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## THE ROLE OF GENE THERAPY IN PREMATURE OVARIAN INSUFFICIENCY MANAGEMENT

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**Abstract:**

Premature ovarian insufficiency (POI) is a highly prevalent disorder, characterized by the development of menopause before age of 40. Most cases are idiopathic; however, in some women the cause of this condition (e.g. anticancer treatment, genetic disorders, and enzymatic defects) may be identified. Although hormone replacement therapy, the principal therapeutic approach for POI, helps to alleviate the related symptoms, this does not effectively solve the issue of fertility. Assisted reproductive techniques also lack efficacy in these women. Thus, the effective approach to manage the patients with POI is highly warranted. Several mechanisms, associated with POI, have been identified, including lack of FSH receptor functioning, alterations in the apoptosis control, mutations in Sal-like 4 genes, thymulin or basonuclin-1 deficiency etc. The above-mentioned may be good targets for gene therapy in order to correct defects, leading to POI. The goal of this review is to summarize the current experience on the POI studies, that employed gene therapy, and to discuss the possible future directions in this field.

**Goal of the review:** to summarize the current experience of gene therapy use in treatment of premature ovarian insufficiency.

**Keywords:** Premature ovarian insufficiency, POI; Gene therapy; Menopause; SAL-like 4 genes, SALL4; Follicle-stimulating hormone (FSH); Basonuclin-1; Replication-incompetent adenoviral vector, Ad; Stem cells, SC.

## Introduction

Premature ovarian insufficiency (POI) affects 1% of women by 40 years of age and less than 0.01% of patients younger than the age of 35 (1, 2). It is defined by the development of menopause before 40 years of age. Although the majority of cases remain idiopathic, a detailed history and physical in addition to a workup should be initiated to investigate identifiable etiologies(3). The different etiologies of POI are described in Table 1. Follicle-stimulating hormone receptor (FSHR) gene polymorphisms, chromosomal defects, autoimmune and enzymatic disorders are among the known causes. Much effort has been made in order to identify genes, responsible for POI development, and today genetic cause of this condition is found in up to 25% of patients (4). FMR1 (5), FIGLA (6), BMP15 (7), FSHR (8), FOXL2 (9), GDF9 (4), NOBOX (10), INHA (11), STAG3 (12) are the examples of genes, mutations of which are seen in some POI women.

It is important to mention also, that the most recent report of the American Cancer Society for 2016-2017, has estimated that female cancer survivors of 49 years of age and younger are estimated to be over 1 million as of January 1<sup>st</sup> 2016. This emerging population increases significantly the prevalence of women with POI and renders it a major health care problem that warrants therapy and management. (13).

**Table 1.** Causes of premature ovarian insufficiency.

Idiopathic
Primary
Chromosomal disease
FSHR gene polymorphism
Mutation of inhibin B
Autoimmune disorders
Enzymatic defects
Secondary
Cancer treatment (chemotherapy, radiotherapy)

Ovarian surgery
Uterine artery embolization
Infections (mumps etc)

Clinical manifestations of POI, similarly to menopause, are signs of hypoestrogenism due to ovarian dysfunction. Those include oligomenorrhea with menopausal symptoms such as vaginal dryness, decreased libido, and vasomotor symptoms. However, the absence of these symptoms does not rule out the diagnosis in the presence of appropriate laboratory findings. Since menstrual irregularities are the most common presenting manifestation of POI, other etiologies of secondary amenorrhea should be excluded. Those are illustrated in Table 2.

**Table 2.** Other causes of secondary amenorrhea that should be excluded.

Physiologic
Pregnancy
Intrauterine adhesions
Asherman syndrome
Tuberculous endometritis
Hypothalamic
Functional hypothalamic amenorrhea
Pituitary
Prolactinoma
Empty sella syndrome
Sheehan syndrome
Cushing syndrome
Ovarian
PCOS
Others
Hypothyroidism

Ovarian tumors
Congenital adrenal hyperplasia
Adrenal tumors

Early onset hypoestrogenism has deleterious effects on a woman's general health and wellbeing. In addition to symptoms affecting the woman's quality of life, such as vasomotor symptoms and vaginal atrophy, accelerated bone loss, leading to osteoporosis, increased cardiovascular morbidity and mortality are the main consequences of estrogen deficiency, especially when it occurs in women of reproductive age. To add, recent reports suggest a role of estrogen in mental health as well, with increased rates of dementia and neurologic disorders such as Parkinson's disease in patients with early onset menopause. (14-16). Interestingly, spontaneous ovulation can still occur in women with POI with ovaries in situ (17). Patients should be counseled that the chances of spontaneous conception, although low for age-matched controls, are still present, and can reach a lifetime probability of 10% (18).

Hormone replacement therapy (HRT) is the mainstay in the treatment of POI from the time of diagnosis until the average age of menopause, at least. Contemporary studies and reports do not fully support its use beyond that age. HRT helps counteract the effects of hypoestrogenism by promoting bone and cardiovascular health, in addition to improved quality of life through the resolution and vasomotor symptoms and vaginal atrophy. In terms of reproductive outcomes, HRT may improve spontaneous ovulation rates, but is not the standard of care for women desiring conception. Assisted reproductive technologies are seldom futile due to diminished ovarian reserve in addition to poor ovarian response. Adoption or oocyte donation are commonly recommended for patients who haven't completed child bearing at time of diagnosis (19). In women with newly diagnosed cancer, in vitro fertilization with oocyte and embryo banking is an option prior to initiation of medical or radiation cytotoxic therapy and the induction of POI (20, 21). Cryopreservation of fresh ovarian tissue prior to cytotoxic treatment with subsequent

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transplantation is not fully implemented yet, but results of pilot studies have been promising (22).

Nevertheless, the concern of simultaneous cryopreservation of carcinogenic cells in the ovarian cortex, mainly in the case of lymphomas and leukemia, leading to cancer recurrence after autologous transplantation has always been a major concern with this technique.

The data on effectiveness and teratogenicity of other agents for POI patients (bisphosphonates, raloxifene, strontium ranelate, herbal remedies) is currently lacking (23).

Thereby, effective treatment of POI is needed, especially to restore reproductive function. Gene therapy is a potentially promising avenue, which has been attracting interest recently. The goal of this review is to summarize the available information on gene therapy attempts in the management of POI and to underline the potential target genes that may be influenced in future studies.

## GENE THERAPY FOR FSH RECEPTOR DEFECT CORRECTION

Females are born with approximately one million primordial follicles, arrested at prophase of the first meiotic division. The majority will undergo atresia and only 600,000 are present at puberty.

When the girl is born, all the follicles are arrested at the early stage of development, this changes with puberty, when stimulation of the ovaries by follicle-stimulating hormone (FSH) occurs (24).

FSH signaling through its receptor (FSHR) is essential for this process, as well as for spermatogenesis in adult males (25). Folliculogenesis is a lengthy process, involving growth and maturation of the follicle from primordial to the preovulatory stage. Approximately 400 follicles of the total ovarian follicle pool will ovulate during the reproductive years of a woman.

Aittomaki et al. identified a point mutation (C566T) in FSHR gene and showed the substantial decrease of FSH/FSHR binding and, thus, failure to increase intracellular cAMP levels in mutated FSHR transfection experiments (24). Males with C566T mutation of both alleles had decreased fertility, whereas homozygous females had POI due to resistant ovary syndrome (ROS) (25).

Usually this is seen as primary amenorrhea with high serum FSH, somewhat decreased secondary sex features, normal karyotype and genitalia (24). Similar signs are seen in other mutations of

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FSHR gene (8, 26-28). The follicles in these women are not developing and continuous atresia occurs (24). There is no effective therapy for these conditions nowadays and chance of spontaneous pregnancy is very low. FSH stimulation of the ovaries is ineffective (29). The only method that allows to become pregnant is in vitro fertilization (IVF) using donated eggs, which is very expensive, ethically unacceptable for many women and results in genetically unrelated fetus. One of the most commonly used vectors for gene therapy are replication-incompetent adenoviruses (Ad) that are proved to be safe (30). Al-Hendy et al. used Ad to transfect both human and Eker rat uterine fibroid cells (ELT3) with dominant negative estrogen receptor (ER) to inhibit estrogen pathway and observed shrinkage of leiomyoma size (31-33). No safety issues have been observed. Ghadami et al. developed Ad vector, carrying full-length human FSHR (hFSHR) gene (Ad-hFSHR), and demonstrated its ability to restore FSH activity in C566T mutated cells (34). Interestingly, follitropin receptor knockout mice (FORKO), a good model of hypergonadotropic hypogonadism with infertility and hypoplastic internal genitalia secondary to deleted FSHR gene and resembling human ROS, when injected with Ad-hFSH bilaterally into ovaries, demonstrated folliculogenesis, 2-3-fold rise in estrogens, serum FSH reduction, body and genitalia weight increase. In addition, ovaries of these animals started to show FSHR expression (35-37). The intraovarian injection of Ad-LacZ didn't show systemic viral spread or fertility disturbances in mice, corroborating previous observations. Viral genes were not detected in pups of mice, injected into the ovaries with Ad-LacZ, thus germ line transmission was also excluded. Unfortunately, ovulation or pregnancy was not achieved in injected mice after 12 weeks of observation. This didn't occur also after injection of both, study and control groups, with PMSG, followed by hCG. Mice were mated with normal males, but no pregnancies were observed. This may be explained by the use of strong dominant CMV5 promoter in Ad-hFSHR vector, not allowing downregulation of FSHR in the later stages of follicular development (luteal phase). This may be fixed with the rebuilt Ad-hFSHR, using authentic human promoter. The data from this study show that Ad-hFSHR injection into the ovaries of FORKO mice lead to partial hormonal

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correction and mobilization of follicles up to antral phase with subsequent arrest, thus not reaching ovulation (35). This is a promising direction of future investigations in the area of POI, related to defective ovarian FSH action, where gene therapy may play an important role.

### **SAL-LIKE 4 GENES AS A TARGET OF GENE THERAPY IN POI**

Sal-like 4 (SALL4) genes are highly expressed in vertebrates' embryonic and adult stem cells, giving rise to their stemness. Postpartum they are found only in adult stem/stemlike cells of bone marrow and gonadal origin (38-40). As these genes are involved in cell growth and development, they may be a reasonable target for gene therapy. It's worth mentioning that some disorders, including POI, may be seen in patients with mutated Sall4 gene (38). The sequence screening for Chinese patients with SALL4-related syndromes (ventricular septal defects and POI) has identified several distinct variants of SALL4 genes (41, 42). The chromosomal locus of human SALL4 is 20q13.13-q13.2, for mice it's chromosome 2H3 (39, 40).

Aguila et al. have shown that SALL4 is able to facilitate the regeneration of bone marrow and division of hematopoietic stem cells (HSCs) in vitro and in vivo (43).

Interestingly, C.541G>A(p.Val181Met) and c.2449A>G(p.Thr817Ala) SALL4 mutations were found in 100 women with POI versus 300 healthy controls (41). The thorough look into the SALL4 pathway and study of its client proteins may clear up the appropriate approach for stem cell-based treatment of many disorders, including POI, in future. However, caution must be used as SALL4 is known to function as oncogene in various germ cell-related tumors (44-47) and neoplasms of gastrointestinal origin (48-52). Sall4-related cell stemness concept for example is used in Sall4-HSC transplantation. Nevertheless, the data on the connections between SALL4 functions and the possibility of their clinical use are still lacking.

### **PROGRAMMED CELL DEATH AND SPHINGOMYELINASE GENE**

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According to the recent data, apoptosis is considered to be the leading mechanism of oocyte loss, both developmental and secondary to malignancy treatment (53). This information may provide us with the new tools to delay menopause, reducing apoptosis-related follicular atresia, or protect ovarian function during anti-cancer treatment (54, 55). Apoptotic pathway is complex, it includes multiple steps, that can potentially be targeted by therapeutic intervention (56). However, several studies reported that targeting final apoptotic stages (e.g. inhibiting caspases) lead to switch from apoptotic pathway in cells, destined to death, to the process similar to primary necrosis, putting the potential benefit of such measures under doubt (57-59). Thus, inhibiting the earlier steps of programmed cell death may theoretically be more effective in cell preservation.

One of such potential targets is ceramide, a secondary messenger, involved in proapoptotic signaling (60). The multistep process of ceramide utilization is partially regulated by sphingosine kinase and finally results in sphingosine-1-phosphate (SP) production, which counteracts ceramide, blocking apoptosis progression (61-63). Morita et al. (2000) studied sphingomyelin pathway in ovaries with the goal to develop a new approach of early-step apoptosis control in prospect (64). Sphingomyelin phosphodiesterase 1 (SPD1) is a crucial enzyme needed for programmed death initiation, as it hydrolyzes sphingomyelin, thus unblocking ceramide signaling. The neonatal female mice deficient in SPD1 (SPD1<sup>-/-</sup>) had significantly higher content of primordial follicles per ovary compared to the wild type controls, primary and small preantral follicles' hyperplasia and greater egg reserve were also observed in SPD1<sup>-/-</sup> mice. Moreover, explainable symptoms, resembling human Niemann-Pick syndrome were observed in the murine study group in postnatal life (65). When fetal ovaries from both groups of mice were collected for in vitro culture, wild-type fetal ovaries demonstrated time-dependent apoptosis initiation in germ cells, whereas in SPD1<sup>-/-</sup> mice it was significantly retarded, serving as a logical explanation for larger egg pool in newborn mutant mice (64).

It's worth mentioning that sphingomyelin degradation is crucial for apoptosis initiation rather than de novo ceramide synthesis, which was proved in experiments with fumonisin-B1, a selective

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blocker of ceramide synthase (66, 67). Morita et al. (2000) also observed similar results in fetal ovarian culture morphology in both SPD1-deficient and non-deficient, but treated with SP, samples (64). When isolated oocytes from SPD-/- and wild type mice were cultured in the presence of doxorubicin (anticancer drug) the study group demonstrated resistance to it, whereas the control group showed robust apoptosis (64, 68). Several studies showed that protective action of SP on neurons and oocytes was not dependent on GI-coupled endothelial differentiation and growth receptors (69-71). SP has also a radioprotective action on gonads, as its administration prior to radiation therapy in mice showed dose-dependent preservation of follicular reserve, whereas almost complete loss of primordial follicles was observed in control group. Observation for two weeks after treatment proved that the follicles in SP-treated mice were totally functional and viable (64).

## NEONATAL THYMULIN GENE THERAPY

Congenitally athymic (nude) mice have pituitary-gonadal axis developmental abnormalities, resulting in delayed sexual maturation, decreased fertility and shorter reproductive period, related to accelerated ovarian follicular atresia with subsequent POI (72-75). When neonatal mice undergo thymectomy similar features occur (76, 77). Thymulin is known to demonstrate gonadotropin-releasing action, regulate sexual development in females and modulate gonadotropin-dependent steroidogenesis in the gonads (78-82).

Reggiani et al. (2012) showed that immunoneutralization of thymulin in normal mice postnatally results in lower levels of gonadotropins at puberty. They also used neonatal thymulin gene therapy (NTGT) in nude mice, which resulted in thymulin production and release into bloodstream in these animals and prevented gonadotropin deficiency, that typically occurs in nude mice (83, 84). NTGT used recombinant adenovirus (RAd), carrying methionine-FTS (5'-ATGCAGGCCAAGTCGCAGGGGGGTCG-AACTAGTAG-3') gene (metFTS). RAd-green fluorescent protein (GFP) was used for control group. Both groups were injected with

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corresponding RAd on day 1 after birth. Nude mice were tested at day 70 and active circulating serum thymulin was increased only in the study group of both homo- and heterozygous nude mice. The levels of thymulin were only slightly lower in RAd-FTS nu/nu mice compared to nu/+ controls, and RAd-FTS heterozygotes had comparable thymulin levels with controls. NTGT was able to prevent gonadotropin-releasing hormone (GnRH) neuronal deficiency (anterior hypothalamus and preoptic nucleus), typical for nude mice. Nu/+ mice had normal gonads with follicles in all stages of development and normal corpora lutea, whereas nu/nu controls had anomalous ovaries with low follicle count, no preovulatory follicles and high amount of atretic follicles compared to normal controls. Treated nu/nu showed ovarian picture comparable to nu/+ controls. Also nu/nu, who received NTGT, had normal serum estrogens versus untreated nu/nu. NTGT was also able to attenuate the vaginal opening delay, observed in nude mice (83).

## BASONUCLIN-1 DEFICIT AS A CAUSE OF POI

Huang et al. (2018) identified another causative gene, responsible for POI development in some of the patients. BNC1 is located on chromosome 15 and is known to be expressed in oocytes. Its defect produces truncated protein, leading to haploinsufficiency or gain of abnormal function. This abnormal basonuclin-1 causes reduced meiosis in oocytes.

Using whole-exome sequencing, the above-mentioned group of scientists identified a 5 bp deletion in BNC1 gene, encoding basonuclin-1. This frameshift mutation has an autosomal dominant type of inheritance and causes POI, running in families. In vivo experiments support these data: *Bnc1*<sup>tr/tr</sup> mice did not produce pups after mating with wild-type males, *Bnc1*<sup>tr/+</sup> group were subfertile, whereas *Bnc1*<sup>++</sup> mice (control group) showed normal fertility. Hormonal level of the latter three groups of animals showed no difference at 8 weeks, however, at 36 weeks significantly lower estrogens and higher FSH and LH in *Bnc1*<sup>tr/tr</sup> and *Bnc1*<sup>tr/+</sup> groups were detected, compared to controls. Expectedly, *Bnc1* mutant mice showed smaller ovaries with lower follicular count (85).

## FUTURE DIRECTIONS

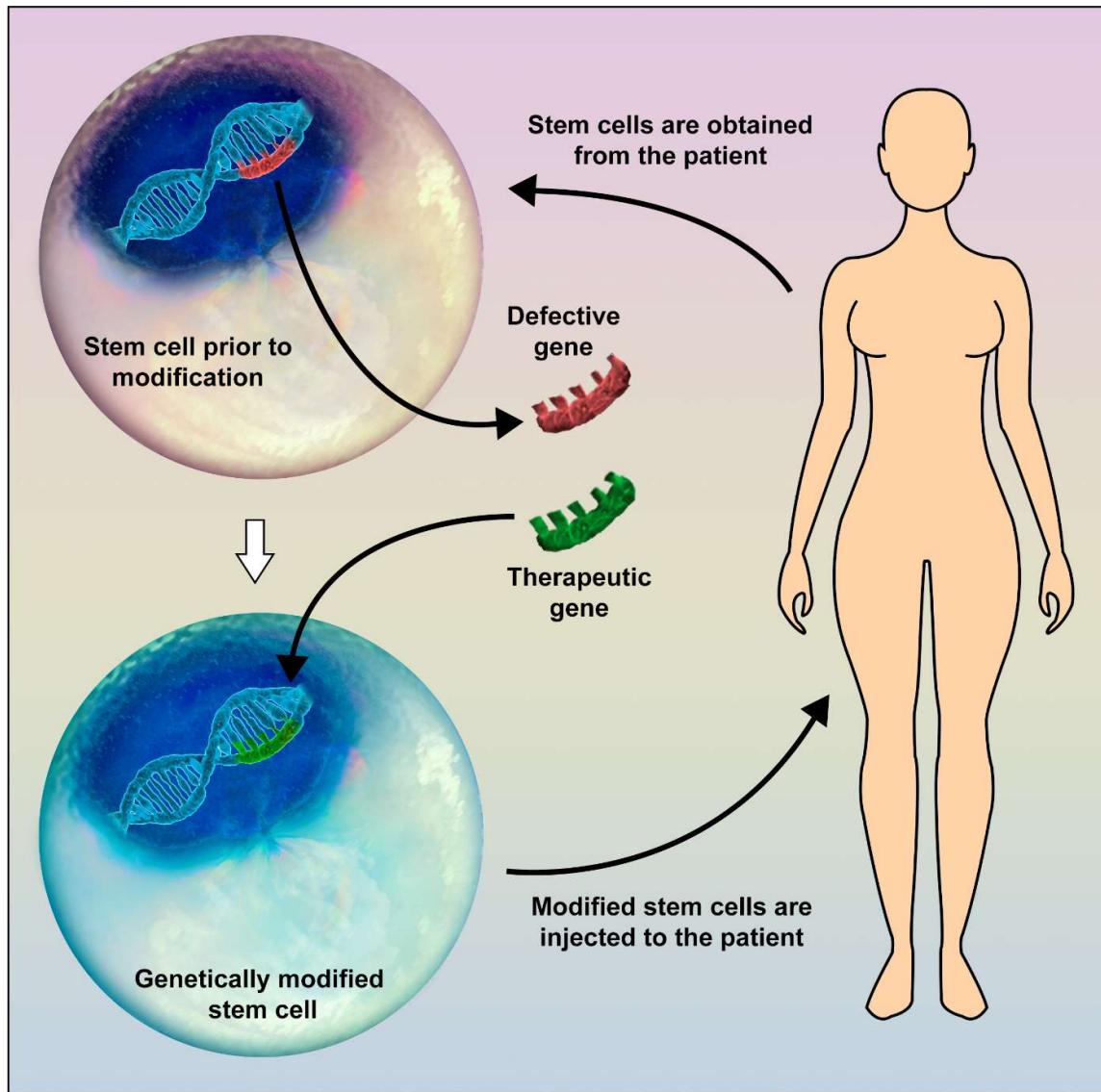
Ad vector and its delivery were safe and well tolerated by mice. Still, as was said earlier, the pregnancy did not occur in treated animals, probably due to the dominance of Ad-hFSHR vector promoter, leading to the lack of FSHR downregulation in the further stages of follicular development and, thus, arrest at the antral stage. The future work is likely to be done on the modification of this vector in order to unblock the later stages of follicular development and reach ovulation.

Distinct mutations of SALL4 genes, associated with POI development, are theoretically good targets for gene therapy. Not much is known about SALL4 functioning and signaling pathways, which need to be studied thoroughly. In addition, the caution should be used, as SALL4 are powerful proto-oncogenes and are known to play role in various tumors.

Targeting genes, responsible for apoptosis, may potentially prevent POI, as it is known that programmed cell death is a leading mechanism of follicular atresia. Undoubtedly, this must be very specific and well-controlled, as apoptosis is the major process of every cell's functioning and its defect may lead to variety of pathologic conditions, including neoplasia.

The other genes, known to be involved in POI development may be also targeted by gene therapy. However, more investigation of their functioning and ways of correction of their defects are needed.

We believe that much effort should be made in future in the field of stem cell (SC) therapy. Since they have multiple mechanisms of affecting the tissues, including paracrine regulation of cell functioning, stimulation of cellular growth and division, ability to differentiate into target cells, SC attract great interest of many researchers all over the World. Theoretically, target genes of SC may be altered in vitro and re-injected back, thus giving the opportunity to avoid the use of viral vector (Fig.1). The resultant SC may potentially give rise to a new, modified pool of cells, functioning in desired way. This would open an exciting field in regenerative medicine. Unfortunately, today the available data is deficient, thus, motivating us for new investigations.



**Figure 1. Gene therapy in combination with the use of stem cells may open a new field in regenerative medicine.** The possible way to modify human genes without using the viral vector is SC therapy. Different types of SC may be obtained from various tissues of human body. The target gene is replaced with the therapeutic one and the modified SC then will be injected back into the patient's body (e.g. peripheral blood, target organ). These altered SC may produce a colony of new specific cells or influence the surrounding tissue functioning.

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