SZENT ISTVÁN UNIVERSITY

OOCYTE TRANSPLANTATION IN FISH (FOLLICLE TRANSPLANTATION IN ZEBRAFISH (*DANIO RERIO*))

Thesis

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1 PRELIMINARIES AND OBJECTIVES

1.1 Preliminaries

Oocytes and follicles has long been studied in fish as knowing morphological, histological and biochemical parameters are essential for improving artificial breeding of commercially important fish species. Although there is a large amount of data on the biology of oocytes, many questions still remain:

i) Time needed for the transition of follicles from one stage to the other, enabling the determination of optimal physiological and environmental parameters for oocyte development, is unknown.

ii) Cryopreservation methods for fish sperm have long been developed and will presumably soon be used in the hatchery breeding of some species. However techniques are available for the cryopreservation of follicles too, there is still no method for the maturation of cryopreserved oocytes, so they are not useable in artificial breeding.

iii) Fish as developmental biological models have become more and more popular lately, especially in maternal effect research. Several methods have been described for studying maternal effects, but all of them are complicated and require strong technical background. Techniques used so far are unable to affect or study maternal factors directly in the oocyte, so new oocyte manipulation methods are needed.

Problems outlined above could be solved with a new transplantation and micromanipulation technique developed for early stage follicles.

1.2 Objectives

The primary objective of my work was to develop a possible egg maturation method for zebrafish (*Danio rerio*), using early-stage follicles. I have set myself the following aims within:

- One of my aims was to investigate the integration of transplanted follicles in the recipient ovaries.
- My aim was also to generate viable offspring from transplanted follicles.
- I aimed to examine the ratio of follicles at different developmental stages in the ovaries following propagation, depending on time.
- I wanted to determine -as accurately as possible- the time needed for the follicles to reach certain developmental stages in zebrafish.
- I wanted to find out if controlled protein synthesis or gene manipulation is possible by introducing mRNA or DNA into early-stage follicles.

2 MATERIALS AND METHODS

2.1 Fish maintenance

All experiments were performed on zebrafish (*Danio rerio).* Fish were maintained in a special fish room, in Tecniplast ZebTEC (SZIU, Gödöllő) or AquaSchwarz (KIT, Karlsruhe) recirculation systems on 27,5˚C, 14 hour light-10 hour dark cycle. 25 sexually mature individuals were kept in each tank, according to their sex.

Adults were fed twice a day with complete fish food supplemented with fresh *Artemia* nauplii twice a week. Spawn after the free swimming stage were fed three times a day and from the 10 dpf (days post fertilization) feed was supplemented with *Artemia.* Fish were propagated in pairs (1 male and 1 female), in breeding tanks of 1 liter.

2.2 Zebrafish strains

AB: wild-type, short finned strain. In the transplantation experiments, females were used as recipients, while in fertility tests, males were used as crossing partners.

Gold (*gol*^{b1}): In the *gold* mutant strain, melanophores are smaller and paler or transparent on average and contain less melanophores than the wild-type individuals (LAMASON et al. 2005). The strain was used as recipient in the transplantation experiments.

β-actin:YFP: The strain (ALAM et al., 1996; HWANG et al., 2003) was generally provided by the KIT, ITG (Karlsruhe) and was used as donor in the experiments. YFP is predominantly skeletal muscle specific, however it also shows activity in the skin, oocytes and in other organs.

2.3 Determination of follicle sizes

In order to determine the size of follicles, the dissected ovary was fixed in 4% formalin, then the sizes of oocytes were measured by ocular micrometer or using images of the follicles, size was measured precisely

with the Image J software (Research Services Branch, National Institute of Mental Health, USA).

2.4 Follicle collection

Sexually mature donor females (*β-actin:yfp*) were overnarcotized in MESAB solution and ovaries were surgically removed. For a few minutes, ovaries were rinsed in mPBS containing Petri dishes, then suspended with a Pasteur pipette until follicles were detached. Follicles in the range of 50-150 μm diameter were collected under a stereomicroscope equipped with an ocular micrometer. Collected follicles of the right size were placed in Holtfreter or Zebrafish Ringer solution until transplantation or microinjection.

2.5 Preparation and transplantation of follicles into recipient females

Recipient (AB or *gold*) females were bred on the day of the experiment. Recipients, following breeding, were anaesthetized in MESAB and ovaries were irrigated twice with Holtfreter or Zebrafish Ringer solution. The region of the genital papilla was disinfected with 70% ethanol prior to the transplantation procedure.

For transplantation of follicles, a special, modified pipette tip was used. Follicles were sucked into this prepared pipette tip by an automated pipette and the capillary was gently introduced into the ovaries of the recipients through the genital papilla. At one time, about 25-100 follicles in 25-30 μl volume were introduced into the recipient ovaries.

2.1 In situ hybridisation

Samples were taken from donor, recipient and transplanted ovaries and *in situ* hybridisation was carried out according to the standard protocol of HAUPTMANN (1999), but omitting the permeabilization step.

2.1 Microsatellite analysis

Samples of non feeding larvae derived from presumably transplanted follicles, whole half-siblings and fin samples of donor and recipient females and males were kept in 96% ethanol on -20˚C until use.

DNA was isolated with the traditional phenol-chloroform method (SAMBROOK et al. 1989). Concentration and quality of the isolated DNA was determined with photometer (NanoPhotometer, IMPLEN, Germany). DNA was then diluted to 10ng/µl.

For the analysis, two microsatellites were chosen per chromosome (one on each arm) from the Ensembl genome database (http://www.ensembl.org/Danio_rerio/Info/Index) (50 microsatellites altogether). All microsatellites were tested on the parents. Microsatellites suitable for distinguishing males, donor and recipient females were used for the analysis of half siblings and individuals from presumably transplanted oocytes.

2.2 Preparing constructs for oocyte microinjection, microinjection procedure

In the microinjection process, *DsRed* mRNA, *pβ-actin:LacZ* and p*CMV:GFP* constructs were introduced to *β-actin:YFP* and AB strain oocytes. *DsRed* mRNA was *in vitro* transcribed from the pCS2+ *DsRed* plasmid using Message Machine mRNS kit (Ambion, TX, USA). The injection solution contained 5 µl 10% phenol red in 10 µl final volume, according to the kit protocol. Already prepared injection solutions of the p*β-aktin:LacZ* and p*CMV:GFP* constructs were provided by Ferenc Müller (KIT, ITG).

During microinjection, follicles were immobilised in 2mm deep grooves cut into 2% agarose gels in Petri dishes filled with Ringer solution. Free-hand microinjection was carried out under a stereomicroscope using a microinjector (Tritech Research, USA) and glass pipettes (Cat# 5242 952.008, Eppendorf Germany), introducing 1-2 nl injection solution into an oocyte.

2.3 Statistical methods

Data were analysed by SPSS 13.0 for Windows program. For studying oocyte developmental dynamics Tukey or Dunnet's test was used for data evaluation, depending on the results of ANOVA, ($P<0,05$), a Test of Homogeny. In follicle integration studies ($P\leq0.05$), I used the Kruskal-Wallis test.

2.4 Experiments

2.4.1 Studying oocyte developmental dynamics

50 females were spawn and placed back to their tanks in five groups, 10 by 10. Individuals of one group were terminally anaesthetized immediately after spawning and weekly in the following four weeks. Body weight and ovarian weight was measured and ovaries were fixed in 4% formalin. Oocyte sizes in were measured under a microscope and on the basis of the ratio of cells in each developmental stage was determined. GSI% was quantified from the body ovarian weight.

2.4.2 Detection of transplanted follicles and examination of integration efficiency in the recipient ovaries

In the experiment 20-25 transgenic follicles were transplanted into the ovary of 5 AB and 5 gold females.

On the fourth day after transplantation recipient AB females were terminally anaesthetized, ovaries were surgically removed and examined under in bright field and under a fluorescent microscope. Ovaries were fixed in BT fix and transgenic follicles were detected in the fixed tissue by *in situ* hybridization (ISH). Images were taken of oocytes including bright field images as well of those in which signal was detected.

Recipient *gold* females were anaesthetized with MESAB on the second day after transplantation and images were taken of the transplanted follicles through the abdomen without dissection in normal and in fluorescent light.

2.4.3 Examination of follicle integration efficiency

20-40 transgenic follicles were transplanted into the ovary of 100 AB females. Number of transgenic follicles was determined in dissected ovaries under a fluorescent microscope every day in the first week and on the 14th day following transplantation.

2.4.4 Analysing the growth of transplanted follicles in the recipient ovary

About 50 Stage I follicles were transplanted at once into the ovaries of 20 recipient females. Before transplantation and after isolation from manipulated ovaries (1-2 weeks following transplantation), pictures were taken and size of follicles was determined by the Image J software.

2.4.5 Fertility examination of transplanted follicles

About 100 transgenic follicles were transplanted to the ovaries of 100 AB females and mated every week for six weeks. Eggs were investigated in the fluorescent microscope at the 24 and 48 hpf (hours post fertilization) stage. Spawns probably originating from transplanted transgenic follicles were collected and at the 10 dpf (days post fertilization) stage, fluorescent images were taken. Samples for DNA isolation were taken from all individuals for further microsatellite analysis.

2.4.6 Oocyte micromanipulation

Stage I and II oocytes were collected from the ovary of transgenic females for mRNA (*DsRed*mRNA) and from AB females for DNA (p*β-actin:LacZ* és *pCMV:GFP* construct) microinjection. Oocytes injected with *DsRed*mRNA were transplanted to AB recipients, and ovaries were removed after 24 hours incubation. Oocytes into which the p*β-actin:LacZ* and the p*CMV:GFP* was introduced were incubated on 27,5 ˚C for 24 hours. Follicles were examined with fluorescent microscopy.

3 RESULTS

3.1 Results on oocyte developmental dynamics

GSI showed to be the lowest (6,8%) after spawning (0. week) while it tripled to the second week (18,4%). Statistical difference ($P<0,05$) could only be detected in the GSI values of the first three samplings.

Due to formalin fixation, frequency of oocytes in different developmental stages was only determined according to the Selman's scale (SELMAN et al. 1993).

Follicles with the maximum size of 140 µm (in diameter) were considered as Stage I follicles, so the group was not subdivided into prefollicular and follicular stages.

Percentage of Stage I follicles in the ovary was constantly very high (about 60%) all through the experiment. Compared to other stages their number increased slightly until the second week after spawning and showed a little decline to the end of the fourth week. There was no statistical difference ($P<0,05$) between the results of each week.

Follicles of 140-340 µm in diameter are in the cortical alveoli stage or Stage II. Ratio of these were between 20-25% during the experiment, and appeared to be the highest (26,37%) immediately after spawning. No statistical difference ($P<0,05$) was found between the results of each week.

Size of follicles considered to be in the stage of vitellogenesis ranged between 340-690 µm. However the size range of this group is the highest, their number is relatively low (about 10%) compared to other stages. Statistical difference ($P<0,05$) could only be found between the second and the third week.

Follicles between 690-730 µm were categorized as Stage IV (maturation). Ratio of this group was the lowest (lower than 1,5%). Number of Stage IV oocytes was only 0,3% following spawning, which was statistically significant ($P<0,05$) compared to other weeks except for wwek four. From the second week, no statistical difference ($P<0,05$) was observed.

Follicles larger than 730 µm were classified as Stage V (mature) oocytes ignoring ovulation. Ratio of Stage V follicles increased steadily in the experiment. After spawning, when nearly all mature eggs were laid,

follicles in this stage were hardly detectable (0,12%), while by the end of the fourth week their ratio was about 5,7%, however some of them atretised. Number of these oocytes showed significant difference ($P<0,05$) regarding the results of the 0. and the 4. week, compared to those of the 1.-3. week.

3.1 Detection of transplanted follicles, integration efficiency, results

In bright field, no difference was observed, all ovaries looked the same. Under the fluorescent microscope (using YFP filter), all follicles in the transgenic ovary glowed in yellowish-green, while in recipient ovaries, only a few follicles (donor, transplanted follicles) showed marker gene activity. Presence of transgene mRNA could also be detected in positive controls and in the donor follicles of the recipients, using *in situ* hybridisation.

In the second part of the experiment glowing transplanted follicles were detected in vivo through the abdominal wall of melanophre mutant (*gold*) recipients.

3.2 Follicle integration results

The number of donor follicles in the recipient ovaries, compared to the initial follicle number, halved to the first day after transplantation (47,5±20,5%). Decrease continued until the end of the first week, the rate, except the fourth and fifth days, was statistically verifiable($P\leq0,05$). However, donor follicles were still detectable (2,6±2,6%) in the recipient ovaries at the end of the second week post transplantation.

3.3 Determination of follicle development, results

After transplantation the number of donor follicles significantly reduced, as in the previous experiment, however, integrated, viable follicles were found in every test. Some follicles did not resume oogenesis, while the size of a few increased compared to the size of follicles before transplantation. In case of three, size increase could statistically be proven ($P\leq0,05$). By the end of the third week, the largest

of these could reach stage III. (486μm) according to the Selman scale, while an other nearly matched the size limit for stage IV. follicles (680μm).

The images taken of donor follicles show that follicles which continued oogenesis are intact and develop normally. In the recipient ovaries, only donor, transgenic follicles glow in the fluorescent light, as control donor follicles. I also observed that the later the developmental stage the weaker the fluorescent signal.

3.3.1 Fertility examination of transplanted follicles, results

During the tests, a total of four follicles showing YFP activity were obtained from two recipient females. Among the progeny of one of these females, two transgenic individuals were found on the third week post transplantation. The other female also had two transgenic offspring, while in this case, one originated from the third and one from the fourth crossing. Where the transgene was expressed, spawn originated from transplanted follicles produced the yelow-green YFP signal in fluorescent light, just like the transgenic control larvae. The signal could not be detected in larvae developed from the recpient's own follicles. All fries from donor follicles showed adequate, normal development. Three of the four ofspring were used for further DNA studies. One was grown to a female, that after reaching sexual maturity was mated. All the fries developed normally.

From the selected independent microsatellite loci, 8 were able to distinguish parents and could provide reliable information on the origin of the offspring. The result clearly demonstratedthat the transgenic larvae shared their genotype with the transgenic donor female and the wild type male but not with the recipient female.

3.4 Results of oocyte micromanipulation

In the first part of the experiment, oocytes were injected with *in vitro* synthetised *DsRed*mRNA. Microinjected oocytes that translated the mRNA into reporter protein showed *DsRed* activity under the microscope. In bright field, all injected and control follicles were shown to be intact and normal, in accordance with their developmental stage.

Following incubation, 58 of the 477 injected, transplanted oocytes could be recovered, 6 of which (10,3%) showed both *DsRed* and *YFP*

activity. Regarding efficiency, the fifth series showed to be best, as 21,4% of the recovered oocytes expressed the *DsRed* protein.

In the second part of the experiment, I introduced DNA constructs into the oocytes. GFP was expressed from the encoding construct in four (6,7%) injected oocytes. No GFP activity was detected in non-injected oocytes and in those which were injected with the *lacZ* construct. In the bright field, both injected and control follicles were shown to be intact and normal, in accordance with their developmental stage.

3.5 New scientific results

1. I developed a transplantation method for early stage (I.-II. stage) follicles in zebrafish, first in the World. It was undoubtedly shown that transplanted follicles can integrate and survive in the recipient ovaries, continue oogenesis and are able to generate mature eggs and viable offspring.
2. Using the new transplantation method I was the first to give experimentally validated description of the time dependence of oocyte development in zebrafish, maintained on 27.5˚C.
3. It was confirmed that zebrafish follicles may rest in any of the first four developmental stages for a shorter or longer period, then resume oogenesis.
4. I determined the ratio of follicles of different stages immediately after spawning and in the following four weeks in the ovaries.
5. I demonstrated that mRNA or DNA constructs are expressed in the injected follicles so microinjection could be a feasible tool to manipulate gene expression in early-stage zebrafish embryos.

4 CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions from studying oocyte developmental dynamics

Results suggest that oogenesis in zebrafish -like in other tropical fish (SZABÓ 2000b)- is fast and continuous. Ratio of Stage I. and II. follicles in the ovary is high, however trends specific for the early stage follicles of cold and warm water fish of the temperate zone (LEFLER et al. 2008) can not be observed.

A zebrafish female, under laboratory conditions, can produce 100-150 eggs at weekly intervals. However, much more follicles can be found in the ovary of a mature female suggesting that some follicles may arrest in a certain developmental stage. It is still unclear which follicle from the hundreds, how and why will continue the oogensis. Deeper understanding of this phenomenon would be of great importance in increasing the egg production of farmed fishes.

Zebrafish in nature have a definite breeding season (rainy season). They spawn anytime within, depending on the frequency of rainfall. The spawning season is followed by a reproductively less active period (dry season (MUNRO 1990). Hence results on the dynamics of oocyte development are only true for the AB strain of laboratory zebrafish, where because of multi-generation laboratory maintenance individuals of favorable reproductive parameters were selected.

Taken together the results suggest that Stage I. follicles can be isolated from mature females at any time, however this experiment does not tell about the time dependence of follicle development in zebrafish.

4.2 Conclusions from detecting and examining integration of transplanted follicles

Viability of donor follicles were verified by *in situ* hybridisation. Results suggest that some donor follicles indeed invade the recipient ovaries and retain viability.

The *gold* mutant zebrafish could be used for *in vivo* studies as well, however the viability and size of follicles can not be determined by transabdominal inspection.

4.3 Conclusions from integration efficiency studies

Results indicate that transplanted follicles found in the ovaries at the end of the first week are integrated, and will probably continue oogenesis.

Many parameters may account for the rapid and considerable decrease in the number of transplanted follicles. The most likely is that the isolation method used in the experiment was not optimal, so follicles might have damaged when being suspended strongly reducing their viability.

The donor transgenic strain was developed from the same wild-type AB strain that were used as recipients in the studies, thus these two strains are genetically homogeneous, so there is minimal risk of rejection. No signs of acute or chronic rejection reactions were observed in the recipient ovaries (GERGELY és ERDEI 2000). Optimal conditions could also help the survival of donor follicles and reduce the possibility of rejection reactions (BLY és CLEM 1992, NEVID és MEYER 1993). In my opinion, rejection reactions did not play role in reducing the number of follicles, though it was not evaluated by direct methods.

4.4 Conclusions from analysing the growth of donor follicles

This study provided insights into the dynamics of follicle development in zebrafish. According to the results, intervals in which a follicle may develop from one stage to the other could be determined. Stage I. follicles may reach Stage III. in a week, and stage IV. in another seven days. Time needed for the whole maturation process could not be defined.

Some donor follicles did not grow, while growing follicles of the same stage could also be observed on both dates. Thus the previous hypothesis that follicles may rest for a shorter or longer period in any developmental stage, has also been confirmed.

4.5 Conclusions from the fertility tests

Results suggest that intraspecific transplantation can successfully be implemented and the developed method is suitable for the intraovarian transplntation of Stage I. follicles.

It was confirmed that trasplanted follicles are able to develop into mature eggs resulting in viable offspring and in optimal conditions grow to fertile adults. The above suggest that the transplantation process did not impact negatively on the individuals originating from transplanted follicles.

4.6 Conclusions from oocyte microinjections

In the first part of the experiment, mRNA was injected into the cytoplasm of *β-actin:YFP* oocytes. Results indicate that oocytes can be manipulated by mRNA microinjection and 1 day incubation is sufficient for protein expression, although microinjection eficiency shown to be very low. It can also be concluded that the chance for an injected follicle to integrate is much lower than that of non-injected ones, as the injection process may reduce the viability of follicles because of the mechanical damage caused by the capillary.

In the second part of the experiment, DNA constructs were microinjected ito wild-type follicles. Results suggest that the reporter protein can be synthetised in a 1 day *in vitro* incubation. The *lacZ* reporter can not be detected directly by fluorescent microscopy, indicating that the fluorescent signal is not a consequence of the injection process or any the components of the injection solution.

In summary the microinjection experiments demonstrate that early stage follicles can directly be manipulated by nucleic acid injection, that combined with the newly developed transplantation method could provide a new technique for developmental biology or other kinds of research based on follicles.

4.7 Conclusions from the two methods

The transplantation technology may have several advantages over existing methods of manipulating maternal genes. Recent methods require the establishment of maternal mutants for the effective manipulation of maternal genes, like PGC (primordial germ cell) transplatation (TAKEUCHI et al. 2003), somatic rescue of zygotic mutants or four generation maternal effect studies (DRIEVER et al. 1996), all of these need zygotic mutants. The new follicle transplantation technology opens the possibility to manipulate the wild-type oocyte directly with reverse

genetic methods and may alleviate the need for a zygotic mutant to generate a loss of function phenotype. It allows for the direct manipulation of oocytes by gene silencing („gene knock down") approaches (like RNA interference, overexpression of dominant-negative protein variants or morpholino techniques). Future work will be required however to establish knock down technologies in follicles.

Obviously oocyte transplantation has several advantages over four-generation maternal mutant starins. The former is based on the classic approach thus allow the identification of oocyte specific genes that do not have somatic role.

The reverse genetic approach in turn enable the identification of any gene that is active in the oocyte and allow to find out its oocyte specific functions.

Reverse genetics however does not provide sufficient information for the determination of the functions of genes expressed in oocyte development. So techniques that enable the manipulated oocyte to develop into an embryo could highly be informative when studying embryogenesis from fertilisation through the maternally to zygote controlled embryogenesis to tissue and cell specification and differentiation.

Considerable progress has been made in the *in vitro* maturation of late stage follicles (SEKI et al., 2008), but for the effective manipulation of maternal genes, early stage follicles are needed that has not yet been successfully incubated *in vitro.* The optimisation of the new transplantation technlogy could provide additional information to the maternal effect studies using classical genetic approach. Additionally, the oocyte transplantation if combined with efective gene knock down techniques could also enable to determine at what level mRNA and proteins of maternal genes active in the zygote play a role in embryogenesis.

In case of some genes for which zygotic or maternal mutants are available (*one eyed pinhead* or *dicer*), the loss of maternal gene function drastically improved the moderate phenotype of the zygotic mutant(GIRALDEZ et al. 2006; GRITSMAN et al. 1999). A recent study shown that about 20% of genes expressed in the blastula or morula is already present as maternal gene product (MATHAVAN et al. 2005). On the basis of this, RNA translated from thousands of genes are maternally present and so the phenotype of zygotic mutants can be improved by the inhibition of maternal effects during early embryogenesis. For this, oocyte manipulation could be a feasible tool.

4.8 Recommendations

4.8.1 Recommendations regarding follicle transplantation

- Although follicle transplantation is a feasible technique, the efficiency needs to be further increased. Improving the tools and the follicles isolation technique used in the procedure could make the efficiency of the method acceptable, so further on I recommend these improvements to be carried out.
- Survival of oocytes in the recipient ovaries should be studied in semi-transparent or transparent zebrafish strains. Fate of reporter expressing transgenic oocytes could easily be followed all along in the ovaries providing much more information about the oogenesis of fish. Later on I recommend the use of the *gold* or *nacre* mutants as semi-transparent recipients, or the *casper* (WHITE et al. 2008) strain that is transparent in the whole life cycle.
- I also recommend to carry out interspecific transplantation experiments and to transplant cryopreserved, thawed follicles.

4.8.2 Recommendations on oocyte micromanipulation

- The oocyte micromaipulation technique also needs to be further improved by intranuclear injection, optimisation of injection conditions, using a precision micromanipulator or immobilising the oocytes during the injection procedure.
- According to the above I also recommend the siRNA or morpholino injection of oocytes.

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