

## ARTICLE

# A Preliminary Technique for the Isolation and Culture of Brown Trout (*Salmo trutta macrostigma*, Dumeril, 1858) Spermatogonial Stem Cell

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### ABSTRACT

This study was aimed to find a practical technique for isolation and culture spermatogonial stem cells from male brown trout (*Salmo trutta macrostigma*). Twelve wild juvenile male were obtained from Kılıç Trout Fish Farm (Kahramanmaraş, Turkey). The juveniles were taken alive to the aquaria unit and stored in a 1000-liter capacity fiberglass tank. In order to identify the best size, age and testis structure of *S.t. macrostigma* for spermatogonial stem cell isolation and culture. Morphological and histological testis conditions were assessed. Fish were anesthetized with 0.04% 2-phenoxethanol. The surface of the fish was sterilized with 70% ethanol. Twelve fish were divided into two groups for enzyme digestion, and each group was divided into two replicates (three fish per replicate). Testis tissue of group one were digested by 0.25% trypsin-EDTA, and testis tissues of group two were digested by 0.05% trypsin-EDTA. At the end of the trial, first, the best age, size and weight of the male fish for spermatogonial stem cell isolation and culture were identified as 5+ month old, 12.13±1.5 cm, 19, 25±7.05 g respectively. Then, the highest spermatogonial stem cells were measured in the stage one and two of the testes. Finally, isolation and culture conditions were optimized for male *S.t. macrostigma*. Spermatogonial stem cell isolation and culture techniques were defined for fish in order to be used in surrogate reproduction technologies and gene transfer systems.

## 1. Introduction

Spermatogenesis defined as the process by which haploid spermatozoa develop from germ cells in the seminiferous lobules of the teleost fish testis [1,2]. This process starts with the mitotic division of the primordial germ cells (PGCs) laid close to the basement membrane of the lobules. Primordial germ cells differentiate into ovary or testis after reaching the gonadal anlage [1,3,4,5]. Histologically, the spermatogonia still resemble the PGCs with only a minimal size difference where the sper-

matogonia are smaller than the PGCs [1].

The isolation and culture conditions were well optimized for *O. mykiss* [3,4,6,7]. These authors concluded that, in vitro and in vivo studies on *O. mykiss* spermatogonial stem cells can differentiate into mature egg and sperm in the recipient's (*O. mykiss*) gonads [4,7]. Although, *O. mykiss* and Anatolian mountain trout *Salmo trutta macrostigma* (*S. t. macrostigma*) are belongs to the same family, Salmonidae family, its unique morphology, phenotip and immune system (sexual differentiation, maturation age, weight, size and each fish species has its own immune system) assurances to

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identify isolation and culture conditions for *S.t. macrostigma*. *S. t. macrostigma* is commercially important and is found in Turkey's natural inland water habitats. The scientific name of *S. trutta macrostigma* is still under discussion and somehow remains controversial<sup>[8,9]</sup>. Tougard and his colleagues suggested the name, Brown trout, *S. trutta*<sup>[10]</sup>. As it previously mentioned above *S.t. macrostigma* has unique phenotypic characteristics Therefore, the scientific name *S.t. macrostigma* has been used throughout this study and distribution of *S.t. macrostigma* in Turkey is given in Figure 1A<sup>[11,12,13,14]</sup>.

Females reach sexual maturity at the age of four whereas males reach sexual maturity at the age of 2 years<sup>[11]</sup>. Commercial value of *S. t. macrostigma* when compared to *O. mykiss*, is four to ten-folds higher. Maximum body weight is 25Kg in *S. t. macrostigma* whereas *O. mykiss* has body weight of only 10Kg. It is difficult to spawn in captivity, is unable to stand stress, it cannot tolerate high water temperature and comparatively it takes longer time to reach sexual maturity than *O. mykiss*. Therefore, gamete production for this species is expensive in terms of time, cost, labor and space. However if, *S. t. macrostigma* spermatogonial stem cells could be transplanted into the *O. mykiss*, which is a closely related species that reaches sexual maturity only in just 1,5 years at a body weight of 1Kg, able to stand stress, high water temperature *S. t. macrostigma* gametes might be more easily and rapidly produced. In addition, the natural stock of *S. t. macrostigma* is declining and it is also an endangered species<sup>[15]</sup>. Therefore, after isolation and culture of spermatogonial stem cells it could be possible to preserve the cells by cryopreservation<sup>[16]</sup>.

Although, spermatogonial germ cell isolation and culture is well established for *O. mykiss*, there is only one study using this technology for *S. t. macrostigma*. The study was performed by Lujic' and his colleagues<sup>[16]</sup>. As stated above, *S. t. macrostigma* have different phenotype, morphology and immune system. Therefore, the main objective of the present study was to isolate and culture of spermatogonial stem cells from the *S. t. macrostigma*.

## 2. Materials and Methods

### 2.1 Experimental Design

Wild mature, immature and juvenile male *S.t. macrostigma* were obtained from Kılıç Trout Fish Farm (Kahramanmaraş, Turkey). The males were taken alive to the aquaria unit and stored in three 1000-liter capacity fiberglass tanks. Water was supplied from a tap. Mature, immature and juveniles were fed three times a day with commercial trout feeds during the experiment (IDL ALFA, 2.2mm; Inve, Aquamaks, Turkey). The water was

continuously aerated with a pump. The fiberglass tanks were housed inside an experimental room with a natural photoperiod (12 h dark, 12 hour light). A static water system was used, and 80% of the water in each tank was changed weekly, before the morning feed. One day before gonadal sampling the fish starved. The average weight and length of fish recorded. The testis structure of *S.t. macrostigma* were morphologically and histologically studied in order to identify the best size, age and testis structure of *S.t. macrostigma* for spermatogonial stem cell isolation and culture.

### 2.2 Histological Procedures

Males were anaesthetized in 0.04%, 2-phenoethanol (Sigma Chem. Dorset, UK). The testes were dissected from three sacrificed males for each week. The testis tissue was divided in two lobes and half of the testes lobes was fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and then sectioned at 5µm thickness and stained with hematoxylin and eosin (MERCK) for histological evaluation<sup>[17,18]</sup>. The second lobe of the testes tissue was cut into small pieces for spermatogonial cell isolation. After histological work, all slides were examined under a light microscope (CH-2 Olympus-Japan). Photomicrographs were taken to illustrate the most abundant number of spermatogonia in the testes of *S.t. macrostigma*<sup>[1]</sup>. Spermatogenesis and spermatogonia were classified by developmental stages adapted from Çek and Yılmaz<sup>[2]</sup>.

### 2.3 Isolation of Spermatogonial Stem Cells

Before sacrificing of the male *St. macrostigma*, the whole fish body was sterilized with 70% isopropanol to prevent any possible contamination. *S.t. macrostigma* was dissected using a sterilized dissection set and the testes were placed in HBSS in a sterile petri dish. The used tubes, lids and dissection set were kept in a continuous burner to prevent possible contamination. The testes were chopped into very small pieces with pre-sterilized scalpel were cleared of blood vessels and peritoneum in the sterile cabinet (uv system was turned on 15 minute before the study started and burner flame was used continuously). For proper enzymatic digestion and maximum dispersion of the testes, 0.25% and 0.05% Trypsin-EDTA (ethylene diamine tetra acetic acid) added in two different groups (each containing 3 male fish). EDTA decouples cell-cell connections and allows the cells to be homogeneously dispersed. The trypsin was neutralized by 10% fetal bovine serum (FBS).

After washing the testes in streptomycin, gentamycin

and fungizone containing HBSS, the testes in petri dish were left in the bleach solution in pH 7.4 (1/10 clorox bleach, 9/10 ultra-distilled pure water) for 2 minutes, followed immediately by 3 times washed with HBSS<sup>[19,20,21]</sup>. Minced testes tissues of each fish were transferred into one 50 mL autoclaved glass flask, which contained a stir bar. All the samples were incubated on ice for 30 min followed by 1 hr. at 20 °C with a magnetic stirrer to achieve higher digestion efficiency. In order to eliminate cell clumps, and to obtain single cell suspension, the cell suspension from each replicate was then filtered using a 40 µm cell strainer (nylon mesh, Falcon, BD Falcon) and centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet resuspended in 2 mL HBSS. The trypan blue dye exclusion tests were used to determine the number of viable spermatogonia cells. It was based on the principle that live cells possessed intact cell membranes that excluded trypan blue, whereas dead cells did not. Therefore, a viable cell had a clear white cytoplasm whereas a non-viable cell had a dark blue cytoplasm. Five µL of cell suspension were gently mixed with 45 µL Trypan blue.

## 2.4 Spermatogonial Stem Cell Culture

After centrifugation, the cells were washed 3 times with 50µg / ml penicillin, 50µg/ml streptomycin, 50µg/ml gentamycin and 50µg / ml fungizone containing HBSS. Following resuspension of cells in 2 mL HBSS, 20µl of prepared cell suspension was transferred to each culture dish and 60µl culture medium (L-15) was added (BIO-CHROM AG). Stock solution of culture medium prepared as 25mM HEPES, antibiotics, 1.0µg/ml NaHCO<sub>3</sub>, 0.3µg/ml L-glutamine, 10% FBS, 5% *S.t. macrostigma* serum, 50µg/ml insulin and 1ng/ml bFGF. Previously six compartments polystyrene sterile culture plates were well coated with poly-L lysine, and then the cells were seeded into these culture plates including culture medium (GRENIER BIO-ONE CELLSTAR). Culture media pH was maintained at 7.45. Cells were cultured at 19 °C in a refrigerated incubator. Osmolality adjustment was done by using an osmometer (Automatic semi-micro osmometer, model A0300, Knauer). They were checked daily under an inverted microscope (OLYMPUS CKX41SF). Spermatogonia were removed by medium change daily in the first week and daily dilutions were made.

## 3. Results

### 3.1 Morphological Suitability of the Testes

The best age, size and weight of the male *S.t. macrostigma* for spermatogonial stem cell isolation and culture were

identified as 5+ month old (age), 12.13±1.5 cm (in length), 19,25±7.05 g (in weight) respectively. At these age, size and weight, the testes paired and attached to the dorsal lateral lining of the peritoneal cavity. They were inactive and the experience needed to separate testis from the ovaries. At 5 months old, testes were creamy white in color. Thin ribbon like translucent structure observed (Figure 2A). Morphological characteristics of the testes are given in Table 1.

### 3.2 Histological Suitability of the Testes

Histologically testes conditions were assessed as seen in Figure 2B and C. The highest spermatogonial stem cells were measured in the stage one and two of the testes. This stage was characterized by a large nucleus in the central position, surrounded by little cytoplasm, at five months old (Figure 2B and C). Histological characteristics of the testes are given in Table 1.

### 3.3 Isolation of Spermatogonial Stem Cells

The 0.25% Trypsin-EDTA enzymatic digestion indicated the best efficiency and the best amount of cell isolation (Figure 3A). Isolation conditions were optimized for *S.t. macrostigma*. Viable spermatogonial stem cells and non-viable spermatogonial stem cells were shown (Figure 3B). In the figures the germinal vesicle of the viable cells which carry the genetic material were bright and white in color whereas the germinal vesicle of the death spermatogonial cells were dark blue (Figure 3B). Trypsin-EDTA enzymatic dissociation was terminated by adding 5% sterilized *S.t. macrostigma* 's serum and culture medium.

### 3.4 Culture of Spermatogonial Stem Cells

After testicular germ cell isolation, culturing of these cells were performed. Cell filtration carried out through a 40 µm cell stainer, and then the cell suspension from each testis was centrifuged at 500 g for 10 min. The supernatant discarded and the pellet resuspended in culture medium. Cells seeded in attachment factor and poly-L lysine coated 6- well plates. Using poly-L lysine was successful and the cells attached to the plate during the first five days of the culture (Figure 3C). Plates were incubated at 19°C in an incubator without 5% CO<sub>2</sub>. Cells were sub cultured when 80% culture surface was covered by cells. After changing the 50% of the media, cells and tissue were observed under an inverted microscope and images were taken (Figure 3C and D). In the following days, colonization of these cells was detected (Figure 3E). Culture conditions were optimized for *S.t. macrostigma*. Survival rate of spermatogonia and mitotic activities in L-15 culture media, with 5% serum of *S.t. macrostigma* (Culture media

pH, 7.45; temperature 19°C, Osmolality was maintained at  $235 \pm 0.5$  mOsm  $\text{kg}^{-1}$ ) were developed.

#### 4. Discussion

The best age, size and weight of the male *S.t. macrostigma* for spermatogonial stem cell isolation and culture in *S.t. macrostigma* has not been comprehensively investigated. Previously, Kise and her colleagues<sup>[22]</sup> and Sato and his team<sup>[23]</sup> studied in a closely related species, Rainbow trout, *Oncorhynchus mykiss* at 10°C and recommended 11-12 months old fish for spermatogonia isolation and culture. In their study, the best total length and weight were  $13.9 \pm 1.4$  cm and  $42.4 \pm 11.5$  g, respectively. Hayashi and his colleagues<sup>[24]</sup> used 10-15 months old transgenic *O. mykiss*. In the present study, the best age, size and weight of the male *S.t. macrostigma* for spermatogonial stem cell isolation and culture were identified as 5+ month old (age),  $12.13 \pm 1.5$  cm (in length),  $19.25 \pm 7.05$  g (in weight) respectively. When compared those studies with the age and weight of *S.t. macrostigma* in the present study is much smaller. Although *S.t. macrostigma* and *O. mykiss* are belong to the same genus, both are quite different phenotypically. In previous studies, the temperature was 10°C whereas the temperature in our study was 19°C. The differences between current and other studies may be explained by the differences in phenotype and temperature.

In the present study, the highest spermatogonial stem cells were measured in the stage one and two of the testes.

Therefore, the stage one and two of testes development was suggested to be used for spermatogonial stem cell isolation and culture. This finding was similar to that of Lujic and his colleagues<sup>[16,25]</sup>.

In fish, the first isolation of spermatogonial stem cells was in *O. mykiss*. Okutsu and his team<sup>[3]</sup> cultured *O. mykiss*. Testes of immature male *O. mykiss* were incubated in PBS (pH, 8.2) with 0.5% trypsin and spermatogonia were isolated<sup>[3]</sup>. In catfish, to determine the enzymatic efficiency in testicular tissue trypsinization, Shang<sup>[19,20,21]</sup> compared two different concentration of trypsin. First concentration was 0.05% trypsin-EDTA and the second one was 0.25% trypsin-EDTA. Based on their studies the 0.25% trypsin-EDTA showed higher efficiency and a higher amount of cell isolation than 0.05% trypsin-EDTA enzymatic digestion. Trypsin has been successfully used for dissociation of spermatogonial stem cells in *O. mykiss*<sup>[3,6]</sup>, blue catfish *Ictalurus furcatus*<sup>[19,20,21]</sup>, goldfish, *Carassius auratus*<sup>[26]</sup>, and Neotropical catfish, *Rhamdia quelen*<sup>[27]</sup>. However, Lujic and her colleagues<sup>[27]</sup>, investigated the efficiency of different concentration of collagenase on gonadal dissociation of *S.t. macrostigma*. The highest total yield was recorded in two groups without trypsin

(2 and 6 mg/ml collagenase). The protocol using 6 mg/ml collagenase displayed the lowest number of viable cells. Therefore, Lujic and her colleagues<sup>[27]</sup>, suggested the use of 2mg/ml collagenase for the dissociation of *S.t. macrostigma* spermatogonial stem cells. In the present study, the protocol described by Shang<sup>[19]</sup> was modified. In the present study, the 0.25% trypsin-EDTA enzymatic digestion was found to be more efficiency than the 0.05% trypsin-EDTA and amount of cell isolation was satisfactory (pH was maintained at 7.45 and temperature at 19°C).

Spermatogonial stem cell culture had been studied in *O. mykiss*<sup>[27]</sup> and blue catfish *Ictalurus furcatus*<sup>[19,20,21]</sup>, testicular cell suspensions were prepared from six to 9 month old pvasa-Gfp transgenic *O. mykiss*. Testes were minced and incubated with 1ml of L-15 containing 2 mg/ml collagenase and 500IU/ml dispase for 7-9 hours at 10 °C. The resultant cell suspension was filtered through a 20-mm pore-size nylon screen to eliminate cell clumps and blood vessels. Testicular germ cells isolated from two-year-old juvenile blue catfish. The cells were easily cultured in L-15 medium at 27 °C in air and had the same morphological characteristics as channel catfish testes cell line. The cell suspension was filtered using a 42  $\mu\text{m}$  cell strainer<sup>[19,20,21]</sup>. The authors stated that spermatogonial stem cell culture was more difficult than oogonial stem cell culture.

In the present study, the cells were cultured in L-15 culture media, with 5% serum of *S.t. macrostigma* (Culture media pH, 7.45; temperature 19°C, Osmolality was maintained at  $245 \pm 2$  mOsm  $\text{kg}^{-1}$ ). The main difference between present study and the studies done by Okutsu and his colleagues<sup>[3]</sup>, Hayashi and his colleagues<sup>[24]</sup> and Lujic<sup>[16]</sup> was the temperature. In the present study, the temperature of the incubator was 19°C, whereas the temperature of the incubator in those studies was 10 °C. In the current study sterilized 5% serum of *S.t. macrostigma* were used very often therefore, the media was quite rich. In conclusion, The current study suggest that the best age, size and weight of *S.t. macrostigma* for spermatogonial stem cell isolation and culture should be 5+ month old,  $12.13 \pm 1.5$  cm,  $19.25 \pm 7.05$  g respectively. The testes should be at the stage one and two. The environment in the optimum culture media should maintained at pH, 7.45; temperature 19°C, Osmolality at  $245 \pm 2$  mOsm  $\text{kg}^{-1}$  (with 5% sterilized serum of *S.t. macrostigma*). The spermatogonial stem cell isolation and culture technique was developed for *S.t. macrostigma* in order to be used in surrogate reproduction technologies and gene transfer systems.

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**Statement of Conflict of Interest**

The authors declare that they have no conflict of interest.

**Statement on the Welfare of Animals**

Fish experiments were approved by the Mustafa Kemal University in Turkey and were conducted in agreement with the guidelines of Republic of Turkey University of Mustafa Kemal Laboratory Animal Ethics Committee.

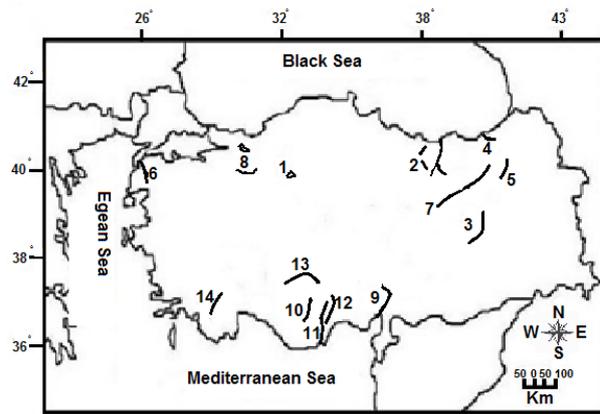
**Appendixes**

*Tables*

**Table 1.** Description of Testes Stages. Classification was based on the histological criteria adapted from Cek et al. 2001

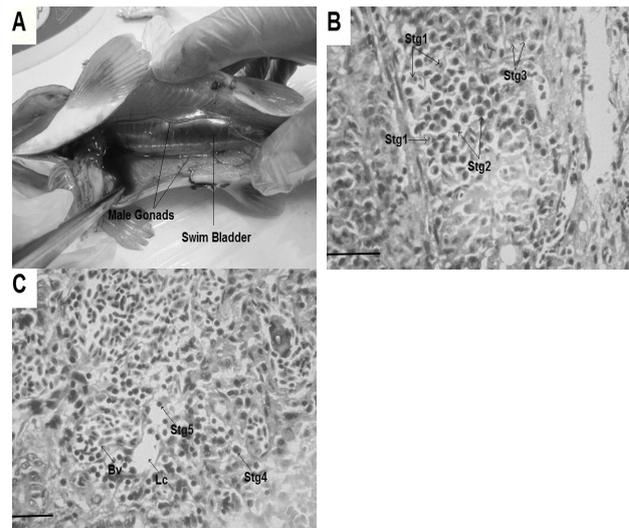
Stages	Morphological Description	Histological Description
<b>Stg1 (Spermatogonia)</b>	Testes were colorless, thread like structure, thin and were like ovaries. Elongated and close to the vertebrate column. No morphological differences between the testes and ovaries were visible.	Spermatogonia was visible and, was characterized by a large nucleus in the central position which surrounded by little cytoplasm. Somatic cells were also abounding.
<b>Stg2 (Primary spermatocytes)</b>	Testes were slightly larger and distinctively longer than the previous stage. They were white and smooth	Few primary spermatocytes and at the periphery, many spermatogonia were detectable. Somatic cells were greater in number. Primary spermatocytes were smaller than the spermatogonia.
<b>Stg3 (Secondary spermatocytes)</b>	Testes were larger and elongated, more distinctive in color, which was white. Two lobes begin to conjugate.	Secondary spermatocytes were detected at 4 months of age. Nucleolus was not clear. Secondary spermatocytes were morphologically similar to Primary spermatocytes; however, the size was somehow smaller than the Primary spermatocytes.
<b>Stg4 (Spermatids)</b>	Red spots were visible on both sides of the testes. They were very white. Milt was not observed.	At this stage, secondary spermatocytes divided meiotic ally and turned into spermatids. The cells were irregular in shape and extremely basophilic.
<b>Stg5 (Spermatozoa)</b>	Red spots were still visible. Drops of milt were observed under pressure.	At this stage, cell division was not observed. Just before maturation stage, all stages of spermatogenesis were detectable.

**Figure Legends**



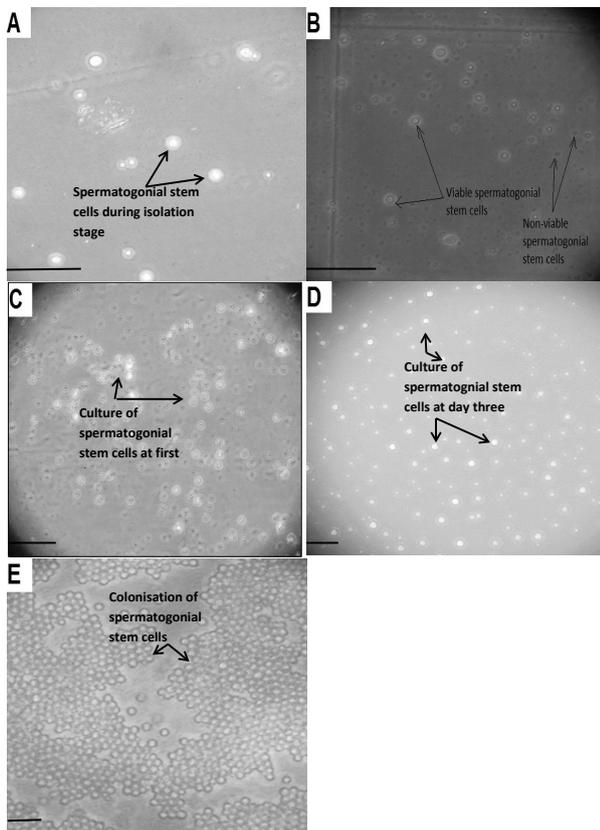
**Figure 1.** Map showing distribution of *Salmo trutta macrostigma* in Turkey

**Note:** Numbers indicates the location of the species. 1. Bolu (Yedi Göller National Park); 2. Trabzon (Uzun Göl Supplies, Uçarsu-Çatak, Arpalı-Sultanmurat); 3. Tunceli (Munzur Stream); 4. Rize (Ovit Mountain); 5. Erzurum (Tortum Stream); 6. Çanakkale (Kaz Mountains); 7. Gümüşhane (Siran Stream) <sup>[11]</sup>; 8. Sapanca (Sapanca Lake, Mahmudiye Stream) <sup>[12]</sup>; 9. Ceyhan (Ceyhan Stream) <sup>[13]</sup>; 10. Antalya (Alakır Stream); 11. Antalya (Köprüçay Stream); 12. Antalya (Alara Stream); 13. Karaman (Ermenek Stream); 14. Fethiye (Eşen Stream) <sup>[14]</sup>.



**Figure 2.** Morphological and histological suitability of the testis for spermatogonial stem cells isolation and culture

**Note:** (A) Morphology of the testis; (B) Histology of the testis; (C) Matured testes and blood vessels, which is less suitable for spermatogonial cell isolation and culture. Stg1, Spermatogonia; Stg2, Primer spermatocytes; Stg3, Seconder spermatocytes; Stg4, Spermatids; Stg5, Spermatozoa; Bv, blood vessels; Lc, Luminal cavity. Scale bar = 100 µm (B, C), Stained with Hematoxylin & Eosin.



**Figure 3.** Isolation and culture of spermatogonial stem cells

**Note:** (A) Viable spermatogonial stem cells are shown at the isolation stage. (B) Counting of viable and non viable spermatogonial stem cells by hemocytometer. (C) Culture of spermatogonial stem cells, first day. (D) Culture of stem cells, Day 3. (E) Culture of spermatogonial stem cells day seven. Colonization of cells is shown. Scale bar= 125µm (A, B, C, D and E).

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