidogenic pathway by ERK, AKT, calcium signaling, and other intracellular signaling effectors, leads in a complex and dynamic signaling network that characterizes the sex- and stage-specific gametes maturation.[44]

Even though the FSH-mediated signaling network has not yet been discovered in its entirety, its complete understanding is of significant physiological and therapeutic importance owing to the hormone's essential function in regulating reproduction.

The FSH receptor was enhanced by the CRISPR/dCas9 technology, according to the findings. Due to FSH promotes several signaling cascades, activation of FSHR affected many molecules.

FSH can promote cell growth and survival at the intracellular level, in contrast to steroidogenic signals connected to apoptosis. [77] In the experiments, it was seen that the

increase of FSHR and the administration of FSH hormone increased the level of mitogen-activated kinase 1 and 8 genes. Mapk1 (ERKs) directly correlates with mitogenic signals. Increasing ERKs also upregulates steroidogenic signals.

FSH stimulates AKT and ERK1/2 pathways, and the proliferative and anti-apoptotic roles of these signaling cascades are reflected during folliculogenesis, oocyte maturation, and ovarian development. [44] The AKT/mTOR signaling module activated by FSH is a positive regulator of cell cycle progression and cell proliferation[78]. According to the results, this explains the increased expression of the AKT molecule and, accordingly, mTOR after dcas9 activation.

After FSH-FSHR increased, gene expression levels of aromatase and ESR1 increased. Estrogens are the last products necessary for the normal growth of the dominant follicle at the expense of scarifying other follicles, causing them to become atretic. Overexpression of the FSHR results in caspase 3 cleavage and the induction of apoptosis. [79] Apoptotic signals are essential for controlling atresia of non-dominant ovarian follicles[80]; nevertheless, they should be temporary and balanced by mitogenic signals following FSHR contact with opposing transducers, as shown in this study.

When examining at the protein level, the CRISPR dcas9 system was shown to have enhanced the amount of FSHR protein in the cells. It was also discovered that gene expression levels were associated with a rise in the p-38 MAPK, AKT, and aromatase proteins. Enhanced protein concentrations are associated with increased pro-apoptotic, steroidogenic, and mitotic signals. The stage-specific impacts of signals and the temporary influence on gene expression may both be attributed to the variety in outcomes seen in time-dependent treatments.

One of our goals in this work was to determine if CRISPR dcas9 technology might be used to enhance the number of FSH receptors in HGRC1 cells. At the conclusion of the

investigation, we discovered that dcas9 technology can be used to effectively increase FSHR at the gene and protein levels.

We demonstrated that enhanced FSH receptor activity impacts a variety of sub-pathways in which FSH is connected with a variety of steroidogenic, life-sustaining, and death signaling events. In addition, it has been clarified that this impact is temporary and that it may change over time. Because of the influence of FSH on oocyte maturation in HGRCs, it is possible that our work may give a fresh viewpoint on in vitro maturation technology in the in vitro area, as previously stated. Given the fact that HGRC cell lines and hormones are now being used to assist oocyte maturation in current technology, this is a potential outcome that should be investigated further in the context of dcas9 triggered HGRC IVM technology.

One of the striking results can be considered as having similar E2 levels although an increase in FSHR has been obtained. This is most probably due to the fact that granulosa cells need androgens to product estrogen and as no androgen source has been provided. The slight but non-significant increase can be explained by the culture medium having FBS and some amount of testosterone can be found in all serums.

A clinical study using genetically modified FSH receptor, polycystic ovary syndrome (PCOS), ovarian hyperresponsiveness, and hyporesponsiveness, as well as cancer patients, may be helpful in assessing FSH sensitivity issues that are particularly frequent in female populations. The poor ovarian response may be related to advanced maternal age and a reduced ovarian reserve, among other factors.[81] Some young women with normal ovarian reserve, as measured by baseline blood FSH levels and antral follicle count, do, nonetheless, present with a poor response to ovarian stimulation when they should not. It has recently been revealed that the FSH receptor is critical in defining the ovarian response, as described in cases of mutation of the FSH receptor. [82] According to some reports, women with FSHR inactivating mutations have ovulation failure,

amenorrhea, and other symptoms. [82] In vitro investigations have shown that these unique, particular mutations of the FSHR cause a significant reduction in receptor expression and a disruption in normal signal transduction. Moreover, increasing the FSHR in granulosa cells with dcas9 systems may contribute to studies, especially for male infertility, to increase FSHR or similar receptors in Sertoli cells.

Obviously although dCas9 systems do not cause gene knock out as CRISPR/Cas 9, viruses are still used. Further studies may include dCas9 activation of different types of receptors and also use of virus like particles (VLP) for same purposes can be tried, as well.

Chapter 5:

CONCLUSION

Our current study showed that FSHR gene can be activated in HGRC1 cells with CRISPR/dCas9. Compared to other CRISPR systems, dCas9 used for activation is safer because it integrates into the genome and does not cause any damage. CRISPR/dCas9 system can be used to prevent the diagnosis of poor ovarian response that may occur due to a mutation in the FSHR gene in granulosa cells and can restore receptor function. Increase in FSHR gene can also increase all downstream pathway related genes efficiently.

The increase in the aromatase gene with the activation of FSHR in granulosa cells indicates that it increases steroidogenesis. Since androgens play an essential role in folliculogenesis by acting primarily as a source for granulosa cell estradiol synthesis and providing various other physiological benefits to the follicle during its growth, particularly in the early stages of developmental stages prior to follicular selection, this genome edition may be useful for such interventions.

VITA

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