Lentivirus vectors mediated eGFP transfected into rat ovary in vivo

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Summary

Purpose: To evaluate the optimum dosage and time-effect relationship of lentivirus vectors mediated enhanced green fluorescence protein gene (lenti-eGFP) transfection into the rat ovary in vivo. *Materials and Methods:* Lenti-eGFP was microinjected into rats' ovaries with different dosage (2×10^6 TU and 10×10^6 TU virosome respectively, n = 5 rats). The expression of eGFP was examined by the fluorescence microscope at injection at five days. The fluorescence intensity of different dose groups was calculated and determined the optimum dosage. The authors observed the expression of eGFP in ovaries and others tissues at days 5, 15, 30, 45, 60, and 75 after the rats' ovaries were microinjected with the optimum dosage of lenti-eGFP. Reserve transcription-polymerase chain reaction (RT-PCR) and RT-quantitative (q) PCR (RT-qPCR) were used to qualitative and quantitative analyze the expression of eGFP in different tissues and organs of transfected rats. *Results:* The expression of eGFP in both ovaries of every rat was seen at five days of transfection. Semi-quantitative assessment of green fluorescence for the two-dosage group was 0.2311 ± 0.0203 and 0.2307 ± 0.0199, respectively. There was no significant difference in both groups ($p = 0.976$). The expression of eGFP enhanced with transfection time prolongation and continued with 75 days of transfection (the fluorescence density in different time was 0.2307 ± 0.0199, 0.3119 ± 0.0213, 0.3462 ± 0.0264, 0.3568 ± 0.0127, 0.3496 ± 0.0133, and 0.3513 ± 0.0172, respectively). Furthermore, there were efficient and durable expressions of eGFP in other tissues and organs of rats. RT-PCR and RT-qPCR proved these results. *Conclusion:* Lenti-eGFP may successfully transfect ovary tissues and other organs in vivo simultaneously, the expression of eGFP is highly efficient and durable.

Key words: Lentivirus vectors; Enhanced green fluorescence protein; Ovary; In vivo; Animal experiment.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder. The incidence is 4%-12% [1-4]. In some areas of China, the epidemiological survey showed its incidence rate to be 6%-8% [5, 6]. It is one of the most common causes of sterility in young women. The pathogenesis for the disease is not clear. Environment and inheritance (gene mutation) working together is accepted by most of researchers. There is yet no satisfactory effect in clinical treatment. There could be a breakthrough in its treatment through an appropriate intervention of the mutant gene by transgenic method in concomitance with lifestyle change (diet and exercise). Hence, the authors attempted to explore a transgenic treatment for PCOS. There are less researches regarding gene transfection into ovary in vivo. In the present study, the authors used lentivirus vectors mediated enhanced green fluorescence protein (eGFP) gene to transfect rat ovary in order to verify its effectiveness.

Materials and Methods

Animals

All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee at Chongqing Medical University in Chongqing city (China). Forty specific pathogen-free (SPF) degree Sprague-Dawley female rats two months of age (weighing 160 to 180 g) were provided by Animal Center of Chongqing Medical University and housed in a 12-hour light/dark cycle at a controlled temperature and humidity with free access to food and water.

Rats were randomly assigned to the following eight groups: control, low-dose, high-dose, 10 days, 15 days, 30 days, 45 days, 60 days, and 75 days (n = 5, respectively). At five days of transfection, the optimum dosage was to be selected between the two doses by infection efficiency observed with a fluorescence microscope.

Lenti-eGFP transfection into rat ovaries and microinjection

The animals were fasted overnight but were allowed free access to water. The body weight for each rat was calculated prior to surgery. Anesthesia was induced with 10% chloral hydrate at a dose of 100 mg/kg by intraperitoneal injection. Rats were subjected to the prone position and a microtubule nick about 1.0 - 1.5 cm at right back of body surface projection for ovary. Hypodermal, muscular layer, and peritoneum were cut and the ovary was drawn out from abdominal cavity.

Different dosage viral particle of lenti-eGFP was microinjected into sub-envelope of ovary. The low-dose group was 2×10^6 TU and high-dose group was 10×10^6 TU virosome every rat respectively. The surgery of microinjection is shown in Figure 1.

The expression of eGFP

Frozen and paraffin-imbedding sections were prepared to fluorescence microscope for each group of rat ovaries. The green fluorescence density for ovary sections of every group was calculated and the statistic difference was analyzed.

RT-PCR and real time quantitative PCR

Total RNA was extracted from ovary tissues stored at -80°C using the RNAiso plus extraction method as directed. Briefly, total RNA was extracted with RNAiso plus and precipitated with isopropyl alcohol, washed in ethanol, and re-suspended in

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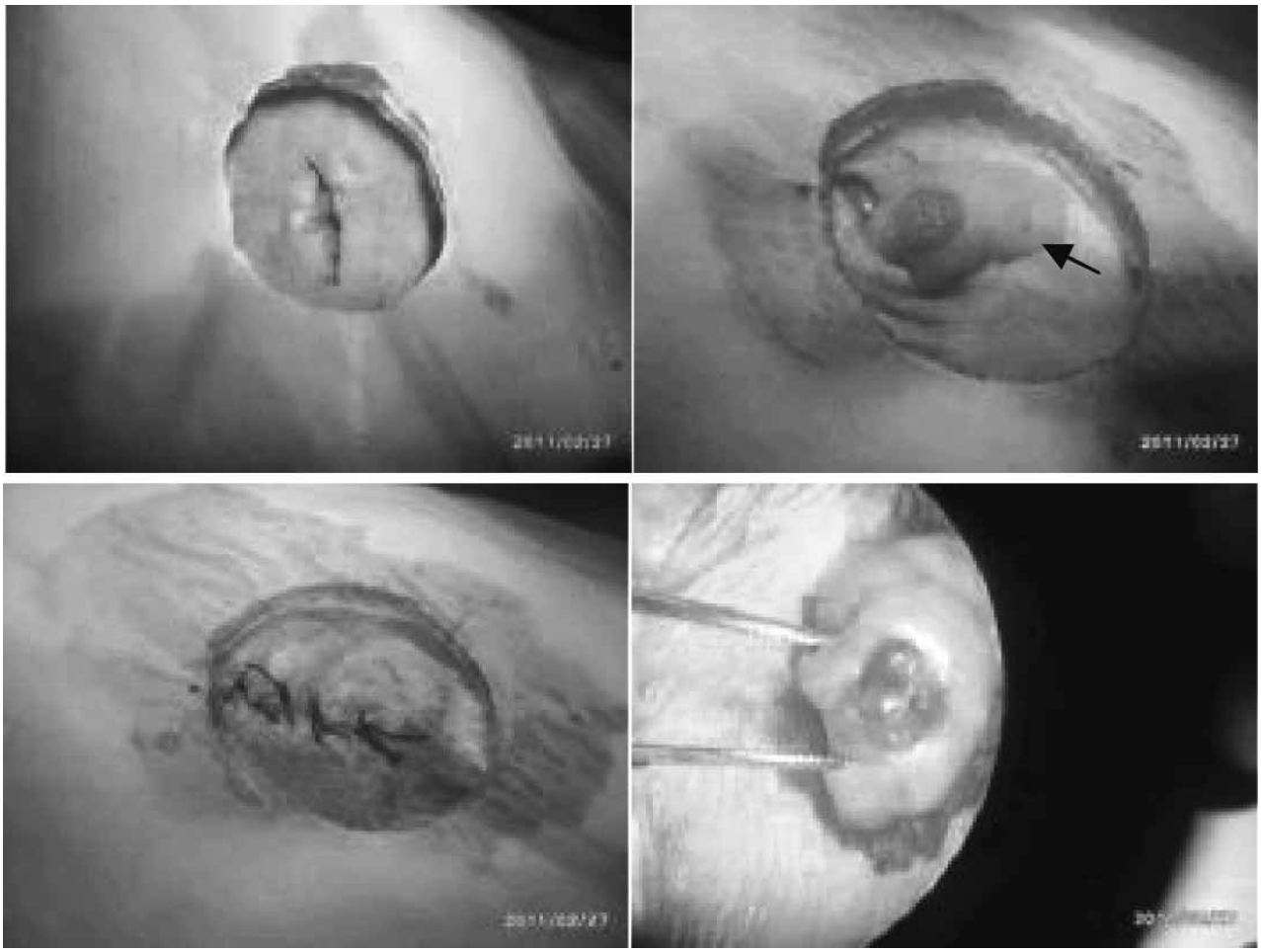


Figure 1. — Surgery of rat ovary microinjection. The arrow indicates rat ovary.

RNase-free water. RNA quantity and quality were determined by spectrophotometry. Two micrograms of total RNA were used for reverse transcription (RT) and the RT reagent kit method as described. CDNA was used for polymerase chain reaction (PCR) and quantitative PCR (qPCR) and repeated three times. PCR and a qPCR products were synthesized. Each 20 μl SYBR Green reaction contained 1.0 l cDNA, 0.1 mol/l: forward primer 0.75 μl and 0.1 μmol/l reverse primer 0.75 μl. For amplification of both eGFP and the reference gene actin-beta, the following PCR protocol was applied: 95°C for 60 s, 95°C for 5 s, 55°C for 30 s, 40 cycles. The specific primers for qPCR of eGFP gene were F1 5'-ctttcggttatggtgttcaatg-3', R1 5'-tgtcttgtagt tcccgtcatctt-3', and product size was 137 bps; the specific primers for actin-beta gene were F1 5'-cacccgcgagtacaaccttc-3', R1 5'-cccatacccaccatcacacc-3', and product size was 207 bps. The fluorescence spectra were recorded during the elongation phase of each PCR cycle. The results were analyzed by the delta Ct (ΔCt) method, which reflects the difference in threshold for the target gene relative to that of actin-beta in each sample. The specific primers for PCR of eGFP were F1 5'-gcgagggcgatgccacctac-3', R1 5'-cggttcaccagggtatctcc-3', and product size was 267 bps.

Data analysis

IPP 6.0 software was used to calculate the fluorescence density of ovary tissue sections for every group. Data were presented as means ± Std. deviation. SPSS 16.0 was used to all data statistics. Statistical differences among the various groups were assessed by one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

Results

Green fluorescence in the rats' ovary cortex, stroma, and corpus luteum cells was stronger and was less in complete follicles of different developmental stages at five days of transfection. There was no significant difference for the fluorescence density between the two dose groups. The lower dosage (2×10^6 TU) resulted in a better transfection effect. The expression of eGFP increased as the extension of transfection time reached its peak at 30 days. The green fluorescence was still seen in the ovary at 75 days of transfection (Figure 2). Semi-quantitative outcome for fluorescence density according to different time points is shown in Table 1 and Figure 3.

The eGFP expression was not only in the side ovary of virosome injection but also in other tissues and organs. The authors observed the green fluorescence at 30 days of transfection in homolateral fallopian tube, contralateral ovary and fallopian, uterus, brain, cornea, retina, liver, muscle, lung, heart, and fat tissue (Figure 4).

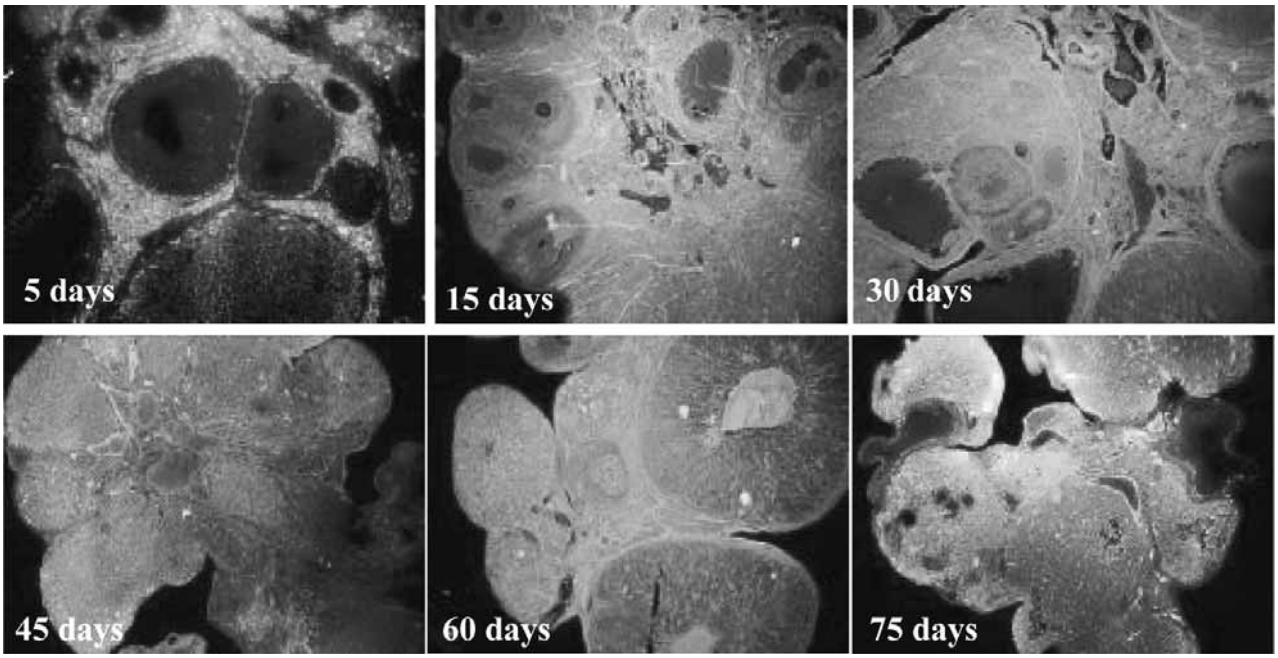


Figure 2. — Fluorescence image of different time intervals for transfected rat ovaries. Virosome of lenti-eGFP for every rat is 2×10^6 TU. Original magnification is 10 × 20.

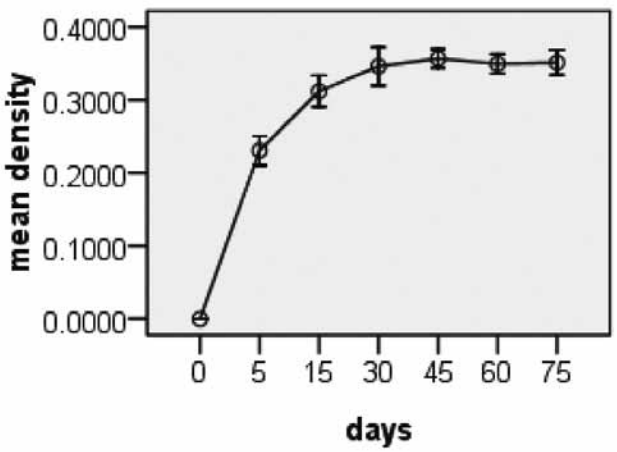


Figure 3. — Fluorescence density curve for eGFP expression in rat ovaries at different time intervals.

Table 1. — *Statistics of semi-quantitative eGFP expression in rat ovaries at different time intervals*.

N = 5	5 days	15 days	30 days	45 days	60 days	75 days
Mean density	0.231	0.312	0.346	0.357	0.350	0.351
Std. deviation	0.020	0.021	0.026	0.013	0.013	0.017
p value		0.000	0.005	0.353	0.526	0.879

The *p* value is an outcome compared to the previous time interval.

The outcome of RT-PCR (Figure 5) was in accordance with the information depicted in the fluorescence photograph, in which there is no expression of eGFP mRNA in control rats' ovary but in transfected rats' various organs and tissues.

RT-qPCR was prepared to the relative quantity of expression eGFP mRNA. Different expression levels in transfected rat ovaries and other organs and tissues are shown in Figure 6.

Discussion

The conception of transgenic treatment began in the middle of the 20th century. It was praised as a revolutionary technology for those diseases of basic molecular level abnormity. It mainly aims to carry the exogenous therapeutic genes into target cells with specific vectors. The gene makes a target treatment through correcton of mutation sequences of pathogenic gene or reprogramming host cell function [7, 8]. The lentivirus vectors basic of HIV-1 render the target gene expression stabilized and durable because they may integrate the gene into host cell chromosomes. Several researches indicate the reorganized vector has no cytotoxicity for animals in vivo [9, 10].

Currently, the gene therapy animal models basic of lentivirus vectors includes various cancers and some metabolic diseases [11-14]. Even the foreign gene can be targeted and expressed into specific cells and tissues [15] and it is also developing the direction for future gene therapy.

There are less reports regarding ovarian-related disease gene transfection in vivo. In the experiment the authors used lentivirus vectors mediated report gene-eGFP to observe its expression in rat ovaries and other organs in vivo through ovary-microinjection.

Transfection efficiency is related to different cells and tissues [16, 17]. The larger the carried gene, the lower the transfection efficiency will be. In this experiment, the same fluorescence density in the two different dose virosome ovaries was seen. The authors believe that the lower dosage is adequate for rat ovary.

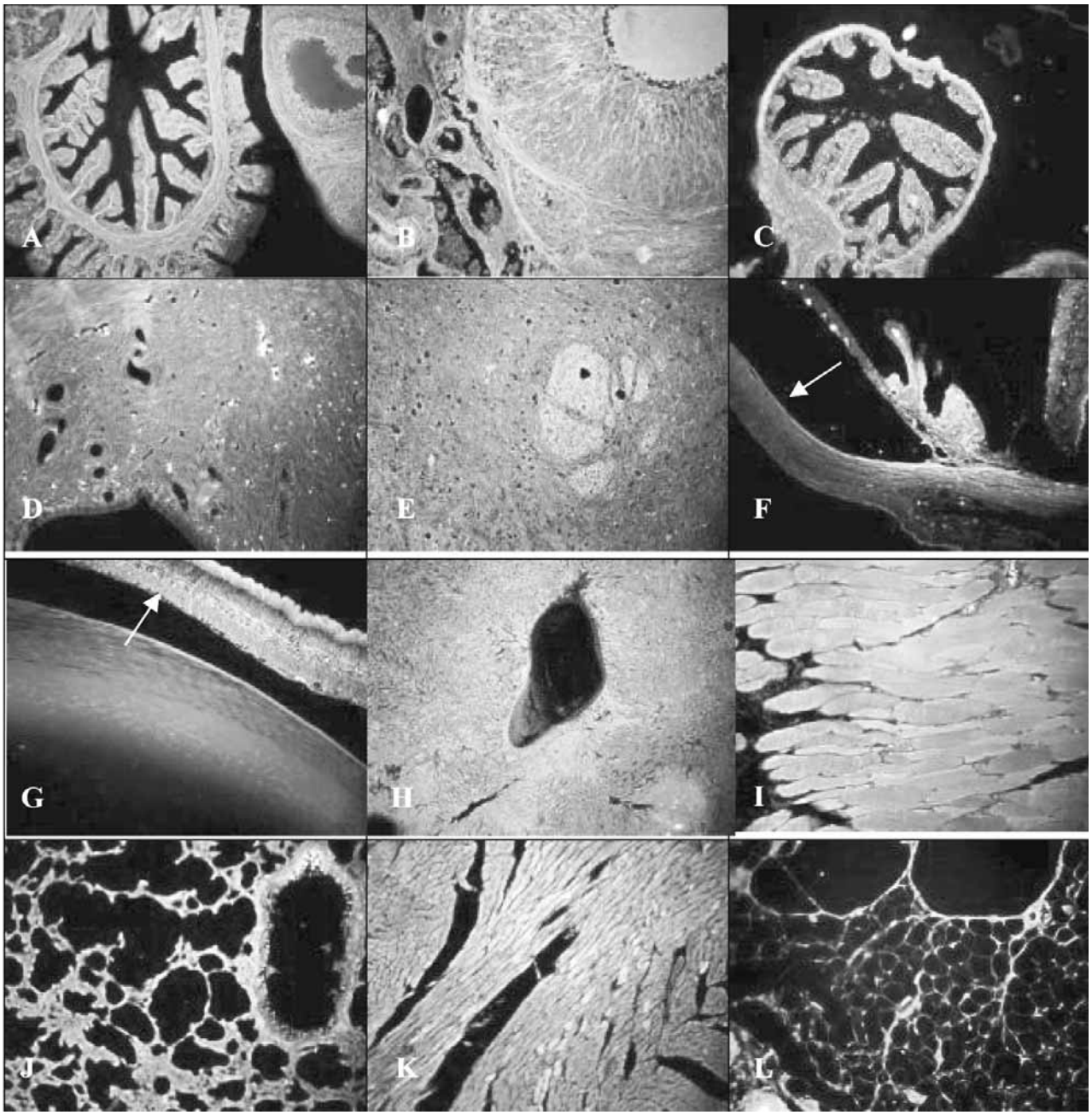


Figure 4. — The expression of eGFP in rats various organs and tissues at 30 days of transfection. A: homolateral fallopian tube, B: contralateral ovary, C: contralateral fallopian tube, D: uterus, E: brain, F: cornea (arrow), G: retina, H: liver, I: muscle, J: lung, K: cardiac muscle, L: fat. Original magnification is 10 × 20.

They observed on transfection five days that eGFP was highly and efficiently expressed in several ovarian cells and tissues including epithelial cells, thecal cells, granular cells, and ovarian medulla.

An interesting phenomenon found is that eGFP expression is less in complete follicles but more in corpus luteum. An essential factor for successful transfection is direct cell-to-cell contact. So the authors presume the complete follicular walls could have a barrier function to delay the virosome into follicles and the barrier could be broken when the follicle ruptures to develop corpus luteum. There is no apparent difference between follicles and corpus luteum for eGFP expression at 15 days of transfection.

During the time-effect observation of eGFP expression, the authors found the expression of eGFP was at peak and durable at 30 days transfection. There is no significant difference for the fluorescence density in ovaries between at 30 and 75 days transfection. The authors suppose lentivirus vectors mediated exogenous genes could

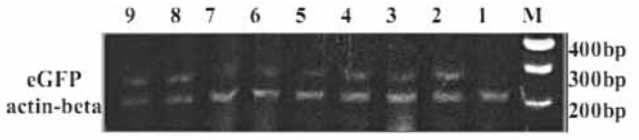
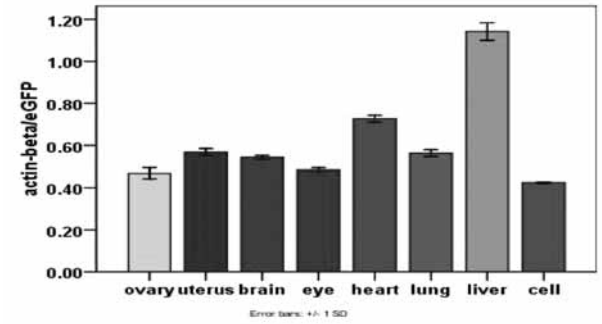


Figure 5. — RT-PCR outcome. M: marker; 1: control ovary; 2-9 is transfected rat organ and tissues. 2: ovary, 3: liver, 4: brain, 5: eye, 6: lung, 7: heart, 8: fat and 9: uterus. The products size for eGFP is 267 bps and actin-beta is 203 bps.



Figuer 6. — RT-qPCR statistic outcome. The cell as a positive control in the figure is lenti-eGFP transfected granular cells.

express at long-term and in a stable form in ovarian tissues in vivo. They also discovered eGFP expression in other several organs and tissues including brain and eye. An ideal transgenic therapy would be to target transfection into specific cells [15], but the current vectors are unsuccessful in this. Nonetheless, the non-choice transfection could play an important role in treatment of several genetic and endocrine and metabolic diseases such as PCOS. The authors now believe that the cause of PCOS is the "co-operation" of several genes [18]. Gene mutation occurred not only in the ovary but in other tissues, namely non-choice mutation. The non-specific transfection is just for the non-specific mutation.

Moreover, the authors created a new method for rat ovary microinjection. Traditionally, abdominal incision serves as a belly-cavity operation. There are two main shortcomings for ovarian microinjection. The rat ovaries are located in deep abdominal cavity and near posterior peritoneum, so it is difficult to withdraw them. Furthermore, the abdominal incision must be large enough to complete the operation. However, these problems can be resolved if the incision is selected on the back of the rats where the surface projection of the ovary is located. The advantages for this include less incision, less lesion, and facilitated ovary exposure.

In a conclusion, lentivirus vectors mediated gene could express stable in a form at long-term in rat ovary.

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